Australian Bat Lyssavirus

Thesis submitted by:

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Declaration of Originality

The work presented in this thesis is, to the best of my knowledge and belief, original, and my own work except as acknowledged in the text. This material has not been submitted, either in whole or in part, for any other degree at this or any other university.

Janine Barrett

Date: Friday, 26 March 2004

Acknowledgements

The work carried out in this thesis was done as a collaborative project between the School of Veterinary Science at the University of Queensland (St Lucia campus) and the Animal Research Institute, Yeerongpilly of the Queensland Department of Primary Industries, with valuable contributions by the CSIRO Australian Animal Health Laboratory, Geelong, the Queensland Health Scientific Services division of the Department of Health, and the Rabies Laboratory of the Centres for Disease Control and Prevention, Atlanta, USA. Research funding was provided by the Queensland Treasury New Initiatives Program and subsequently a NHMRC grant. I am very grateful to Wayne Robinson and Peter Young for creating an initial scholarship fund, to John MacKenzie for bridging funds when this ran out, and finally The University of Queensland Graduate School Award (UQGSA) that allowed me to complete this work.

Throughout this project I have enjoyed the support and assistance of colleagues and staff at The University, the DPI, the CSIRO AAHL, QHSS and CDC Atlanta. As far as possible, specific contributions are acknowledged in the sections where they applied. In particular I am deeply grateful to Peter Young and Roger Kelly who, as my supervisors gave me invaluable assistance and support during the research work and the preparation of this thesis.

At the University I would like to acknowledge the support and advice of RH (Dick) Sutton and the technical assistance of the staff in the veterinary histology laboratory, notably Chris Cazier for the preparation of histological slides and sections for immunoperoxidase staining. I am particularly grateful to Tom Steginga and Ivor Harris of the Central Animal Breeding House, for having taught me everything I needed to know about handling, caring for, and anaesthetizing mice, for allocating large numbers of mice to a breeding program in order to provide the age specific mice used, and for loaning a large number of mouse cages and a cage rack. Without this logistical support, the work involving mice would have been severely limited. I am also very grateful for having had the fabulous opportunity to collaborate with Paul Prociv and Melissa Carlisle to characterize samples of *Angiostrongylus* sp. It was also a great pleasure to be able to discuss bat ecology with Les Hall and benefit from the expertise of Morag Wilson and Annie Rose for the radiographs.

Most of the work was done at the ARI, where I enjoyed support as part of the ‘bat research’ team of Hume Field, Kim Halpin, Craig Smith, Natasha Smith, and Stirling Tavener under the direction of Peter Young, which contributed as a group to the parallel Hendra virus and Australian bat lyssavirus projects. In particular Craig was essential to the success of this project contributing to the bat necropsies, data entry, bat catching, maintenance of the bat colonies, blood sampling, and sample management. So many things simply could not have been done without him. It was always a pleasure working with Barry Rodwell, who as the head of the diagnostic laboratory did hundreds of diagnostic fluorescent antibody tests, and verified research results done by the candidate. I am also very grateful for the logistical support provided by Geoff Dawson and Andrew

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Through Peter Young's support I was able to travel to CDC, Atlanta and present preliminary results there and at the 48th Annual Wildlife Disease Association Conference. This gave me the opportunity to discuss the project with staff at the CDC rabies laboratory, in particular Charles Rupprecht, and receive additional training in mouse inoculations and fluorescent antibody test interpretation. Later, CDC contributed by generously performing serological tests to complement those done at AAHL.

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Abstract

In Chapter 1, the literature relating to rabies virus and the rabies like lyssaviruses is reviewed.

In Chapter 2 data are presented from 1170 diagnostic submissions for ABLV testing by fluorescent antibody test (Centocor FAT). All 27 non-bat submissions were ABLV-negative. Of 1143 bat accessions 74 (16%) were ABLV-positive, including 69 of 974 (7.1%) flying foxes (*Pteropus* spp.), 5 of 7 (71.4%) *Saccolaimus flaviventris* (Yellow-bellied sheathtail bats), none of 151 other microchiropteran bats, and none of 11 unidentified bats. Statistical analysis of data from 868 wild Black, Grey-headed, Little Red and Spectacled flying foxes (*Pteropus alecto, P. poliocephalus, P. scapulatus,* and *P. conspicillatus*) indicated that three factors; species, health status and age were associated with significant ($p < 0.001$) differences in the proportion of ABLV-positive bats. Other factors including sex, whether the bat bit a person or animal, region, year, and season submitted, were not associated with ABLV. Case data for 74 ABLV-positive bats, including the circumstances in which they were found and clinical signs, is presented.

In Chapter 3, the aetiological diagnosis was investigated for 100 consecutive flying fox submissions with neurological signs. ABLV (32%), spinal and head injuries (29%), and neuro-angiostrongylosis (18%) accounted for most neurological syndromes in flying foxes. No evidence of lead poisoning was found in unwell (n=16) or healthy flying foxes (n=50). No diagnosis was reached for 16 cases, all of which were negative for ABLV by TaqMan® PCR.

The molecular diversity of ABLV was examined in Chapter 4 by sequencing 36 bases of the leader sequence, the entire N gene, and start of the P gene of 28 isolates from pteropid bats and 3 isolates from Yellow-bellied sheathtail (YBST) bats. Phylogenetic analysis indicated all ABLV isolates clustered together as a discrete group within the *Lyssavirus* genera closely related to rabies virus and European bat lyssavirus-2 isolates. The ABLV lineage consisted of two variants; one (ybst-ABLV) consisted of isolates only from YBST bats, the other (pteropid-ABLV) was common to Black, Grey-headed and Little Red flying foxes. No associations were found between the sequences and either the geographical location or year found, or individual flying fox species.

In Chapter 5, 15 inocula prepared from the brains or salivary glands of naturally-infected bats were evaluated by intracerebral (IC) and footpad (FP) inoculation of Quackenbush mice in order to select and characterize a highly virulent inoculum for further use in bats (Inoculum 5).

In Chapter 6, nine Grey-headed flying foxes were inoculated with $10^{5.2}$ to $10^{5.5}$ $MICED_{50}$ of Inoculum 5 divided into four sites, left footpad, pectoral muscle, temporal muscle and muzzle. Another bat was inoculated with half this dose divided into the footpad and pectoral muscle only. Seven of 10 bats developed clinical disease of 1 to 4 days duration between PI-days 10 and 19 and were shown to be ABL-positive by FAT, HAM immunoperoxidase staining, virus isolation in

mice, and TaqMan PCR. Five of the seven bats displayed overt aggression, one died during a seizure, and one showed intractable agitation, pacing, tremors, and ataxia. Viral antigen was demonstrated throughout the central and peripheral nervous systems and in the epithelial cells of the submandibular salivary glands (n=4). All affected bats had mild to moderate non-suppurative meningoencephalitis and severe ganglioneuritis. No ABLV was detected in three bats that remained well until the end of the experiment on day 82. One survivor developed a strong but transient antibody response.

In Chapter 7, the relative virulence of inocula prepared from the brains and salivary glands of experimentally infected flying foxes was evaluated in mice by IC and FP inoculation and TaqMan assay. The effects in mice were correlated to the TaqMan C_T value and indicated a crude association between virulence and C_T value that has potential application in the selection of inocula.

In Chapter 8, 36 Black and Grey-headed flying foxes were vaccinated with one (day 0) or two (+ day 28) doses of Nobivac rabies vaccine and co-vaccinated with keyhole limpet haemocyanin (KLH). All bats responded to the Nobivac vaccine with a rabies-RFFIT titer > 0.5 IU/mL that is nominally indicative of protective immunity. Plasma from bats with rabies titres >2 IU/mL had cross-neutralising ABLV titres >1:154. A specifically developed ELISA detected a strong but transient response to KLH.

Publications

Refereed Journal Articles

Australian bat lyssavirus infection in a captive juvenile Black flying fox
Emerging Infectious Diseases 1999: 5 (3) 438-440
H Field, B McCall, and J Barrett

Potential exposure to Australian bat lyssavirus, Queensland 1996-1999
Emerging Infectious Diseases 2000: 6 (3) 259-264
B J McCall, J H Epstein, A S Neill, K Heel, H Field, J Barrett, G A Smith, L A Selvey, B Rodwell, and R Lunt

Neuro-angiostrongylosis in wild Black and Grey-headed flying foxes (*Pteropus* spp.)
Australian Veterinary Journal 2002: 80 (9) 554-558
J Barrett, M Carlisle, and P Prociv

Conference Proceedings

Australian bat lyssavirus in Queensland
48th Annual Wildlife Disease Association Conference, The University of Georgia, Athens, Georgia USA 8- 12 August 1999
J Barrett, P Young, H Field, B Rodwell, G Smith, and P Hooper

Australian bat lyssavirus
Australian Society for Veterinary Pathology Annual Conference The University of Melbourne, Parkville, Australia 15-16 May 1999
J Barrett, P Young, H Field, N Smith, C Smith B Rodwell, and G Smith

Australian bat lyssavirus in Queensland
1st Australian Virology Group Meeting, Fraser Island 5 – 9th December 2001 (P1.12)
J Barrett, H Field, P Young, B Rodwell, G Smith, and P Hooper

Vaccination of captive flying foxes against Australian bat lyssavirus
1st Australian Virology Group Meeting, Fraser Island 5 – 9th December 2001 (P3.37)
J Barrett, R. Lunt, and P Young

Other publications

When a bat is in the waiting room: Practical advice to veterinary practitioners regarding euthanasia and submission of bats for Australian bat lyssavirus testing
Australian Veterinary Association Queensland News 2000: Autumn Edition 1-2
J Barrett and H Field
Reprinted in
Advantage: Newsletter of the Australian Veterinary Association Northern Territory Division June 2000 (3)
and *Australian Veterinary Association Queensland News* 2002: Summer Edition 3

Rat lung worm in flying foxes and **A reminder about Australian bat lyssavirus**
Orphan Native Animal Rear & Release Association Inc (ONARR) Newsletter December 2001 (available via WWW.ONARR.org.com.au)
Janine Barrett

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List of Abbreviations

AAHL	CSIRO Australian Animal Health Laboratory, Geelong, Victoria, Australia
ABLV	Australian bat lyssavirus
ABLV-RFFIT	rapid fluorescent focus inhibition test (RFFIT) using an ABLV isolate as the challenge virus
AEC	3-amino-9-ethylcarbazole, an immunohistochemical chromogen that oxidizes in the presence of peroxidase to form a red end product
ARI	Animal Research Institute, the Queensland Department of Primary Industries, Fairfield Road Yeerongpilly, Queensland, Australia 4072
BHK	baby hamster kidney (cell line used for virus culture)
bp	base pair
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CVS	challenge virus standard, well characterized fixed laboratory strain of rabies virus
dH_2O	deionised water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
EBL	European bat lyssavirus
ELISA	enzyme linked immunoabsorbent assay
ERA	Evelyn-Rokitnicki-Abelseth strain of rabies virus, a well characterized fixed laboratory strain of classical rabies
FAT	fluorescent antibody test
FFD	fluorescing focus doses
IC	intracerebral (inoculation)
IM	intramuscular (inoculation)
Kb	kilobase
KLH	keyhole limpet hemocyanin
$MFP_{90}ED_{50}$	90 day mouse footpad mean effective dose, a measure of peripheral virulence
$MICED_{50}$	mouse intracerebral mean effective dose $\approx LD_{50}$, 50% lethal dose
n/a	not applicable
ONARR	Orphan Native Animal Rear and Release Association Inc., a volunteer wildlife carer organization in Southeast Queensland
ORF	open reading frame
PC2	Physical Containment Level 2, as defined in the Australian/ New Zealand Standard AS/NZS 2243.3:1995
PC3	Physical Containment Level 3, as defined in the Australian/ New Zealand Standard AS/NZS 2243.3:1995
PCR	polymerase chain reaction
PI-day	post-inoculation day

ppm	parts per million, equals mg/kg
pt-ABLV	variant of ABLV characteristic of that found in *Pteropus* spp (flying foxes)
PV-day	post-vaccination day
rabies-RFFIT	rapid fluorescent focus inhibition test (RFFIT) using CVS-11 rabies virus and standardized against international standard anti-rabies serum.
RFFIT	rapid fluorescent focus inhibition test (see Appendix 2)
RNA	ribonucleic acid
SG	salivary gland or salivary glands
TAE	tris-acetate ethylenediaminetetraacetic acid electrophoresis buffer
QABC	Queensland Agricultural Biotechnology Centre, a unit of the Queensland Department of Primary Industries located at The University of Queensland, Brisbane, Australia.
QHSS	Queensland Health Scientific Services, 39 Kessels Rd Coopers Plains, Queensland, Australia 4108
UTR	untranslated region (of genome)
5'UTR	untranslated region at the five prime (carboxy terminal) end of a gene
3'UTR	untranslated region at the three prime (amino terminal) end of a gene
WHO	World Health Organization
W/v	weight per volume (g/mL)
YBST	Yellow-bellied sheathtail bat (*Saccolaimus flaviventris)*
TCID	Tissue culture infective dose
ybst-ABLV	variant of ABLV characteristic of that isolated from *Saccolaimus flaviventris* (Yellow-bellied sheathtail bat)

Introduction

Australia was considered free of rabies virus and the rabies-like viruses of the genus *Lyssavirus,* family *Rhabdoviridae,* until the recognition in 1996 of Australian bat lyssavirus (ABLV) as the cause of rabies-like disease in a Black flying-fox (*Pteropus alecto*) and a wildlife carer (Allworth *et al.* 1996; Fraser *et al.* 1996). Initial characterisation of the isolates from the flying fox and human indicated that ABLV was very closely related to rabies virus and European bat lyssavirus-2, which are present in insectivorous Microchiropteran bats of the Americas and Europe. No endemic lyssavirus had previously been isolated in Australia, although other Rhabdoviruses of the genera *Ephemerovirus* including Ephemerovirus (Bovine ephemeral fever), Adelaide River virus, Berrimah virus, and Kimberley virus are present (ICTV 2000).

The presence of a virus closely related to rabies virus had important implications for human and animal health and threatened Australia's WHO rabies-free status. This allows Australia to enjoy considerable economic and trade advantages and is pivotal to Australia's live animal import restrictions (AQIS). Dog-variant (urban rabies) is associated with the deaths of over 30,000 humans each year, particularly in India, South-east Asia, South America, and Africa. Bat-variant rabies is associated with human deaths and the loss of thousands of cattle in South America, and with occasional human deaths in North America (World Health Organization 1992). Large flying fox colonies are present in Australian capital cities including Brisbane, Sydney, and Melbourne, from which they disperse nightly to feed on urban and native fruit, blossom and leaves (Hall and Richards 2000). The diagnosis of fatal ABL infection in a volunteer bat carer less than 4 months after the first recognition of ABL in bats, the urban distribution of thousands of bats, the large number of volunteer wildlife carers rehabilitating sick, injured, and orphaned native animals including bats, and the strong similarities between ABLV and rabies virus, suggested that there was potential for large numbers of people to be infected with ABLV.

To effectively manage human exposure to ABLV and defend Australia's rabies-free status required information about the epidemiology, natural history, and molecular diversity of ABLV. The geographical and host species ranges of ABLV were unknown, as were the modes of transmission, susceptibility, clinical course, and serological responses in bats. The susceptibility of domestic and other native animals to ABLV and the risk infection of these secondary hosts posed to humans were also unknown.

Pending specific information about ABLV, and given the close similarity between ABLV and rabies virus, it was presumed that protocols for the management of rabies were appropriate. Initial responses of the relevant authorities included pre-and post-exposure rabies vaccination of persons known or likely to be exposed to bats and or ABLV (McCall *et al.* 2000; NHMRC 1997; Thompson 1999; Torvaldsen and Watson 1998). This included wildlife volunteers, veterinarians, researchers, staff involved in the care of captive bat colonies, and members of the public bitten or

scratched by bats. The death of the wildlife carer received considerable media attention and there was rapid pubic awareness that bats posed a threat of fatal disease and should not be handled. Testing and surveillance programs were set up at Australia's WHO rabies reference laboratory (The CSIRO Australian Animal Health Laboratory, Geelong, Victoria) and were incorporated into the pre-existing Hendra virus (previously equine morbillivirus) research program investigating bats as the natural Hendra virus reservoir at the Animal Research Institute (ARI), Yeerongpilly, Queensland.

The presence of a lyssavirus in Australia had substantial cultural implications with respect to attitudes towards wildlife. It required responses from government and non-government organisations involved in human and veterinary health. A clearer understanding of the natural history of ABLV was required if these responses where to be appropriate and effective. Given the importance of ABLV to Queensland, and as surveillance of bats was being undertaken for Hendra virus, it was decided that concurrent research into ABLV should be undertaken. Funding was allocated for the targeted epidemiological surveys, diagnostic investigations, molecular characterisation, experimental infections, and vaccination trial described in this thesis that characterize ABLV.

1 Literature Review

1.1 Lyssaviruses

Lyssaviruses, which make up the genus *Lyssavirus*, are a group of related, unsegmented, negative sense, single-strand, ribonucleic acid (-ssRNA) viruses within the family *Rhabdoviridae*. The genus includes rabies virus (the type virus) and the rabies-like viruses; Lagos bat virus, Mokola virus, Duvenhage virus, European bat lyssaviruses 1 and 2 (EBL-1, EBL-2) and Australian bat lyssavirus (ABLV) (Amengual *et al.* 1997; Fraser *et al.* 1996; Shope *et al.* 1970; Tignor *et al.* 1977). All lyssaviruses can cause rabies or rabies-like diseases with severe, generally fatal, encephalitis. Lyssaviruses are generally maintained in bat (Order *Chiroptera*) or carnivore (Order *Carnivora*) principle host species, with spill-over and endpoint infection of individuals of other species including man and livestock.

Lyssaviruses are bullet-shaped with a helical nucleocapsid consisting of the ribonucleic acid (RNA) genome, nucleoprotein (N), phosphoprotein (P, formerly M1 or NS) and polymerase protein (L, large). The nucleocapsid is surrounded by an envelope derived from a host-cell lipoprotein membrane and is studded with viral glycoprotein (G) trimers (Cox *et al.* 1977; Dietzschold *et al.* 1978; Gaudin *et al.* 1992). A structural membrane protein (M, previously M2) supports the viral envelope.

The organization of the rabies virus, Lagos bat virus, Mokola virus, and Australian bat lyssavirus (ABLV) RNA genomes is similar and is presumably the same for all lyssaviruses. It comprises ~12 kilobases (Kb) of nucleotides in a gene order of 3'-N-P-M-G-L-5' (Bourhy *et al.* 1993a; Mebatsion *et al.* 1993; Warrilow *et al.* 2002). With a large (423 base), non-protein coding region (intergene or pseudogene, Ψ) occurring between the G and L genes of the genome (Tordo *et al.* 1993).

1.1.1 Classification of the lyssaviruses

The lyssaviruses were originally classified into serotypes defined by serum cross-neutralization studies (which reflect similarities in neutralising glycoprotein antigens), biochemical, and histopathological comparisons. These were:

- Serotype 1 Rabies virus
- Serotype 2 Lagos bat virus
- Serotype 3 Mokola virus – least closely related to serotype 1 Rabies virus
- Serotype 4 Duvenhage virus – most closely related to serotype 1 Rabies virus.

Since a major component of vaccine efficacy is based on antibody responses to neutralizing epitopes on the glycoprotein, a consequence of the variation in neutralizing glycoprotein antigens,

as reflected by the different serotypes, is that vaccination against rabies virus provides poor or negligible protection against Lagos bat and Mokola viruses (Fekadu *et al.* 1988b).

A new classification of seven genotypes has been adopted as a result the development of antigenic (Wiktor *et al.* 1980) and molecular methods (Bourhy *et al.* 1993a; Sacramento *et al.* 1991; Tordo *et al.* 1993) to characterize lyssaviruses, and the recognition of two rabies-like viruses in European bats and the rabies-like virus of Australian bats. Genotypes 1-4 correspond to the original serotypes 1 to 4 with genotypes 5, 6 and 7 corresponding to European bat lyssavirus-1 (EBL-1), European bat lyssavirus-2 (EBL-2) and Australian bat lyssavirus (ABLV) respectively (Bourhy *et al.* 1993a; Gould *et al.* 1998; Tordo *et al.* 1993). The seven genotypes super-segregate in to two main phylogroups; phylogroup I includes genotypes 1, 4, 5, 6, and 7, phylogroup II consists of genotypes 2 and 3 (Badrane *et al.* 2001). The viruses that make up the 7 genotypes have been formally recognised as distinct virus species by the International Committee on Taxonomy of Viruses (ICTV 2000).

The relationship of the lyssaviruses to their serotype, genotype, and phylogroup classification is shown in Table 1-1.

Table 1-1 Comparison of the serotype, genotype, and phylogroup classifications for the lyssaviruses

Serotype	Lyssaviruses	Genotype	Phylogroup
1	Rabies virus	1	I
1	Australian bat lyssavirus (ABLV)	7	I
2	Lagos bat virus	2	II
3	Mokola virus	3	II
4	Duvenhage virus	4	I
4 *	European bat lyssavirus-1 (EBL-1)	5	I
5 *	European bat lyssavirus-2 (EBL-2)	6	I

* EBL-1 and EBL-2 are not generally described in terms of serotype. Early antigenic studies identified EBL-1 as serotype 4 and a 5th serotype was proposed for EBL-2 but not subsequently adopted (King *et al.* 1990).

For the purposes of this thesis the term rabies will only be used in reference to serotype 1/ genotype 1 rabies virus or the disease rabies that is caused specifically by rabies virus variants.

1.2 Rabies virus

Rabies appears to have been recognized as early as ~2300 BC in the pre-Mosaic Eshnunna Code of Babylon which contains a reference to humans being killed by mad/vicious dogs. The term rabies is derived from the Sanskrit *rabhas,* meaning ‘to do violence’, and *lyssa* (Greek) means ‘frenzy’ or ‘madness’, both terms referring to the disturbing effects of the disease on the

behaviour of affected animals (Wilkinson 2002). While it had long been recognized that the disease developed as a result of a bite from an infected animal, it was not until Pasteur in 1885 developed a crude but effective vaccine from the nervous tissue of inoculated rabbits that the potential for clinical disease could be managed. Negri's 1903 identification of "endocellular Negri bodies" and recognition of their diagnostic value formed the basis of rabies diagnosis until the development of immunofluorescent techniques (Tierkel 1973).

In all species clinical rabies is almost invariably fatal and poses a considerable public health problem with over 30,000 human deaths and 4 million post-exposure treatments reported each year (World Health Organization 1992). More than 99% of human deaths are the result of dog bites in the developing world, and the cost of pre- and post-exposure vaccination is a burden worldwide. Where dog rabies has been controlled with dog quarantine and vaccination programs, as in Europe and the United States, wildlife poses the greatest threat of rabies to humans.

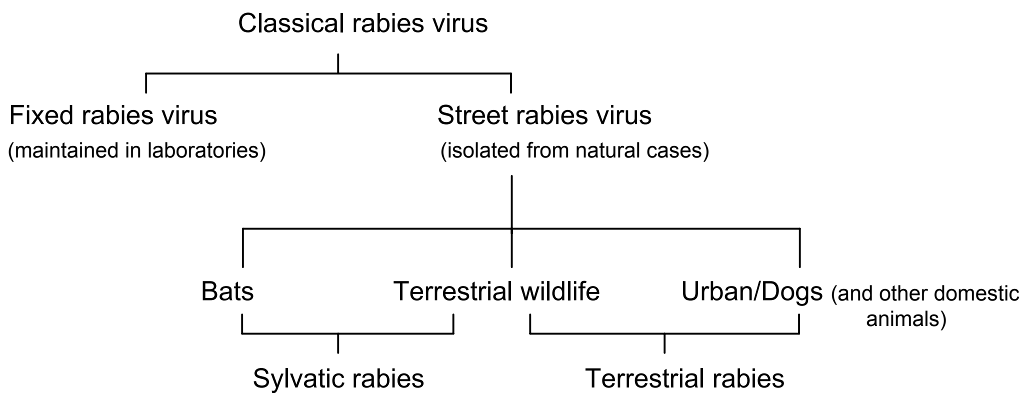
There are a number of terms used to describe different 'types' of rabies virus and disease. These terms as used in this thesis are defined below and their relationships are illustrated in Figure 1-1.

- *Fixed rabies virus* – rabies virus isolates that have been stabilized or 'fixed' by multiple passages by intracerebral inoculation in laboratory animals or in cell culture. Passaging alters the pathogenicity of the virus isolate, typically enhancing its neurotropism. As a result, fixed rabies virus strains have certain characteristics that usually include a shorter incubation period, more consistent clinical signs, less Negri body formation, and more rapid death following intracerebral inoculation, but attenuated pathogenicity by other routes. Attenuated fixed rabies viruses can be effective as live vaccines. The mechanism by which an isolate becomes 'fixed' is not understood.
- *Street rabies virus* – rabies virus isolates from naturally occurring cases of rabies that are not 'fixed'. These isolates may be adapted for cell culture or have undergone limited passage in laboratory animals. The point at which the pathogenicity of a rabies virus isolate from a naturally-infected animal, which is subsequently passaged *in vivo* or *in vitro* in the laboratory, becomes partially 'fixed' is indistinct. This ambiguity complicates the interpretation of some experimental reports.
- *Classical rabies virus* – serotype 1/genotype 1 rabies. This term differentiates 'true' rabies virus from the rabies-like viruses.
- *Dog rabies*– rabies in dogs, also referred to as *urban rabies* as the high concentration of dogs and people in endemic urban environments results in high case numbers in unvaccinated dogs, humans and other domestic animals.
- *Sylvatic rabies* – rabies in wildlife, including bats and terrestrial animals such as raccoons, foxes and skunks, but not including rabies in domestic animals, notably dogs.
- *Terrestrial rabies* – rabies in terrestrial mammals, including carnivores such as dogs, raccoons, foxes, and skunks but not including non-terrestrial animals such as bats. While birds can be infected with rabies, this is exceedingly rare, so that in practice the term

terrestrial rabies differentiates between rabies in terrestrial animals and in bats. The distinction is of epidemiological significance from a human health perspective.

- *Bat rabies* – rabies in bats. A form of sylvatic rabies distinct from rabies in terrestrial animals.

Figure 1-1 Terms used to describe rabies virus



1.2.1 Terrestrial Rabies

1.2.1.1 Antigenic and molecular typing of rabies virus: recognition of variants

Prior to the development of monoclonal antibodies, all rabies viruses were considered antigenically indistinguishable due to the inability of (polyclonal) anti-rabies serum to differentiate strains. When plaque reduction neutralization tests were developed, some minor antigenic differences were detected (Wiktor and Koprowski 1980). Considerable antigenic differences among street and fixed rabies virus isolates were recognized when monoclonal antibody panels were used (Fekadu *et al.* 1988b; Smith *et al.* 1984a; Smith *et al.* 1984b; Smith *et al.* 1986; Smith 1989; Wiktor and Koprowski 1978). A number of studies have been done to type isolates of rabies virus and other lyssaviruses using one or other of the following panels of monoclonal anti-nucleoprotein or anti-glycoprotein antibodies.

- **ERA series**: produced using the Evelyn-Rokitnicki-Abelseth (ERA) vaccine strain at CDC, Atlanta USA (Smith *et al.* 1984a),
- **Wistar series**: prepared from ERA, CVS, and Kelev rabies virus and Mokola virus isolates at the Wistar Institute, Philadelphia, USA (Wiktor and Koprowski 1978; Wiktor *et al.* 1980),
- **CVL series**: prepared from Mokola, Duvenhage, EBL-1 (Danish bat), EBL-2 (Finland man), and Lagos bat virus isolates at the Central Veterinary Laboratory, Weybridge, UK (King *et al.* 1990).

In particular, the ERA and Wistar series have been used to type American rabies virus isolates. Reactivity to panels of these monoclonals indicates antigenic relatedness to the isolates used for antibody production, and implies relatedness between virus isolates with similar profiles. The reactivity of isolates across the United States indicated that:

- There are antigenic differences between isolates of rabies virus from different geographic/outbreak areas, such that in the United States there are multiple endemic patterns of antigenically and geographically discrete rabies virus variants (Smith *et al.* 1984b; Smith 1989; Smith *et al.* 1995).
- The antigenic profile of isolates from a given area is stable over time (8-18 years) (Smith *et al.* 1984b; Smith 1989).
- The majority of isolates with the same antigenic profile are derived from a single species in each region that is primarily responsible for the maintenance of each rabies virus variant (for example raccoons along the Mid-Atlantic coast, red foxes in New York state, and skunks in central USA) with 'incidental' spill over to other local species (Smith 1989).
- Two or more independent cycles involving different rabies virus variants maintained in different principle host species occur in some areas,for example bats, grey foxes, and skunks in Texas. (Smith 1989).
- During multiple laboratory passages, some fixed rabies virus strains developed unique antigenic profiles (Wiktor and Koprowski 1978). However, the antigenic profile of street isolates was preserved through limited passage in cell culture and laboratory animals (Smith 1989).
- Antigenic typing may be used to determine the likely source of new outbreaks, human infection, or isolated cases of rabies in non-endemic areas (Smith *et al.* 1984b; Smith 1988, 1989; Smith *et al.* 1995).

The geographical and species compartmentalization of rabies virus variants has also been demonstrated using polymerase chain reaction (PCR), nucleotide and amino acid sequencing techniques, and phylogenetic mapping (Smith *et al.* 1995; Tordo *et al.* 1993).

1.2.1.2 Antigenic variation and epidemiological observations of terrestrial rabies

Within given endemic areas characterized by a single antigenic strain of rabies virus, the majority of cases occur in one species, even when more than one apparently suitable host population is available (Parker and Wilsnack 1966; Smith *et al.* 1984b; Smith 1989). The principal species for each area/rabies variant include (Smith 1989; Tordo *et al.* 1993):

- Arctic foxes (*Alopex lagopus*), Grey foxes (*Urocyon cinereoargenteus*), Red foxes (*Vulpes vulpes*), coyotes (*Canis lantrans*), striped skunks (*Mephitis mephitis*), and raccoons (*Procyon loctor*) in the United States and Canada (Smith *et al.* 1995).
- Dogs and wolves (*Canis* spp.) in Africa, the Middle East, Latin America, and Asia.
- Red foxes, raccoon dogs (*Nyctereutes procyonoides*) and wolves in Europe.

- Yellow mongoose (*Cynictis penicillata*), dogs, Bat-eared fox (*Otocyon megalotis*), jackals (*Canis mesomelas*), and Kudu (*Tragelaphus strepsiceros*) in Africa (Nel *et al.* 1993).

There is evidence to suggest that strains of rabies virus have specifically adapted to the unique biological and social characteristics of their principal host (Parker and Wilsnack 1966). It is presumed that the virus is maintained in the principal species with opportunistic, dead-end infection of individuals of other species, notably humans. The mechanism of interaction between variants and the biological and social characteristics of their preferred host, and how that interaction limits transmission among incidental species are unknown.

A possible exception involves African Kudu (an antelope). Rabies in herbivores is rare and secondary or tertiary transmission by herbivores is even rarer. However, an apparent rabies endemic exists among Kudu which is thought to have originated from the local jackal strain of rabies virus (Barnard and Hassel 1981; Rupprecht *et al.* 1987). The ability of this jackal-variant to persist in the Kudu has been attributed to relatively high viral excretion in Kudu saliva, contamination of food and water sources with saliva, lesions in the Kudu's oral cavity due to browsing on thorny acacia trees, and high herd densities (Barnard and Hassel 1981; Hubschle 1988; Smith 1989).

1.2.2 Rabies in bats

1.2.2.1 Bats

Bats (Class *Mammalia*, Order *Chiroptera*) are the only mammals capable of true, self-sustained flight and there are approximately 900 species of bat worldwide (Hall and Richards 1979; Richards 1998). All bats are nocturnal. They are classified into two suborders:

- *Megachiroptera* (megabats) consisting of a single family, *Pteropodidae*. All megabats are fruit, blossom or nectar feeders. Most megabats do not echolocate but navigate by sight and sound and have relatively large eyes. Bats of the genus *Rousettus* navigate with the aid of audible clicks produced in the mouth (not the larynx).

- *Microchiroptera* (microbats) consisting of many families. All microbats echolocate with laryngeal sonar. While not blind their eyes are usually very small. Most microbats are insectivorous, others eat fruit or are carnivorous, and three species of true vampire bats feed on blood from living animals.

1.2.2.2 Bat rabies virus

Vampire bats, frugivorous and insectivorous microbats have been demonstrated to be capable of transmitting rabies virus (Humphrey *et al.* 1960; Miller and Nathanson 1977). In North America the epidemiology of bat rabies appears to be independent of terrestrial rabies (Constantine 1967;

Smith 1989; Tordo *et al.* 1993). Unlike terrestrial rabies, rabies in bats is not confined to particular geographic areas. Rates of rabies virus infection in surveys of clinically normal bats in the United States were 0-3%, while the positive proportion of rabies-suspect bats submitted for rabies diagnosis was of the order of 10-12% (Constantine 1967). Although not initially evident (Smith *et al.* 1984b), antigenically and phylogenetically discrete variants of rabies virus appear to circulate in principal bat hosts (Smith 1989 ; Smith *et al.* 1995), and are distinct from the well-characterized terrestrial variants (Smith *et al.* 1984b; Smith 1989). Relative to rabies viruses endemic in terrestrial animals, there is greater variation in the antigenic and molecular profiles of bat rabies virus isolates, and their relationship to particular bat species is more complex (Tordo *et al.* 1993). Rabies virus has been reported in most but not all (30/39) bat species in the United States and Canada (Constantine 1979), but is most commonly reported in one or two species of bat in a given area. Principal bat species (all *Microchiroptera*) include (Smith *et al.* 1995):

- Vampire bat (*Desmodus rotundus*) Central America
- Big brown bat (*Eptesicus fucus*) USA and Central America
- Mexican freetail bat (*Tadarida brasiliensis mexicana*) USA and Central America
- Red bat (*Lasiurus borealis*) USA
- Hoary bat (*Lasiurus cinereus*) USA
- Yellow bat (*Lasiurus intermedius*) USA
- Silver-haired bat (*Lasionycteris noctivagans*) USA

Rabies virus is rarely identified in *Myotis* spp, which are equally prevalent in affected areas. It is likely that principal bat hosts incidentally infect these and other species of bat. While bats do infect humans with rabies, and it is suspected that bats infect occasional terrestrial animals (Charlton *et al.* 1982; Rupprecht *et al.* 1987; Smith 1988), bats are not believed to play an important role in establishing or maintaining terrestrial endemics (Smith 1989).

There is a unique situation in Central America where blood-feeding vampire bats transmit rabies virus to large numbers of apparently ‘incidental’ cattle hosts (>10,000 cases in cattle per year) (Tordo *et al.* 1993). However there is no evidence for maintenance of vampire rabies in cattle by cow-cow transmission. Nor has it been suggested that cattle play a role as intermediate hosts in the maintenance of rabies among vampire bats.

“Rabies virus” has been reported in megachiroptera (fruit bats) in Thailand (Smith *et al.* 1967), and India (Pal *et al.* 1980), however no antigenic or molecular characterisation of these isolates has been done. Without this it remains unclear whether these are truly isolates of genotype 1 rabies virus or another rabies-like virus.

1.3 The rabies-like viruses

As a result of the recognition of rabies virus in bats in the Americas, surveys of African bats were conducted (Swanepoel and King 1992). Three rabies-related lyssaviruses, Lagos bat virus (serotype/genotype 2), Mokola virus (serotype/genotype 3) and Duvenhage virus (serotype/genotype 4) have been isolated in sub-Saharan Africa. There are only a few isolates of each virus, and cases in domestic animals or humans are rare. This suggests that these viruses circulate unnoticed in wildlife hosts with sporadic spill-over into other mammals.

European bat lyssavirus-1 (genotype 5), and European bat lyssavirus-2 (genotype 6) have been isolated in Western Europe, including countries that are considered free of classical (genotype 1) rabies.

Three other rhabdoviruses, Kotonkon, Obodhiang, and Rochambeau, which are more distantly related to rabies and the rabies-like viruses, were isolated during surveys of insects (Shope 1982). The Kotonkon virus and Obodhiang virus, previously considered lyssaviruses, are now listed as unassigned viruses as serological and molecular analysis has linked them with viruses of the genus *Ephemerovirus*. The assignment of Rochambeau virus as a lyssavirus remains to be confirmed (ICTV In press). These viruses are not considered further.

1.3.1 Lagos bat virus

Lagos bat virus was first isolated in 1956 from pool of six brains from Straw-coloured fruit-bats (*Eidolon helvum, Megachiroptera*) at Lagos Island, Nigeria (Boulger and Porterfield 1958). Encephalitis but no Negri bodies were seen in the brains of intracerebrally infected mice. The isolate was shown to be a bullet-shaped rhabdovirus morphologically indistinguishable from rabies virus that could be differentiated from rabies virus on the basis of complement fixation and mouse neutralization tests, leading to its serotype 2 classification (Shope *et al.* 1970). Subsequent antigenic typing and molecular analysis supported the distinction of Lagos bat virus from other lyssaviruses (Bourhy *et al.* 1993a; Fekadu *et al.* 1988b; Flamand *et al.* 1980; King *et al.* 1990; King 1993).

Lagos bat virus has been isolated from two other species of bat (a Dwarf Epauletted fruit-bat, *Micropterus* (*sic*, *Micropteropus ?*) *pusillus,* Central African Republic, and a *Nycteris gambiensis,* an insectivorous bat, Senegal) during virological surveys, and from Common Epauletted Fruit-bats (*Epomophorus wahlbergi*, South Africa) showing abnormal signs or found dead (Swanepoel *et al.* 1993). It has also been diagnosed as the cause of paralytic rabies-like disease in cats from South Africa and Zimbabwe (King and Crick 1988; Swanepoel and King 1992) and a dog in Ethiopia (Mebatsion *et al.* 1993). Routine diagnostic tests do not differentiate between rabies virus and the rabies-related viruses. The cat cases were recognized because the fluorescence with anti-rabies conjugate was uncharacteristically weak, the cats had been vaccinated against rabies, and the

absence of aggression was considered atypical of rabies. The dog case was recognized by concurrent use of a monoclonal antibody panel. Antigenic typing, neutralization tests and molecular analysis differentiated the dog isolate from rabies virus and other rabies-related viruses (Mebatsion *et al.* 1993). Details of the differentiation of the cat isolates do not appear to have been published.

While laboratory vaccination trials suggest that some rabies vaccines confer protection against Lagos bat virus (Fekadu *et al.* 1988b; Mebatsion *et al.* 1993), the cases in rabies-vaccinated cats clearly demonstrate that those particular rabies vaccines in those individuals did not confer cross-protection against Lagos bat virus. This raises veterinary and public health concerns. The distant antigenic relationship between the glycoproteins of rabies virus and Lagos bat viruses was also illustrated by the failure of many anti-rabies-glycoprotein monoclonal antibodies to cross-react with Lagos bat isolates (Fekadu *et al.* 1988b). Molecular techniques have further demonstrated this relatively distant relationship (Bourhy *et al.* 1993a).

Infection with Lagos bat virus appears to be rare but may be underestimated if bats are not being submitted for testing, and because routine tests do not differentiate infection by Lagos bat virus from other lyssavirus infections. Lagos bat virus is the only lyssavirus that has not been isolated from humans.

1.3.2 Mokola virus

Mokola virus was first isolated from three shrews (*Crocidura* sp.) in Mokola, Nigeria in 1968 (Kemp *et al.* 1972; Shope *et al.* 1970). The morphology and mode of replication of Mokola virus as seen with electronmicroscopy was indistinguishable from that of rabies virus and Lagos bat virus. Complement fixation and mouse neutralization tests demonstrated that Mokola was related to but distinctly different from rabies virus and Lagos bat virus, hence its serotype 3 classification. Antigenic typing of Mokola with monoclonal antibodies subsequently supported this distant relationship (Flamand *et al.* 1980; King 1993; Wiktor *et al.* 1980; Wiktor *et al.* 1984). Analysis of the derived nucleoprotein sequence confirmed that, of the lyssaviruses, Mokola was the most distantly-related to rabies virus (Bourhy *et al.* 1993a).

There have been two cases of Mokola virus infection reported in Nigerian children. Mokola virus was isolated from the cerebrospinal fluid (CSF) of the first which presented with fever and convulsions and made a full recovery (Kemp *et al.* 1972). However, for unspecified reasons, this isolate is no longer regarded as valid (Swanepoel and King 1992). A further isolate was recovered from the brain of the second child who died from poliomyelitis-like encephalitis (Familusi *et al.* 1972). The absence of an acute excitement phase appears to have lead to the unjustified conclusion that the human case of Mokola virus infection did not show symptoms of ‘classical rabies’.

Mokola virus was the cause of fatal rabies-like disease in six cats and a dog in Zimbabwe (Foggin 1982, 1983; Wiktor *et al.* 1984) and a cat in Ethiopia (Mebatsion *et al.* 1993). The diagnoses were confirmed by mouse neutralisation tests, antigenic typing with monoclonal antibodies, and molecular analysis. Mokola virus is the only lyssavirus not to have been isolated from bats.

Poor cross-neutralization in mouse serum neutralization tests and mouse vaccination trials suggested neither rabies vaccine nor antiserum would be protective (Fekadu *et al.* 1988b; Mebatsion *et al.* 1993; Shope *et al.* 1970; Wiktor *et al.* 1984). Moreover the Mokola-infected dog had been vaccinated with a potent inactivated rabies vaccine (Rabisin; Institut Merieux) six months previously (Foggin 1982). These observations are consistent with the low homology between antigenic sites in the glycoproteins of rabies and Mokola viruses demonstrated using molecular and antigenic techniques (Bourhy *et al.* 1993a; Wiktor *et al.* 1984). However, DNA-based immunization using a chimeric construct of the glycoproteins of Pasteur virus (genotype 1 rabies) and Mokola virus in a plasmid (with *in vivo* transcription and translation) produced virus-neutralizing antibodies against rabies, Lagos bat and Mokola viruses. This technology may produce an effective pan-lyssavirus vaccine.

There have been few other isolations of Mokola virus, including one from a myomorph rodent, *Lophuromys sikapusi* (Swanepoel *et al.* 1993). It is assumed the cats and dog were infected by shrews and/or rodents during an epizootic in these wild animals. It would appear that Mokola virus is a poorly recognized or rare cause of disease.

1.3.3 Duvenhage virus

In 1970, a further rabies-related virus was isolated from a man (after whom the virus was named) in South Africa who died from a furious form of rabies-like encephalitis approximately five weeks after being bitten on the lip by an insectivorous bat (Meredith *et al.* 1971). While the fluorescent antibody test (FAT) with anti-rabies antibodies was negative, Negri bodies were present in the brain of the patient and the brains of mice inoculated with material from the patients brain (Meredith *et al.* 1971). As had been the case for the other rabies-related viruses, it was shown that Duvenhage virus was related to, yet distinctly different from the other lyssaviruses and it was subsequently designated as serotype 4 (King 1993; Tignor *et al.* 1977). It has since been isolated from two insectivorous bats (*Miniopterus schreibersii* and *Nycteris thebaica*) but appears to be rare (Swanepoel and King 1992; Swanepoel *et al.* 1993).

1.3.4 European bat lyssaviruses (EBL-1 and EBL-2)

Between 1954 and 1984 there were rare reports of ‘rabies’ in insectivorous European bats (King and Crick 1988; King *et al.* 1990; Schneider and Cox 1994). Isolation in 1984 of ‘bat rabies’ from insectivorous bats in Denmark and a man bitten by a bat in Finland (Lumio *et al.* 1986; Mollgaard 1985) eventually led to the recognition of two discrete endemics of rabies-like viruses in European bats that are independent of the prevailing rabies endemic in European foxes.

The most common, EBL-1 is found principally in insectivorous European house bats (*Eptesicus serotinus*), with occasional cases in other insectivorous bats in northern and western coastal Europe. The other, EBL-2 is found only in the insectivorous *Myotis daubentonii* and *M. dasycneme* (Amengual *et al.* 1997; Bourhy *et al.* 1992).

Initially the virus in these bats was presumed to be bat rabies (i.e. serotype 1 rabies) like that recognised in insectivorous American bats (Lumio *et al.* 1986). Then early German and Danish isolates were shown to have such a close serological and antigenic relationship with Duvenhage virus that they were considered variants of serotype 4 Duvenhage virus (Fekadu *et al.* 1988b; Schneider and Cox 1994). It was at one stage proposed that the two European bat lyssaviruses be grouped together into a separate 5th serotype (King *et al.* 1990). However, subsequent antigenic and molecular characterisation of EBL-1 and EBL-2, Duvenhage, and the other lyssaviruses has shown that EBL-1 and EBL-2 are closely related yet discrete viruses that form two distinct genotypes within the lyssavirus genus, with EBL-1 being more closely related to Duvenhage virus than EBL-2, which is most closely related to rabies (Amengual *et al.* 1997; Bourhy *et al.* 1992; Bourhy *et al.* 1993a, b; Bourhy *et al.* 1995; King 1993; Kissi *et al.* 1995; Roine *et al.* 1988; Tordo *et al.* 1993).

The debate regarding classification was resolved by referring to the original lyssavirus serotypes 1 to 4 as genotypes 1 to 4, EBL-1 is referred to as genotype 5, and EBL-2 is referred to as genotype 6 (Amengual *et al.* 1997; Bourhy *et al.* 1993a; Bourhy *et al.* 1995; Kissi *et al.* 1995; Tordo *et al.* 1993). EBL-1 and EBL-2 were recognised as distinct species in the lyssavirus genus by the International Committee on Taxonomy of Viruses (ICTV 1995).

From the original molecular data Bourhy *et al.* (1993a) derived a definition of a (lyssavirus) genotype as a group of isolates which have a minimum nucleoprotein (N) amino acid similarity of 97.1% (maximum intra-genotype diversity of 2.9%) and have a maximum similarity of 93.3% with isolates in other genotypes (minimum inter-genotype difference of 6.7%, observed between Duvenhage virus and EBL-1). However, a subsequent investigation of the intrinsic molecular diversity of the nucleoprotein (N) among isolates of genotype 1 rabies, showed that there was as little as 92.2% amino acid homology among rabies virus isolates (maximum intra-genotype diversity of 7.8%) (Kissi *et al.* 1995). This indicates there is a greater N protein heterogeneity (7.8> 6.7%) within genotype 1 than between the two most closely related genotypes Duvenhage

and EBL-1, which suggests that Duvenhage and EBL-1 could be considered a single genotype/serotype as originally proposed. This appears to be acknowledged in the following:

> "Some of the lineages, containing only one or two isolates each, may not warrant consideration as distinct groups. However, the robustness of the bootstrap resampling and their phylogenetic distance to other clusters obtained by two independent phylogenetic methods, establishing them as distinct entities, should be emphasized (Kissi *et al.* 1995)."

The low level of N protein amino acid similarity between strains of EBL-2 and strains of rabies (similarity = 86-87%) is of the same order as that between Lagos bat and Mokola virus (87.1%), Duvenhage and Lagos bat viruses (85.8%), and rabies and Duvenhage viruses (87-88%). Differentiation of EBL-2 (genotype 6) from rabies virus (genotype 1) is as justified as the undisputed differentiation of rabies, Lagos bat, Mokola and Duvenhage viruses from each other.

The EBLs appear to be widespread in European bat populations, suggesting that they have been present and undetected for a considerable time (Amengual *et al.* 1997). The United Kingdom appeared to be free of bat rabies and European bat lyssavirus until a *M. daubentonii* at Newhaven was diagnosed with EBL-2 (Amengual *et al.* 1997; Anonymous 1996b; Whitby *et al.* 1996). This species of bat is not common in the United Kingdom and it has been suggested that this individual had 'blown in' from Europe.

Human infection with European bat lyssavirus is rare with only four reported cases. There have been two human cases of bat associated rabies-like disease in Russia (Selimov *et al.* 1989). In 1977, a 15 year old girl in Voroshilovograd developed hydrophobia and died 35 days after being bitten on the finger by a bat (Selimov *et al.* 1986). The virus of this case was isolated but does not appear to have been characterized further. In 1985 an 11 year old girl in Belgorod became ill 21 days after being bitten on the lip by a bat that flew away, and died 6 days later (Selimov *et al.* 1986). This isolate (Yuli virus) was characterized as EBL-1 (Amengual *et al.* 1997; Bourhy *et al.* 1992; Selimov *et al.* 1989).

European bat lyssavirus-2 was isolated in 1985 from a bat biologist in Finland who died of a rabies-like disease. He had a history of being bitten by bats on three occasions, the most recent occurring 51 days before he became ill involving a *M. daubentonii* that behaved oddly (Haltia *et al.* 1989; Lumio *et al.* 1986; Roine *et al.* 1988). EBL-2 was also isolated from a 55-year old male wildlife artist trained, licensed, and employed as a bat handler in Scotland that died in November 2002 after a 19 day rabies-like illness (Fooks *et al.* 2003; Nathwani *et al.* 2003). He had a 12 to 14 year history of handling bats and multiple bat bites, the last had occurred 40 days prior to clinical signs and involved a bat suspected but not confirmed to have also been a *M. daubentonii*.

There are discrepancies in the literature as to whether rabies vaccines protect against challenge with EBLs. Humans do occasionally get bitten by EBL-positive bats, but as yet there have been no cases of EBL in those who have received pre- or post-exposure rabies vaccination.

Transmission of European bat lyssaviruses to terrestrial animals appears to be rare. Without EBL-specific antigenic or molecular testing, infection of terrestrial animals would be diagnosed as genotype 1 rabies. A review of 501 French rabies cases found no evidence of EBL in other animals (Bourhy *et al.* 1992). There have since been four reported cases of EBL-1 virus in sheep and in a stone marten (Ronsholt 2002; World Health Organization 1998).

No European bats have been shown to have serotype 1 rabies virus, however an isolate from a *Vespertilo murinus* from Western Siberia (Asia, isolate Omsk 1150, ~1988) had an antigenic profile very similar to that of rabies virus from foxes from European USSR and Kazakhstan. The Omsk 1150 profile using the Wistar and CVL antibody panels was quite unlike that of the Yuli (EBL-1) isolate (King *et al.* 1990; Kuzmin and Botvinkin 1996). More definitive molecular phylogenetic typing of the Omsk 1150 isolate does not appear to have been published. This antigenic profile suggests that either genotype 1 rabies virus, genotype 7 ABLV, or a new genotype virus is present in insectivorous bats in Siberia.

1.3.5 Tentative lyssavirus species from Asian and Eastern European bats

The number of recognised lyssavirus genotypes / virus species may soon be expanded as a result of recent isolations of bat lyssaviruses from Central Asia and Eastern Europe. New genotypes have been proposed for each of four lyssavirus isolates from bats. They are:

- Aravan virus isolated from a Lesser Mouse-eared bat (*Myotis blythi*) from Aravan district, Kyrghyzstan in 1991, GenBank AY262023 (Arai *et al.* 2003; Kuzmin *et al.* 2003).
- Khujand virus isolated from a Whiskered bat (*Myotis mystacinus*) from Khujand, northern Tajikistan in 2001, GenBank AY262024 (Kuzmin *et al.* 2003).
- Irkut virus isolated from a Greater tube-nosed bat (*Murina leucogaster*) from Irkut, Eastern Siberia in 2002, GenBank AY333112 (Botvinkin *et al.* 2003), and
- West Caucasian bat virus isolated from a Common bent-winged bat (*Miniopterus schreibersi*) from the Western Caucasus Mountains in 2002, GenBank AY333113 (Botvinkin *et al.* 2003).

Phylogenetic analysis of the entire N gene sequence indicated that Khujand virus is most closely related to EBL-2 but separated by a distance longer than the distance between Duvenhage virus and EBL-1 (Botvinkin *et al.* 2003; Kuzmin *et al.* 2003). The position of Aravan virus changed depending on the phylogenetic method and whether nucleotide or amino acid sequence data was used, but it appears to be most closely related to EBL-1, EBL-2, and Duvenhage (Arai *et al.* 2003; Kuzmin *et al.* 2003). Phylogenetic analysis of limited N gene sequences (400 bases) of the Irkut and West Caucasian bat virus isolates placed Irkut virus closest to Duvenhage virus, and West Caucasian bat virus closest to Lagos bat and Mokola viruses with weak bootstrap support (Botvinkin *et al.* 2003). All four bat viruses have been listed as tentative virus species by the International Committee on Taxonomy of Viruses (ICTV In press).

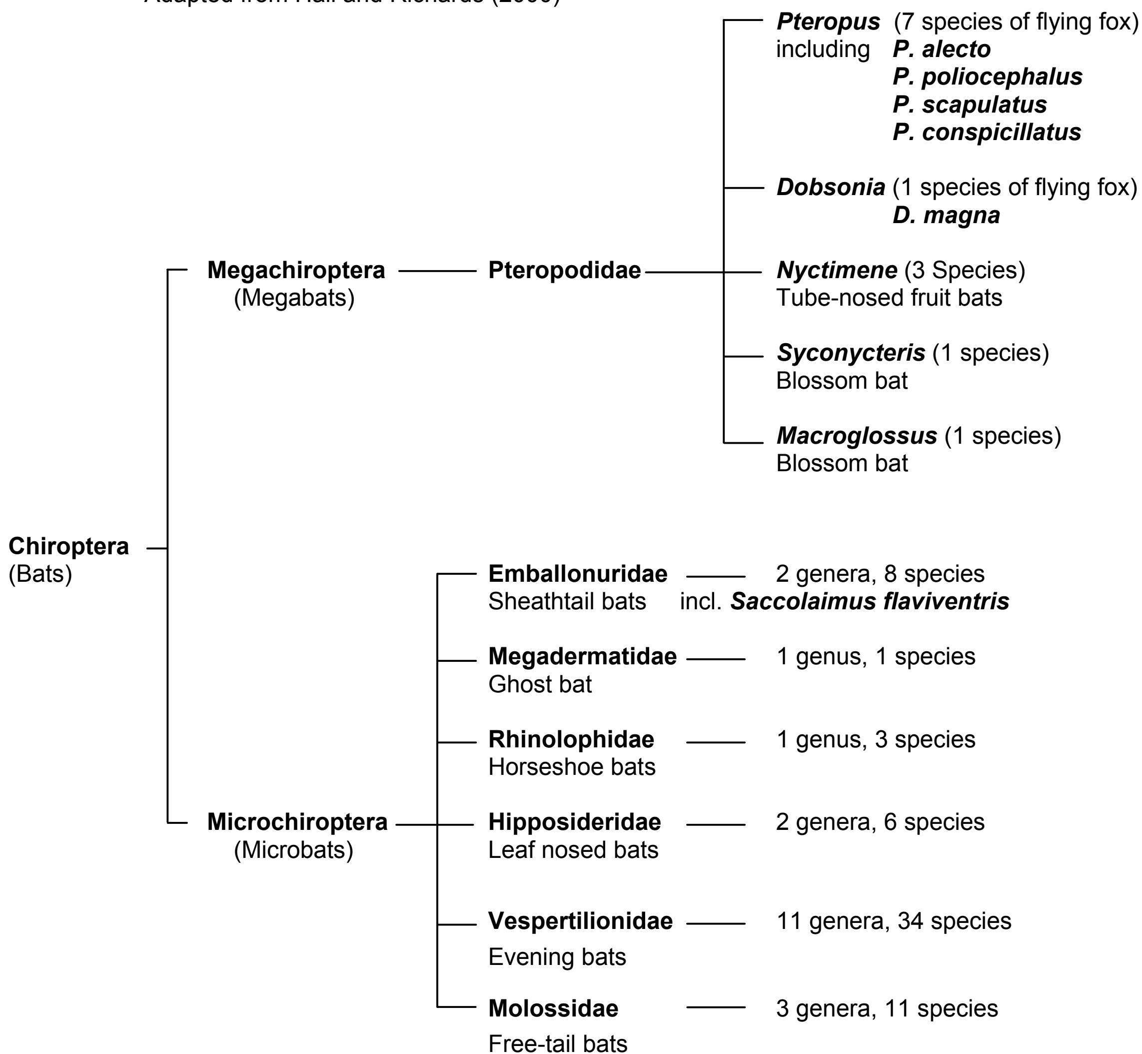
1.4 Australian bat lyssavirus

1.4.1 Bats of Australia

Australia has 13 Megachiropteran and 63 Microchiropteran bat species (Hall and Richards 1979; Richards 1998). A cladogram summarising the structure and relationships within the Order *Chiroptera* in Australia is shown in Figure 1-2.

Australian megabats include flying foxes (*Pteropus* spp and *Dobsonia* sp.), tube-nosed fruit-bats (*Nyctimene* spp) and blossom-bats (*Syconycteris* sp and *Macroglossus* sp.). While the flying foxes are also called fruit-bats, for clarity and the purposes of this thesis, they will be referred to by their more specific common name of flying foxes. Flying foxes of the family *Pteropodidae* are referred to as pteropid bats.

Figure 1-2 Bats of Australia
Adapted from Hall and Richards (2000)

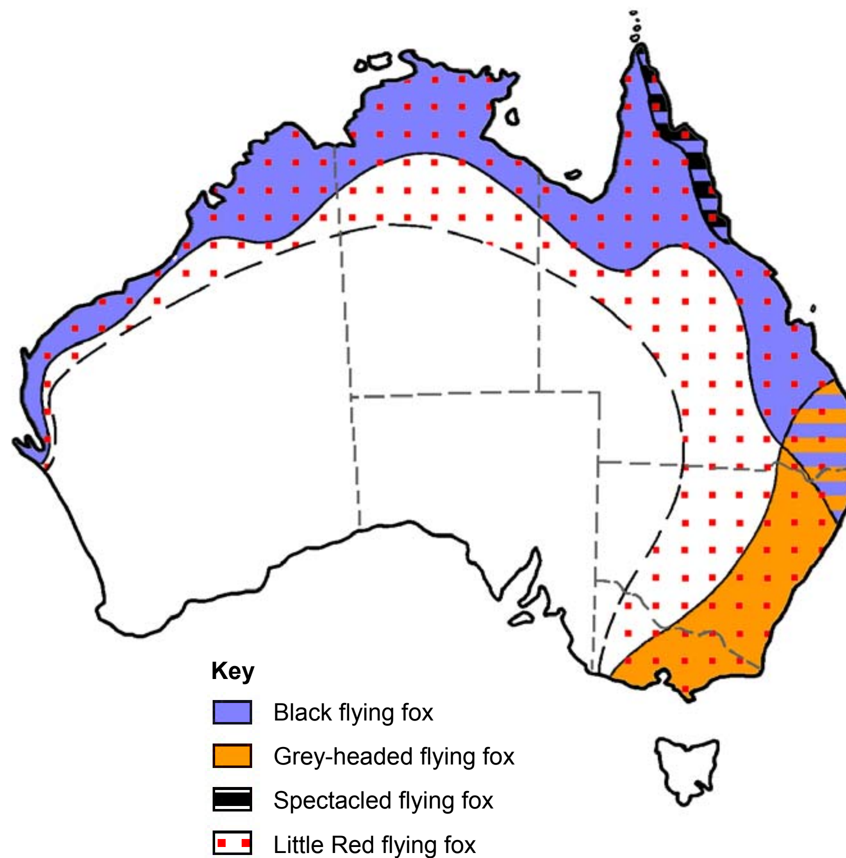


1.4.1.1 Flying foxes

The Black flying fox (*P. alecto*), Grey-headed flying fox (*P. poliocephalus*), Little Red flying fox (*P. scapulatus*), and the Spectacled flying fox (*P. conspicillatus*) inhabit the northern and eastern coasts of Australia. They are large, social bats (adults weigh 310 to 1,000 g), which roost in trees during the day. Camps can contain hundreds to hundreds of thousands of bats. Their nocturnal feeding on blossoms and fruit is important for the dispersal of seeds and pollen, and plays an important role in forest regeneration. Unfortunately, consumption of cultivated fruit crops when native food sources are scarce, and the noise, smell, and damage to trees associated with large camps have brought them into conflict with humans.

The distribution of these flying foxes is shown in Figure 1-3.

Figure 1-3 Distribution of the four common species of Australian flying fox
Adapted from Hall and Richards (2000)



Three other species of *Pteropus* and *Dobsonia magna* (Bare-backed flying fox, previously *D. moluccense*) are not considered further in this study as they are uncommon or absent from mainland Australia. *P. macrotis epularis* (Large-eared flying fox) and *P. banakrisi* (Torresian flying fox) are confined to the Torres Strait Islands, *P. brunneus* (Dusky flying fox) has not been reported since a single specimen was collected at Percy Island in 1874, and *D. magna* is only found north of Cooktown to Cape York (Hall and Richards 2000).

1.4.1.2 Microbats

Australia has over 63 species of microbats weighting 2.7 to 165 g that are broadly classified on the basis of tail morphology into three groups; freetail-bats, enclosed-tail-bats and sheathtail-bats. All are insectivorous and the largest, the Ghost bat (*Macroderma gigas*) is also carnivorous.

Particularly relevant is the Yellow-bellied sheathtail bat (YBST bat, *Saccolaimus flaviventris*, previously *Taphozous flaviventris*), a solitary, rarely encountered, and relatively large microbat (30 to 60 g) that feeds on insects and roosts in tree hollows of coastal and inland Australia. The contrasting colours of its jet-black dorsal fur and pale (white to yellow) belly fur readily differentiate it from other Australian bats. The general public has more frequent contact with other species of microbats that roost in colonies in houses, other buildings and/or caves.

1.4.2 Recognition of a lyssavirus in Australia

In 1996 flying foxes were the focus of research by virologists looking for the natural host of Hendra virus (previously equine morbillivirus). A five-month old female Black flying fox (*P. alecto*) found under a tree, unable to fly, at Ballina, New South Wales in May 1996, was found to have a severe nonsuppurative encephalitis (Fraser *et al.* 1996; Speare *et al.* 1997). While tests for Hendra virus were negative, immunoperoxidase staining of paraffin-embedded tissues with an anti-rabies virus monoclonal antibody (Clone HAM) was positive. Plasma from this flying fox was negative for rabies virus neutralizing antibodies. Virus was isolated from a weanling mouse inoculated with a homogenate of the flying fox kidney (Ballina isolate). The mouse developed hind limb paraplegia, had nonsuppurative encephalitis, and was positive for rabies virus antigens using the fluorescent antibody test (FAT) and immunoperoxidase staining (Fraser *et al.* 1996). Sequence analysis of polymerase chain reaction (PCR) products (size not specified) amplified by nested primers for lyssavirus nucleoprotein from tissue culture virus (presumably mouse derived) and formalin-fixed brain (presumably the Ballina flying fox) showed that they were identical. Electron-microscopic examination of second passage mouse brains revealed classic bullet-shaped rhabdoviruses. In an island continent proud of its rabies-free status, such a discovery had profound implications.

Retrospective examination of archival bat tissues identified a further two infected Black flying foxes which died in January and March 1995 (Fraser *et al.* 1996; Speare *et al.* 1997). The scarcity of appropriate material from flying foxes prior to 1995 limits investigations into how long the virus has been present in Australia.

In a flurry of bat testing, it was soon revealed that the new lyssavirus was present in all four common *Pteropus* species (Black, Grey-headed, Little Red and Spectacled flying foxes) and the insectivorous Yellow-bellied sheathtail bat (*S. flaviventris*). Cases were identified in New South Wales, Queensland, Victoria and the Northern Territory, a range broadly correlating to the

distribution of flying foxes (Hooper *et al.* 1997b). Unwell bats in Western Australia are yet to be tested. It appears that Australian bat lyssavirus is endemic in bat populations and there is little to suggest it is a new arrival.

Initially called pteropid lyssavirus (Fraser *et al.* 1996) it was renamed Australian bat lyssavirus when it was isolated from the non-pteropid Yellow-bellied sheathtail bat (Hooper *et al.* 1997b). The original name was also inappropriate as other lyssaviruses, reported as "rabies virus", had previously been isolated from a flying fox in India (Pal *et al.* 1980) and dog-faced fruit bats (*Cynopterus brachyotis*) in Thailand (Smith *et al.* 1967). Differentiation of these Asian viruses as genotype 1 rabies rather than ABLV requires antigenic and/or molecular phylogenetic analysis. The relationship of the Indian, Thai and Australian isolates is unclear due to the limited characterization of the Indian and Thai isolates. Interestingly, although the Indian bat was reported to be a *Pteropus poliocephalus*, this species is not present in India and it is likely to actually be a *Pteropus giganteus* (Les Hall *pers. comm.*).

The range of some Australian bats, including *P. alecto* (Black flying fox), *P. scapulatus* (Little Red flying fox) and *Dobsonia magna* (Bare-backed flying fox), extends north into Papua New Guinea. The current name, Australian bat lyssavirus (ABLV), has been criticized because this host distribution means it is likely that ABLV will be identified in Asia (St George 1997). In any case, there is a precedent for naming viruses after the place from which they were first recognized /characterized and the name Australian bat lyssavirus accurately reflects what is known of the current isolates.

1.4.3 Classification of Australian bat lyssavirus

1.4.3.1 Electron-microscopy and Serotyping[1]

The electron-microscopic appearance of the Ballina isolate was typical of the family *Rhabdoviridae* (Gould *et al.* 1998; Hooper *et al.* 1997b). Positive staining with the anti-rabies monoclonal HAM (Feiden *et al.* 1988), the pan-lyssavirus monoclonal W502-2 (Wiktor *et al.* 1980) and fluorescein-labelled Centocor ® antibodies indicated that the Ballina isolate was antigenically related to the lyssaviruses (Fraser *et al.* 1996).

In a modified rapid fluorescent focus inhibition test (RFFIT), isolates of Australian bat lyssavirus (ABLV) were neutralized by ERA-rabies antiserum. The neutralising titres of anti-CVS-11 rabies serum and anti-ABLV serum against ABLV were similar (Fraser *et al.* 1996). Commercial human and animal rabies vaccines conferred complete protection to mice challenged with Australian bat

[1] For the purposes of this thesis, the term serotyping will be restricted to the classification of viruses using polyclonal serum. A form of antigenic typing, seroyping is done principally, but not exclusively, by using serum neutralisation tests which differentiate between viruses on the basis of the presence or absence of common neutralising antigens. All the neutralizing antigens of the lyssaviruses are present on the glycoprotein.

lyssavirus, but none to those challenged with Mokola virus (Hooper *et al.* 1997b). These results suggest high antigenic, immunogenic and functional homology between the neutralizing G protein antigens of the fixed rabies virus and ABLV isolates. Using the old classification system, these results indicate that Australian bat lyssavirus is a serotype 1 lyssavirus. All previous serotype 1 lyssaviruses were called rabies virus.

1.4.3.2 Antigenic typing of Australian bat lyssavirus

Antigenic typing with a panel of anti-nucleocapsid (N) protein monoclonal antibodies showed that Australian bat lyssavirus was most closely related to 'classical' (genotype 1) rabies virus but was unique from previously described lyssavirus isolates (Fraser *et al.* 1996; Gould *et al.* 1998; Hooper *et al.* 1997b). While the antigenic profiles of ABLV isolates are indeed unique, analysis of the profiles indicates that the differences in comparison to rabies isolates are slight.

- The W502-2-positive, W422-5-negative profile of Australian bat lyssavirus is common to all tested strains of rabies virus (Charlton *et al.* 1982; Flamand *et al.* 1980; Smith 1989; Wiktor *et al.* 1980; Wiktor and Koprowski 1980; Wiktor *et al.* 1984). The negative reaction to W422-5 only differentiates Australian bat lyssavirus from Lagos, Mokola, and Duvenhage lyssaviruses (Wiktor *et al.* 1980).
- The reactivity of the monoclonal antibody HAM (Feiden *et al.* 1988) has not been extensively evaluated and is of limited use for virus characterization.
- Four isolates of Australian bat lyssavirus were shown to have variable (negative, weak, or positive) reactivity to monoclonal antibodies 3-1 and 24-10 (Hooper *et al.* 1997b). Consequently, these antibodies are not useful in differentiating ABLV from other serotype 1 (rabies) virus isolates.
- The Ballina isolate profile described by Gould *et al.* (1998) and Hooper *et al.* (1997b) includes positive reactions to 17 antibodies, and a negative reaction to 3-1 (synonym 62-3-1) that are common to 178 isolates of raccoon variant rabies virus (Smith 1989).
- The sole consistent antigenic difference between Australian bat lyssavirus (ABLV) and *all other* serotype 1 strains of rabies virus is the negative reaction with the monoclonal antibody 62-15-2 (apparent synonym C15-2) (Fraser *et al.* 1996; Gould *et al.* 1998; Hooper *et al.* 1997b). This antibody produced positive reactions to five strains of rabies virus when originally cloned (Smith *et al.* 1984a). However, its reactivity against larger numbers of rabies virus isolates has not been clearly included in subsequent papers (Fekadu *et al.* 1988b; Smith *et al.* 1984b; Smith *et al.* 1986; Smith 1989), possibly because its pan-rabies reactivity precluded its use for differentiation of rabies virus variants. It may have been one of six pan-rabies monoclonal antibodies used by Smith *et al.* (1984b) that were not specifically identified. It may be the pan-rabies antibody referred to as MAB-N 4 in Smith (1989), and may have been mislabelled as pan rabies antibody 15-3 in Fekadu *et al.* (1988b). Without clarifying the reactivity of other rabies virus strains to monoclonal antibody 62-15-2, the significance of the negative reaction to 62-15-2 by ABLV is unclear. The 62-15-2-negative profile of Australian

bat lyssavirus is consistent across five isolates from pteropid bats (Ballina/96-591, 95-2, 96-390, 96-0963, 96-648), two isolates from YBST bats (97-0747, 96-1293), and the human isolate of YBST-variant ABLV (96-1256) (Gould *et al.* 2002; Hooper *et al.* 1997b).

If is assumed that the 62-15-2-negative profile of Australian bat lyssavirus is characteristic of all ABLV isolates, then this unique antigenic profile differentiates Australian bat lyssavirus from all previously characterized variants of rabies. By definition, each antigenic variant of rabies virus has a unique antigenic profile. The antigenic homology of Australian bat lyssavirus to the ERA vaccine strain of rabies virus used to produce the 62-x-x ERA series of monoclonal antibodies is extremely close. If the ABLV reactivity to 62-3-1 is discounted due to its variability, only one of 19 ERA-strain epitopes (that for monoclonal antibody 62-15-2) is apparently absent on isolates of Australian bat lyssavirus. This single epitope difference is less than the two to seven differences demonstrated among 11 other rabies virus variants (Smith 1989), which suggests that Australian bat lyssavirus is even more closely related to ERA-rabies virus than are many other rabies virus variants. An absence of at least 10 ERA epitopes has been demonstrated for all other rabies-related viruses (Lagos bat, Mokola, Duvenhage, EBL-1 and EBL-2) (Fekadu *et al.* 1988b). As such this antigenic data suggests Australian bat lyssavirus is a unique variant of genotype 1 rabies virus and does not suggest it is a new lyssavirus genotype.

However, further ABLV antigenic data recently published by Gould *et al.* (2002) is inconsistent with that previously published by his laboratory colleagues. The antigenic profiles of five isolates in Gould *et al.* (2002), suggest ABLV is more distinct with between five and 11 negative results per isolate using the same panel. However, whereas Hooper *et al.* (1997b) reported positive responses by isolate 95-2 to nine monoclonal antibodies (3-1, 52-2, 64-4, 71-2, 97-3, 97-11, 141-1, and 146-3) and a weak response to antibody 24-10, their reactivity with isolate 95-2 is reported as negative in Gould *et al.* (2002). As the antigenic results for the same isolate are inconsistent, it is difficult to interpret the panel results for the other isolates.

1.4.3.3 Molecular analysis of Australian bat lyssavirus

Initial phylogenetic analysis of nucleotide and amino acid sequences of PCR product (size and sequence not indicated) derived from the nucleocapsid (N) protein gene showed that the Ballina isolate of ABLV was most closely related to European bat lyssavirus-1 (EBL-1) and street stains of classical rabies virus (Fraser *et al.* 1996). However the analysis was not shown and neither the method nor program used was clearly indicated.

The amino acid sequences of this limited and relatively conserved section portion of the N protein (amino acids 298 to 426 inclusive, that is 28% of N) showed 92 to 93% similarity with that of two isolates of EBL-1, French street fox rabies, and Pasteur vaccine rabies virus. Subsequent determination of the complete N protein sequence, including the more variable amino and carboxy termini, allowed recalculation of the similarity between the Ballina isolate and the other lyssaviruses. The similarity to Pasteur virus remained 92%, the similarity to other genotype

isolates (Lagos, Duvenhage, EBL-1 and EBL-2) using the same GenBank accessions used previously differed by 0 to 3%, ranging from most different (Lagos bat virus) 82%, to most similar (EBL-1) 90% (Gould *et al.* 2002).

Further sequencing of the Ballina isolate permitted analysis of the deduced nucleoprotein (N), phosphoprotein (P or M1), matrix protein (M or M2) and glycoprotein (G) amino acid sequences, confirming the significantly closer relationship of the Ballina isolate to rabies viruses compared to the other lyssaviruses (Gould *et al.* 1998; Hooper *et al.* 1997b). Phylogenetic analysis of N protein sequences indicated that ABLV formed a separate clade on the taxonomic tree relative to other rabies viruses but was closely related to rabies virus variants (Gould *et al.* 1998; Hooper *et al.* 1997b).

Analysis of nucleoprotein (N) gene sequences from 16 flying foxes showed low nucleotide sequence variation (0.5-2%) and no amino acid substitutions (Hooper *et al.* 1997b). Details of the isolates and the size of the 16 compared sequences (whole or partial N gene) were not indicated, but it is likely to be the same partial, highly conserved, N sequence region (nucleotides 443 to 892) as that published later for a larger number of isolates (Gould *et al.* 2002). In this conserved region at least, the 16 ABLV isolates were reported as having sufficiently high amino acid homology (100 ≥ 97.1%), and were sufficiently different from isolates of other lyssavirus genotypes (7 to 8 ≥ 6.7%) to satisfy Bourhy's original definition of a genotype. Use of this definition justifies classification of ABLV as a new lyssavirus genotype (genotype 7 within serotype 1) rather than as a new variant of genotype 1 rabies. However, the 92% homology between the deduced amino acid sequence for the N proteins of ABLV and isolates of genotype 1 rabies (Hooper *et al.* 1997b), is no less than that within genotype 1 (92.2%) (Kissi *et al.* 1995). It is also considerably higher than that between undisputedly distinct genotypes (< 89%) suggesting again that ABLV is simply a new variant of bat rabies virus (Bourhy *et al.* 1993a; Gould *et al.* 1998; Kissi *et al.* 1995). As acknowledged in Gould *et al.* (1998), the degree of difference and justifiability for separate genotype classification between Australian bat lyssavirus and its closest relative rabies virus, is only similar to that between EBL-1 and its closest relative Duvenhage virus.

Hooper *et al.* (1997b) indicated that the sequences for the nucleocapsid (N) and glycoprotein (G) proteins of two isolates from Yellow-bellied sheathtail bats differed from those from pteropid bats, but remained within the single 7th genotype. A more detailed report of phylogenetic analysis of partial N gene sequences (nucleotides 443 to 892) of isolates from three YBST bats and at least 29 flying foxes showed that the ABLV isolates separated into two clades, one comprised of isolates from flying foxes (pteropid-variant ABLV), the other of isolates from YBST bats (YBST variant ABLV), with no other temporal or geographic distinctions (Gould *et al.* 2002). More comprehensive sequencing of one isolate from a YBST bat (AF081020) allowed comparison of the entire YBST variant N coding sequence with that of the Ballina isolate, indicating an 85% similarly at the nucleotide level and 96% similarly at the amino acid level (Gould *et al.* 2002). The origins of this YBST sequence were erroneously published in Figure 3 of that paper as being from

case 96-1256. While 96-1256 is actually YBST variant ABLV from the brain of a human case (see below), the published sequence AF081020 is derived from case 96-1293, a YBST bat found on the ground at Murphy's Creek, Queensland in November 1996, being the first recognised case of ABLV in a YBST bat (Allen Gould, AAHL *pers. comm.*) It has been suggested that genetic divergence between the viruses from flying foxes and the Yellow-bellied sheathtail bats indicates that Australian bat lyssavirus has been circulating in these populations for sufficient time to allow specific adaptation to each suborder of bat (Gleeson 1997).

ABLV has been formally recognised as a discrete virus species by the International Committee on Taxonomy of Viruses (ICTV 2000).

1.4.4 Two human cases of Australian bat lyssavirus infection

1.4.4.1 Rockhampton 1996

In October 1996, a woman wildlife carer in Rockhampton became ill and died from a 'paralytic' rabies-like progressive central nervous system disease (Allworth *et al.* 1996; Samaratunga *et al.* 1998). As a wildlife carer she had had close contact with a number of marsupials, cockatoos, dogs and cats. For the 2 to 4 weeks prior to her illness she had been caring for a number of flying foxes from which she had sustained scratches, but there was no history of a flying fox bite. While the first detailed report attributed the source of the infection to flying foxes (fruit bats) (Samaratunga *et al.* 1998), the virus was later sequenced and shown to be the variant identified only in Yellow-bellied sheathtail bats (*S. flaviventris*) (Gould *et al.* 2002). The patient was bitten severely on the left hand by a sick insectivorous bat either 4½ (Hanna *et al.* 2000) or 6 weeks (Speare *et al.* 1997) before her illness. The bat was aggressive and agitated and because of the bite and intractable behaviour, it was released onto a tree in a nearby park. The bite wound was painful and the woman sought medical attention shortly after the incident (Helen Luckoff ONARR, recollections of interview with patient's husband).

The woman re-presented with pain and numbness of her left arm. For 2 to 3 days she had non-specific signs of fever, headaches, dizziness and vomiting. Despite symptomatic treatment she developed diplopia, difficulty in swallowing, bulbar palsy, muscle palsies, progressive limb paresis and was eventually areflexic and unresponsive with electroencephalographic signs of diffuse encephalitis (Allworth *et al.* 1996; Samaratunga *et al.* 1998). The woman died after an illness of approximately 20 days despite the administration of rabies immunoglobulin in the late stage of her disease. At post-mortem examination she was found to have meningoencephalomyelitis with neuronal eosinophilic inclusion bodies and chronic sialadenitis with eosinophilic cytoplasmic inclusion in occasional salivary acinar cells, but no myocarditis (Samaratunga *et al.* 1998).

Anti-lyssavirus group antibodies were identified in serum (collected before rabies-immunoglobulin treatment) and ABLV RNA was amplified from cerebrospinal fluid using ABLV-specific primers.

Acute phase serology for a number of other viruses, including Hendra virus was negative. It was an alarming coincidence that a person died from ABLV-infection barely 16 weeks after the virus was first recognized in bats in Australia.

There was immediate concern for bat handlers/carers, veterinarians, and others who came into contact with bats. A task force of infectious disease experts drew up protocols for managing further lyssavirus exposure (Anonymous 1996a). State health authorities produced vaccination protocols for pre-and post-exposure situations (NHMRC 1997), and advice for veterinarians was published (NHMRC 1997). Not all who received appropriate vaccination developed or maintained adequate titres of anti-rabies antibodies (Crome *et al.* 1998).

1.4.4.2 Mackay 1998

A second human case of ABLV infection caused the death of a 37-year old woman from Mackay, Queensland in December 1998. She had presented with a 5 day history of fever, vomiting, anorexia, left shoulder pain, paraesthesia of the dorsum of her left hand, a sore throat and difficulty in swallowing (Hanna *et al.* 2000). When initially examined she was well oriented, but febrile, drooling saliva and unable to fully open her mouth or speak easily. Within 12 hours she was agitated, had dysphagia and dystonia with frequent and severe muscle spasms and required paralysation and ventilation. Unless controlled by muscle relaxants, she developed facial grimacing, rolling eye movements, and arching of the back. There were also marked fluctuations in body temperature and blood pressure. On day 14 of hospitalization, 19 days after the onset of her illness, she died following withdrawal of ventilator support.

The diagnosis of ABLV infection was made following detection of ABLV RNA in saliva, brain, salivary glands, and adrenal glands by hemi-nested-PCR (Heaton *et al.* 1999), intense staining of fresh brain impression smears by fluorescent antibody staining, and virus isolation in cell culture from brain and spinal cord (Hanna *et al.* 2000). Serum and CSF were negative for antibodies to rabies and other encephalitic viruses. Subsequent sequencing of the isolate indicated it was of the variant characteristic of flying foxes (Warrilow *et al.* 2002).

As in the first human case, there was extensive and severe encephalitis and some inclusion bodies in the hypothalamus. Unlike the first case, there was a diffuse pancarditis and no inclusion bodies in the parotid or submandibular salivary glands (Hanna *et al.* 2000).

The only history of contact with bats had occurred 27 months before the onset of illness. At an evening barbecue in late August 1996, a flying fox had suddenly landed on the back of a young child and was removed by the patient who sustained a bite at the base of her fifth left finger. The patient had sought medical treatment 2 days after the attack and received tetanus toxoid and antibiotics. Unfortunately, ABLV had only first been recognized in bats in May 1996 and in August 1996 the potential zoonotic risk of a rabies-like disease from bats was not appreciated. However, in early March 1997, some months after the death and media attention of the first human case,

she had represented requesting a blood test for the “bat virus”. She was advised that post-exposure vaccination was available, but did not return to receive the vaccine that was ordered and sent to her doctor, apparently due a misunderstanding that she was required to travel 300 km to Townsville to receive it (Roberts and AAP 1998).

It has been stated that had she received the vaccination in March 1997, 6 to 7 months after exposure, her fatal illness may not have been prevented due to the long interval between exposure and vaccination and because guidelines at the time would have led to her receiving rabies vaccine only and not rabies immunoglobulin (Hanna *et al.* 2000). Clinical rabies has been reported in humans where post-exposure vaccination was delayed or did not include rabies immunoglobulin (Wilde *et al.* 1989). However, where post-exposure vaccination for rabies has failed, this has been in cases where the incubation period was as short as 19 to 25 days and where the delay has resulted in the vaccination being initiated only 14 to 19 days before the onset of clinical signs (Wilde *et al.* 1989). It is likely that the problem with delayed vaccinations and not providing passive immunity with rabies immunoglobulin is not so much the elongated exposure to vaccination interval, but rather the reduction in the interval between vaccination and clinical onset, depriving the patient of sufficient time to respond to the vaccine before infection was irreversibly established. In this case, while the delay to the opportunity to be vaccinated was long (6-7 months), the vaccination would still have been received 19 to 20 months prior to clinical onset, ample time for completion of the vaccination schedule, a typical antibody response and subclinical resolution of the at that time latent infection.

There is also the possibility that the disease was not the result of exposure in August 1996, but of some other, more recent, less conspicuous bat/virus contact, that may have occurred after the request for vaccination in March 1997. Within 12 hours of being admitted the patient’s capacity to contribute to an accurate history was greatly diminished, and the details of the August exposure and history of absence of more recent exposure were provided only by the patient’s family. Certainly the aberrant behaviour of the flying fox landing on the child is highly suggestive that the bat was ABLV-positive, but it is not conclusive. The long incubation period the August 1996 exposure would imply (27 months) is atypical of lyssaviruses. Only 1% to 6.8% of human rabies cases occur more than a year after the purported exposure (Hattwick 1974; Wang 1956; Wilde *et al.* 1989). However, histories suggesting similarly long incubation periods for human rabies infections have been reported (Grattan-Smith *et al.* 1992; Smith *et al.* 1991).

It does seem remarkable that the only two recognized cases of ABLV in humans should both result from contacts with bats in August-September 1996, just 3 to 4 months after the virus was first recognized in bats. Also that the first should be the result of contact with a species of bat with which humans have comparably little contact (the YBST bat), and that the other should become ill after a remarkably long and uncharacteristic incubation period. It is particularly poignant that both patients had sought appropriate medical attention as a result of the bites, the second on two occasions, yet neither received potentially life saving vaccination in the pre-clinical period. It is

perhaps of great significance that both bats to which the infections are attributed, bit people while displaying overtly aggressive and uncharacteristic behaviour.

1.4.5 History of human rabies in Australia

Since the differences between Australian bat lyssavirus and rabies virus are very subtle, and standard diagnostic tests do not differentiate between them, an obvious question was whether or not Australians diagnosed with rabies prior to the recognition of ABLV had actually been infected with ABLV.

There have been three recorded outbreaks/incidents of rabies in Australia. In 1866-7, four or five dogs, a pig, and a child bitten by one of the dogs were provisionally diagnosed with classical rabies in Tasmania on the basis of clinical and epidemiological observations (Pullar and McIntosh 1954). Unfortunately, as there are no pathological samples from the outbreak, definitive diagnosis and differentiation from ABLV is not possible. However, while there are six species of microbats in Tasmania, the typical urban rabies presentation combined with the fact that there are no flying foxes or Yellow-bellied sheathtail bats in Tasmania (Hall and Richards 1979; Richards 1998) make it extremely unlikely this outbreak involved ABLV. As there were poor quarantine restrictions at the time, it is assumed a ship introduced a dog incubating the disease.

In 1987 a boy in Queensland died of rabies nine months after returning from eight months travelling in countries in which rabies is endemic (Anonymous 1988; Faoagali *et al.* 1988). The diagnosis was based on; a dramatic rise in rabies virus antibody titre between days 1 and 19 of his illness, nonsuppurative meningoencephalitis with cytoplasmic eosinophilic inclusion bodies in neurons, and the demonstration by electron microscope of bullet-shaped viral particles in the brain. The virus does not appear to have been isolated. This level of virus characterization is consistent with both rabies virus and ABLV. There are grounds to suspect this infection was due to ABLV:

- ♦ The long (9-17 month) incubation period required for infection outside Australia is atypical of (but not inconsistent with) dog or sylvatic rabies. In Taiwan and Thailand, only 6.8% and 6.45% of 707 and 235 human rabies cases respectively, occurred after an incubation period of more than a year (Wang 1956; Wilde *et al.* 1989).
- ♦ Initial inquires uncovered no history of a bite during his travels. A recollection that the boy received a minor bite injury from a monkey was only revealed when relatives were re-interviewed after the boy’s death and so could not be confirmed by the patient.
- ♦ As ABLV had not then been recognized, it is likely that inquiries would have focused on animal contacts outside Australia rather than recent bat contact at home, and the significance of any reported bat exposure would not have been realized.

Differentiation would require determination of the antigenic profile (notably reactivity to monoclonal antibody 62-15-2), and/or molecular analysis using ABLV-specific primers and/or sequence analysis. Such a review of this case has not been published.

The most obvious candidate for an earlier case of ABLV infection is a 10 year old Vietnamese girl who developed clinical rabies after living continuously in Australia for 5 years (Bek *et al.* 1992; Grattan-Smith *et al.* 1992; McColl *et al.* 1993). At the time, sequence analysis of polymerase chain reaction (PCR) products from the patient's brain indicated that the highest homology (92.5-93%) was to isolates of dog rabies virus from Southeast Asia suggesting exposure prior to immigration to Australia. This is a similar degree of homology to that demonstrated between ABLV and Pasteur vaccine rabies virus (Fraser *et al.* 1996). However, the sequence data has been reviewed and does not suggest this was a case of ABLV (Ken McColl, AAHL *pers. comm.*). Other cases of apparently long incubation rabies in humans have been reported in patients that died 11 months, 4 years or 6 years after leaving rabies endemic areas (Smith *et al.* 1991). Antigenic and sequence characterization of the rabies isolates of these cases strongly suggested that they had been exposed in Asia or Mexico prior to immigrating to the USA.

There was also concern that human cases of ABLV had gone undiagnosed. A review of case records at the Gold Coast Hospital, Queensland from 1980-1996, identified 21 patients who died with encephalitis or meningitis. It was concluded that none had a clinical picture consistent with viral encephalitis (Gerrard 1997). However, it remains possible that some of these cases were infected with ABLV as there is no reference to brain sections being re-examined or to specific testing for lyssaviruses. It has been shown that typical histological lesions may be absent in ABLV-infected bats (Hooper *et al.* 1999a) and that evidence of an alternative aetiology may be present (Skerratt *et al.* 1998; Speare *et al.* 1997). Despite this, it is generally considered unlikely that there have been unrecognized human cases of ABLV infection. This suggests that either infection in bats is uncommon, or the virus is not readily transmitted to humans, or human infection is not usually fatal, and/or the virus is new in the bat and human populations (Gerrard 1997).

1.5 The pathology of lyssavirus infection

The pathology of lyssaviruses has largely been studied using rabies virus as the type virus for the genus.

1.5.1 Gross pathology

Despite the dramatic clinical course of lyssavirus-induced disease, there is a noticeable absence of gross lesions such that gross post-mortem examination rarely even suggests the diagnosis.

Most infections occur as the result of a bite or scratch from an infected animal; however these traumatic lesions heal unremarkably during the incubation period. Approximately 50% of humans cases reported pain, burning, numbness, itching or tingling at the site exposure during the prodromal stage of the disease (Hemachudha 1994; Mrak and Young 1994), but no characteristic gross or histological lesion has been identified at these sites.

Occasionally the meninges are congested. Cerebral oedema is rare in rabies (Fekadu *et al.* 1982a). Reduced weight gain, failure to thrive, wasting, and lymphoid depletion have been reported in experimentally infected mice, rats, rabbits, cats and cows (Torres-Anjel *et al.* 1988) but are noticeably non-specific indicators of the aetiology.

1.5.2 Histopathology

The most consistent histopathological changes produced by lyssavirus infection occur in the central nervous system and ganglia and are similar in humans and animals.

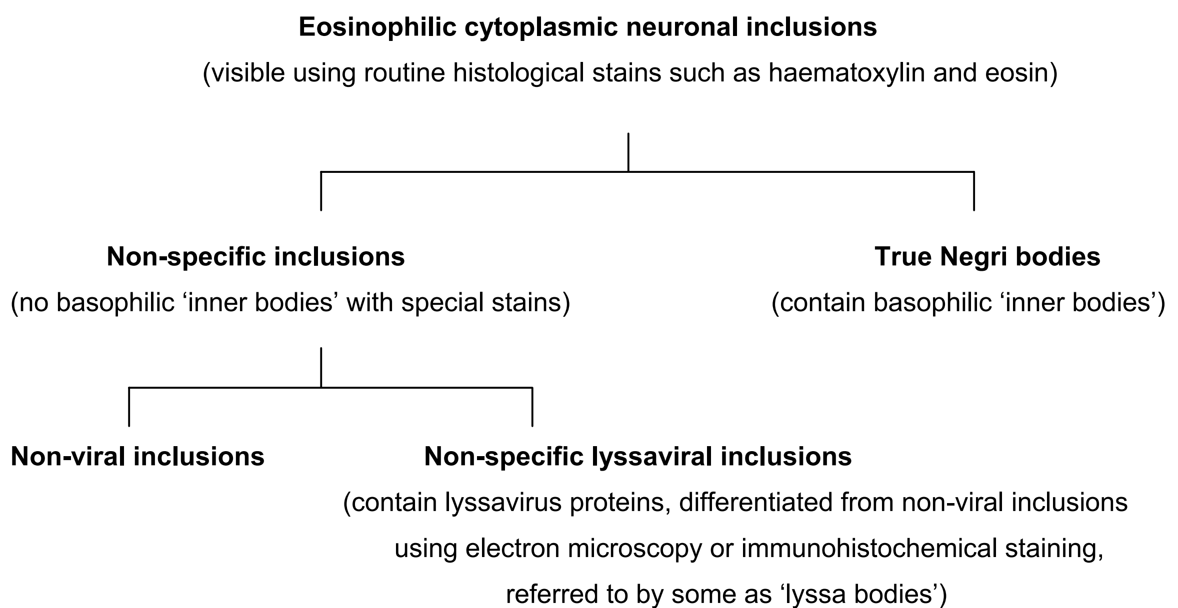
1.5.2.1 Negri bodies

The pathognomic histological lesion in rabies is the presence of Negri bodies, which are characteristic eosinophilic cytoplasmic inclusions within neurons. Adelchi Negri's recognition of these bodies in 1903 using Mann's stain (based on eosin and methylene blue) demonstrated an interaction between the aetiological agent and neurons (Negri 1903). He noted that they were particularly prominent in the hippocampal neurons of Ammon's horn and their detection at this site formed a basis for rabies diagnosis. Negri believed these inclusions were a specific parasite like the then recently recognised *Plasmodium* of malaria (Kristensson *et al.* 1996). The nature of Negri bodies was hotly disputed until electron microscopy showed that they contained granular or filamentous matrix of viral proteins (Miyamoto and Matsumoto 1965).

It should be noted that Negri bodies are not the only eosinophilic cytoplasmic inclusions of neurons. Non-rabies inclusions have been observed in the neurons of a number of normal

animals including cats, sheep, elk (Feiden *et al.* 1988), in human senescence and degenerative diseases such as myotonic dystrophy, but are relatively rare. Specific identification of inclusions as Negri bodies requires the use of specific stains (such as Mann's or Seller's stains) with which Negri bodies appear as magenta-coloured inclusions containing basophilic 'inner bodies'. Not all the aggregates of viral proteins in lyssavirus infection are large enough to be seen using routine histological stains, and only a proportion of those which are, have the characteristic 'inner bodies' of true Negri bodies. The relationship between Negri bodies and other eosinophilic inclusions in neurons is illustrated in Figure 1-4.

Figure 1-4 Relationships of terms used to describe Negri bodies and other intracytoplasmic eosinophilic inclusions in neurons



Electron-microscopy suggests that the basophilic 'inner bodies' of Negri bodies consist of whole or partial virions and cellular organelles such as basophilic ribosomes 'trapped' within the eosinophilic aggregate of filamentous viral nucleocapsid proteins (Miyamoto and Matsumoto 1965, 1967; Murphy *et al.* 1973a). Negri bodies have also been reported in cells of the adrenal medulla and the cornea (Houff *et al.* 1979).

The role of Negri bodies in lyssavirus infections remains obscure. While evident in most (approximately 75%) of infections with 'street' rabies, they are rare in infection with 'fixed' rabies. Their characteristic appearance may be the result of the accumulation of aberrant, non-infectious viral proteins.

Inclusion bodies were not observed in the original cases of Lagos bat virus (Boulger and Porterfield 1958), however typical viral inclusions too small to be see by light microscopy were

revealed by electron microscopy (Shope *et al.* 1970), and inclusions were seen in experimentally infected hamsters (Murphy *et al.* 1973b).

In the report of the original isolation in mice of Mokola virus (from shrews), the awkward statement that “Inclusion bodies compatible with Negri bodies were not observed” leaves open the possibility that inclusions may have been present, which for unspecified reasons did not fulfil the criteria for Negri bodies (Shope *et al.* 1970). Inclusions which were “quite different in size and appearance from the Negri bodies of rabies” were seen in the fatal human case of Mokola virus (Familusi *et al.* 1972). However, rabies Negri bodies are known to be very variable in size and shape, so this description is insufficient justification for the subsequent suggestion that inclusion body morphology could be used to differentiate between rabies virus and Mokola virus infections. No inclusions or Negri bodies were seen in the brains of shrews inoculated with Mokola virus (Kemp *et al.* 1973). Only “poorly defined, irregular material interspersed in the cytoplasm of degenerate neurons” and no Negri bodies were seen in the neurons of dogs inoculated with Lagos bat and Mokola virus (Percy *et al.* 1973). Negri bodies were seen with Seller’s stain in the central nervous system of monkeys inoculated with Mokola virus, and it is unclear from the text whether they were also present in monkeys inoculated with Lagos bat virus (Percy *et al.* 1973).

Negri bodies were “clearly visible” using “special staining” in the human case of Duvenhage virus infection (Meredith *et al.* 1971).

While many eosinophilic inclusion were seen in one of the first two bats reported with ABLV (Fraser *et al.* 1996), they were rare or absent in a further 19 naturally occurring cases (Hooper *et al.* 1999a).

The presence or absence of non-specific intracytoplasmic inclusion bodies or Negri bodies in neurons can not be used to differentiate between infections with rabies virus and the other lyssaviruses. The importance of identifying and characterizing Negri bodies or other inclusions for the diagnosis of rabies virus and other lyssavirus infections has been entirely superseded by the use of monoclonal antibodies and molecular techniques.

1.5.2.2 Other nervous system lesions

Cytopathology and inflammatory lesions are generally more prominent in animals inoculated with fixed-rabies virus, Lagos bat and Mokola viruses, than those infected with street-rabies virus (Murphy 1977; Percy *et al.* 1973).

The dominant lesion of lyssavirus infections is non-specific nonsuppurative viral encephalitis, meningitis, and/or ganglioneuritis. No one lesion is consistently present and the severity of inflammatory and/or degenerative lesions is highly variable. Indeed, death may occur when virtually no histological lesion is detectable. The range of lesions seen is similar in dogs, other principle host species, humans, and other dead end hosts. Generally ‘fixed’ strains of rabies virus

and passaged rabies-like viruses produce more severe inflammatory lesions with fewer (or no) Negri bodies than does 'street' rabies virus, however there is considerable individual variation (Murphy 1977; Percy *et al.* 1973; Smart and Charlton 1992; Tignor *et al.* 1977). When inflammatory lesions occur, they may be widespread (Fekadu *et al.* 1982a), but are usually most severe in the brainstem. In any one case none, any, or all of the following lesions may be present (Baer *et al.* 1982; Bundza and Charlton 1988; Charlton and Casey 1979a; Charlton 1984; Charlton *et al.* 1987b; Charlton 1988; Fekadu *et al.* 1982a; Mrak and Young 1994; Percy *et al.* 1973).

- Perivascular cuffing with mononuclear inflammatory cells, predominantly lymphocytes, plasma cells, and some macrophages
- Focal gliosis
- Nonsuppurative meningitis
- Neuronal degeneration with increased eosinophilia, chromatolysis, nuclear pyknosis, or spongiform change and neuronal necrosis
- Babes' nodules – clusters of neuronophagic cells (microglia and macrophages) ingesting necrotic neurons, seen principally in the ganglia
- Demyelination of spinal cord
- Peripheral neuritis, with infiltration of lymphocytes and plasma cells, and Wallerian degeneration, for example of optic nerve.
- Spongiform change, particularly of grey matter neuropil.

Most neurons infected with street rabies (those containing antigen) are morphologically normal when examined by microscopy. Neuronal degeneration may be more common in some species (for example cattle and foxes), and is generally more common after prolonged clinical signs (Charlton 1988). Massive necrosis was reported in the brain of a man bitten by a bat who died after five weeks of vigorous medical therapy (Dolman and Charlton 1987). Such a prolonged clinical course and histological outcome is rare in animals.

Early reports of ABLV lesions were consistent with those of rabies virus, and it was noted that infected bats may have mild or no histological signs of encephalitis (Fraser *et al.* 1996; Speare *et al.* 1997), and may indeed have evidence of other causes of neurological disease, notably lead poisoning (Skerratt *et al.* 1998; Speare *et al.* 1997). Clearly, neither the absence of encephalitis nor the presence of lesions that suggest an alternative diagnosis excludes a diagnosis of ABLV infection.

A detailed report of the lesions associated with ABLV in 21 naturally infected bats demonstrated how very similar the distribution and severity of lesions are to those of rabies virus, including that there is considerable variation between individuals. Lesions included neuronal necrosis and neuronophagia, cytoplasmic vacuolation, eosinophilic inclusion bodies consistent with Negri bodies, gliosis, lymphocytic perivascular cuffs, and subarachnoid meningitis, which were most frequent and severe in the brain stem (Hooper *et al.* 1999a).

1.5.2.3 Beyond the nervous system

Lyssavirus lesions outside the central nervous system are generally non-specific and have not been intensively investigated. Myocarditis has been reported in cases of human rabies (Araujo *et al.* 1971; Metze and Feiden 1991; Ross and Armentrout 1962). Mild to moderate sialoadenitis with infiltration of mononuclear cells and scattered foci of acinar cell necrosis has been reported in skunks and foxes experimentally infected with rabies virus (Balachandran and Charlton 1994). Adrenal medullitis with heavy accumulations of mononuclear cells has been recorded in humans, skunks and foxes (Almeida *et al.* 1986; Balachandran and Charlton 1994). Ocular pathology including lymphocyte and plasma cell infiltration of the ciliary body, choroid, and retinal nerve fibre layer and loss of ganglion cells was reported in the human case of EBL-2 infection (Haltia *et al.* 1989). Similar ocular lesions were reported in a dog inoculated with Mokola virus and monkeys inoculated with Lagos bat or Mokola virus (Percy *et al.* 1973). The involvement of the adrenal gland and eye may reflect the close embryological and functional relationships of these organs to the central nervous system (Almeida *et al.* 1986). While viral antigen can be demonstrated in a wide range of other tissues including the cornea and skin, no histological lesions have been recorded in these tissues.

No extensive examination of non-neural tissues of bats naturally infected with ABLV has been published. Lymphocytes were present in the aortic body of a Little Red flying fox, and in two of eight salivary glands (Hooper *et al.* 1999a).

1.6 The pathogenesis of lyssavirus infection

Most of the considerable body of work on the pathogenesis of lyssavirus infections has been done using strains of terrestrial rabies virus. However, there remain significant gaps in our understanding of host-lyssavirus interactions and the subsequent development of disease.

Much of the work on lyssaviruses has used fixed strains of rabies virus or variants of street rabies virus in mice or other laboratory animals. The recognition of species-specific variants of lyssaviruses suggests that the natural pathogenesis of lyssaviruses involves unique interactions between the host species and virus variant that at this time are not understood. It had been presumed that the findings from rabies disease models involving laboratory animals and ‘mismatched’ virus variants/host pairs applied to all susceptible species. However, differences in experimental design, host species, virus variant/strain, and route of inoculation have produced considerable differences in incidence, incubation period, duration, and form of clinical disease. Identification of subtle aspects of the pathogenesis and mechanisms for maintaining infection in particular ecological niches is likely to require modelling in natural hosts with appropriate street virus variants. The largest body of such work appeared to have been done at the Animal Diseases Research Institute in Canada with skunks (*Mephitis mephitis*) and inocula derived from naturally

infected skunk salivary glands by K. Charlton and co-workers (Balachandran and Charlton 1994; Charlton and Casey 1979a, 1981; Charlton *et al.* 1983, 1984a, 1987a; Charlton *et al.* 1987b; Charlton 1994; Charlton *et al.* 1996; Charlton *et al.* 1997; Smart and Charlton 1992). Unfortunately molecular studies of rabies isolates from naturally infected Canadian skunks have since shown Canadian skunk rabies is actually derived from Arctic fox variant rabies that is believed to have reached south-eastern Canada via Canadian Arctic foxes in the north and is very closely related to isolates from Arctic foxes in Greenland and Russia. While the variant has persisted in red foxes and skunks in the area, it is clearly not a skunk-specific variant. Indeed it is possible that only the Red fox, a close relative of the Arctic fox, is the new principal host for the Arctic fox variant rabies and that large numbers of skunks are only affected as ‘incidental’ dead end hosts, much as cattle are in areas with endemic vampire bat rabies. That skunks are frequent incidental rather than maintenance hosts in the eastern United States, where raccoon variant rabies virus is enzootic, is suggested by spatio-temporal analysis of rabies surveillance data (Guerra *et al.* 2003).

Inoculation of rabies virus and rabies-like viruses (Lagos bat and Mokola viruses) in comparative studies with experimental animals produced remarkably similar results (Murphy *et al.* 1973a; Murphy *et al.* 1973b). The clinical signs, gross and histological lesions, and virus distribution in dogs and monkeys inoculated with Lagos bat or Mokola viruses were similar to those described for rabies encephalitis in these species (Percy *et al.* 1973; Tignor *et al.* 1973).

There are few reports of experimental work with bat lyssaviruses in bats. Most of the work using bat variant rabies and bat host species was done using vampire bats in the 1930’s (Pawan 1936) or insectivorous bats around the 1960’s (Baer and Bales 1967; Constantine 1966a, b; Constantine and Woodall 1966; Constantine *et al.* 1968a; Constantine *et al.* 1968b; Stamm *et al.* 1956; Sulkin *et al.* 1960). Related studies used bat-variant rabies in non-bat species (Constantine 1966a, b, c; Constantine and Woodall 1966; Constantine *et al.* 1968a; Constantine *et al.* 1968b). These early studies suffer from the limited technologies available at the time (e.g. reliance on detection of Negri bodies or isolation in mice for positive diagnosis), and not having screened experimental animals for pre-existing infection or immunity. There have since been only occasional papers on bat-variant lyssaviruses using modern virological methods to revisit earlier findings (Kuzmin and Botvinkin 1996; Moreno and Baer 1980). A recent review of bat lyssavirus infections has been published (McColl *et al.* 2000). Legal protection of European bats restricts the study of European bat lyssaviruses (EBL-1 and EBL-2) in bats to those that are dead or dying of natural disease. There have been no investigations of Lagos bat or Duvenhage viruses in African bats despite them being the presumed natural hosts of these viruses.

1.6.1 Transmission

1.6.1.1 Natural transmission

Natural human and canine rabies virus infection is usually the result of a bite from a rabid animal with deposition of virus-laden saliva into tissues. Transmission is rarely observed among wildlife, but as the bites of rabid terrestrial animals and bats led to rabies in man, presumably bites are also the principal means of maintaining sylvatic rabies. In unvaccinated humans approximately 15 to 50% of people bitten by rabid animals subsequently develop rabies. Multiple severe bites to the face or head may cause rabies in 50 to 80% of exposed persons (Hattwick 1974).

In the United States, post-exposure vaccination is readily available to people who are aware that they may have been exposed to rabies. As a result, despite the occurrence of multiple terrestrial endemics, human rabies in the United States is now rare. It occurs principally in those who were unaware they had been exposed to rabies virus and hence had not requested post-exposure vaccination. From 1980 through 1997, 36 cases of human rabies were diagnosed in the United States and 21 (58%) were characterized on the basis of nucleotide sequencing and phylogenetic analysis as bat-variant rabies virus. Of the 21 people infected with bat-variant rabies, only one had a definite history of being bitten. In 10 cases the patients, their family, or friends could recall the patient having physical contact with a bat but could not recall any bite or wound, and in the remaining 10 cases there was no history of physical contact with a bat at all (CDC 1994, 1996a, b, 1997a, b, 1998, 1999; Messenger *et al.* 2003). Clearly, the mode of transmission of rabies virus from bats to humans is frequently unapparent, suggesting exposure by means other than a bite.

Oral transmission is generally considered to be rare; however as most natural hosts are carnivores, it is possible that ingestion is a significant means of transmission in nature.

Rabies was reported in two people who had been in a bat caves, including Frio Cave, Texas who strongly denied being bitten by bats or other animals (Constantine 1962; Humphrey *et al.* 1960; Irons *et al.* 1957). Foxes, coyotes, and a ring-tailed cat developed rabies after experimental exposure to the atmosphere of the same bat cave (Constantine 1962), and virus was later isolated from the cave air (Winkler 1968). Rabies virus has been isolated from the kidneys, salivary glands, and nasal mucosa of naturally infected Mexican free-tail bats and it is assumed that virus liberated in urine, saliva and/or nasal mucous contaminates the atmosphere of densely populated caves (Constantine *et al.* 1968b; Constantine *et al.* 1972). There was an outbreak of rabies among a group of experimental animals (coyotes, foxes, and raccoons) that had not been intentionally exposed to rabies virus, but were individually caged in the same room as clinically well and rabid animals that had. Transmission was attributed to airborne dissemination of bat-rabies virus (Winkler *et al.* 1972). Rabies virus infection occurred in laboratory workers exposed to viral aerosols when preparing homogenates of rabid goat brain, or when spraying suspensions of modified live SAD rabies virus (CDC 1977; Conomy *et al.* 1977; Winkler *et al.* 1973). These

human and animal cases provide good evidence of aerosol transmission via mucous membranes, which may be important in the maintenance of some sylvatic lyssavirus variants, particularly bat lyssaviruses among dense colonies of cave dwelling bats.

Natural transplacental infection has been reported in a cow and a skunk on the basis of fluorescent antibody detection of rabies virus antigen in the foetal calf and a skunk embryo, and virus isolation from foetal calf tissues (Howard 1981; Martell *et al.* 1973). The clinically rabid, five-months pregnant cow was submitted for rabies diagnosis 20 days after being bitten by a rabid dog. The skunk was submitted for rabies diagnosis although whether it was clinically rabid was not indicated. In neither case was the infected foetus/embryo viable. As such neither case represents transmission to a viable individual but simply indicates non-specific centrifugal spread to the uterus and its contents. Effective transplacental transmission would require demonstration of rabies virus, antigen, or disease in young removed from their mother immediately after birth. For such transmission to be anything other than incidental would require the young be capable of transmitting rabies virus to others. Virus or virus antigen has been demonstrated in the aborted foetus, intrauterine foetus, and newborns of experimentally infected bats (Constantine *et al.* 1968a; Sims *et al.* 1963). However, the high dose of inoculated virus and the possibility of post-parturient contamination of the aborted foetus may have affected the findings, and surveys of free-living pregnant bats and suckling bats less that 5 days of age failed to find evidence of prenatial infection (Constantine 1986). While evidently possible, the role of transplacental transmission in the maintenance of natural rabies virus endemics is unknown.

There is no clear evidence of natural human-to-human transmission of rabies, despite rabies virus being excreted in human saliva. Up to two-thirds of rabies patients develop furious rabies with violent episodes of aggression, and many cases receive intensive care by multiple people until their death. Possible instances of human to human transmission have been reported but occurred where rabies is highly endemic and other sources of exposure could not be discounted (Fekadu *et al.* 1996). This failure of human rabies patients to transmit rabies is consistent with humans being dead-end, incidental rabies virus hosts. While hardly natural, human-to-human infection via surgical corneal transplants has been recorded (Baer *et al.* 1982; CDC 1981, 1999; Houff *et al.* 1979). This is consistent with reports of rabies virus antigen, viral matrix and rabies virions in corneal epithelium, indeed corneal scrapings have been used for anti-mortem diagnosis of rabies (Balachandran and Charlton 1994; Schneider 1969).

Human infections with Duvenhage virus, EBL-1, EBL-2, and Australian bat lyssavirus have occurred after recent bat bites. The mode of transmission in the single human case of Mokola virus infection is not known, however shrews, the presumptive natural host were plentiful in the house where the patient lived. It is assumed she was infected by a shrew bite; however there was no evidence of a wound at the time of her illness (Familusi *et al.* 1972).

1.6.1.2 Experimental transmission

Numerous methods have been used to experimentally inoculate animals with rabies. These include:

- **Intracerebral inoculation** (IC)– commonly used in mice for rabies diagnosis (mouse inoculation test) and determining virus titres expressed as 50% mouse inoculation lethal dose (LD_{50}). This method produces the highest proportion of clinically affected animals after a minimal incubation period. It is an efficient model for virus recovery and amplification, but is somewhat contrived as it does not approximate natural transmission and bypasses the processes that occur before the virus reaches the brain. HEP-Flury and mutant strains of CVS and ERA rabies virus that have been selected using monoclonal antibodies, are non-pathogenic or have attenuated pathogenicity by IC inoculation in adult mice, and the alterations in virulence are associated with substitutions of the glycoprotein amino acid residue arginine (R) 333 or lysine (K) 333 with other residues (Coulon *et al.* 1994; Dietzschold *et al.* 1983; Seif *et al.* 1985). Virulence by IC inoculation of at least one CVS mutant has been shown to host species specific, IC inoculated mice were unaffected, IC inoculated skunks died (Tolson *et al.* 1990). All lyssavirus variants, strains, and mutants are pathogenic by IC inoculation of suckling mice.
- **Bite inoculation** – using lyssavirus infected animals to infect others by biting is rare, presumably due to logistical and ethical considerations, but it most closely replicates natural bite transmission, except that in some instances extraordinary numbers of bites per animal (>400) have been used (Constantine 1966a). The bites of experimentally infected vampire bats (*Desmodus rotundus*) (Pawan 1936), red bats (*Lasiurus borealis*) (Constantine and Woodall 1966), Mexican freetail bats (*Tardardia brasiliensis mexicana*) (Constantine 1966a), leaf-nosed bats (*Macrotus waterhouseii*) (Constantine *et al.* 1968a), and shrews (Kemp *et al.* 1973) have been shown to transmit rabies (bats) or Mokola virus (shrews) to other species. Uninoculated wild-caught bats have also transmitted rabies by laboratory managed bites (Bell *et al.* 1969; Pawan 1936).
- **Intramuscular, subcutaneous, intraperitoneal, footpad inoculation, or application of saliva to scarified skin** – forms of peripheral inoculation, which to varying degrees, mimic the exposure that occurs during natural bites. The incidence and incubation period following inoculation by these routes is more variable than after intracerebral inoculation, consistent with the variability observed after natural exposure. Some mutant strains of rabies virus (e.g. CVS-tsG1) and Mokola and Lagos bat virus isolates (phylogroup II), which are pathogenic for adult mice when injected IC, were not pathogenic when injected IM (Badrane *et al.* 2001; Coulon *et al.* 1994; Prehaud *et al.* 1989; Tidke *et al.* 1987). This difference in pathogenicity has been associated with substitutions of the glycoprotein amino acid residues leucine (L) 132 for phenylalanine (F) (CVS-tsG1), and arginine (R) 333 for aspartic acid (D) (Mokola and Lagos bat viruses). It appears however, that adult mice inoculated intramuscularly with Mokola or Lagos bat virus, were only observed for a period as short as 17 days.

- **Intravenous inoculation** – one of the earliest methods used. Viraemia is not believed to be a feature of natural rabies virus infection and this relatively artificial approach is now rarely used. No studies have been done to determine whether exposure of the host's blood to rabies virus when bitten by a rabid vampire bat plays a role in natural infections.
- **Oral and intranasal inoculation** – models for transmission via mucous membranes. Mucosal infection has been demonstrated in a number animal species including bats, skunks, and mice (Baer and Bales 1967; Charlton and Casey 1979b, c; Correa-Giron *et al.* 1970; Hronovsky 1971; Soave 1966) and led to the development of oral vaccines for foxes (Barrat and Aubert 1993).

Experimental infection and transmission studies of Mokola virus in shrews demonstrated that they could be infected by subcutaneous or intramuscular inoculation of relatively high doses ($\geq$ 30,000 mice intracerebral LD_{50}) of infected mouse brain suspension (incubation period 10 to > 59 days) or by ingesting live, clinically ill, infected mice (incubation period 4 to 32 days). Adult mice could be infected by the bite of an infected shrew, with incubation periods ranging from 6 to 17 days (Kemp *et al.* 1973).

1.6.2 Spread from the site of inoculation

1.6.2.1 Passage via peripheral nerves

The prevailing dogma is that rabies virus is transported from the site of exposure to the central nervous system via peripheral nerves by passive, retrograde axoplasmic flow. This mechanism is implicated by a number of experiments in which:

- Neurectomy or amputation proximal to the site of inoculation (prior to or shortly after inoculation) prevents the development of clinical disease (Baer *et al.* 1965; Johnson 1965)
- Virus or antigen is first detected in areas of the central nervous system containing the cell bodies innervating the inoculation site, for example ventral motor neurons or dorsal root ganglion cells in relevant sections of the spinal cord following limb inoculation (Baer *et al.* 1965, 1968; Johnson 1965).
- Virus ascended peripheral nerves at rates that exceeded their ability to undergo replication and cell-to cell transfer within nerve support tissue, suggesting passive transfer (Johnson 1965)
- Removal of the nerve fasciculus (preserving the perineural structures) conferred protection whereas removal of the perineural structures (preserving the nerve fasciculus) did not (Baer *et al.* 1965, 1968).
- Application of vinblastine or colchicine, which impair axoplasmic flow, to sciatic nerves 0 to 36 hours after footpad inoculation of mice or rats with fixed or street rabies virus reduced the incidence of rabies (Bijlenga and Heaney 1978; Tsiang 1979).

- Cytochalasin B, vinblastine, and colchicine blocked fixed rabies virus transport in cultured ganglion cells *in vitro*, indicating axonal transport is a function of microfilament and microtubule networks (Lycke and Tsiang 1987).

It has been proposed that the P protein interacts with dynein to use the microtubule-associated motor protein complexes during retrograde axonal transport (Raux *et al.* 2000). Inoculations at various sites, early demonstration of viral antigen in motor neurons and sensory and autonomic ganglia, and the failure of selective neurectomy to prevent disease, indicate that peripheral motor, sensory, and autonomic nerves can be equally involved.

While electron microscopy and immunofluorescence have detected virus/antigen in peripheral axons, it has not been detected early enough post-inoculation to specifically demonstrate primary centripetal transfer (Baer *et al.* 1965; Murphy *et al.* 1973a). Indeed the inability to demonstrate the virus in nerves prior to its demonstration in the ganglia and/or spinal cord has been referred to as an eclipse period. Presumably the amount of centripetal antigen is below the detectable threshold for immunological techniques or virus titration, and the concentration of recognizable viral particles is too low for them to have been observed by electron microscopy. Failure to definitively demonstrate antegrade axoplasmic transport is a weak link in the theory of rabies pathogenesis.

Whether haematogenous transport is important in the pathogenesis of rabies has been a matter of contention. Intravenous inoculation can induce rabies. Viraemia has been recorded within 1 hour of footpad inoculation in rats (Kitselman and Mital 1967), but was not detected after three hours in mice (Baer *et al.* 1965). Failure of prior/early neurectomy to confer protection suggests haematogenous spread can be important in some cases (Baer *et al.* 1968; Charlton and Casey 1981; Kitselman and Mital 1967). It has been suggested that haematogenous spread is more significant in young animals and particularly susceptible species such as the hamster. Viraemia is not a feature of the clinical disease in humans. The current consensus is that haematogenous spread from the site of infection is minor compared to that via peripheral nerves.

1.6.2.2 Direct uptake by axons versus prior replication at the inoculation site

There has been considerable debate as to whether inoculated virus enters peripheral nerves directly, undergoes prior replication in non-neural tissues (namely muscle), or both.

There is substantial evidence for direct entry of inoculated virus into nerves. Amputation at the ankle as early as 2-12 hours post-footpad-inoculation with fixed rabies virus may fail to protect mice (Baer *et al.* 1968; Johnson 1965). The minimum *in vitro* replication time has been estimated as 6-15 hours, so the rapid escape of virus from the site of inoculation appears to preclude replication at the site prior to nerve entry. Viral RNA was detected by PCR in the trigeminal ganglia 18 hours post-inoculation, and in the brain stem after 24 hours in mice inoculated in the masseter muscle with fixed rabies virus (Shankar *et al.* 1991). In this study viral RNA was detected in muscle 1 hour post-inoculation (the inoculum), but not again until 36 hours post-

inoculation. Rapid transit of virus to the CNS had occurred in the absence of evidence of replication at the inoculation site, strongly suggesting direct entry of fixed rabies virus into the nervous system.

Assessment of replication in non-neural tissues requires careful differentiation between tissue involvement before and after centrifugal distribution. This differentiation is difficult because in many animal models centrifugal distribution occurs early, prior to clinical disease. There is however good evidence that inoculated virus can be taken up and replicate in local myocytes (Shankar *et al.* 1991) and viral antigen has been demonstrated early at neuromuscular junctions (Charlton and Casey 1979a, 1981; Murphy *et al.* 1973a; Murphy *et al.* 1973b). The observation that replication in local muscle occurs prior to that in the distant nervous system (Charlton and Casey 1979a; Murphy *et al.* 1973a; Murphy *et al.* 1973b) does not prove that invasion of the nervous system occurs after myocyte amplification. Unfortunately, it has not been possible to differentiate neural uptake of progeny virus from direct uptake of inoculated virus, and so the role of muscle-progeny virus in invasion of the nervous system is unclear.

It has been suggested that sequestration of virus in local muscle may be important in cases with long incubation periods (Charlton 1994; Murphy 1977). However, rabies virus antigen in denervated muscle had only been detected up to 28 days post-infection, far less time than that which would account for incubation periods of > six months (Charlton and Casey 1981).

In skunks inoculated with a low dose of skunk origin (street) rabies virus to produce a long incubation model for rabies, positive immunoperoxidase staining and recovery of viral RNA by PCR from the inoculated muscle of clinically normal skunks demonstrated that viral genome and antigen could be sequestered at the injection site in skunks for up to 62 days, in the absence of evidence that the virus had entered the nervous system (Charlton *et al.* 1997). It is probable that both inoculation dose and type of virus (variant and fixed versus street) influence the extent of prior muscle replication versus direct entry into the nervous system. The prior intracerebral passage of fixed viruses may have selected for proficiency in entering nerve endings/cerebral neurons directly and high doses of virus in inocula may increase the likelihood of direct entry to the nervous system. If this is the case, direct entry of inoculated fixed rabies virus may be the norm in most short incubation animal models, while localisation and prior replication in local muscle may be more common following natural exposure to street viruses.

1.6.2.3 Cellular uptake of virus

The precise mechanism by which transmitted rabies virus enters cells, in particular peripheral nerve processes, is unknown, but is presumed to involve attachment between cell surface receptors and functional regions of the rabies glycoprotein. The role of the glycoprotein in receptor recognition and membrane adhesion is suggested by the loss of virulence in adult mice by IC inoculation of escape mutants of rabies virus that have point mutations at glycoprotein amino acid positions 132 (virulent phenotype leucine, L132) and 333 (virulent phenotypes arginine R333 or lysine K333) (Coulon *et al.* 1994; Dietzschold *et al.* 1983; Seif *et al.* 1985). Interestingly, substitution in rabies virus strains of R333 or K333 with other residues confers a total loss of virulence in adult mice by IC and IM inoculation, whereas wild-type Mokola virus and Lagos bat virus, with a D333 (aspartic acid) phenotype, remain pathogenic in adult mice by IC inoculation but are avirulent by IM inoculation (Badrane *et al.* 2001). It appears that Mokola and Lagos bat viruses remain virulent by the IC route despite ‘substitution’ of the positively charged R333 residues because they possess compensatory positively charged residues at positions 331 and 334 that are not present in rabies virus.

A number of cell surface molecules have been implicated in *in vitro* binding of rabies virus including nicotinic acetylcholine receptors, neural cell adhesion molecule, and low affinity nerve growth factor receptor (p75NTR), although which is significant *in vivo* remains unclear (Langevin and Tuffereau 2002; Lentz *et al.* 1983; Spriggs 1985; Thoulouze *et al.* 1998; Tuffereau *et al.* 1998; Tuffereau *et al.* 2001).

Electron microscopy has shown that rabies virus enters cells by pinocytosis, internalizing the whole virion within a coated vesicle (Iwasaki *et al.* 1973; Lentz *et al.* 1983). The nucleocapsid of virions within coated vesicles is discharged into the cytosol as a result of fusion of the coated vesicle with a lysosome and the action of the lysosomal enzymes. Free nucleocapsids also enter cells by pinocytosis (Gosztonyi 1994).

1.6.2.4 Transit in peripheral nerve fibres

It is not known in what form the virus travels centripetally within nerves. Possibilities include whole virions, nucleocapsids, or negative, positive, single or double stranded RNA. As the long neural processes of peripheral motor, sensory, and autonomic nerves are axons that have very few ribosomes, viral replication *en route* is unlikely. In an *in vitro* model there was internalization of virus into the neurites of cultured dorsal root ganglion cells exposed to fixed rabies, and retrograde axoplasmic transport was recorded at a rate of 12 to 24 mm/day (Lycke and Tsiang 1987). Fixed virus was shown to reach the lumbar cord within 24 to 48 hours in footpad-inoculated mice (Johnson 1965). The first opportunity for viral replication in the nervous system occurs at the perikaryon (cell body) at the source of the peripheral nerve process.

1.6.3 Pathogenesis of lyssaviruses in ganglia and the central nervous system

Having ascended peripheral nerves, virus reaches the perikaryon of motor neurons in the spinal cord or brain stem, and sensory neurons in the dorsal root or cranial sensory ganglia, or autonomic ganglia. Early detection of viral antigen at these sites indicates they are the first central nervous system sites for antigen amplification by viral replication (Baer *et al.* 1965, 1968; Charlton and Casey 1979a). It is unclear whether all the inoculated virions replicate here, and only progeny virus subsequently ascends to the brain and adjacent neurons, or whether inoculated virus can continue to higher segments of the central nervous system directly via the central process of the primary neuron.

1.6.3.1 Replication of lyssaviruses

The replication of rhabdoviruses, particularly vesicular stomatitis virus (VSV) and rabies virus, has been well documented. The morphology and replication of rabies and the rabies-like viruses are essentially the same (Gosztonyi 1994). Replication is confined to the cytoplasm with neither virus nor viral antigen occurring in the nucleus. Briefly, viral negative sense ribonucleic acid (-RNA) is transcribed by the RNA dependent viral RNA polymerase (L protein) into five positive sense, polyadenylated monocistronic messenger ribonucleic acid (mRNA) species. Each mRNA species is translated by cellular ribosomes into its respective viral protein (nucleoprotein, phosphoprotein, membrane protein, glycoprotein, and polymerase protein). The polymerase (L) protein also transcribes a single full-length positive sense complimentary string (+ cRNA) from which multiple negative sense genomes are replicated.

The assembly of replicated genomes with transcribed nucleoproteins (N), phosphoproteins (P) and polymerase (L) proteins forms viral nucleocapsids. Randomly oriented strands of viral proteins and/or nucleocapsid form cytoplasmic bodies of viral matrix, which when large enough are visible by light microscopy as eosinophilic inclusions or Negri bodies. Nascent virions are produced on cisternal or plasma membranes by synchronous coiling of nucleocapsids and incorporation of viral membrane (M) proteins and glycoproteins (G) into the host cell membranes as the nucleocapsid buds through it (Gosztonyi 1994; Murphy *et al.* 1973b). Virions budding through the cell plasma membrane are released directly into the extracellular space (Miyamoto and Matsumoto 1967). Virions formed on internal cisternal membranes are released when either the cell dies and disintegrates (not a feature of most street rabies virus infections), or the cisternal membrane fuses with the plasma membrane (Gosztonyi 1994).

1.6.3.2 Dissemination in the central nervous system

Following initial replication there is rapid dissemination of rabies virus throughout neurons of the central nervous system (Baer *et al.* 1965; Jackson and Reimer 1989; Murphy *et al.* 1973b), and simultaneous centrifugal spread to non-nervous tissues via peripheral nerves. Rabies antigen can be detected in several groups of neurons during the incubation period indicating there is no specificity for particular neuron types (Charlton *et al.* 1996). There appears to be negligible involvement of the glia, ependyma, choroid plexus, meningeal or vascular cells (Jackson and Reimer 1989; Johnson 1965; Murphy *et al.* 1973b). However, rabies virus replication has been observed in astrocytes by electron microscopy (Fekadu *et al.* 1982a) and rabies virus nucleoprotein antigen has been detected in oligodendroglia (Feiden *et al.* 1988). *In vitro* studies have demonstrated that, compared to astrocytes and other non-neuronal cells, neurons have a much greater susceptibility to infection and a greater propensity to sustain fixed and street rabies virus growth (Tsiang *et al.* 1983).

Trans-neuronal and trans-synaptic cell-to-cell transfer of whole virus particles has been observed by electron microscopy and is implied by the stepwise spread of the virus revealed by sequential tissue titration and immunofluorescence (Charlton and Casey 1979a; Iwasaki and Clark 1975; Iwasaki *et al.* 1975; Murphy *et al.* 1973b). Trans-synaptic passage of infective RNA, rather than complete virions, may be important in the rapid dissemination of lyssaviruses in the central nervous system (Gosztonyi 1994). An immunoperoxidase study of viral antigen distribution in pre-clinical, hind-limb inoculated skunks indicated that virus entered the lumbar spinal cord, replicated at L2 and L3, spread locally via propriospinal neurons, and gained early access to the brain via long ascending and descending fibre tracts (Charlton *et al.* 1996). Unlike earlier hind-limb inoculation studies where there was sequential involvement of the lumbar, thoracic, cervical spinal cord and brain (Baer *et al.* 1965; Johnson 1965; Murphy *et al.* 1973b), this recent study suggested that rabies virus reached the brain early by bypassing the grey matter of proximal segments of the spinal cord.

As neuronal degeneration is not a feature of early stages of the disease, release of virions from disrupted neurons is not considered important for dissemination. Virus isolation from the cerebrospinal fluid (CSF) is generally rare, usually occurs late in the disease, and there is minimal viral antigen in ependymal and pial cells, suggesting CSF is not an important vehicle for virus transport (Murphy *et al.* 1973a; Murphy *et al.* 1973b). Transit in the CSF may be important in some intracerebrally inoculated mice (Jackson and Reimer 1989).

By the time clinical signs develop, the virus is generally widespread in neural and non-neural tissues. In laboratory animals it can take as little as 24 hours for rabies, Mokola and Lagos bat viruses to spread throughout the central nervous system (Murphy *et al.* 1973b). In skunks street virus took a minimum of 4 to 5 days for virus to widely disseminate from the lumbar cord and cause clinical disease (Charlton 1994). In humans the incubation period is usually 1 to 2 months

(Miller and Nathanson 1977), however far longer incubation periods have been reported (>> 1year). How and where virus is sequestered during these long incubations is not known, but has important implications for post-exposure prophylaxis.

1.6.3.3 Clinical disease

The most detailed records of naturally occurring clinical rabies involve human patients because the source of exposure and entire clinical progression is often recalled or observed. After an incubation period typically of 1 to 2 months, but up to several years, non-specific prodromal symptoms occur for a period of 1 to 10 days, often with paraesthesia of the bitten area, followed by clinical signs specifically indicating CNS involvement (Hemachudha and Phuapradit 1997; Warrell 1976).

As in animals, human rabies is described as manifesting as either the furious (encephalitic) or dumb (paralytic) form of the disease depending on whether hyperactivity or paralysis dominates the acute neurologic phase. The clinical signs of furious rabies are mainly indicative of cerebral dysfunction. The most characteristic sign of furious rabies in man is hydrophobia: terror, excitement, and spasms of the inspiratory muscles of the larynx and pharynx triggered by the sight of water, attempts to drink, and other stimuli. Similar signs can be triggered by a draught of air on the skin (aerophobia). Other typical signs of furious rabies include intermittent episodes of excitement, agitation, hallucinations, aggressive or maniacal behaviour, tachycardia, pyrexia, and hyperreflexia, and signs of autonomic dysfunction including hypersalivation, fixed dilated pupils, anisocoria, and piloerection. Usually paralysis, generalized areflexia and coma supervene after a few days and patients die within 7 days depending on the extent of medical support (Hemachudha 1994; Hemachudha and Phuapradit 1997; Warrell 1976).

Dumb or paralytic rabies is mainly indicative of spinal cord and peripheral nerve dysfunction and is characterised by ascending flaccid paralysis that often starts with the bitten limb and progresses to generalised paresis with sphincter involvement, loss of deep tendon reflexes, and sensory disturbances. Death usually occurs from respiratory and bulbar paralysis after a longer illness than furious rabies, particularly when respiratory function is supported by medical intervention. In some cases hydrophobia can develop late in the course of otherwise paralytic rabies (Hemachudha and Phuapradit 1997; Warrell 1976).

Humans bitten by vampire bats infected with genotype 1 bat rabies virus more typically develop paralytic clinical signs without an acute excitement phase (Pawan 1936). However, the few cases of other bat lyssavirus infections in humans have often exhibited furious encephalitic signs. No human case of Lagos bat virus infection has been identified. A child infected with Mokola virus died of a poliomyelitis-like disease characterised by symmetrical paralysis and drowsiness (Familusi *et al.* 1972). The only case of human Duvenhage virus infection was highly agitated with spasms and aggression on presentation, responded to water with typical hydrophobia, and went

“berserk” despite repeated sedation (Meredith *et al.* 1971). One of two cases of bat-associated lyssavirus infection in Russia manifested “a typical clinical picture of hydrophobia”, the other, which was confirmed as EBL-1 (Yuli isolate), presented with flabbiness and sleepiness, and developed aero-hydrophobia with spasms of the face, chewing and deglutitory muscles and extremities (Selimov *et al.* 1986). The only human to become ill with EBL-2 died after presenting with an ascending paralysis radiating from his right arm and neck and developed hyperexcitability, overt delirium, hyperventilation, muscle spasms and convulsions (Haltia *et al.* 1989; Lumio *et al.* 1986; Roine *et al.* 1988). The two women to die with ABLV infection also first presented with pain and/or numbness in the bitten limb. The first developed progressive weakness and depressed conscious state and was eventually areflexic and unresponsive. On one occasion she became extremely agitated then lapsed back into depressed consciousness (Allworth *et al.* 1996). The second developed increased agitation and frequent and severe muscle spasms that led to the induction of medical paralysis and ventilator support, ameliorating and obscuring the subsequent clinical course (Hanna *et al.* 2000).

The clinical signs in animals are similar to those in man, and are also described as occurring as furious or dumb rabies. Furious rabies in animals is characterised by altered behaviour, unusual aggression, or tolerance of humans. Hydrophobia is not a feature of animal rabies. Rabid vampire bats in Brazil were observed flying during the day and shrieking and fighting with one another (Pawan 1936). Paralytic rabies is characterised by ascending paralysis. Rabid animals may also manifest with a change in voice, pica, and cranial nerve deficits (de Mattos *et al.* 2001). As for human rabies, after a short clinical period rabid animals become moribund and die. Because of the threat of transmission of rabies virus to humans and others, naturally infected animals suspected of being rabid are usually killed quickly rather than treated or observed.

Rabies in experimentally infected mice is almost invariably paralytic, although mice that die without preceding signs of paralysis (e.g. dying after a period of sluggish inactivity a hunched back and/or weight loss) are described as having encephalitic rabies (Iwasaki *et al.* 1977; Sugamata *et al.* 1992). Clinical signs in skunks infected with street (arctic fox variant) rabies typically included hyper-responsiveness to external stimuli, biting attacks, and mild incoordination and paresis, while the clinical signs of CVS-infected skunks were instability and hind limb incoordination, dullness, and lack of response to external stimuli (no biting attacks) (Smart and Charlton 1992). However, in neither animals nor man are the clinical signs associated with infection with any one lyssavirus genotype, variant, isolate, or strain, sufficiently distinctive to allow differentiation of the type of lyssavirus involved.

1.6.3.4 The cellular basis of disease: a missing link

Immunofluorescence and immunoperoxidase studies in experimental animals have documented regional variations in lyssavirus antigen distribution. For example, heavy rabies virus antigen loads occur in the hippocampus and cerebellar Purkinje cells, while neurons of the cerebellar molecular and granular layers are spared (Johnson 1965; Murphy *et al.* 1973b). In contrast, Mokola and Lagos bat viruses invaded all levels of the cerebellum including the white matter (Murphy *et al.* 1973b).

Studies of human encephalitic (furious) and paralytic (dumb) rabies found that the distributions of neither antigen nor inflammatory lesions correlated with clinical observations (Hemachudha 1994). In the human studies this may reflect the fact that the brains were examined at the 'end-stage' of the disease, by which time any significant early regional variation is likely to have been obscured. Similarly, studies of naturally occurring disease in animals are typically done late in the clinical course of the disease (Feiden *et al.* 1988). However, even in studies where experimentally infected animals were examined at various stages of the disease, no simple association between antigen, inflammation, and forms of the disease was observed (Charlton *et al.* 1996; Smart and Charlton 1992; Sugamata *et al.* 1992).

Using immunofluorescence, Johnson observed early selective involvement of the limbic system rather than the neocortex and later considered this correlated with the early onset of behavioural changes and relative sparing of motor functions (Johnson 1965; Murphy 1977). However, he made this often-paraphrased observation in mice inoculated with fixed rabies virus (CVS) that developed paralytic rather than furious rabies, and his findings have not always been supported by subsequent immunoperoxidase studies. Studies using fixed rabies virus (CVS-11) in mice and skunk street virus in skunks found viral antigen in the cerebral cortex before it was detected in the hippocampus (Charlton *et al.* 1996; Jackson and Reimer 1989). Pre-clinical involvement of the medial reticular formation (modulates sensory impulses) and relative sparing of small primary sensory neurons and dorsal horn neurons (involved in pain) were associated with the hyper-responsiveness to external stimuli in skunks (Charlton *et al.* 1996).

Differences were found between the early distribution of viral antigen in the brains of skunks that developed paralytic rabies after intranasal inoculation with fixed rabies (CVS) compared to those which developed furious rabies following intramuscular or intranasal skunk street virus inoculation (Smart and Charlton 1992). No conclusions were drawn as to the significance of involvement of any specific part/s of the brain with respect to clinical signs. It was concluded that virus variant/strain, not route of inoculation, was an important factor in the early distribution of rabies virus and clinical signs. However, clinical expression does not appear to be simply a function of the virus variant as a single dog transmitted furious (encephalitic) rabies to one human patient and paralytic rabies to another (Hemachudha *et al.* 1988), and molecular sequenceing of the N, G and P protein genes from furious and paralystic human rabies cases found only minor nucleotide

differences, none of which encoded amino acid changes in functionally important areas of the proteins (Hemachudha *et al.* 2003). Evidently virus and host factors, and characteristics of the exposure (dose, site) have impacts on disease expression.

It should be noted that the amount of antigen/virus in the brain *per se* may not correlate with the degree of dysfunction. As viral antigen is widely disseminated *prior* to clinical signs (Hronovsky 1971), clearly neither the simple presence of replicating virus, nor the involvement of a large proportion of the central nervous system is the trigger for clinical disease. Non-fatal mutants of fixed rabies virus strains ascend to the central nervous system, replicate, disseminate widely, reach titres of up to 10^4 to 10^5 $MICLD_{50}$ (mouse intracerebral 50% lethal dose) and are subsequently cleared in immunocompetent adult mice either without causing clinical disease (HEP-Flury, CVS-ts2) or in association with persistent (7 months) hind limb paralysis (ERA/BHK) (Fischman and Strandberg 1973; Kaplan *et al.* 1975; Smith 1981). Two mice inoculated with dog rabies virus and immunosuppressed by repeated injections of cyclophosphamide remained clinically normal until killed 20 days after centrifugal spread was detected by the presence of antigen in corneal touch impressions (Smith *et al.* 1982). High titrations of virus were recovered from the brains of these mice and massive amounts of antigen were detected in their peripheral nervous systems. Clearly, following infection of a considerable proportion of the central and peripheral nervous systems, some other trigger or threshold must be met for dysfunction and clinical disease to occur. Perhaps it is the absence/delay of this trigger, rather than a delay in the progression of the virus to the central nervous system, that is important in long incubation rabies. If a threshold viral load is important in cellular dysfunction, it is likely that different populations of neurons vary in their sensitivity to virus accumulation. Large accumulations of virus in some groups of neurons may be less significant than smaller quantities of virus in other neurons. Electron microscopy has not revealed any morphological basis for neural dysfunction (Murphy *et al.* 1973b). Levels of inducible nitric oxide synthase messenger ribonucleic acid (mRNA), undetectable in the normal brain, were present in the brains of experimental animals infected with rabies virus, Borna disease virus, herpes simplex virus and experimental allergic encephalitis (Koprowski *et al.* 1993). This suggests that nitric oxide, derived from increased levels of inducible nitric oxide synthase, may be a toxic factor for cell damage in neurological diseases. However, the precise mechanism by which rabies virus causes cellular dysfunction is not known. This constitutes a considerable ‘missing-link’ in our understanding of rabies pathogenesis.

1.6.4 Involvement of non-neural tissue

Centrifugal spread of rabies virions and/or matrix is believed to occur by anterograde axoplasmic flow in potentially all nerves of the body. Viral antigen and virus has been detected in peripheral nerves at times and locations consistent with centrifugal spread (Baer *et al.* 1965; Hronovsky 1971; Murphy *et al.* 1973a). Remarkably, despite electron microscopic demonstration of viral matrix in axons and virus budding from intra-axoplasmic and axonal plasma membranes, no

image of viral uptake by non-neuronal cells has been captured. The mechanism by which virus and/or viral matrix are transferred from peripheral nerves to non-neural cells is not known. It is significant that direct inoculation of the salivary gland did not result in continued infection of the salivary gland (Charlton *et al.* 1983). Sequential immunofluorescence showed that the inoculated virus was rapidly cleared from the site and the salivary glands remained negative until fluorescence was also detectable in the brain stem and salivary ganglia consistent with centripetal spread via nerves. Apparently, substantial involvement of non-neural tissues is dependent upon uptake from neural networks and implies prior involvement of the associated ganglia or central nervous system. Centrifugal dispersion of ABLV is strongly suggested by the isolation of ABLV from the kidney of an unwell naturally infected bat (Ballina isolate) (Fraser *et al.* 1996).

The involvement of non-neural tissue is relatively unimportant in the progression of clinical disease *within* an individual. Its importance lies in ensuring that virus is excreted for transmission to other individuals. It is likely that virus-variant and host-species factors influence transmissibility by affecting the rate at which salivary gland (or other relevant organ such as nasal mucosa) involvement occurs, how readily virus infects salivary epithelium, and the titre at which virus is excreted in saliva. Notably fewer organs outside the CNS contained European bat lyssavirus-1 or Duvenhage virus in intracerebral- and footpad-inoculated mice compared to mice inoculated with bat-variant rabies (Fekadu *et al.* 1988a).

Infection of salivary gland epithelium, particularly mucinous acinar cells, results in replication and directional virion budding across the apical plasma membrane into duct secretions and budding into secretory granules (Balachandran and Charlton 1994; Murphy *et al.* 1973b). Virus titres in salivary glands can be greater than in the brain (Parker and Wilsnack 1966). In skunks inoculated intramuscularly with skunk street virus, virus titres in submandibular salivary glands and the extent of immunofluorescence were inversely correlated to serum and tissue neutralizing antibody titres respectively (Charlton *et al.* 1987a). In dogs inoculated with dog street rabies, virus has been detected in saliva up to 7 days, and 13 days before clinical disease (Fekadu *et al.* 1982b). On rare occasions virus has been detected in the saliva of naturally infected, clinically normal dogs which either did not subsequently develop clinical rabies, or did so after many months (Fekadu 1972, 1975). Virus has been detected in the saliva of experimentally infected bats up to 8 days (*Desmodus rotundus*) (Moreno and Baer 1980) and 12 days (*Tadarida braziliensis mexicana*) before the onset of clinical disease (Baer and Bales 1967). Three rabbits and three calves died of rabies as a result of bites they received from inoculated vampire bats, 14 and 43 to 83 days before the vampires were ill (Pawan 1936). Considerable involvement of neuroepithelium in the olfactory end organs and taste buds has been demonstrated in bats, hamsters, and skunks (Charlton *et al.* 1984b; Constantine *et al.* 1972; Murphy *et al.* 1973b). The contribution by these organs to viral load in oronasal secretions and aerosol contamination of the environment may be significant in transmission for some host species including bats.

The involvement of non-neural tissues following centrifugal spread is generally not associated with dysfunction of those organs. Hypersalivation is most likely the result of autonomic dysfunction regulating the gland, or an inability to swallow rather than a disorder of the epithelium itself. Rabies virus antigen has been detected in the skin, buccal cavity, cornea, pancreas, and other epithelia, with no dysfunction of the infected organs (Fekadu and Shaddock 1984; Smith *et al.* 1972). Viral matrix, virions and anomalous viral products have been detected by electron microscopy in the salivary glands, adrenal gland, cornea, nasal mucosa and glands, indicating that active replication, rather than passive accumulation of virus, occurs in these tissues (Balachandran and Charlton 1994).

Antigen is commonly present in the adrenal medulla and while adrenal function is undoubtedly altered during clinical rabies (Hemachudha 1994), it has not been suggested either that altered adrenal dysfunction is a direct result of adrenal infection or that adrenal dysfunction is important in the pathogenesis of rabies.

The heart and pituitary gland may be exceptions to the rule that viral dissemination is not related to non-CNS organ failure. Viral antigen in myocardial cells, myocarditis, and atrial ganglioneuritis have been reported in the hearts of rabies patients and cardiovascular failure is often a feature of the final stages of the human disease (Hemachudha 1994; Metze and Feiden 1991). Further work is required to determine if this failure is the result of direct involvement of the myocardium and/or pacemaker mechanism or is simply secondary to the failure of cardiovascular regulation by the brain. Rabies antigen has been demonstrated in the pituitary gland associated with reduced staining for growth hormone, failure to thrive, and wasting, suggesting infection of the pituitary gland produces primary growth dysfunction (Torres-Anjel *et al.* 1988).

There has been some interest in the possible role of brown fat in long incubation and latent rabies, particularly in the hibernating microbats of the United States (Baer and Bales 1967; Kuzmin and Botvinkin 1996; Moreno and Baer 1980; Sulkin *et al.* 1957; Sulkin *et al.* 1959). While centrifugal spread can certainly involve brown fat, it remains unclear how this may be involved in the pathogenesis or epidemiology of rabies.

1.6.5 The role of the immune system

While the effectiveness of pre- and post-exposure vaccination schedules demonstrates the potentially protective role of the immune system, it may also be involved in the pathogenesis of rabies and might modulate or trigger clinical disease.

1.6.5.1 The immune system in defence

In the 1880's, Louis Pasteur demonstrated that subcutaneous injections of desiccated spinal cords from rabbits infected with fixed rabies virus protected dogs against natural virus infection. In

1885, he used the same approach to treat 9-year old Joseph Meister who had received multiple bites from a rabid dog (Dietzschold *et al.* 1996). Rabies vaccines have undergone a number of improvements, including use of avirulent vaccine rabies virus strains and growth in cell culture to reduce the incidence of rabies due to inadequate inactivation of pathogenic vaccine virus and neurological disease resulting from allergic reactions to the vaccine's animal CNS tissue base.

Current human protocols recommend either vaccination (e.g. with human diploid cell vaccine, HDCV), prior to potential exposure, or vaccination and treatment with rabies immunoglobulin within 48 hours of suspected exposure (CDC 1999; NHMRC 1997). Post-exposure treatment has also been shown to be effective in patients where treatment was delayed for as long as 3-14 days (Bahmanyar *et al.* 1976). Failures of post-exposure treatment have occurred where the incubation period was short, the post-exposure management was flawed, and/or patients were immunosuppressed (Wilde *et al.* 1989).

The rabies glycoprotein is the only rabies virus antigen that induces neutralizing antibodies (Cox *et al.* 1977). The production of neutralizing IgG antibodies to rabies virus antigen has been shown to be T-cell dependant (Iwasaki *et al.* 1977; Miller *et al.* 1978) and vaccination with the G protein alone can confer protective immunity (Fekadu *et al.* 1992; Lodmell *et al.* 1991). The N protein also plays a significant role in inducing protective immunity, and alone can confer immunity to peripheral, but not IC challenge, against heterologous rabies or rabies-like viruses (Dietzschold *et al.* 1987b; Fekadu *et al.* 1992; Lodmell *et al.* 1991; Lodmell *et al.* 1993; Sumner *et al.* 1991).

Naturally occurring fatal infection of hosts (including humans) does not lead to the development of neutralizing rabies antibodies until late in the disease, if at all, and active and/or passive immunization is not effective once clinical signs have developed (Hemachudha 1994). Animals inoculated with high doses of virulent rabies virus may develop serum neutralizing antibodies just prior to or early in the course of clinical disease, but the disease progresses rapidly and they die (Charlton *et al.* 1987a; Niezgoda *et al.* 1997). Most animals inoculated with low to moderate doses of virulent rabies virus develop antibodies late in the clinical disease, or not at all, similar to the situation in naturally infected cases (Charlton *et al.* 1987a). Homologous antibody given to mice before and/or after inoculation with street rabies or Lagos bat virus reduces or eliminates mortality (Sugamata *et al.* 1992; Tignor *et al.* 1974).

The effectiveness of vaccination schedules is often attributed to virus neutralisation by an early, and adequate immune response *prior* to involvement of the CNS (in the pre-clinical period), particularly as transport of neutralising antibody to the CNS is limited by the blood-brain barrier. However, as animal models indicate that virus can reach local ganglia within 18 hours (Shankar *et al.* 1991) it is likely that in some cases virus has reached and replicated in the CNS prior to receipt of, or response to, post-exposure treatment.

There is evidence that effective immunity can occur following CNS involvement. In strains of mice resistant to intraperitoneal inoculation with street rabies virus, virus titres were detected in the

spinal cord but dropped steadily after the appearance of prominent serum neutralizing antibody titres (Lodmell and Ewalt 1985). Intracerebral inoculation of adult mice with attenuated rabies virus (HEP-Flury, ERA/BHK, CVS-ts2) induces protective humoral and cell mediated responses capable of clearing infection from the CNS. In immunodeficient or immunosuppressed mice, these immune responses are absent or poor and inoculation is fatal (Fischman and Strandberg 1973; Kaplan *et al.* 1975; Miller *et al.* 1978; Smith 1981; Wiktor *et al.* 1977b). Mice lethally infected with street rabies failed to develop cytotoxic T cells and had suppressed immune responses to concurrent viral infection (Wiktor *et al.* 1977a).

It would appear that the pathogenesis and virulence of natural (street) rabies infections is related to failure (avoidance and/or suppression) of the immune response, and that pre- and even post-exposure vaccination circumvents this. The protective functions of the immune system are abrogated in immunodeficient or immunosuppressed individuals as indicated by increased mortality in mice inoculated with street or fixed rabies virus, or Lagos bat virus (Iwasaki *et al.* 1977; Smith *et al.* 1982).

Even when the immune response is insufficient for survival, a positive role of the immune system in the pathogenesis of rabies is indicated by:

- More rapid onset and progression of clinical signs in cyclophosphamide immunosuppressed skunks infected with street virus (Charlton *et al.* 1984a).
- Tissue neutralising antibodies are associated with less immunofluorescence and lower virus titres in salivary glands (Charlton *et al.* 1987a), and immunosuppression facilitates recovery of rabies virus from salivary glands (Charlton *et al.* 1984a)

1.6.5.2 The role of immunological responses in the pathogenesis of rabies

The role of the immune system as a contributing factor in clinical rabies has also been investigated but there is as yet no clear understanding of how immune responses influence pathogenesis.

Histological evidence of mononuclear cell infiltration and neuronophagia indicates that there are cellular immune responses to rabies virus infection that could play a role in neuronal dysfunction. However the low level or absence of inflammatory lesions in many rabies cases suggests that these visible immune responses alone are not the mechanism by which clinical disease is produced.

There is evidence to suggest that the immune system influences the incubation time, clinical expression and mortality following infection with lyssaviruses in numerous studies examining the responses of normal, immunosuppressed, or immunodeficient animals, generally mice. That the immune system can have negative effects on the host during lyssavirus infection is indicated by:

- Immunosuppression delayed the onset of clinical signs after footpad-inoculation of mice with street rabies viruses by 1 to 2 weeks, albeit with increased mortality from 27 to 100%. The onset of paralysis after limited immunosuppression corresponded with a return to immune responsiveness, and passive transfer of homologous immune serum led to early onset of paralysis and death. There was an absence of clinical disease in two mice in which T- and B-cell responses were suppressed with cyclophosphamide for 40 days post-inoculation, despite detection of rabies virus antigen in corneal touch impressions on days 21, 27 and 36, and recovery of high titres of virulent virus from their brains and demonstration of "massive" amounts of viral antigen in their peripheral nervous systems when sacrificed on day 40 (Smith *et al.* 1982).
- Immunosuppression with cyclophosphamide or by thymectomy and sublethal irradiation delayed the onset of clinical signs in mice following IC inoculation with Lagos bat virus, and death was accelerated by immune serum transfer (Tignor *et al.* 1974).
- An 'early death' phenomenon, where inadequately immunized animals die after a shorter incubation period than unvaccinated controls, occurs in primates and mice challenged with CVS rabies virus and may be involved in the deaths of some humans in whom post-exposure vaccination fails (Blancou *et al.* 1980; Sikes *et al.* 1971). 'Early death' has been shown to be dependant on B lymphocytes, neutralising antibody, and/or T-cells (Prabhakar and Nathanson 1981; Smith *et al.* 1982; Sugamata *et al.* 1992; Tignor *et al.* 1974). The mechanism/s by which antibody and/or T-cells precipitate the early onset of rabies in these situations is not known.

Evidence for immune involvement in the paralytic form of lyssavirus infections includes:

- Encephalitic rather than paralytic clinical signs prior to death in immunosuppressed or immunodeficient mice inoculated with fixed or street rabies virus (Iwasaki *et al.* 1977; Smith 1981; Sugamata *et al.* 1992).
- Paralysis of street rabies virus-infected mice was shown to be dependant on T lymphocytes and associated with inflammation including perivascular accumulations of CD4+ (helper) and CD8+ (cytotoxic) T cells, and the presence of CD8+ T cells in brain parenchyma. It was suggested cytotoxic elimination of infected neurons induces paralysis (Sugamata *et al.* 1992).
- Immunosuppression by cyclophosphamide or thymectomy and irradiation reduced the incidence of paralysis in mice inoculated with Lagos bat virus (Tignor *et al.* 1974).
- Onset of paralysis was associated with the return to immunocompetence in mice that had been inoculated with dog or fox rabies virus variants after temporary immunosuppression with cyclophosphamide (Smith *et al.* 1982).
- Lymphoproliferative responses to rabies antigen were detected in human patients with encephalitic rabies who died after a shorter clinical period, whereas patients with paralytic rabies showed no response to rabies antigen (Hemachudha *et al.* 1988). Patients with paralytic rabies tended to have diminished numbers of B lymphocytes compared to those with encephalitic rabies (Hemachudha *et al.* 1988; Sriwanthana *et al.* 1989).

There is also conflicting evidence that paralysis is independent of the immune system. Nude (athymic T-cell deficient) mice developed paralysis after inoculation with fixed (CVS) but not street rabies virus (Charlton 1994), and disease expression in immunocompromised skunks did not differ from that in competent skunks (Charlton *et al.* 1984a).

It is apparent that there is a complex relationship between the influences of the immune system, the host species, virus variant/strain, dose, and route of inoculation that complicates interpretation of results using different methods. The role and mechanisms by which the immune system influences the pathogenesis of rabies, neural dysfunction, and the expression of clinical disease remain unclear.

1.6.6 Evidence of recovery and subclinical infection

Clinical rabies in mammals is generally considered fatal. There are however occasional reports of asymptomatic carrier animals and recovery from clinical disease. Reports of rabies-neutralizing serum in clinically normal animals and humans suggest subclinical resolution of viral infection and/or recovery from clinical disease.

1.6.6.1 The carrier state

There are reports indicating that on rare occasions apparently healthy animals, with no known previous or subsequent signs of rabies, excrete infective rabies virus in their saliva. True carrier animals need to be differentiated from those excreting virus in the immediate (generally <14 days) pre-clinical period.

The earliest reports of a carrier state involved vampire bats (*Desmodus rotundus*) (Pawan 1936). Wild-caught bats that appeared healthy transmitted rabies to calves on which they were allowed to feed. Bats inoculated with passaged rabies virus of human origin (variant unknown) that did not develop clinical signs also transmitted rabies to calves. A bat that was “restless and fierce’” when caught in the wild remained fierce and excited for nine days, then became normal, and three-to-six days after its apparent recovery transmitted fatal rabies to a calf on which it was allowed to feed. The bat survived for at least a further 2 ½ months. These findings suggest that clinically normal wild bats and those who recover from clinical disease can be carriers of rabies virus. Unfortunately, these early reports suffer from a reliance on clinical signs and the detection of Negri bodies for rabies diagnosis. No evidence of virus excreting ‘carriers’ was found in a more recent study of experimentally inoculated vampires using suckling mice, cell culture and fluorescent antibody tests for virus detection (Moreno and Baer 1980). In these bats virus was only isolated from salivary swabs up to 8 days before and during clinical disease.

Early studies in which dog-variant rabies virus was recovered from the brown fat but not the brain of apparently well, IM inoculated Mexican freetail bats suggested that brown fat may play an important role in the persistence of rabies virus in asymptomatic carriers and allow latent rabies infection to ‘over winter’ in bats while hibernating (Sims *et al.* 1963; Sulkin *et al.* 1957; Sulkin *et al.* 1959). However no special role for brown fat was indicated in later studies in which virus was only recovered from the brown fat of unwell bats that also had virus in the brain, and where virus was not recovered any more frequently from bats kept in hibernation for 60 days post-inoculation (Baer and Bales 1967; Kuzmin and Botvinkin 1996; Moreno and Baer 1980). In the absence of data on the prevalence of lyssaviruses, using modern techniques, in the brown fat of clinically well wild animals, the role of brown fat in long incubations, hibernation, and carrier status remains unclear.

Outbreaks of EBL-1 in a colony of captive bats (Egyptian flying fox, *Rousettus aegyptiacus*) 9 days after transfer from a closed Dutch colony, and of clinical rabies in captive *E. fuscus* 3 to 4 weeks after importation from the USA, suggest that persistent, subclinical lyssavirus infections in bats may be common, and that clinical outbreaks may occur following stress such as transport and relocation (Ronsholt *et al.* 1998; Wellenberg *et al.* 2002). That bats may survive subclinical infection either as asymptomatic ‘carriers’ or having cleared the infection is supported by the detection of neutralising antibodies (n=3) or nucleoprotein antigen (n=2), and by amplification of EBL-1 RNA (n=30) from 40 remaining, apparently healthy *R. aegyptiacus* in the source colony (Wellenberg *et al.* 2002).

In India, a boy died of rabies 44 days after being bitten by a clinically normal dog. Rabies virus was isolated by mouse inoculation from the saliva of the dog on 13 occasions over four years, yet it remained asymptomatic, had no serum-neutralizing antibodies, and no virus was recovered from its brain when it died, although viral antigen was detected by fluorescent antibody test. The cause of the dogs death was not indicated (Nanavati 1973; Veeraraghavan *et al.* 1970). The death of the boy suggests the dog carried virulent rabies virus, yet in the absence of neutralizing antibody; unrecognized host factors either protected the dog from developing clinical rabies, or had enabled the dog to recover from clinical disease prior to biting the child.

In Africa, atypical strains of rabies virus appear to circulate in dogs that can produce a chronic carrier state. In Ethiopia rabies virus was repeatedly isolated from the saliva of five of 1,083 clinically normal dogs submitted for isolation after biting, scratching, or licking humans or as in the case of one bitch after four of her two-month old pups died of rabies. The bitch died of bronchopneumonia after nine months and a dog died of furious rabies 19 months after virus was first isolated from saliva. Three others remained asymptomatic for at least four years (>50, >62 and >70 months) and had rabies-neutralizing antibodies, yet rabies virus was intermittently isolated from their saliva by mouse brain inoculation (Fekadu 1972, 1975).

In Nigeria, swabs of saliva were collected from 1500 dogs presenting for routine veterinary examination over 5 years. Of these, four were positive by fluorescent antibody test and mouse inoculation. The four cell-adapted isolates showed identical reactivity when typed by a panel of anti-nucleocapsid and anti-glycoprotein monoclonal antibodies, which was clearly distinct from the LEP-Flury strain used for vaccination and other street rabies variants in Nigeria (Aghomo and Rupprecht 1990).

If involvement of the salivary glands in these dogs and bats was the result of centrifugal spread, then at least limited infection of the central nervous system must have occurred. The question that remains is why this had not resulted in fatal clinical rabies. The possibility that the asymptomatic shedding carrier state occurs after recovery from clinical disease is suggested by one dog inoculated with a rabies isolate from the saliva of a naturally asymptomatic carrier dog (Fekadu and Baer 1980; Fekadu *et al.* 1981; Fekadu *et al.* 1983). Virus was recovered from the saliva of this dog 42, 169, and 305 days after recovering from clinical signs consistent with rabies and developing high serum and CSF rabies neutralising antibody titres. Sixteen months after recovery the dog died following the stillbirth of two pups, at which time virus antigen was detected in the brain, spinal cord, and tonsils, and virus was isolated from the palatine tonsil.

The prevalence of true carriers, the mechanisms that prevent them succumbing to clinical disease, and the significance of their role in the maintenance of rabies within bat and other species populations remain unclear.

1.6.6.2 Recovery from clinical disease

While approximately 60,000 clinical cases of human rabies are reported each year, there are only five well-documented reports of human survival of clinical rabies.

- ♦ A 6-year old boy bitten by a Big Brown bat (*E. fuscus*) recovered completely within six months (Hattwick *et al.* 1972).
- ♦ Recovery of a 45-year old woman bitten by her dog was 'nearly complete' after 13 months (Porras *et al.* 1976).
- ♦ A 32-year old male rabies laboratory worker apparently exposed to aerosols of modified live (SAD strain) rabies showed 'gradual improvement' from a deep coma, no longer needing respiratory assistance but unable to respond to verbal commands or communicate with others (CDC 1977).
- ♦ A 9-year old Mexican boy bitten by a rabid dog survived at least 2 years with severe neurological sequelae that included quadriplegia and amaurosis with the capacity to breath unassisted via a tracheostomy and injest food by mouth (Alvarez *et al.* 1994).
- ♦ A six-year old Indian girl bitten by a street dog was comatose for 3 months then showed gradual improvement over a further 2 months to be able to respond to verbal commands and

feed orally. She remained alive after 18 months with little further improvement and persistent rigidity, tremors, and involuntary movements of the limbs (Madhusudana *et al.* 2002).

The merit of these reports lies in the validity of the diagnoses of rabies infection. The bat and the boy's dog were confirmed as rabies-positive by fluorescent antibody test and both the woman's and girl's dogs died 4 days after the bite with clinical signs suggestive of rabies but no laboratory confirmation was made. Antemortem diagnosis of rabies is notoriously difficult and definitive diagnosis requires virus isolation or the demonstration of viral antigen or viral RNA, none of which was demonstrated in these cases. However, brain biopsies are rarely taken and tests on nuchal skin biopsies (Smith *et al.* 1972), corneal scrapings, and saliva are not very sensitive (World Health Organization 1992). In all five cases, the diagnosis of rabies was made on the basis of each developing extraordinarily high serum (1: 63,000, 1:640,000, 1: 64,000, 1:34.800, and 1:265,000) and CSF (1:3,200. 1: 160, 000, 1:16,225, 1:78,125, and 1:124,000) rabies antibody titres. All five had received rabies vaccination, in four cases as post-exposure treatment (duck embryo vaccine (bat boy), suckling mouse brain vaccine (woman), VERO vaccine in the preclinical period and HDCV when acutely ill (Mexican boy), or chick embryo cell vaccine in the preclinical period and HDCV when acutely ill (Indian girl)), and in one cases as pre-exposure vaccination (man - primary course 9 years earlier followed by yearly boosters inducing a rabies titre of 1:32, suggesting protective immunity, 4 months before exposure). However the serum and particularly the CSF titres are far in excess of those recorded in response to these vaccines either in patients that remain well, or in those who develop Guillain-Barré syndrome in response to vaccination.

High rabies virus neutralising titres in CSF, are considered evidence of recovery from clinical rabies because in cases of fatal rabies and in vaccinated animals CSF titres are comparatively low (Arko *et al.* 1973; Bell *et al.* 1971; Bell *et al.* 1972; Fekadu and Baer 1980; Fekadu *et al.* 1988a). The presence of high neutralising titres in the CNS is believed to reflect *in situ* antibody production rather than 'leakage' of serum antibodies across a damaged blood-brain barrier as lead nitrate induced encephalitis in vaccinated dogs with high rabies neutralizing serum titres did not produce high rabies neutralising titres in the CSF (Arko *et al.* 1973). Evidence of intrathecal production of rabies antibodies in the CSF was demonstated in the Mexican boy and Indian girl. The relationship between high CSF titres and recovery deserves further investigation.

Since Pasteur there have been reports of recovery from clinical rabies in *experimentally* infected animals including bats (Pawan 1936), mice (Bell 1964; Iwasaki *et al.* 1977; Lodmell *et al.* 1969; Lodmell and Ewalt 1985; Mifune *et al.* 1980; Sugamata *et al.* 1992), dogs (Arko *et al.* 1973; Bell *et al.* 1972; Constantine 1966c; Fekadu and Baer 1980; Fekadu *et al.* 1988a; Fekadu *et al.* 1992), a ferret (Niezgoda *et al.* 1997), and a donkey (Ferris *et al.* 1968). A monkey intramuscularly inoculated with Largos bat virus developed persistent unilateral paresis on post-inoculation day 22 but survived until killed on day 108 (Tignor *et al.* 1973). In these reports the extent to which the diagnosis of rabies virus infection was confirmed varied.

Reports of recovery from *naturally* occurring rabies, such as that reported in a wild caught vampire bat and four of six pigs bitten by a rabid skunk (Baer and Olson 1972; Pawan 1936), are much rarer. In no naturally occurring case of clinical recovery has the diagnosis been confirmed by recovery of virus or viral antigen. There is little in the literature to suggest that recovery from *clinical* rabies is common in nature.

1.6.6.3 Serological evidence of lyssavirus infection in normal animals

Rabies neutralizing serum has been reported in clinically normal domestic and wild animals and humans from rabies-endemic areas. Interpretation of anti-lyssavirus titres in clinically normal hosts is difficult. Of primary consideration is the validity of the results, false-positives are a feature of any biological test. Serum neutralizing tests such as the mouse neutralization and rapid fluorescence focus inhibition test (RFFIT) are not entirely specific for rabies neutralizing antibodies and there should be concern that positive reactions, particularly those of low titre (e.g. less than 1:10) are due to non-specific virus neutralizing substances in serum. Results could also reflect the presence of cross-reacting antibodies to other unrecognized antigens. Presuming the results do indicate the presence of rabies (or other lyssavirus) antibodies, the possibility of prior anti-rabies vaccination should be considered, particularly when interpreting data from humans and dogs. Where prior vaccination is unlikely, often too little is known of the history and subsequent progress of animals to allow meaningful interpretation of whether the presence of antibodies indicates recovery from prior clinical disease, subclinical resolution of infection, or impending disease (tested during an incubation or latent period). In none of the studies have ‘wild caught’ seropositive animals been challenged to assess the effectiveness of their apparent immunity.

In most experimental studies, inoculated animals that fail to develop clinical rabies do not develop neutralising titres, although 11% of Mexican freetail bats that survived inoculation with bat rabies were seropositive 181 days later (Baer and Bales 1967). Studies of inoculated denervated skunks indicate that infection restricted to the inoculation site can induce serum neutralizing titres without disease (Charlton and Casey 1981). The development of neutralizing titres and subclinical resolution may occur in wild animals as a result of factors/mechanisms that produce ‘abortion’ of the infection at the inoculation site, peripheral or central nervous system.

In humans, rabies neutralizing serum was reported in 15 of 226 (6%) students and staff at a veterinary college (Ruegsegger *et al.* 1961), three of 200 cave explorers (Doege and Northrop 1974), and one of 26 Alaskan fox trappers (Follmann *et al.* 1994) who denied exposure or contact with rabies vaccines.

Wildlife surveys have found prevalences of 2 to 29% of rabies neutralizing serum in apparently normal populations of grey and red foxes, skunks, opossums, mongooses, bobcats, raccoons, and vampire bats, (Carey and McLean 1978, 1983; Doege and Northrop 1974). Seroprevalences

of 18% (n=49) (Yasmuth *et al.* 1974), 30% (n=463) (Ogunkoya *et al.* 1990), and even 80% (n=10) (Mebatsion *et al.* 1992) have been reported in unvaccinated dogs.

The most striking serological evidence of survival following rabies infection occurs in bats. Positive rabies antibody titres were detected 14-80% of apparently normal Mexican Free-tailed bats, yet <1% were positive for rabies antigen and/or virus (Constantine *et al.* 1968b; Steece and Altenbach 1989). Similarly, 2% of Little Brown bats (*Myotis lucifugus*) and 9% of Big Brown bats (*E. fuscus*) in New York State were seropositive for rabies, as were up to 33% of vampire bats (Ortega *et al.* 1987; Trimarchi and Debbie 1977). A four month survey of normal adult female Mexican free-tail bats at Lava Cave, New Mexico (n=750) found a constantly high IgG antibody prevalence of 63-73%, low (2%) prevalence of IgM antibodies and a low (<1%) prevalence of rabies antigen in brains (Steece and Altenbach 1989). The findings in 600 neonatal bats over a three-month period were more variable. Initially the proportion with neutralizing antibodies was high, the prevalence of IgM antibodies was low, and rabies antigen was not detected. As the summer progressed there was a drop in the proportion with neutralizing antibodies, which then steadily rose (reaching 50% by the time the survey was completed) in concert with a rise in the prevalence with IgM antibodies. The number of juveniles which were rabies antigen positive was low (2%) but notably higher than that among adults during that period. In combination these results suggest: prenatal transfer of rabies antibody, the levels of which fell rapidly in the post-natal period; early exposure of young bats to rabies virus with the development of IgM and then IgG antibodies; and persistence of IgG antibodies and loss of IgM antibodies in adult bats. Prenatal transfer of rabies antibody followed by a decrease in the proportion of young bats with antibody being associated with clinical rabies had also been observed in the same bat species in Carlsbad Cave, New Mexico (Constantine *et al.* 1968b). What remains to be determined is whether or not the adult bats that had high antibody titres had recovered from clinical disease or had seroconverted during subclinical resolution of rabies infection.

Interestingly, while Lagos bat virus was first isolated from fruit bats (*Eidolon helvium*), in a survey of shot fruit bats, none had detectable antibodies to Lagos bat, Duvenhage or Mokola viruses, while 5 of 50 (10%) had titres to CVS-11 rabies virus (Aghomo *et al.* 1990). There has been serological evidence of Mokola virus infection in cattle, goats, swine, birds, dogs, and fruit-bats (*Eidolon helvium*)(Kemp *et al.* 1972; Ogunkoya *et al.* 1990). Perhaps surprisingly if shrews are the natural host for Mokola virus, no neutralizing antibodies were detected in a serological survey of shrews (n=91) and only one of 13 experimentally infected shrews developed a neutralizing titre (which was low) (Kemp *et al.* 1972; Kemp *et al.* 1973). Using a mouse neutralization test, neither of the two human cases, nor humans in a serological survey (n=82) had Mokola neutralizing antibodies (Kemp *et al.* 1972). Using a modified rapid fluorescent focus inhibition tests (RFFIT), 7.5% of 158 people were shown to have anti-Mokola antibodies, and 2.5% had antibodies to Lagos bat virus (Ogunkoya *et al.* 1990). An aaverage of 7.8% of 976 sera from microbats in Spain were positive for EBL-1 specific antibodies, with an average seroprevalence of 24% among *M. myotis* in two locations (Serra Cobo *et al.* 2002). Banding and recapture of the Spanish bats

demonstrated that four individuals survived at least 1 to 3 years following detection of positive EBL-1 titres.

The presence of anti-rabies antibodies in humans who have no recollection of prior rabies vaccination, have no history of an illness suggestive of recovery from rabies, and who do not subsequently develop rabies suggests either test failure, cross reactivity with other antigens or prior subclinical resolution of infection from an unapparent exposure. If subclinical resolution occurs it suggests humans (and probably other animals) have an ‘all or nothing’ response to naturally occurring rabies infection. That is that following exposure to rabies, either the patient develops antibodies with subclinical resolution of the infection or the patient develops invariably fatal clinical disease, with or without late production of antibodies. The virtual absence in humans of a spectrum of response, which would include people surviving clinical disease with and without sequelae, is unusual in biological systems. The unlikeliness of an ‘all or nothing’ response, and the fact that many seropositive humans recall no history of likely exposure, such as bites from dogs or other hosts, rabid or not (Ogunkoya *et al.* 1990), suggests that the neutralizing activity detected in serum is not necessarily the result of exposure to lyssaviruses.

1.7 Controlling rabies

A moderate level of rabies virus control among vampire bats is achieved though the application of anticoagulants systemically to cattle and topically to individual vampire bats. Anticoagulant, ingested by bats when feeding on treated cattle or by mutual grooming of treated vampire bats back at the colony, is fatal to vampires (Setien *et al.* 1998). Attempts to control rabies by eliminating litter-bearing (carnivore) principal hosts have been universally unsuccessful, presumably because the fecundity of the hosts quickly restores the minimum population density required to maintain the disease. However, targeting the *susceptibility* of the principal host in a particular area has successfully eradicated or dramatically reduced rabies in some areas. Between 1889 and 1902, restrictions on the movement and importation of dogs, and the use of muzzles when at large, eliminated rabies from the United Kingdom. Between 1907 and 1917 similar measures controlled small outbreaks of rabies in Canada (Pullar and McIntosh 1954; Tabel *et al.* 1974). Presumably, variants of dog rabies virus then present in the United Kingdom and Canada had not adapted to sustain themselves in the fox populations of those countries.

With the development of killed and attenuated parenteral (intramuscular and subcutaneous) animal rabies vaccines, mass canine vaccination programs have virtually eliminated dog rabies from many developed countries. The effectiveness of parenteral vaccination programs in many developing countries has been poor because success is dependant on costly vaccination and effective concurrent control of domestic and stray animals that is difficult to achieve in many social, cultural, religious, political, and economic circumstances (de Mattos *et al.* 2001).

A considerable limitation to control of sylvatic rabies is safe, effective, suitably targeted, and affordable vaccine delivery. While vaccination by injection is not practical for wildlife, vaccination

of red foxes with standard (e.g. SAD) or attenuated (e.g. SAD-B19) rabies virus strains by oral baiting has been effective in some European countries, the USA, and Canada (Barrat and Aubert 1993; de Mattos *et al.* 2001; Wandeler *et al.* 1988) and has led to development of new oral vaccines with increased efficacy, safety and thermostability. Recent advances in animal vaccine technology include vaccinia virus-, raccoon poxvirus-, or adenovirus-vectored recombinant vaccines expressing the rabies virus G protein (Charlton *et al.* 1992; Fekadu *et al.* 1991; Lodmell *et al.* 1991; Pastoret and Brochier 1992) and live cloned cDNA rabies virus vaccines (Pulmanausahakul *et al.* 2001). These new vaccines have the potential to increase the number of host species, lyssavirus genotypes, circumstances, and environments in which rabies control programs can be effective.

Elimination of rabies from areas such as the USA, where different variants are involved in multiple endemics, will require suitable control programs be directed towards rabies virus transmission in each principal wildlife species. While the development of thermostable oral vaccines and improvements in bait design may result in effective control of urban and sylvatic terrestrial rabies, no suitable method of vaccine delivery to insectivorous bats or flying foxes is known.

2 Naturally acquired Australian bat lyssavirus infection in bats

Aims

1. To estimate the prevalence of Australian bat lyssavirus in bats.
2. To identify risk factors for ABLV infection of bats.
3. To characterize the circumstances in which bats with Australian bat lyssavirus are found, as well as the clinical signs, clinical duration and outcome, serological response, and duration / extent of possible human exposure associated with naturally acquired infection in bats.

2.1 Introduction

Recognition and surveillance of ABLV occurred as a direct result of investigations in to the origins of the 1994 outbreaks of Hendra virus (previously equine morbillivirus) (Allworth *et al.* 1995; Hooper *et al.* 1997a; Hooper *et al.* 1997c; Murray *et al.* 1995a; Murray *et al.* 1995b; O'Sullivan *et al.* 1997; Rogers *et al.* 1996; Selvey *et al.* 1995; Ward *et al.* 1996; Westbury *et al.* 1995; Westbury *et al.* 1996). A five month-old Black flying fox (*P. alecto*) with hind limb paresis, submitted to AAHL for Hendra virus testing, was subsequently diagnosed with a lyssavirus infection (Fraser *et al.* 1996). Prior to this it was believed that no lyssaviruses were endemic in Australia. Even more alarmingly, within 4 months a woman that had had contact with bats was confirmed as having died of ABLV encephalitis (Allworth *et al.* 1996; Samaratunga *et al.* 1998).

The recognition that one or more lyssavirus/es was present in Australia, was capable of causing human fatalities, and threatened Australia's WHO 'rabies free' status, through which Australia enjoyed political, economical, trade and quarantine advantages, meant it was urgent that research be done to characterize and quantify the prevalence, distribution, risk factors, and natural history of lyssavirus infection in Australia.

2.2 Materials and Methods

2.2.1 Animal submission and classification

Bats and other species submitted to the Queensland Department of Primary Industries (DPI) between June 1996 and March 2002 were tested as part of a surveillance program for the detection of Australian bat lyssavirus (ABLV). The bats included in this analysis are those submitted for 'diagnostic ABLV-testing' and includes retrospective (pre-mid-1997) and prospective data. This analysis excludes normal wild bats caught and tested as part of concurrent structured surveillance research by the DPI (H. Field *pers. comm.*) and bats in the vaccination and experimental infection trials described in Chapters 5 and 7.

Most bats were submitted dead to the Animal Research Institute (ARI), Yeerongpilly or other regional laboratories of the Queensland Department of Primary Industries. Live flying foxes submitted to the ARI were killed by injection of barbiturate (Lethobarb®, 325 mg/mL pentobarbitone sodium, Virbac) administered intravenously via the uropatagial vein or by intraperitoneal injection. Live microchiroptera were killed by inhalation of CO_2. The submitter completed a standard Department of Primary Industries submission form (Specimen Advice Sheet: Form A, GEN-008) giving their details, details of the bat/s, and the history of their involvement with the bat and their observations. The quality of the information supplied by the submitters varied. Where possible the submitters of ABLV-positive bats and some ABLV-negative bats were later phoned for further details.

In addition, results and details of some Queensland bats submitted to the Department of Health, (QHSS) during 1999 and 2000, including all ABLV-positive bats, were added to those submitted to the DPI (see Section 2.2.4 Evolution of laboratory submission and test protocols). As such this study includes all ABLV-positive bats from Queensland between 1996 to 2000 inclusive, but not ABLV-positive bats submitted to QHSS during 2001 and 2002.

Where possible the following information was recorded:

- Date of death and date submitted
- Species of bat
- Sex (male or female)
- Age (juvenile or adult)
- History of circumstances found
- History of clinical signs observed
- History of human or animal contact with bat
- Location found (town and DPI region, see Map, Appendix 1)

The original intent was to have combined the data of DPI and all QHSS submissions to construct and analyse a complete data set for bats submitted from Queensland. However, information not required by QHSS to perform and report the test, but necessary for this analysis, such as species of bat, sex, age, and histories, was not usually supplied by the referring physician, and issues of privacy and patient confidentiality precluded direct contact with QHSS submitters. Bats were

classified by the candidate on the basis of the history and clinical signs for two independent characteristics;

- **Health status:** the clinical health versus disease status of the bat
- **Contact status:** whether or not it had bitten or scratched any person (Contact Human) or other animal (Contact Animal)

2.2.1.1 Health status

Each bat was classified as one of the following:

1. **Normal bat.** A healthy bat with no indication of being unwell. This group included captive bats with no recent history of being unwell, bats shot as pests in orchards, and apparently healthy microbats found in homes.

2. **Sick, injured, or orphaned.** All other bats which were not normal (sick, injured, or orphaned). These bats were further classified as:

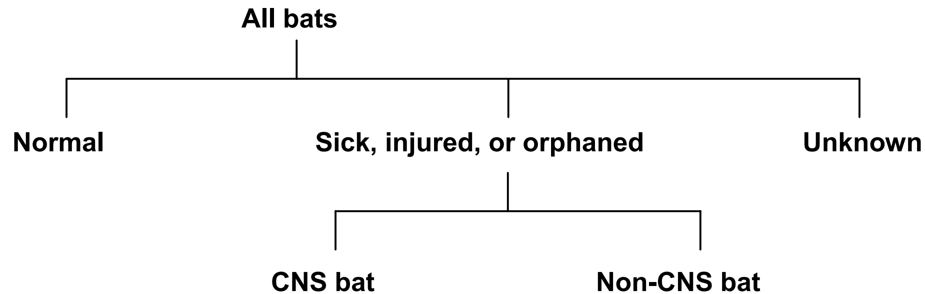
   2a. CNS bat. An ill bat with clinical signs suggesting the central nervous system was primarily involved. This included bats with signs of paresis, paralysis, cranial nerve deficits, and abnormal behaviour including uncharacteristic aggression or tolerance of humans. Most of the bats submitted were wild animals and many were submitted by wildlife carers who were experienced in the normal behaviour of wild-caught bats. Typical aggression and fear was not considered an indication of central nervous system disease. Depressed moribund bats were not classified in this group, as secondary nervous system depression can result from the end stage of many non-nervous system conditions.

   2b. Non-CNS bats. All bats that were neither normal nor showing signs of primary central nervous system disease. This included bats with injuries such as fractures, bats caught in fences, found dead, or bats that were non-specifically unwell. Maternally-dependant orphan bats, even if apparently in good health, were included in this classification as it was presumed that some disease or misadventure involving the dam or the orphan had led to it being taken into care, and that it had been subjected to at least some degree of stress, dehydration, and exposure.

3. **Unknown**. Where the history provided by the submitter was not sufficient to determine whether the bat was normal or sick, injured, or orphaned the health status was recorded as unknown.

All health classifications; Normal, CNS bat, Non-CNS bat, and Unknown are mutually exclusive. The relationship of the health categories is illustrated in Figure 2-1.

Figure 2-1 Health status categories



2.2.1.2 Contact status

Each bat was classified as one of the following:

1. **None.** No human or animal contact (as defined below) reported.

2. **Human only.** Where a bat was reported as being *known* to have bitten or scratched one or more persons**.** Often this occurred when members of the general public attempted to rescue a sick, injured, or orphaned bat, such as when attempting to release a bat trapped on a barb-wire fence, and when wildlife careers raised or rehabilitated sick, injured, or orphan bats. Direct contact alone did not warrant classification in this group as virtually all submitted bats had been handled by humans. Where there was the possibility of people having been bitten or scratched (e.g. bat found injured in school yard) but no bite or scratch was *known* to have occurred (e.g. children denied touching bat, claimed not to have been bitten or scratched and no consistent injuries were found), the bat was *not* classified in this group. Classification in this group was intended as a measure of aggressive/defensive behaviour by the bat rather than the appropriateness of post-exposure vaccination for the relevant humans.

3. **Animal contact.** Where a bat was reported as having had contact with an animal such that it was *known or suspected* that the bat could have bitten or scratched the other animal. Dogs and cats occasionally attack bats. In some cases, members of the public heard or observed bats being attacked, found their pet with a live or dead bat, or found a dead bat in the pet's bed. Unless it was clear that the bat was dead prior to the pet having contact with it (e.g. advanced putrefaction or evidence of having been dug up), it was presumed that that the bat could have bitten or scratched the other animal. Bats submitted with no history of being attacked but with injuries consistent with an animal attack, such as puncture wounds and multiple fractures, were also classified in this group. Classification in this group was intended as a measure of the potential of bats to expose other animals to ABLV.

4. **Dual: Human *and* Animal Contact.** The contact status classifications of None, Human Contact and Animal Contact were not mutually exclusive as a small number of bats had bitten or scratched one or more persons *and* had contact with one or more animals.

The total number of bats which had scratched one or more persons was the sum of those that had bitten or scratched a person (Human only) and those that had bitten or scratched a person *and* had contact with an animal (Dual).

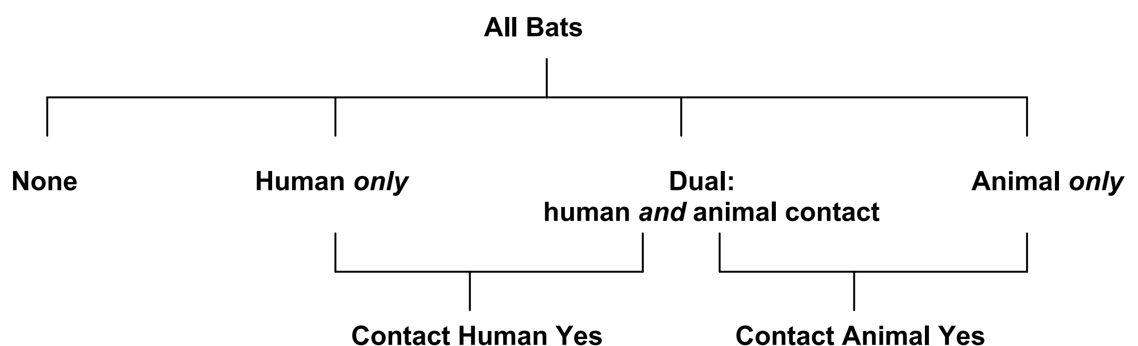
The total number of bats which could have bitten or scratched an animal was the sum of those that had direct contact with an animal (Animal Only) and those that had bitten or scratched a person *and* had contact with an animal (Dual).

The relationship of the contact categories is illustrated in Table 2-1 and Figure 2-2.

Table 2-1 Contact status categories

Contact Human	Contact Animal	Circumstances	Contact Status
No	No	No reported human bite or scratch or animal contact	None
Yes	No	Only human bites or scratches reported	Human only
No	Yes	Only animal bites, scratches or direct contact with animals reported	Animal only
Yes	Yes	One or more persons were bitten or scratched *and* one or more animals had direct contact with the bat	Dual: Human and Animal

Figure 2-2 Contact status categories



Persons bitten or scratched by any bat were considered potentially exposed to Australian bat lyssavirus and referred to the local Public Health Unit of the State Health Authority for medical advice, and where appropriate, post-exposure rabies vaccination. Dogs, cats or other animals known or suspected to have been bitten or scratched by virtue of having contact with a live bat were also considered potentially exposed to ABLV. Neither pre- nor post-exposure vaccination for

lyssaviruses is available for animals in Australia, except as part of preparation for export, typically pets travelling abroad. Animals known or suspected of being in contact with an ABLV-positive bat were managed by the Department of Primary Industries on a case by case basis. Where possible the outcomes for these in-contact animals were determined.

2.2.2 Sample collection

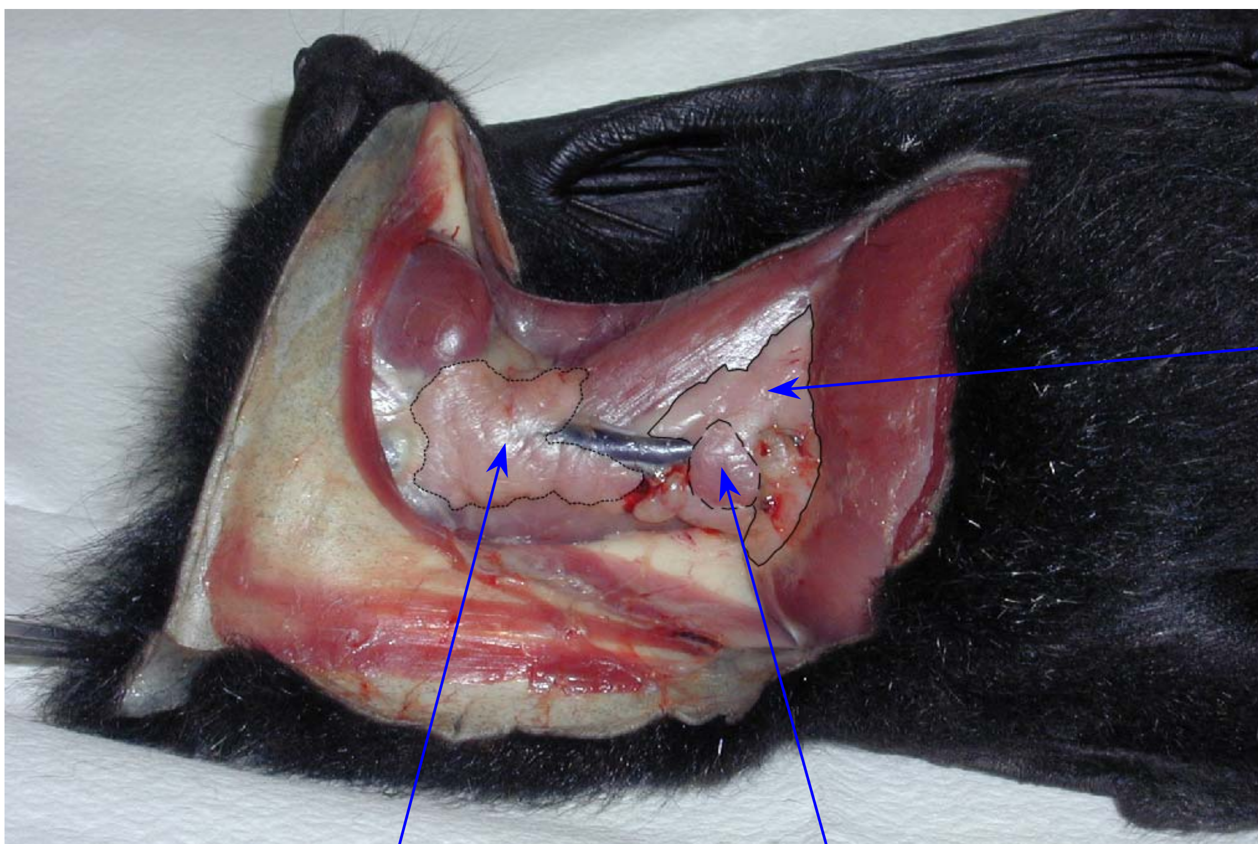
Bats were refrigerated at 4 ^{0}C until examined[1] within a Class II biosafety cabinet within the ARI Diagnostic Virology Laboratory. Prior to September 1998 this was a Physical Containment Level 2 (PC2) laboratory (Australian New Zealand Standard AS/NZS/ 2243.3:1995, 2243.3/Amdt 1/1996, and 2243.3/Amdt 2/1998 in which ABLV is classified as a virus of Risk Group 3, http://www.standards.com.au). In 1998 the ARI laboratory was upgraded to PC Level 3.

The brain was removed and halved longitudinally. One half of each brain was placed in a sterile container and stored fresh at 4 °C for use in the fluorescent antibody test, and subsequently stored at -70°C. The other half of the brain was fixed in 10% buffered neutral formalin for histological examination.

The anatomy of the major salivary glands in Black flying foxes is shown in Figure 2-3. The gross anatomy of these glands is similar in the four common species of Australian flying fox. The gross, histological, and electronmicroscopic morphology of the salivary glands of Black and Grey-headed flying foxes were investigated in detail by another member of the research team (Geisel 1999). The parotid salivary gland from one side was removed and placed in a sterile container and initially stored at 4°C, then transferred to the -70°C freezer. The contra-lateral gland was fixed in 10% buffered neutral formalin. The submandibular and sublingual salivary glands, which occur in close proximity near the clavicle, were removed as a single mass. These two glands from one side were placed together in a sterile container at 4°C and then -70°C, those from the other side fixed in 10% buffered neutral formalin. Other tissues were fixed in 10% buffered neutral formalin as considered appropriate.

[1] most (>90%) bats were examined by a member of the ABLV research team, namely the candidate, Kim Halpin, Craig Smith or Natasha Smith. Occasionally when no member of the team was available, ARI diagnostic veterinary staff examined the bat, in which case only fresh brain for the FAT may have been collected.

Figure 2-3 Anatomy of the major salivary glands of flying foxes (*Pteropus alecto*)
Superficial dissection of the right ventrolateral head and neck. The skin and superficial musculature are reflected dorsally.



Sublingual salivary gland
Pale triangular mucus gland that borders the sternocephalic and pectoral muscles.

Parotid salivary gland
Large pale serous gland that lies immediately caudal to the auditory canal.

Submandibular salivary gland
Round, darker pink, mixed serous and mucous gland. A second larger lobe of this gland lies deep and dorsal to that shown.

2.2.3 Fluorescent antibody test

Centocor FITC (fluorescein isothiocyanate) anti-rabies monoclonal globulin was reconstituted with 5.0 mL of sterile reagent grade water and then further diluted 1:20 in phosphate buffered saline containing 0.75% bovine saline and 0.01% Evan's blue (dilution protocol provided by AAHL). Aliquots of working dilution reagent were prepared by staff of the Diagnostic Virology Laboratory of the Animal Research Institute and stored at -20°C.

Fresh brain touch impressions or brain smears were made on acetone-cleaned glass slides, fixed in acetone at -20°C for a minimum of 1 hour, and then air-dried. The brains were sampled so as to include the hippocampus, medulla and brain stem, usually by making a touch impression of the cut surface following sagittal section of the brain. Sufficient working dilution of the Centocor reagent was applied to cover the impression smear, and the slide incubated within a humidity chamber at room temperature for a minimum of 30 minutes. Excess reagent was washed off with phosphate buffered saline, and the slide cover-slipped with glycerol in 10% phosphate buffered saline. Slides where then examined in a dark room with a florescent microscope using a wavelength of 450 µm. Cellular material was stained reddish by the Evan's blue counterstain. Positive material produced a bright apple-green fluorescence. All positive results were presumed to indicate the presence of Australian bat lyssavirus as Australia is considered free of all other lyssaviruses detected by the Centocor reagent. Most (>90%) of the fluorescent antibody tests were performed by the technical staff of the Diagnostic Virology Laboratory.

2.2.4 Evolution of laboratory submission patterns and protocols

Prior to 1996, Australia was considered free of all lyssaviruses, and lyssavirus (rabies) testing was done on a national basis at the CSIRO Australian Animal Health Laboratory (AAHL), Geelong Victoria, the WHO reference laboratory for rabies in Australia. The number of submissions for rabies/lyssavirus exclusion in Australia was low at approximately 6-8 tests per year, the majority of which were for rabies exclusion following cat or dog deaths in quarantine with only one or two Australian animals tested each year (Ross Lunt AAHL *pers. comm.*).

With the recognition of ABLV and the concurrent research sampling of bats for EMV/Hendra Virus and ABLV it was clear that state based testing in Queensland was required and Barry Rodwell of the Diagnostic Virology Laboratory of the Department of Primary Industries Animal Research Institute (ARI), received training at AAHL to perform the fluorescent antibody test at the then PC2 ARI Virology Laboratory. Samples from bats which were known or suspected of having bitten or scratched a person or animal, had clinical signs suggesting ABLV, were ABLV-positive in the ARI-FAT or where the result was inconclusive, were forwarded to AAHL for confirmation by duplicate

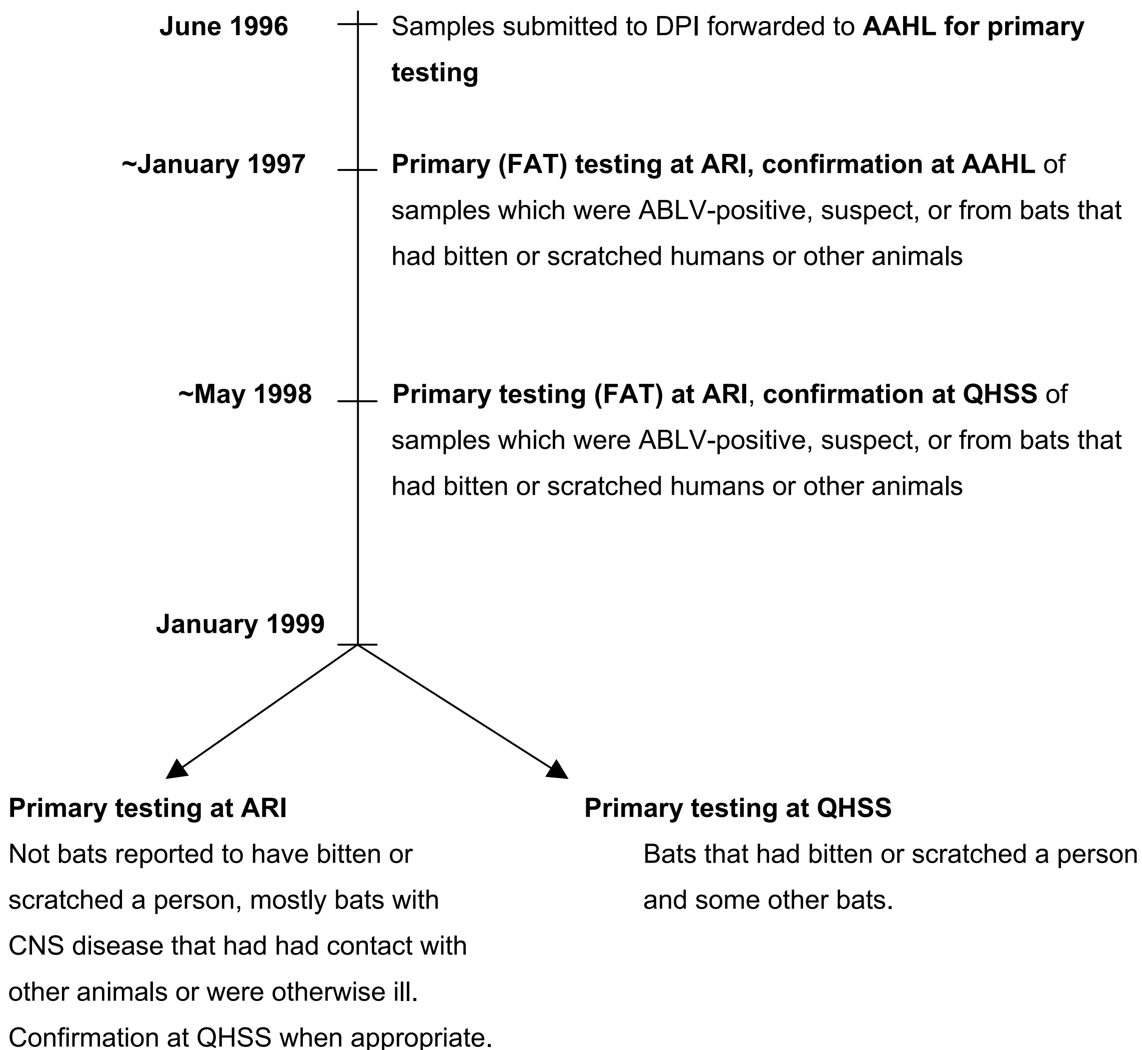
fluorescent antibody testing plus immunoperoxidase staining, virus isolation, and PCR amplification of viral RNA.

Due to the medico-legal implications of the results, the ARI did not report the primary FAT results from bats that had bitten or scratched a person to health authorities. Only after the DPI results had been confirmed at AAHL were the results forwarded to the health authority to be considered in the management of potentially-exposed patients.

However, the time required to submit samples to the ARI, for sampling and initial FAT screening, and then forward them to AAHL for confirmation, resulted in delays, typically of 3-6 days, between the potential exposure of the patient and the results being made known to the health authority. As a result, a large number of patients bitten by ABLV-negative bats that did not require post-exposure vaccination were receiving expensive initial vaccination and immunoglobulin pending the results, and post-exposure treatment of some patients bitten by ABLV-positive bats was delayed by 4-10 days.

During this period Greg Smith of the regional medical diagnostic laboratory (QHSS) in Brisbane developed a diagnostic real-time PCR (TaqMan) test for the pteropid (flying fox) and Yellow-bellied sheathtail bat variants of ABLV (see Appendix 3) and received training in the FAT (from AAHL) and post-mortem sampling (from the candidate). Subsequently either whole bats or a sample from the brains of all bats that had bitten or scratched a person and some ABLV-positive or inconclusive samples were forwarded to the QHSS rather than AAHL for confirmation. Medico-legal implications and NATA (National Association of Testing Authorities) protocols for sample handling meant that by January 1999 all bats which had bitten or scratched a person were sent directly to the QHSS and no longer tested at ARI. A time line illustrating the evolution of the submission and testing processes for ABLV in Queensland is shown in Figure 2-4.

Figure 2-4 Evolution of the submission and testing processes for ABLV testing in Queensland



2.2.5 Opportunistic samples from ABLV-positive bats tested in collaboration with other institutions

When available, serum/plasma usually supplied by the submitting veterinarian or occasionally collected at post-mortem, was submitted to AAHL for rabies-rapid-fluorescent-focus-inhibition tests (modified rabies (CVS)-RFFIT, see Appendix 2).

Urine collected from a small number of bats at post-mortem and a sample of saliva supplied by a submitting wildlife carer were submitted to QHSS for detection of ABLV viral-RNA by TaqMan® assay (see Appendix 3).

2.2.6 Statistical methods

Crude analysis

Differences in proportions of ABLV-positives between major groupings, ignoring other factors, were tested for statistical significance using the chi-square test.

Association with factors (wild flying foxes)

For wild flying foxes, the relationships between proportion of ABLV-positives and factor categories were tested for statistical significance by fitting generalized linear models (McCullagh and Nelder 1989; Tukey 1977). The models used the binomial distribution and logit link. For models with more than one factor, the association with each factor was tested after accounting for the associations with all the other terms in the model.

Preliminary analyses showed highly significant associations with species, health status and age. Associations with other terms were therefore tested in models with these three terms already included. Factors considered were sex, contact status, region, year, season, and reproductive season and all possible interactions between species, health status and age. Generally the terms were tested one at a time, though the interactions were tested jointly and models with both year and climatic season, and year and reproductive season were also considered.

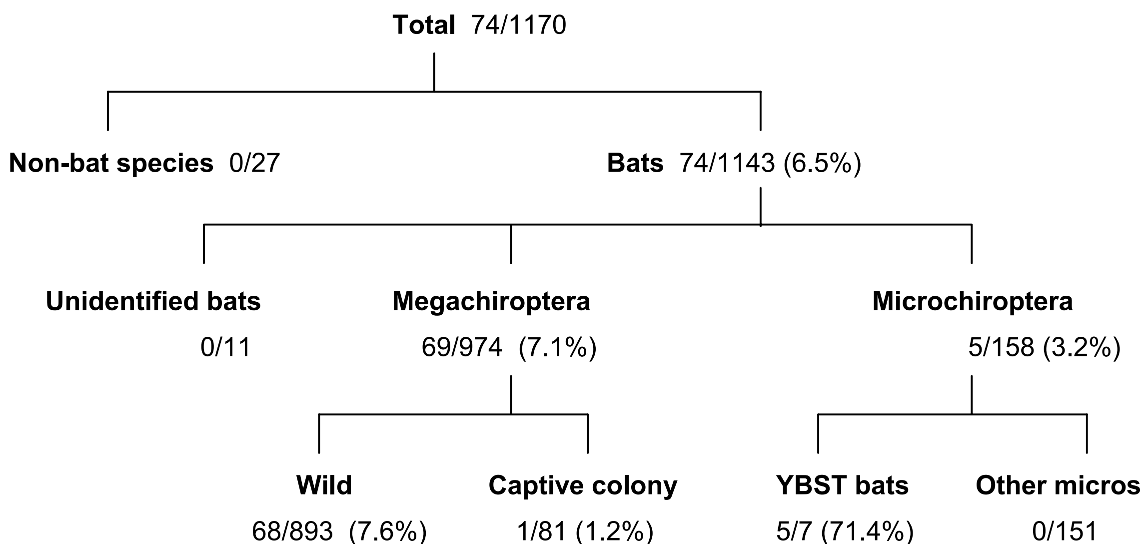
For the significant factors, estimates for each of the categories were calculated from the fitted model and differences between categories tested for significance on a pair-wise basis. The estimates were back-transformed to the percentage scale for presentation.

The statistical package GenStat 2000 was used for all analyses under the direction of Tony Swain (biometrician, ARI).

2.3 Results

A total of 1,170 diagnostic submissions for lyssavirus testing were submitted from Queensland between June 1996 and 31 March 2002. Of these 27 were non-bat species, and 1,143 were bats. Zero of the 27 submissions of non-bat species and 74/1143 (6.5%) of bat submissions were ABLV-positive. Numbers of ABLV-positive / total tested for relevant categories of submissions are shown in Figure 2-5.

Figure 2-5 Crude summary of proportions of ABLV-positive / tested diagnostic submissions
Queensland, June 1996 to 31 March 2002.



YBST bat – Yellow-bellied sheathtail bat (*Saccolaimus flaviventris*)

Wild Megachiroptera (n=68/893)

P. alecto (Black)	37/474	(7.8%)
P. poliocephalus (Grey)	8/175	(4.6%)
P. scapulatus (Little Red)	21/124	(16.9%)
P. conspicillatus (Spectacled)	1/95	(1.0%)
Unidentified *Pteropus* sp.	1/17	(5.9%)
Nyctimene spp. (Tube-nosed bat)	0/2	(0 %)
Syconycteris spp. (Blossom bat)	0/6	(0 %)

2.3.1 Crude analysis

The difference in percent ABLV-positive between the following pairs of categories, ignoring all other factors, was tested using a Chi-square test.

Megachiroptera (n=69/974)		
Microchiroptera (n=5/158)	Not statistically significant	($p > 0.05$)
Wild megachiroptera (n=68/893)		
Colony megachiroptera (n=1/81)	Wild significantly higher	($p < 0.05$)
YBST bat (n=5/7)		
Other micros (n=0/151)	YBST significantly higher	($p < 0.001$)
Megachiroptera (n=69/974)		
YBST bat (n=5/7)	YBST significantly higher	($p < 0.001$)
Wild megachiroptera (n=68/893)		
YBST bat (n=5/7)	YBST significantly higher	($p < 0.001$)

2.3.1.1 Non-bat species submissions

Twenty seven non-bat-species submissions were tested for ABLV. The majority of these cases were examined by diagnositic pathologists of the ARI. These were:

- 5 domestic dogs
- 5 domestic cats
- 2 domestic horses
- 3 domestic ferrets
- 7 wild possums
- 3 wild foxes
- 1 captive Tasmanian Devil
- 1 captive Macaque monkey

None of these animals were ABLV-positive. Two of the dogs and a fox were examined by the candidate. The remainder were examined by ARI diagnostic veterinary pathologists. Case details are provided in Appendix 4.

Most (n=19) were submitted because they had clinical signs of central nervous system disease and were suspected of having ABLV. Five of these animals had also bitten or scratched one or more persons and required testing to determine whether post-exposure vaccination of these persons was indicated.

Two dogs from the same household were submitted because they had been in contact with a bat confirmed as ABLV-positive. These two dogs and a fox were examined by the candidate.

The three unregistered ferrets seized by the Department of Natural Resources were tested because the origin/s of the ferrets was unclear and there was the possibility of them having been illegally imported from a rabies endemic country.

The three submitted foxes had neither signs suggesting central nervous system disease nor histories of having bitten or scratched humans or other animals, but were sick (n=1), injured (n=1) or orphaned (n=1). These animals were presumably submitted because foxes are well recognized as hosts of rabies and it is believed that foxes might be a likely non-bat-species host of ABLV.

2.3.1.2 Unidentified bats, other micros and unidentified Pteropus sp.

Eleven submitted bats were identified only as “bats”, with no indication by the submitter or recipient lab as to species or even order of bat.

Of the 158 submissions of microchiroptera only 5/7 Yellow-bellied sheathtail bats (*Saccolaimus flaviventris*) were ABLV-positive. The 151 other microchiropteran submissions were:

- *Chalinobus* spp. n = 13
- *Miniopterus* spp. n = 23
- *Momopterus* spp. n = 18
- *Myotis* spp. n = 2
- *Nyctinomus* sp. n = 1
- *Nyctophilus* spp. n = 23
- *Pipistrellus* sp. n = 1
- *Rhinolophidae* sp. n = 1
  Scotophilus sp. n = 1
- *Scotorepens* spp. n = 22
- *Taphozous* spp. n = 2
- *Vespadelus* spp. n = 2
- Unidentified microchiropteran n = 42

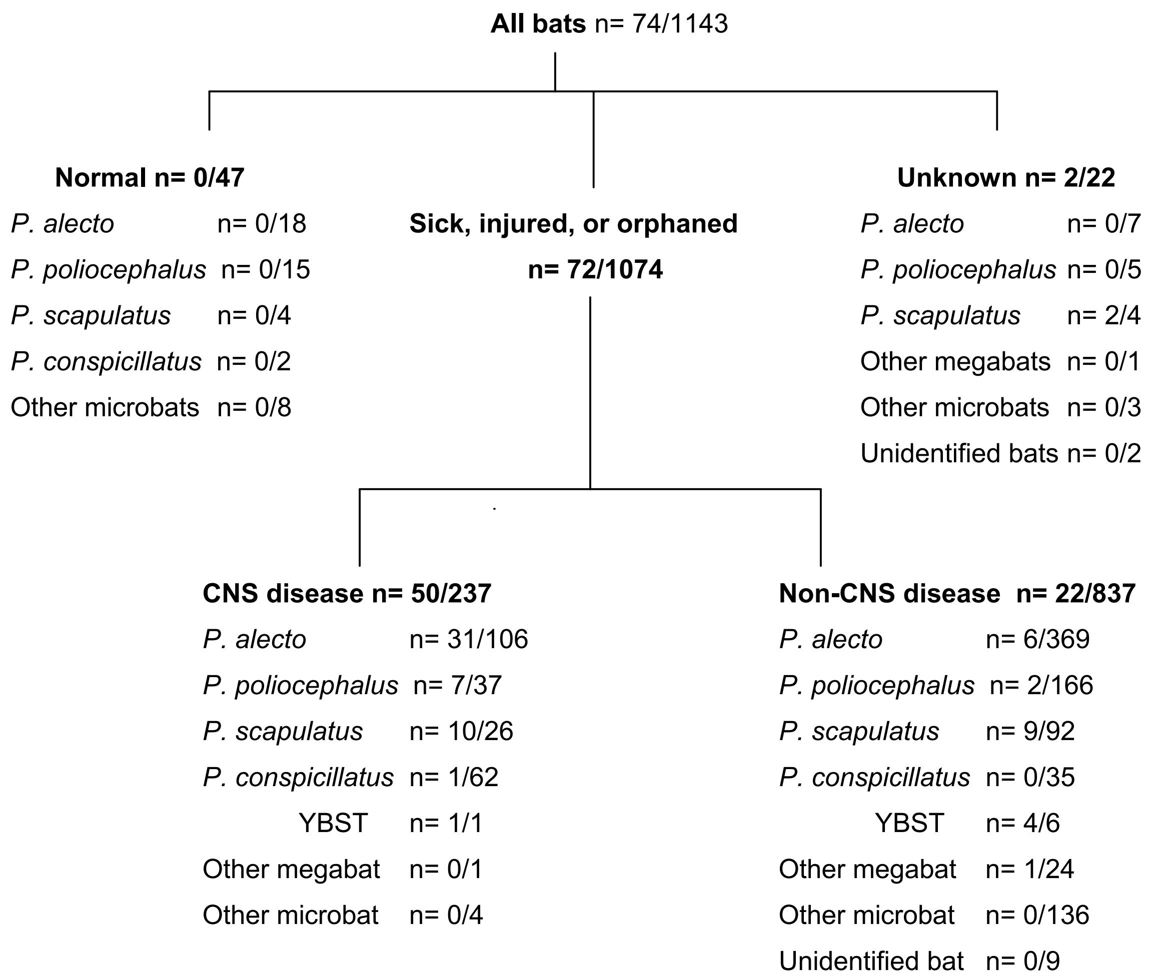
Seventeen submitted wild bats were identified by the submitter/recipient laboratory only as “flying foxes”, with no indication as to the specific flying fox species.

2.3.2 Health status

For each health category and relevant species categories, the numbers of ABLV-positive / total tested bats are shown in Figure 2-6.

Note: in Figure 2-6, Figure 2-7, and Figure 2-8 the term 'other megabat' refers to unidentified *Pteropus* sp. and non-flying fox megachiroptera (*Nyctimene* spp. and *Syconycteris* sp). The term 'other microbat' refers to all microchiropterans not identified as Yellow-bellied sheathtail bats, as listed in Section 2.3.1.2, including unidentified microchiropterans.

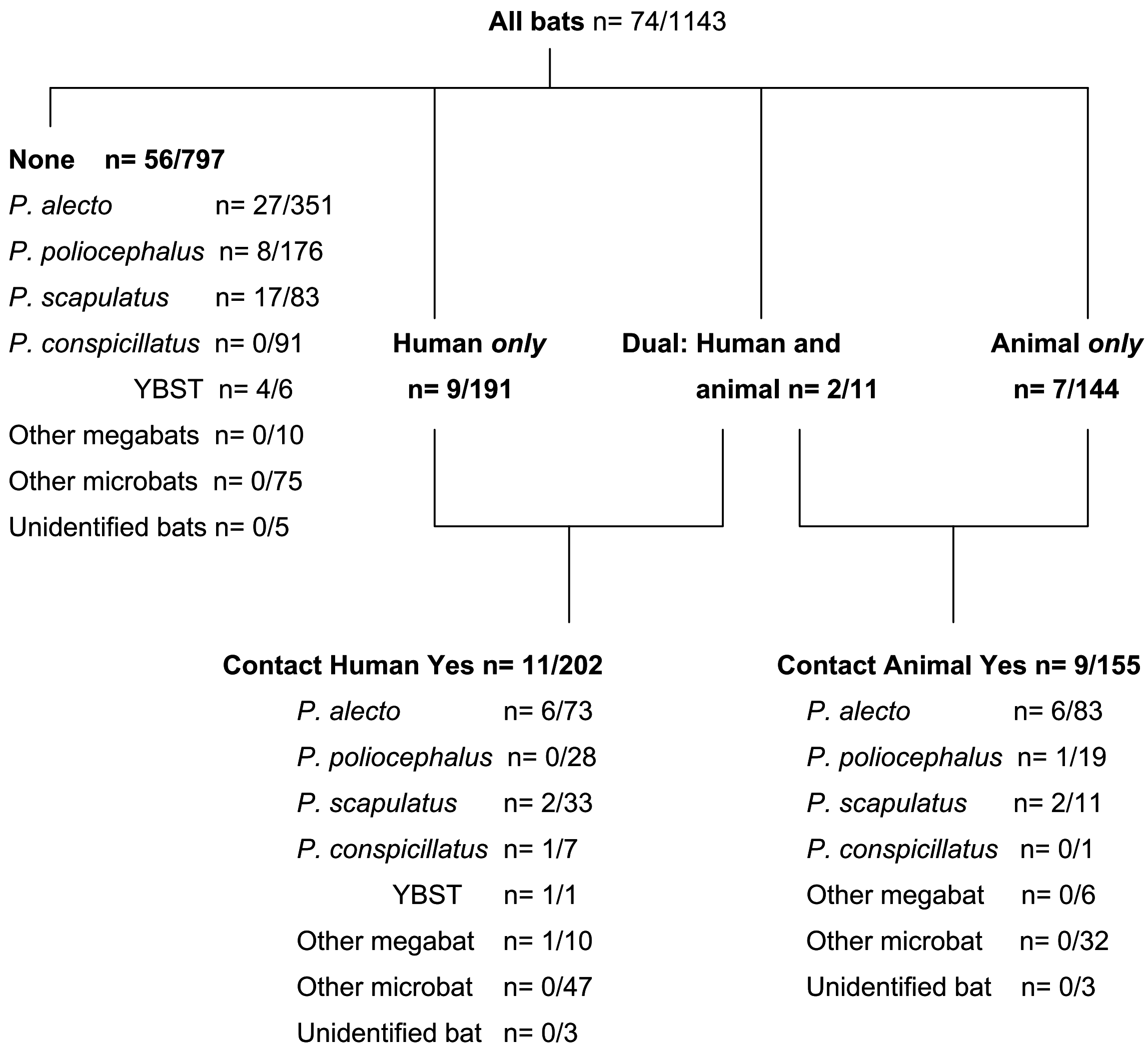
Figure 2-6 Crude summary of the number of ABLV-positive / total tested bats classified by health status and relevant species categories



2.3.3 Contact status

For each contact category and relevant species categories, the numbers of ABLV-positive / total tested bats are shown in Figure 2-7.

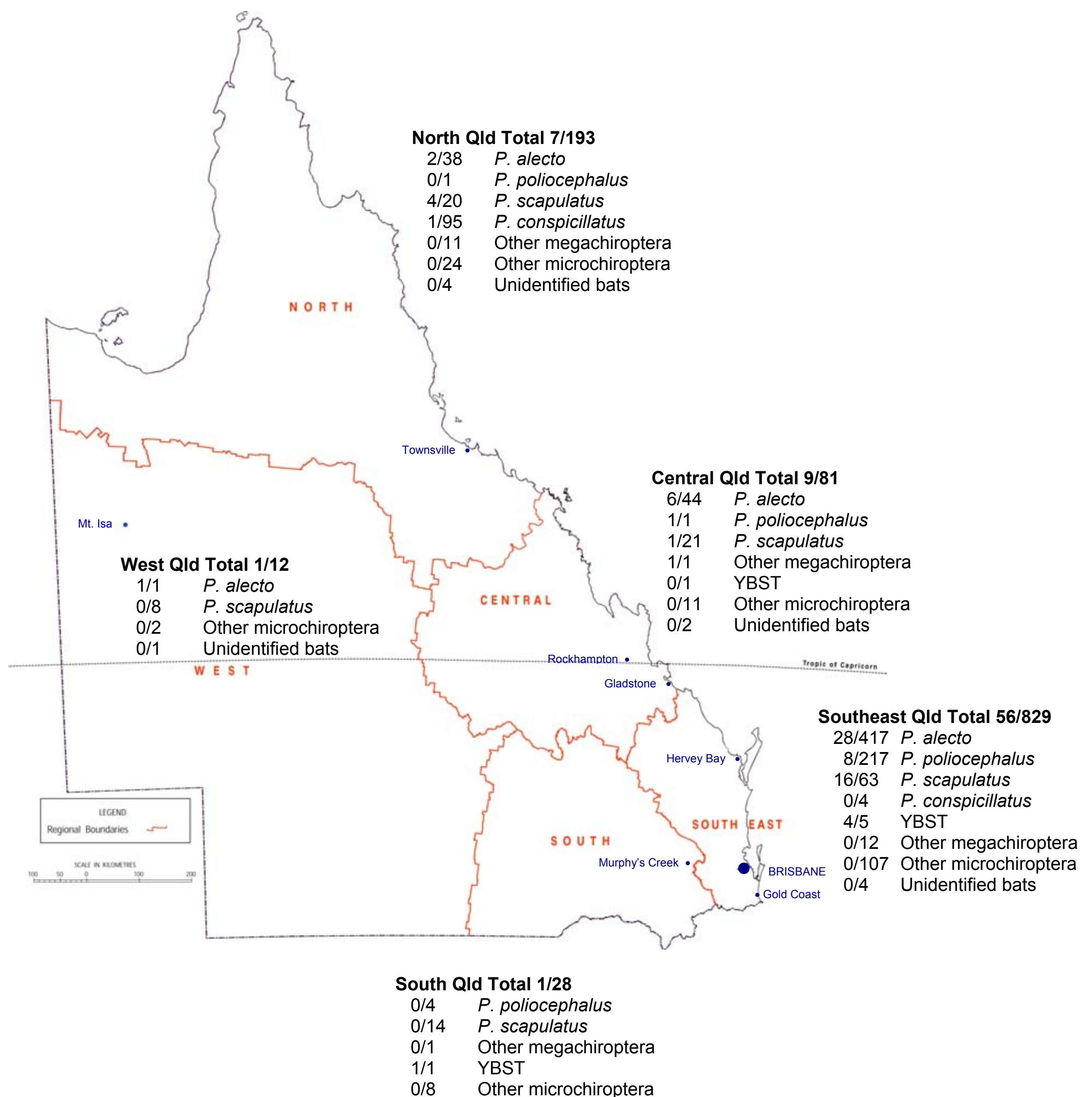
Figure 2-7 Crude summary of the number of ABLV-positive / total tested bats classified by contact status and relevant species categories



2.3.4 Geographical distribution of bat submissions

Bats were accepted from throughout Queensland; however the vast majority (n=>1,090/1,143) were submitted from the coastal strip extending south from Cape Tribulation that corresponds to an area of high population density for humans and flying foxes. The largest number of bats was submitted from South-east Queensland (n=828), an area inhabited by Black, Grey and Little Red flying foxes and numerous species of microchiroptera. Southeast Queensland is highly urbanized and houses more than 70% of Queensland's human population. The geographical distribution of bat submissions is illustrated in Figure 2-8.

Figure 2-8 Geographical distribution of ABLV-positive / total bat submission (n=1,143)



2.3.5 Temporal distribution of bat submissions

There was a noticeably higher rate of bat submission in the early years following the recognition of ABLV. This was a period when wildlife carers were highly motivated to submit bats for testing in order to contribute to an understanding of the disease, and of high to intense media interest. From 1 January 1999, a proportion of bats from Queensland were being submitted directly to QHSS rather than the ARI. However, even when the submissions to both laboratories are combined (see Table 2-2, the number of bats submitted in 2000 and 2001 was noticeably less than in late 1996, 1997 or 1998.

The yearly pattern of bat submission is illustrated in Figure 2-9 and Table 2-2.

Figure 2-9 Yearly pattern of ABLV-positive (n=74) and total (n=1,143) bat submissions

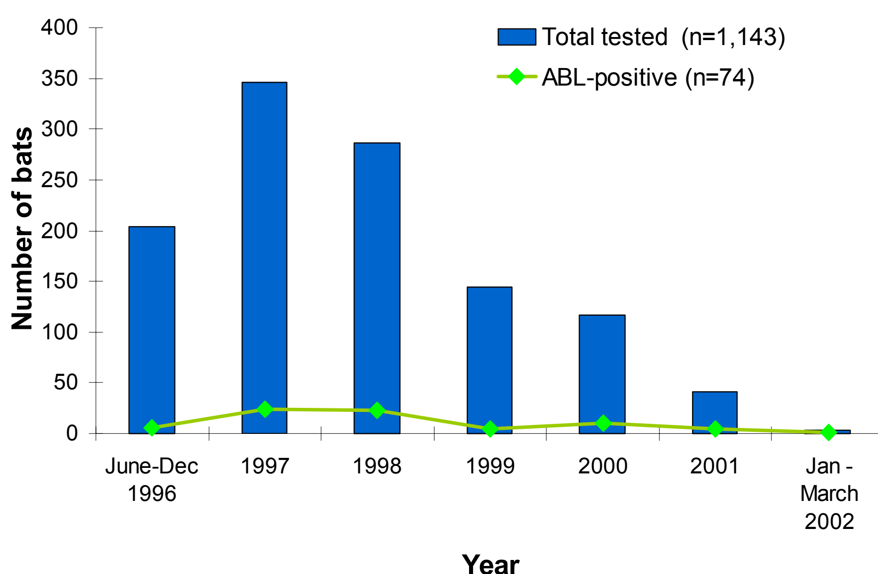


Table 2-2 Yearly pattern of ABLV-positive and total bat submissions

	June-Dec 1996	1997	1998	1999	2000	2001	Jan-March 2002	Total
ABLV-positive	6	24	23	5	10	5 (+3)	1 (+2)	74 (+5)
Total	204	346	287 (+15)	144 (+67)	117 (+62)	41 (+59)	4 (+21)	1143 (+224)

() numbers in brackets are additional bat submissions tested at QHSS, not tested at ARI and not otherwise included in this study. The total number of bat submissions in Queensland during this period was 79 ABLV-positive of 1367 tested.

2.3.5.1 Monthly submissions

The accumulated monthly numbers of ABLV-positive (n=74) and total (n=1143) bat submissions are shown in Figure 2-10 and Table 2-3.

Figure 2-10 Accumulated monthly ABLV-positive (n=74) and total (n=1143) bat submissions (June 1996 to March 2002)

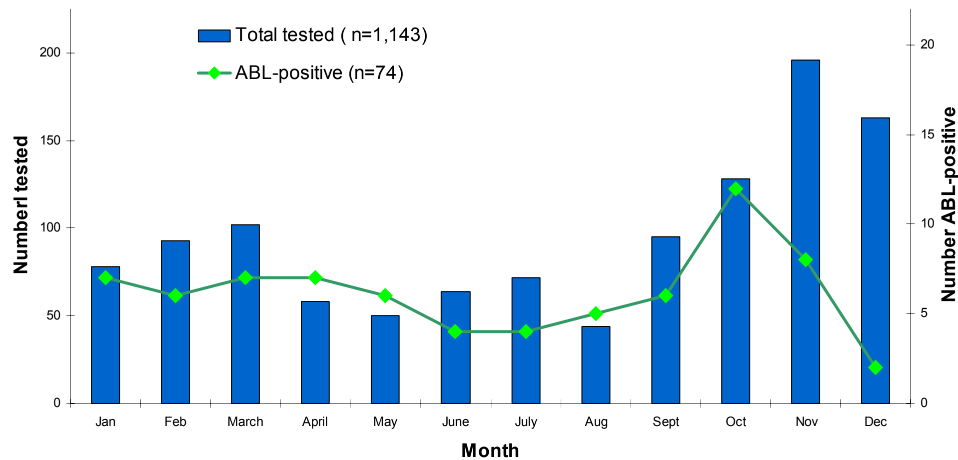


Table 2-3 Accumulated monthly ABLV-positive (n=74) and total (n=1,143) bat submissions (June 1996 to March 2002)

	Jan	Feb	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec
ABLV-pos.	7	6	7	7	6	4	4	5	6	12	8	2
Total	78	93	102	58	50	64	72	44	95	128	196	163

2.3.5.2 Reproductive seasons

The reproductive seasons of bats vary. For flying foxes the year consists of three principal reproductive seasons, although occasional matings and births occur year round.

- **Establishing territories and mating**. During this period testicle size and testosterone levels increase to their maximum, males establish and defend territories and mating occurs. This is a period of comparatively high intra-colony aggression.
- **“Off” season** during which testicular size, testosterone levels and sperm production rapidly decrease, accessory sex glands become non-secretory, and most adult females are pregnant.
- **Giving birth and raising young.** Births occur after a pregnancy of approximately 6 months, followed by a lactation of about 6 weeks. For at least 4 weeks, pups cling to their dam, including during flight. As pups become too large they are left behind at night while the dam forages, and by February/March are usually weaned and fully independent. During this period males usually congregate together in areas of the camp separate from the dams and pups.

Black, Grey-headed and Spectacled flying foxes generally establish territories and mate in January to April, are pregnant in May to August, and give birth and raise young during September to December. For the Little Red flying fox these reproductive seasons are offset by approximately 6 months.

The date of submission for all flying foxes that had been identified to species (n= 868) was translated into one of the three reproductive seasons as shown in Table 2-4.

Table 2-4 Reproductive seasons of flying foxes

Reproductive season	Months of the year submitted	
	Black, Grey-headed and Spectacled flying foxes	Little Red flying fox
Establishing territories and mating	January to April	August to November
“Off” season	May to August	December to March
Giving birth and raising young	September to December	April to July

The total number of flying foxes and the number that were ABLV-positive for each reproductive season are shown in Figure 2-11 and Table 2-5.

Figure 2-11 Number of ABLV-positive (n=67) and total (n=868) wild Black, Grey-headed, Little Red, and Spectacled flying foxes submitted in each reproductive season (June 1996 to March 2002)

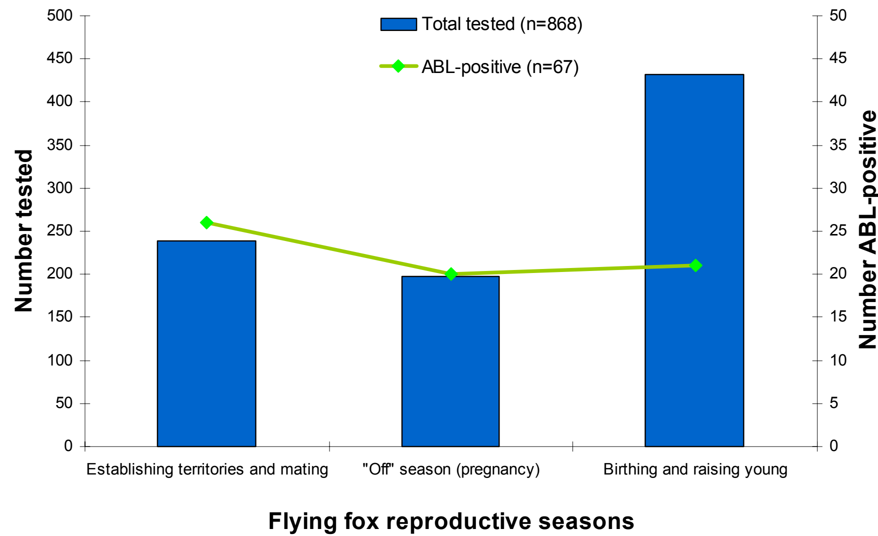


Table 2-5 Number of ABLV-positive (n=67) and total (n=868) wild Black, Grey-headed, Little Red, and Spectacled flying foxes submitted in each reproductive season (June 1996 to March 2001)

	Establishing territories and mating	"Off" season	Giving birth and raising young
ABLV-positive	26	20	21
Total	238	198	432

2.4 Statistical analysis

The following relates to all wild flying foxes that had been identified to species, i.e. Black, Grey-headed, Little Red, and Spectacled flying foxes (n = 67/ 868).

Other categories of bats were excluded because key genus and/or species data was unknown or the numbers of submissions within each species were too small for statistical analysis. Captive colony bats were excluded because it was presumed that the factors affecting exposure and susceptibility in captivity could be different from those affecting wild bats.

Bats that were excluded were; unidentified bats (n =11), each genus of microchiropteran bat (n =1 to 23, and 42 unidentified), each species of captive colony megachiropterans (n = 4 to 48), unidentified wild *Pteropus* spp. (n=18), and non-flying fox wild megachiropterans (*Nyctimene* spp n=2 and *Syconycteris* sp n=6).

2.4.1 Significant factors

Three factors were associated with statistically significant differences in the proportion of ABLV-positive bats, each after taking the other two factors into account (p = conditional probability).

- **Species** $p < 0.001$
- **Health status** $p < 0.001$
- **Age** $p < 0.01$

The ABLV-positive percent predictions (%predns), taking species, health and age into account and assuming the proportions in each category are the same as in the original data, are shown below.

Species	**%predns**
P. alecto	4.6
P. poliocephalus	2.3
P. scapulatus	11.4
P. conspicillatus	0.2

In terms of statistically different proportions of ABLV-positive wild flying fox per species (p=0.05):

P. scapulatus* > *P. alecto* ≈ *P poliocephalus* > *P. conspicillatus

Health	**%predns**
Normal	0.03
CNS disease	19.3
Non-CNS disease	1.8
Health unknown	3.9

In terms of statistically different proportions of ABLV-positive wild flying foxes per health status (p=0.05):

CNS > non-CNS

The proportions of ABLV-positive wild flying foxes with normal or unknown health status were not significantly different from those with CNS or non-CNS disease health status. In the case of bats with a normal health status this was principally due to the small numbers and this group (n=9) and the associated large standard error.

Age	**%predns**
Juvenile	1.7
Adult	4.9
Age unknown	2.5

In terms of statically different proportions of ABLV-positive wild flying foxes per age group (p=0.05):

Adults > Juveniles

The proportion of ABLV-positive wild flying foxes of unknown age was not significantly different from either adult or juvenile bats.

2.4.2 Insignificant factors

Other factors, or combinations of factors listed below, when taken into account with species, health status, and age, were not associated with statistically significant differences in the proportion of ABLV-positive bats (conditional probability $p > 0.05$).
For example; sex, when taken into account with species, health status, and age did not have a statistically significant effect, $p = 0.54$.

- Sex (p = 0.54)
- Contact status (Contact Human Yes p = 0.89, Contact Animal Yes p = 0.92)
- Region (North, West, Central, South and Southeast Qld, p = 0.67)
- Region (Southeast Qld versus rest of Queensland, p = 0.44)
- Year of submission (p= 0.053)
- Season of submission (Summer, Autumn, Winter, Spring, p = 0.13)
- Reproductive season of submission (p=0.15)
- Year and Season (p=0.15 and p=0.39 respectively)
- Year and Reproductive season (p=0.85 and p=0.27 respectively)

2.4.3 Interactions

There were no statistically significant interactions between the three separately significant factors of species, health and age (p = 0.714 to 0.996 for all possible interactions).

2.4.4 Fitted values for percent ABLV-positive for species, health status and age combinations

Where the species, health status, and age of a wild flying fox are known, an estimate of the likelihood of that bat being ABLV-positive is given in Table 2-6, assuming the same submission pattern continues as occurred during this study.

Table 2-6 Fitted values (percent ABLV-positive) and raw data (ABLV-positive/total tested) for wild flying fox species, health status, and age combinations

Species	Age	CNS disease		Non-CNS disease	
		Fitted %	Raw data	Fitted %	Raw data
P. alecto	**Juvenile**	15.4	7/35	1.4	1/137
	Adult	34.9	22/56	3.9	4/187
	Unknown	---	2/11	---	1/32
P. poliocephalus	**Juvenile**	8.2	0/9	0.7	1/52
	Adult	20.8	6/24	1.9	1/73
P. scapulatus	**Juvenile**	32.7	1/3	3.5	0/15
	Adult	58.8	8/16	9.7	6/43
	Unknown	---	1/7	---	3/33
P. conspicillatus	**Juvenile**	0.6	0/10	0.1	0/13
	Adult	1.9	0/48	0.1	0/14
	Unknown	---	1/2	---	0/8

All species and age combinations of wild flying foxes with a normal health status had a fitted value for percent ABLV-positive of < 0.2%.
Juvenile sexually immature, typically 0 to 3 years of age
Adult sexually mature, typically > 3 years of age

2.5 ABLV-positive case data

Case details for each of the 74 cases of ABLV-positive bat are given in Appendix 5, including; species, month died, location found, sex, age, health and contact status, together with a summary of the history, post-mortem examination findings and other comments. Case histories were derived from the original submission forms, and where possible, phone conversations with the people involved. Subsequent references to individual cases are by ABLV case number, e.g. ABLV-1, ABLV-2, as indicated in Appendix 5.

2.5.1 Circumstances in which ABLV-positive bats are found

The circumstances in which 59/74 ABLV-positive bats were found were recorded, or recalled during follow-up phone discussions.

- **38 were found on the ground**, often under trees in places that included; backyards and gardens, grounds of wildlife parks, cleared bushland, a garden nursery, beside the road, in bamboo by a pond, school grounds, caravan parks and city footpaths. ABLV-1, 3, 5, 7, 8, 18, 19, 23, 24, 26, 27, 29, 30, 33, 35, 38 –47, 50, 53, 54, 57, 61-65, 69-71, 74.

- **16 were found in trees, away from camp during the day**, often hanging inappropriately low (< 2 m) from the ground. Normal flying fox behaviour is to return to camp in the early hours of the morning and hang as high as their rank in the colony and the trees will allow, typically > 10 m from the ground. ABLV-4, 6, 13, 28, 32, 48, 49, 51-53, 55, 58, 59, 60, 68, 72.

  Of the 16 ABLV-positive bats initially seen in trees during the day, ABLV-13 was later seen to fly into the path of an oncoming car, ABLV-48, 60, and 72 later fell from the tree, ABLV-49 and ABLV-51 flew from the tree to land on or fly towards a person in what was described by the people as an unprovoked attack, and ABLV-52 and 53 uncharacteristically and repeatedly "ran" / came towards and away from the person who had climbed the tree to rescue them.

- **3 were found on man-made structures, away from camp during the day**, one hanging on the ropes of playground equipment (ABLV-10), one on the roof of a captive bat colony enclosure (ABLV-66), and one on the roof of a house (ABLV-67).

- **1 was found in a canal** (ABLV-17).

- **1 captive colony flying fox became ill within its enclosure** (ABLV-73), one month after a wild ABLV-positive flying fox (ABLV-66) had been removed from the roof of the colony's enclosure.

None of at least 89 bats rescued from entrapment in barb wire or other fences were ABLV-positive.

2.5.2 Clinical signs of ABLV infection

The clinical signs reported by submitters, most of whom were non-professional wildlife carers, are given in more detail in Appendix 5. In summary:

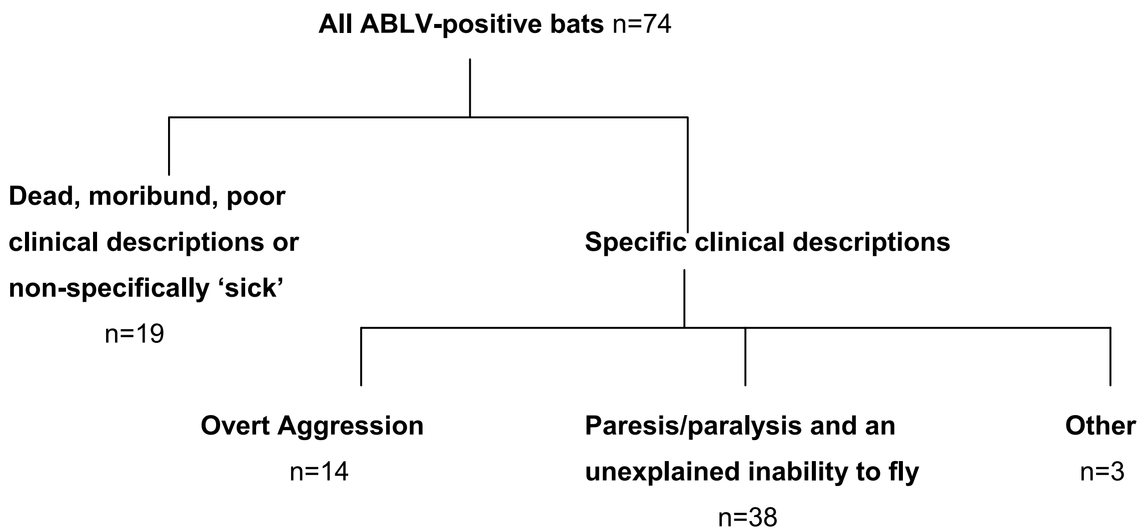
- **38 clinical descriptions were dominated by paresis/paralysis, usually of the hind limbs or generalized paresis, and an inability to fly**. Often these bats were unable to hang properly, and either initially or eventually were recumbent and required support such as a sling. Some bats that could hang were unable to invert to urinate.

- **14 clinical descriptions were dominated by overt aggression,** ABLV-13, 25, 32, 49, 51, 52, 53, 60, 64, 66, 67, 68, 70, 73. This aggression manifested as; flying out of trees in unprovoked attacks on people and a dog (ABLV-49, 51, 64), advancing towards and attempting to bite, rather than retreating from, rescuers during rescue e.g. from trees (ABLV-52, 53, 64, 66), aggression towards cage-mate bats (ABLV-32 and 52), vocalizing and appearing “angry”, agitated, and unable to calm down when placed in a small covered cage in a separate dark room (ABLV-13, 25, 53, 68, 73), repeated aggressive rather than defensive attempts to bite rescuers/carers, even from within a cage (ABLV-60, 64, 66), attacking inanimate objects within a cage or the cage itself (ABLV-32, 60, 64, 67, 68, 70, 73), self mutilation including biting itself (ABLV-67, 70), and head butting a tree and fracturing teeth when biting cage and banging itself against a cage (ABLV-68)

- **19 were either dead** (n=1, ABLV-19), **moribund** (n=7, ABLV-17, 20, 23, 31, 39, 45, 48)**, or were submitted with inadequate or non-specific clinical descriptions** (n=11; ABLV-1, 2, 7, 15, 16, 21, 34, 41, 47, 56, and 61)

- **3 had specific clinical signs or injuries suggesting non-lyssavirus disease.** One Yellow-bellied sheathtail bat (ABLV-5) aborted a foetus shortly after rescue, ABLV-11 was submitted with a one word history of “electrocution”, and ABLV-57 had clear and obvious linear burns to both feet and necrosis of the left thumb, consistent with having received an electric shock.

Figure 2-12 Clinical forms of ABLV-infection in bats



Other clinical signs included;

- **Abnormalities relating to the mouth/pharynx suggesting cranial nerve deficits** (n=11). These were described as; hypersalivation, inability to swallow (n=3), inability to spit out fruit pulp, saliva pooling in mouth, choking, difficulty eating, poking tongue in and out, grinding teeth, tooth bitten through lip, having bit own tongue while drinking, excessive yawning (n=2), and abnormally harsh voice.

- **Clinical signs suggesting CNS or cranial nerve deficits** (n=13). These were described as: "fitting"/ seizures, twitching, shaking and tremors, head tremors (n=2), twitching of head and ears with clamped teeth, eyes "wavering" (nystagmus n=1), reduced corneal, "sneeze" and gag reflexes, reduced blink reflexes, and reports of glazed eyes and dazed appearance.

- **Altered/ difficult respiration** was reported in five cases. On one occasion (ABLV-72), the respiratory changes of a weak and deteriorating bat taken to a veterinarian were thought to indicate pneumonia, no lung lesions were found in the post-mortem examination.

- **Altered appetite** Bats were recorded as not eating (n=4), being able to eat / lap juice (n=3), and also to be ravenous (n=2).

2.5.2.1 Clinical recognition of ABLV

It is necessary for wildlife veterinarians to make some sort of assessment as to whether bats brought to them by carers are sufficiently likely to have ABLV that they should be killed and tested rather than treated. Unfortunately none of the circumstances or clinical signs described above is unique to ABLV (see Chapter 3 and Appendix 6 for clinical descriptions of bats with non-lyssavirus CNS disease). Some ABLV-positive bats have more '*typical'* or specific clinical signs suggesting involvement of the CNS and are more readily recognized as likely to have ABLV than others.

The types of clinical signs seen in ABLV-positive bats, and an assessment of how readily ABLV-positive bats with those clinical signs are recognized are given in Table 2-7.

Table 2-7 Clinical recognition of bats likely to be ABLV-positive

Clinical signs of ABLV-positive bats	Ease of recognition	Comments
Dead or moribund	Difficult	Indistinguishable from other end-stage bats.
Poor or non-specific clinical description	Difficult	The number of bats in this category can be minimized if veterinarians make detailed and specific inquires as to the bats physical and neurological abilities.
Overt aggression	High index of suspicion	Wild bats are generally not aggressive. Most bat bites and scratches are clearly defensive. Any suggestion by experienced wildlife carers that a bat is uncharacteristically aggressive or 'angry' should be considered *very* seriously. These bats are particularly likely to attempt to bite or scratch people and pose a high risk of human infection. Both human lyssavirus deaths involved aggressive bats.
Paresis, paralysis of hind-limbs	High index of suspicion	While there are other causes of paresis (see Chapter 3), virtually none have a good prognosis and all bats that have hind-limb paresis and / or are unable to hang should be submitted for ABLV testing.
Generalized paresis	High index of suspicion	Bats found with unexplained generalized weakness, or with progressive weakness should be submitted for ABLV-testing.
Abnormal function of mouth / larynx	High index of suspicion	These clinical signs are uncommon in ABLV-negative bats (data not shown). All bats with a history that includes difficulty swallowing, abnormal salivation or tongue movements, odd yawning etc. should be submitted for testing.
"Fitting", twitching and tremors	High index of suspicion	All bats showing evidence of CNS and / or cranial nerve involvement should be submitted for ABLV-testing.
History or clinical signs suggest a specific, non-ABLV diagnosis	Low index of suspicion	Perhaps the most difficult ABLV-bat to recognize. Many of the >90% of bats that are ABLV-negative have histories and clinical signs unlike those of most ABLV-bats, such as being caught in barb-wire fences, electrical burns, being found as orphans, etc, which suggest a specific, non-ABLV reason for rescue. ABLV-bats 'masquerading' as one of the common ABLV-negative bats are likely to not be recognized.
Weak bat with respiratory difficulties	Difficult	The presence or development of respiratory abnormalities in a bat with lyssavirus-like weakness should not reduce the otherwise high index of suspicion for ABLV.
Yellow-bellied sheathtail bats	High index of suspicion	Due to the extraordinarily high prevalence of ABLV in this species (71%), and the difficulty associated with safely examining this small animal, all sick, injured, or orphaned YBST bats should be submitted for ABLV-testing.

2.5.3 Post-mortem findings in ABLV-positive bats

At gross post-mortem examination, a dead ABLV-positive bat is usually unremarkable and so indistinguishable from bats dead of other causes.

- No post-mortem examination comment was recorded for 27/74 bats.
- 9 bats were recorded as having no visible lesions.
- The nutritional condition of 20/74 ABLV-positive bats was recorded as poor/lean with little body fat, moderate to good with some but not large fat reserves in 10/74 bats, and one bat was considered fat.
- **7 bats had post-mortem evidence of blunt trauma** which was not previously detected. This included; subcutaneous bruising of the head and neck (n=6; ABLV-13, 28, 45, 51, 55, 74) including bruising over the eye, temporal muscle and underlying cranium, and over the frontal bone, fractures of a rib (ABLV-55) and the frontal process of the zygomatic arch (ABLV-74), bruising of the thorax and lung (n=1), and haemoperitoneum (ABLV-17). These injuries are presumed to be secondary to ABLV-infection, e.g. as a result of falling or crashing. ABLV-13 was observed to have flown into a car.
- **3 bats had small puncture wounds and associated soft tissue damage that could be related to bat, cat or small dog attacks**. ABLV-48 had wing and pectoral muscle injuries and was thought by the carer to have a fractured wing, however no fracture was found. This bat was known to have fallen at least once from a tree. ABLV-52 was found with punctures, bruising and swelling of the right arm and was later found attacking another rescued bat, and so may have been involved in earlier bat fights. ABLV-55 had bruising to the frontal bone, a fractured rib and multiple punctures to the back, pectorals and abdomen with a history of being treated with 20 mL of subcutaneous fluids. It was presumed the head and rib injuries could be the result of a fall and the punctures the result of s/c fluid administration, however a bat, cat or small dog could have been involved. It should be noted that other ABLV-negative bats, submitted after known dog attacks, usually had far more significant injuries than those seen in these bats such as gross disruption/avulsion of limbs and body cavities.
- **4 bats had distended bladders containing urine suggesting visceral paresis** (ABLV-18, 35, 37, 65). The bladders of most bats are empty at post-mortem (data not shown). Bladders continuing urine were most often associated with evidence of neurological dysfunction e.g. ABLV and spinal injuries, see Chapter 3.
- **3 bats had food in their mouth/trachea suggesting dysphagia**
(ABLV-33, 35, 41)

- **2 bats had injuries suggesting an electric shock from over-head power lines.** The patagium of ABLV-31, which had been submitted live and moribund, was contracted, suggestive but not conclusive for an electric shock. The soles of both feet of ABLV-57 had deep, linear burns and the left thumb was necrotic, almost certainly the result of contact with overhead power lines. There was no post-mortem comment recorded for a third bat submitted with a one word history of "electrocution". Power lines are a common cause of flying fox morbidity and mortality, accounting for > 50% of > 1,000 bat rescues / year in southeast Queensland (Helen Luckoff, ONARR pers. comm.)

- Six of 24 adult female bats were pregnant. "Very small numbers of fluorescent particles" (ARI) and "some (1-2) positive cells" (AAHL) were detected by FAT in the brain of the late-term foetus of ABLV-26. While the maternal brain was positive for lyssavirus by cell culture virus isolation, attempts at viral isolation from the foetal brain were negative (AAHL). The brains of the late and mid-term foetuses of ABLV-34 and 42 were FAT-negative. The embryo/early foetus of ABLV-44 was too small for testing. The mid to late term foetuses of ABLV-3 and 10 were not tested.

- Miscellaneous findings include 3 bats with dehydration (ABLV-43, 54, 55), ABLV-49 with gun shot wounds, ABLV-68 with fractured teeth and gum injuries as a result of biting its cage and ABLV-71 with congested meninges.

2.5.4 Incubation period, clinical duration and outcome, and the extent of human exposure to ABLV

The incubation periods (36 to 57 days and 30 days) of only two naturally infected bats are known, as a result of apparently clinically normal bats developing clinical disease while in captivity.

ABLV-32, a male orphan Black flying fox, was rescued as an orphan from low in a tree when 2-3 weeks of age and appeared to grow and develop normally for the following 5 weeks. After 36 days in care (8-9 weeks of age), this pup became suddenly aggressive to his cage mate, and over the next 9 days progressively deteriorated with paresis, dysphagia and weight loss (see Appendix 5 and Field *et al.* (1999)). As no in-contact bat or other animal had or has since been diagnosed with ABLV, and natural lyssavirus (rabies) infection in bats is not known with be the result of *in utero* infection (Constantine 1986), it is presumed that this pup was infected during the 2-3 weeks prior to his rescue. The incubation period in this case was therefore 36 to 57 days.

ABLV-73, a mature aged female Grey-headed flying fox was a long term, indeed dominant resident of a captive colony. In October, an ABLV-infected wild bat (ABLV-66) was found behaving abnormally and aggressively on the colony's cage roof/wall, which consisted of a single 'skin' of wire netting. Thirty days later, colony resident ABLV-73 was observed to be behaving abnormally and aggressively, licking its vulva profusely. It deteriorated rapidly during the next 24

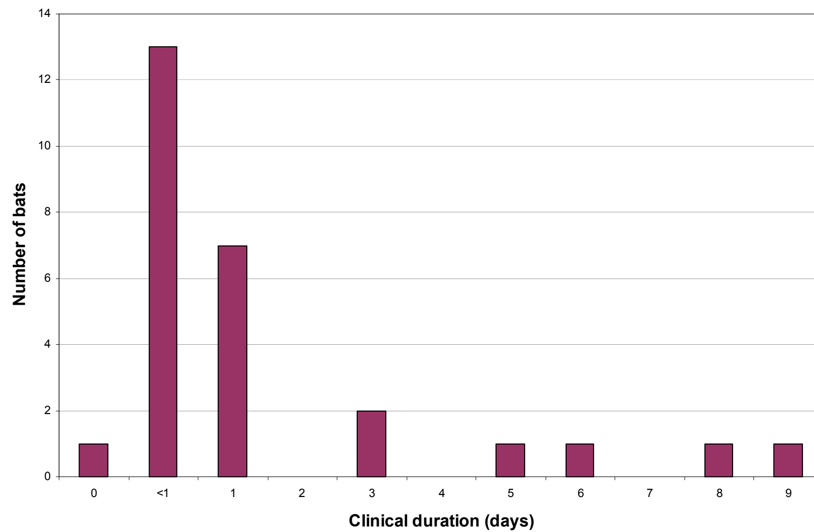
hours and was killed. It is presumed that ABLV-73 was infected when exposed to ABLV-66, and developed clinical disease after an incubation period of 30 days. Details of the public health management of these cases have been published (Warrilow *et al.* 2003).

2.5.4.1 Clinical duration and outcome of ABLV infection

Of 74 ABLV-positive bats, 27 died naturally, 36 were killed, and for 11 the circumstances of their death were not recorded.

The observed clinical duration of those bats that died naturally, i.e. where the clinical duration had not been shortened by euthanasia is shown in Figure 2-13. This data includes ABLV-32, the orphan who developed clinical disease of 9 days duration after ~ 36 days in care, and ABLV-60 who had been observed in a Cocos palm for 2 days prior to being taken in to care for 3 days.

Figure 2-13 Observed clinical duration of ABLV-positive bats that died naturally (n=27)



0 days	Found dead
< 1 day	Indicates that the bat died or was killed on the same day as first observed, not surviving the night to the next morning.
1 day	Indicates bat survived for 12 – 36 hours and died or was killed the day after first observed.
3 to 9 days	Indicates the bat survived until the 3rd to 9th day after first being observed ill.

2.5.4.2 Duration of human exposure to ABLV via clinically ill bats

The number of days that each bat was cared for, prior to its death while infected with ABLV, is shown in Figure 2-14 and Table 2-8. During these periods, in-contact humans may have been exposed to ABLV. Ill bats are often cared for in wire domestic cat cages. The extent of exposure to the bats and ABLV during these periods varied but typically included: handling the bat without gloves, sometimes with the use of a towel e.g. to place bats in and out of cages, holding bats in lap or ‘hung’ on the carers chest clothing to hand feed fruit and juice, attending to wounds and discharges, and cleaning urine and faeces from cages. Minor scratches from the hind leg claws were very common. In one case (ABLV-55), a dedicated carer syringed over 150 mL of saliva from the mouth of an ABLV-positive bat that was unable to swallow (see Section 2.5.6).

Figure 2-14 Number of days clinically ill ABLV-positive bats (n=74) were looked after by wildlife carers and/ or veterinarians (duration of possible human exposure)

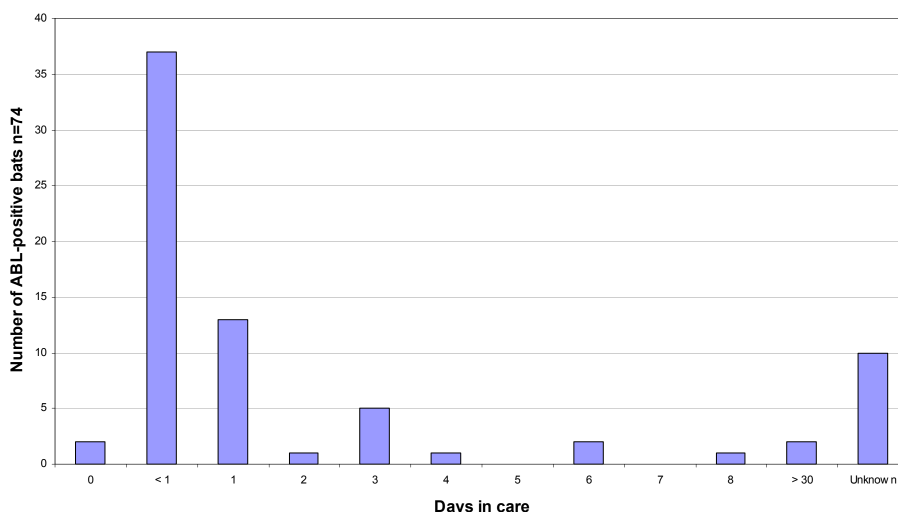


Table 2-8 Number of days bats were in care while infected with ABLV (n=74)

	Number of days in care									
	0	**< 1**	**1**	**2**	**3**	**4**	**6**	**8**	**> 30**	**Unknown**
Number of ABLV bats	2	37	13	1	5	1	2	1	2	10

0 Dead when rescued
< 1 Indicates that the bat died or was killed on the same day as rescue, not surviving the night to the next morning.
1 Indicates bat survived for 12 – 36 hours and died or was killed the day after rescue.
> 30 Two captive bats (ABLV-32 and ABLV-73) were in care for ~ 36 and ~30 days respectively during which time they were incubating ABLV infection. ABLV-32 as a result of exposure prior to being rescued as an orphan, and ABLV-73 after exposure to ABLV-66, a wild ABLV-positive bat.
Unknown The dates of rescue and/or death were not recorded and so the interval while in care is unknown

2.5.5 Serology from ABLV-positive bats

Plasma from 16 ABLV-positive bats was tested for anti-lyssavirus antibodies in the AAHL modified rabies (CVS)-RFFIT. Titres for each case are given in Table 2-9.

Table 2-9 Rabies-RFFIT titres (IU/mL) of sera from 16 ABLV-positive bats.
Data relating to positive titres are shown in bold.

Case No.	Flying fox species	Observed clinical duration (days)	Died or killed	Titre (IU/mL)[1]	Interpretation
ABLV-8	Black	< 1	Not recorded	0	Negative
ABLV-18	Black	< 1	Killed	0	Negative
ABLV-23	**Black**	**< 1**	**Killed**	**1.5**	**Positive**
ABLV-24	Black	Not recorded	Killed	0	Negative
ABLV-27	Grey	< 1	Killed	0	Negative
ABLV-28	Grey	< 1	Killed	0	Negative
ABLV-30	Little Red	3	Not recorded	Plasma toxic to cells	No result
ABLV-33	**Grey**	**< 1**	**Killed**	**0.9**	**Positive**
ABLV-50	Little Red	4	Killed	0	Negative
ABLV-54	**Black**	**< 1**	**Killed**	**0.2**	**Marginally positive**
ABLV-57	**Black**	**1**	**Killed**	**0.2**	**Marginally positive**
ABLV-58	**Black**	**< 1**	**Killed**	**1.9**	**Positive**
ABLV-62	Grey	< 1	Killed	0	Negative
ABLV-65	**Black**	**3**	**Died**	**0.91**	**Positive**
ABLV-68	Black	< 1	Killed	0	Negative
ABLV-72	Black	2	Killed	0	Negative

[1] A titre of zero (0) IU/mL in the AAHL modified rabies-RFFIT corresponds to a count of 20 of 20 high power fields containing fluorescent foci at a plasma dilution of 1:20. Very low titres of anti-rabies activity (< 0.2 IU/mL) may not be detected at this level. Titres of 0.2 IU/mL are estimates that correspond to counts between 10 and 20 of 20 fields containing fluorescent foci, i.e. where the level of antibody activity is marginally below that for accurate calculations, normally ≤ 10 of 20 positive fields.

In addition, serum from one of two ABLV-*negative* Yellow-bellied sheathtail bats (DPI accession 00-164484, adult non-pregnant female August 2000) was tested in the AHHL modified rabies-RFFIT and was found to have a titre of 2.7 IU/mL (test result confirmed in retest).

2.5.6 Opportunistic urine and saliva samples

A sample of urine from ABLV-65 collected by cystocentesis at post-mortem was positive for ABLV by TaqMan® PCR (C_T = 27.18). Similar urine samples from ABLV-63 and ABLV-67 were negative (C_T = 50 and 45.4 respectively, where C_T> 35 = negative).

A sample of >150 mL of bat saliva submitted by a carer that had syringed excess saliva from the mouth of ABLV-55, which could not swallow, was negative for ABLV-RNA by TaqMan® PCR.

2.5.7 The trouble with bats and dogs

Seven ABLV-positive bats were reported to have direct contact with dogs or captive bats such that the dogs or captive bats could have been exposed to ABLV. One other ABLV-positive bat had injuries suggesting involvement of a bat, cat or small dog, and a further ABLV-positive bat was found with similar injuries and was subsequently observed to fight another captive bat. Due to government regulations, pre- and post-exposure rabies/lyssavirus vaccinations are not available for Australian animals, even in the event of known potential exposure to ABLV. The outcome with respect to the potentially exposed dogs and bats is given in Table 2-10.

Table 2-10 Outcomes for dogs and bats in-contact with ABLV-positive bats

Case No	Circumstances	Outcome
ABLV-4	Sept 1996 < 1 yo German shepherd, blood on dog's nose suggested bat bite or scratch.	March 2004, dog alive and well.
ABLV-23	Female Labrador "sniffing around" moribund bat.	Dog subsequently killed in road accident. Not submitted for testing.
ABLV-29	Inadequate original history. During follow up call more than a year later, rescuer reported a "dog had been with bat". Dog not identified.	Dog not identified, outcome unknown.
ABLV-46	Two adult poodles in yard with bat, at least one dog thought to have bitten bat. No evidence of injuries to bat at necropsy.	Both dogs killed at owners request 8 days later. Both dogs FAT-negative.
ABLV-48	Puncture wound and soft tissue damage to bat forearm and other puncture wounds and haemorrhage to pectoral muscles suggest bat, cat or small dog attack. No reference to attack in submitter's history.	Suspect bat, cat or dog attack by unidentified animal, outcome unknown.
ABLV-52	ABLV-52 rescued with bruising swelling and tooth marks on forearm. Later found attacking another injured bat in care. Presumed to have had previously been fighting other wild bats.	Other captive bat recovered uneventfully from original injuries and remained well until released 56 days later. Long term outcome unknown.
ABLV-64	Oct 2000 bat flew from tree on to back of 3 yo female cattle dog, one of four dogs in household.	March 2004, attacked dog and companion dogs alive and well.
ABLV-66 and ABLV-73	Oct 2000 ABLV-66 (wild bat) landed on enclosure of 28 flying foxes. 30 days later ABLV-73 (colony bat) ill with ABLV. Remainder of colony potentially exposed.	March 2004, 26 of remaining colony bats alive and well. 1 bat found dead July 2002, FAT-negative.

2.6 Discussion

2.6.1 Prevalence and risk factors for ABLV in bats

The data presented here represents sampling of animals that were submitted for testing because they were sick, injured, or orphaned, or had potentially exposed a person or animal to ABLV. As such the submissions do not represent the source bat populations as a whole, but rather the sub-population of bats that

1. suffer some sort of illness, injury or misadventure,
2. and come to the attention of human/s
3. and one or more of the persons involved is aware of the possibility that the bat may have a zoonotic disease,
4. and one or more of the persons involved has or does receive appropriate information about the availability of testing,
5. and one or more persons involved is prepared to submit the bat.

Independent of the proportion of ABLV-positive bats in each population, other factors such as the proximity of particular colonies or feeding sites to human activity, the susceptibility of each species to other events that bring them to the attention of humans, the attitude of local people to bats, the attitude of local carers towards ABLV-testing and the enthusiasm of local carers and various agencies (veterinarians, regional DPI offices, etc) for submitting bats for testing may have influenced the numbers of bats of each species submitted and the likelihood that ABLV-positive rather than ABLV-negative bats be submitted.

While the interaction of all these factors may mean that the data collected does not reflect risk factors for ABLV in the *whole* bat population, it accurately reflects risk factors for ABLV among the population of bats with which humans are *in contact*, i.e. the sub-population from which humans are at risk of exposure to ABLV.

While data from all the bat submissions could be considered in the crude analysis, more detailed statistical analysis was restricted to the four common species of wild flying fox, there being insufficient numbers of submissions in other categories.

Crude analysis showed that 6.5% (95% confidence interval of 5.1 to 8.1%) of all submitted bats (n=1,143) were ABLV-positive. However, this overall figure masks significantly different proportions for some meaningful groups of bats. ABLV was identified in YBST bats and at least one of each of the four common species of flying fox, and one flying fox not identified to species. The unidentified wild flying fox was submitted in Rockhampton. Based on species distribution it is highly likely to be either a Black or Little Red flying fox and ABLV-positive bats of both species have been submitted from this area.

2.6.1.1 Health status

While this study shows no significant difference in the crude proportions of healthy bats (point prevalence 0 of 47, 0%) and sick, injured, or orphaned bats (point prevalence 73 of 1075, 6.8%), this is principally due to the low numbers of normal healthy bats submitted and the associated large standard error. However, in a separate but related structured study of the prevalence of ABLV among healthy wild-caught bats by Hume Field (ARI), all of 153 wild-caught flying foxes and all of 181 insectivorous bats were ABLV-negative by FAT (McCall *et al.* 2000). This study suggests that the prevalence of ABLV in the general bat population is less than 1% (95% confidence interval 0 to 1.1%). In combination, these studies show that sick, injured, and orphaned bats have a significantly higher crude prevalence of ABLV infection ($p < 0.001$) than healthy bats.

Sick injured, and orphaned > Healthy (clinically normal)

There was a highly significant association between the presence of clinical signs suggesting CNS involvement and ABLV infection among *all* sick, injured, and orphaned bats (n= 1075, $p < 0.001$). This association was shown to be independent of all other tested factors in the generalized linear model analysis of data from identified wild flying foxes (n=868, $p < 0.001$).

CNS disease > Non-CNS disease

Obviously, this association is dependent on the criteria by which bats were classified as having CNS disease. While involvement of the CNS in many bats was obvious; hind limb paralysis, overt aggression, or specific cranial nerve deficits (e.g. nystagmus, seizures), classification of some bats was more subjective. In particular some bats had a comparatively subtle generalized weakness (not moving about in cage, having voluntary limb movements but difficulty in hanging, holding wings in an unusual “loose” position, or failing to groom etc), yet often retained alert and responsive facial expressions, giving the bat an uncharacteristically calm appearance. Bats with an unexplained weak or calm demeanour (i.e. no obvious wounds or indications of other disease, no history of dog attack, and no suggestion of electrical shock) were interpreted as having CNS rather than metabolic paresis and included in the CNS classification. Moribund bats were not included. As such the criteria for inclusion in the CNS classification was relatively broad. Classifications were generally made at the time of post-mortem examination on the basis of the submitted history and any phone discussions leading to the bats submission, that is prior to the FAT. In some cases (n=13), classification was assisted by the carer submitting the live bat for euthanasia permitting direct observation and neurological examination (see Section 3.2.1 Central nervous system examination). It is also true that in the case of ABLV-positive bats, and not the majority of ABLV-negative bats, follow-up conversations with carers and others allowed for further elaboration of the history and clinical signs, which in some cases (5 of 74) revealed unreported

evidence of CNS involvement and so an ‘upgrading’ from non-CNS to CNS disease status. At this rate of under-reporting of true CNS status (6.7%) it could be extrapolated that approximately 57 ABLV-negative bats classified as having non-CNS disease may have been classified as having CNS disease had further investigations been made. Even after correcting for this level of under-reporting of CNS disease among ABLV-negative bats, the association of CNS disease status with ABLV-positive bats remains highly significant ($p < 0.001$). Recognition of CNS disease, as defined by these broad criteria and based on a thorough history, is a very good indicator of a high risk (crude prevalence 21%, model percent prediction 19.3%) of ABLV infection.

2.6.1.2 Contact status

Perhaps unexpectedly, there was no significant difference in the proportions of ABLV positive bats among the different contact status groups ($p > 0.05$ in both crude and model analysis). As the lyssaviruses are typically thought of as producing aberrant aggressive behaviour, it was expected that ABLV infected bats would have been more likely to bite or scratch humans or animals. However ABLV-positive bats were statistically no more likely to bite and/or scratch than any other submitted bat ($p > 0.05$).

Contact None ≈ Contact Human Yes ≈ Contact Animal Yes

In fact the point prevalence of ABLV-positive bats is lower for both Contact Human Yes (11 of 202, 5.4%) and Contact Animal Yes (9 of 155, 5.8%) than bats with Contact None (56 of 798, 7.0%) status.

Reporting a bat as having bitten or scratched a person led to a demand to kill and test the bat by the Department of Health. Among bat carers, who are dedicated and very attached to their bat patients, such a drastic and uncompromising response to what was very often minor abrasions from a recovering bat or clinically well orphan was often unacceptable. Particularly in the case of orphan flying foxes, there were considerable feelings of loss associated with the destruction of well loved, clinically well, expressive young animals. Virtually all bat carers were vaccinated, and did not consider themselves susceptible to ABLV infection. In addition to individual carers not wishing to relinquish bats for euthanasia, considerable pressure developed within carer communities not to ‘dob in’ a bat. The reluctance to report bites or scratches was exacerbated by the response of some pubic health staff who were unsympathetic and unsupportive of carers and their “hobby” and resentful of the drain on public funded resources associated with testing and vaccination.

On one occasion (ABLV-60) a carer that had to transfer a very aggressive bat in her care to another carer, specifically told the second carer she had not been bitten. They had discussed at some length the unusual aggression shown by the bat and the carer’s high suspicion that the bat may have had ABLV. Despite their suspicions, the bat was cared for for a further 2 days until it died. Only once the positive test results were relayed to the carers did the first ‘admit’ to having

received a bite that drew blood on the abdomen, and belatedly receive post-exposure booster vaccinations. This example demonstrates the reluctance of carers to report potential exposure not only to testing authorities, but to their peers, even when their own suspicions of ABLV-infection are high, and their preparedness to persist with caring for even an aggressive and difficult animal to the point that this results in a delay in providing potentially life-saving post-exposure treatment. While not wise, this near heroic dedication of carers to the welfare of bats in their care is common. This is in part due to a 'selection' effect. The number of people who cared for bats dropped substantially after publication of the first and second cases of fatal ABLV infection in humans (> 160 carers pre-1996, < 40 carers in 2001, Helen Luckoff *pers. comm*. ONARR), with only highly motivated carers that were comfortable with the risk of infection remaining.

In cases involving ABLV-positive bats, where there was a real risk of infection and the possibility that an exposure may have gone unreported, exhaustive post-testing efforts were made to identify all bitten or scratched persons. This was not the case for the majority of ABLV-negative bats. Consequently, it is highly likely that the number of bites and scratches in this data are under-reported only among ABLV-negative bats. The effect of this under-reporting is that the proportion of ABLV-positive bats that did not bite or scratch (Contact Status None) is probably higher than that indicated (i.e. >7.1%), and the proportions of ABLV-positive bats that did bite or scratch (Contact Human Yes and Contact Animal Yes) are probably lower than that indicated (< 5.4% and < 5.8%). That ABLV-positive bats are no more or less likely to bite or scratch may be due to most ABLV-positive bats being found either dead, moribund or with generalized paresis (46/74) and surviving less than 12 hours after rescue (39/64 where the interval was known) and so having a reduced capacity and limited opportunity to bite or scratch.

Of the 14 ABLV-positive bats that did show overt aggression, six (43%) managed to bite or scratch one or more humans or animals. The numbers of aggressive and ABLV-positive bats is too small to demonstrate statistical significance. It is possible that while the majority of ABLV-positive bats are less likely to bite or scratch than an ABLV-negative bat, the small proportion of ABLV-positive bats that are aggressive are more likely to bite and scratch. If so, it is this small proportion of aggressive ABLV-positive bats that poses the greatest risk of ABLV transmission to humans. Both known human cases of ABLV infection, and the one naturally infected bat were the source of infection was identified (ABLV-73), occurred after exposure to overtly aggressive bats (Hanna *et al.* 2000; Speare *et al.* 1997).

Aggressive ABLV-positive bat > ABLV-negative bat > dead or weak ABLV-positive bat

2.6.1.3 YBST bats and other relevant categories of bat submissions

There were significantly different proportions of ABLV-positive bats between distinct species or groups of bats.

YBST bats > wild megachiroptera > colony megachiroptera ≈ other microbats
($p < 0.001$) ($p < 0.05$)

A very high 71% of only seven submitted *S. flaviventris* (YBST bats) were ABLV-positive (95% confidence interval of 29.0-96.3%). As it is improbable that such a high proportion of the total population of YBST bats become ill with ABLV, it is assumed that some interaction between the prevalence of ABLV in this species and other factors such as the distribution, biology, and behaviour of this species and/or the effect of ABLV on individuals resulted in the selective submission of ABLV-positive YBST bats. Regardless of whatever interactions were involved, the fact remains that identification of a sick or injured bat of this species is the strongest risk factor for ABLV infection.

In contrast, none of 151 other microbats was ABLV positive, suggesting ABLV is not present in microbats other than YBST bats. However, the usefulness of these data are limited by the large number of other microbat species (n=62) compared to the number tested. Individuals of only 12 of 19 other genera were submitted and the number submitted of most genera was very low (n= 1 or 2 for seven genera). Consequently the absence of an ABLV-positive bat among such small samples is meaningless with respect to the presence or absence ABLV in each microbat species. Despite this, it remains true that as a group, other microbats pose a low risk of transmission of ABLV to humans due the low prevalence of ABLV in the group and the low frequency with which these bats interact with humans (microbats comprise 62 of 76 bat species (82%) yet contributed only 151 of 1143 (13.2%) of all bat submissions.)

The great majority of bat submissions (974/1143, 85.2%) were Megachiroptera and all but eight of these were flying foxes. While neither of two Tube nosed bats and none of six Blossom bats were ABLV-positive, the numbers of submissions of each species is too small to infer that ABLV is or is not present in these species.

The prevalence of ABLV among *wild* flying fox submissions (68 of 893, 7.6%) was significantly higher ($p < 0.05$) than among *captive* flying foxes (1 of 81, 1.2%). The history of the colony from which the single ABLV-positive bat was identified (ABLV-73) strongly suggests this infection did not arise *de novo* but as a result of contact with a wild ABLV-infected bat (ABLV-66) that had landed on the colony’s cage one month earlier. No other bat in the colony developed clinical signs as a result of contact with either bat, suggesting that ABLV is not readily contagious, even among closely confined flying foxes. Bat ABLV-73 was a long term dominant resident of the colony. Her exposure to ABLV may have been the result of acting defensively to try to drive the wild bat off the

cage. Early recognition of clinical signs and removal of ABLV-73 from the cage by staff may have precluded transmission of ABLV to other colony residents.

2.6.1.4 Species associations among wild flying foxes

Among the four common species of flying fox, there were differences in the number and characteristics of wild bats submitted. The model analysis indicated there was a highly significant independent association between species ($p < 0.001$) and the proportion of ABLV-positive flying foxes.

Both the crude and model analysis showed that the prevalence of ABLV was significantly higher in Little Red flying foxes (crude prevalence 16.9%, model percent prediction 11.4%) than in Black and Grey-headed flying foxes (crude prevalence 7.8% and 4.6% respectively, model percent predictions 4.6% and 2.3%) which were not significantly different. The prevalence of ABLV among Spectacled flying foxes was significantly lower than the other three species (crude prevalence 1.0%, model percent prediction 0.2%).

P. scapulatus* > *P. alecto* ≈ *P. poliocephalus* > *P. conspicillatus

Only 9.4% (95 of 893) wild flying foxes submitted were Spectacled flying foxes, which may simply reflect the relatively small distribution of these bats in Queensland and the lower human population density in coastal far north Queensland relative to southeast Queensland. The Spectacled flying fox submissions were heavily biased towards bats with certain characteristics. Sixty-eight of the 95 Spectacled submissions (72%) were submitted by a single veterinarian during late 1996 and 1997. All were known or clinically suspected of having tick paralysis as a result of poisoning with the toxin of *Ixodes holocyclus*, but had failed to respond to medical treatment, including in some cases anti-tick toxin serum. These submissions are biased with respect to when they were submitted, the clinical syndrome of paralysis and respiratory difficulty, a health status classification of CNS disease, and a high expectation of non-ABLV aetiology. While biased, these samples were highly appropriate, as having showed evidence of CNS disease and having failed to respond to treatment for tick poisoning meant there was a real possibility that the presence of the tick had been incidental or secondary to primary infection with ABLV. None of these 68 'tick' submissions were ABLV-positive suggesting that ABLV is not contributing to the seasonal incidence of tick paralysis in Spectacled flying foxes. The remaining 27 Spectacled submissions spanning December 1996 to June 2000 were individual cases from individual carers as was typical of the submission pattern of the other flying fox species. One of these spectacled flying foxes was ABLV-positive (ABLV-25).

Due to the apparent bias in the Spectacled flying fox submissions, the low prevalence of ABLV in these submissions (1/95) should not be directly interpreted as indicating a low or lower prevalence

of ABLV among this species relative to other flying fox populations. While the prevalence of ABLV in the Spectacled population as a whole may be significantly higher, similar, or lower than that in other species, the combination of this true prevalence, restriction of the spectacled population to a relatively small area of the far north coast near Cairns, the low human population density in the area, and the high incidence of tick paralysis in this species, combine to result in a very low prevalence of ABLV (1/95, 1%) among wild sick, injured, or orphaned Spectacled flying foxes that are in contact with humans. Despite the apparent sample bias, these submissions indicate that Spectacled flying foxes pose a low, indeed the lowest risk of ABLV infection to humans.

Wild Black flying foxes were the most commonly submitted bat species (n= 474). This reflects their coastal distribution that corresponds to the highest human population densities, and their inclination to roost and forage in or near urban development, increasing the likelihood that a sick, injured, or orphaned Black flying fox will come in to contact with humans. While the number of wild Grey-headed flying foxes in Queensland is comparatively low and their distribution is restricted to southeast Queensland only, the high human population density in this region and their use of urban sites for roosting and foraging resulted in the second highest number of submissions (n= 175). In contrast, wild Little Red flying foxes, despite having the widest distribution, including southeast Queensland, contributed only 124 submissions. This may be because they disperse into inland Queensland, where human population density is low, to give birth and raise their young, and fewer of them roost or forage in urban environments.

2.6.1.5 Age and other factors

Generalized linear regression models showed that only three factors, species, health status and age of were associated with statistically significant differences in the proportions of ABLV positive flying foxes.

The association with age indicated that the proportion of adult bats that were ABLV-positive was significantly higher (model percent prediction 4.9%) than that of juvenile bats (<3 years of age model percent prediction 1.7%). The basis for this association remains unclear. As up to 5% of flying foxes have been shown to have neutralizing antibodies to ABLV, it is possible that maternally derived antibodies confer protection to some juvenile bats. Bat colonies are highly structured with sections of colonies consisting of juveniles, young adult males, or pregnant or lactating females at certain times of the year. Differences in the seasonal and territorial aggression among adults and juveniles may influence the transmission of ABLV in certain sections of the colony.

No significant association was identified between any other tested factor (sex, contact status, location found, year, and climatic season or bat reproductive season).

While there was no significant association with year and the *proportion* of ABLV-positive flying foxes, there was certainly a strong downward trend in the *total number* of ABLV-positive

submission (see Table 2-2). In 1996 and 1997, immediately after the recognition of ABLV in bats and the first human fatality, the submission rate of bats was very high (average of 34 and 28 bats per month respectively), due principally to the valuable support of the bat carer community and veterinarians at Currumbin Sanctuary that provided veterinary services to carers in the Gold Coast region. However, as testing required euthanasia of bats and the cost and inconvenience of submitting bats, once it became clear that the vast majority of bats were ABLV-negative, and as carers adapted to minimize or conceal being bitten or scratched, over time fewer bats were being submitted. During 2001 only 100 bats (~ 8 per month) were submitted in Queensland (ARI and QHSS figures combined). Publicity regarding the risks associated with contact with bats meant fewer members of the public became involved with bats. Many carers became selective about the bats they submitted. Live bats were generally only submitted if they had bitten or scratched someone, and often then only if they had bitten or scratched an unvaccinated person (e.g. a member of the public during rescue). Dead bats were often only submitted if the carer suspected the bat was ABLV-positive or they had some other interest in determining the cause of death. This submission pattern was partly in response to changing research focus over the period. Initially a team of three postgraduate students and two research assistants were focused on concurrent ABLV and Hendra virus projects. By 2001, ABLV testing was being done simply as a diagnostic procedure. As funding and staff dissipated so did the submissions. ABLV is a nationally listed notifiable disease, obliging veterinarians or other scientists to notify the nearest veterinary officer of the existence or suspected existence of ABLV (Stock Act 1915). As such the Department of Primary Industries has an obligation to test any bat suspected of having ABLV. While this continues to be the case, without active encouragement and feedback from the research group, submissions dwindled and by 2002 the majority of submissions were as a consequence of reports of people being bitten or scratched.

2.6.2 Characterization of ABLV infection in bats

ABLV infection in bats is not readily recognizable clinically, in part due to an expectation that lyssaviruses will produce overt aggression and because it is difficult to appreciate paresis in bats. Five ABLV-positive bats were examined by veterinarians that did not recognize the condition to the extent that the bats were released back to the carer. A weak, even dead bat is capable of hanging due to the anatomy of their hind limbs. A weak bat is therefore quite able to hang, apparently normally in a cage, with paresis only evident by their inability to move or groom normally. A similarly weak quadruped, such as a cat or dog, would be unable to stand.

The range of clinical presentations and signs is consistent with that reported previously, (Hooper *et al.* 1997b), however it should be noted that the report refers to some of the same cases described here. The ‘classic’ ABLV-positive bat is weak, not aggressive, having been found on the ground or hanging inappropriately low in tree or other structure away from camp during the day. Presumably these bats were recently well enough to fly from camp, but had deteriorated since

and were unable to climb or fly from where they were found. When rescued they often appeared calm and alert. Those that could hang may have appeared quite well. In some cases bats that had died naturally were submitted with the paradoxical history of “appeared healthy”, others were more obviously paralysed and required support. Some showed signs of cranial nerve or cerebral involvement. Abnormal use of the mouth, seizures, tremors, or nystagmus, while not common is highly suggestive of ABLV infection. Most deteriorate or die unexpectedly within 36 hours.

Approximately 19% of ABLV positive bats present with overt aggression. Some fly from an inappropriate roost to attack humans and other animals. Others behave aggressively when being rescued, often vocalizing, and may show intractable aggression towards inanimate objects and approaching humans when caged. This behaviour is readily recognized as abnormal by experienced carers, and their rapid progression from over-active and aggressive to moribund or dead is a strong indicator of ABLV infection.

Most ABLV-negative bats can be recognized as having a particular problem; trapped in a fence, broken limbs, history of dog attack, found near powerlines with electrical burns, or being an orphaned juvenile. Any bat that is unwell enough to be caught but has nothing obviously wrong with it should be considered likely to have ABLV.

Surprisingly, very few ABLV-positive bats suffer secondary misadventures. One infected flying fox flew into the path of a car, and two others appeared to have received electrical shocks from power lines. One had small puncture wounds that may have been the result of a bat attack as no cat or dog interaction had been observed. Remarkably, none were seriously injured by dogs or cats, despite the fact they invariably ended up vulnerable to such attacks.

At gross post-mortem examination ABLV infection is a most unremarkable condition. A dead bat with no gross indicators of cause of death, with low or moderate fat reserves, with or without bruising consistent with a fall, and bladder containing urine, could be considered more likely to be ABLV-positive.

The incubation period of four naturally occurring ABLV infections are believed to be known, those of the two human cases, which were 6 weeks (Rockhampton (Speare *et al.* 1997)) and 27 months (Mackay (Hanna *et al.* 2000)) respectively and those of ABLV-32 and ABLV-73, which were 36 to 57 days and 30 days respectively. The shorter incubation periods are most consistent with those of the majority of rabies cases where 70% occur within one month of exposure, 90% within 6 months and < 7% after more than 12 months (n=707) (Wilde *et al.* 1989).

The clinical duration of naturally-occurring ABLV in most cases is unknown as either there was an unknown period of clinical illness prior to the bat being found and/or the duration was shortened by euthanasia. Of the two bat cases that became ill while under observation, ABLV-32 died after a 9-day illness and ABLV-73 was killed on day 2. Minimum clinical durations as indicated by the

periods that bats were observed sick before dying naturally varied from 0 (found dead) to 9 days. While most died within 36 hours, six survived for 3 (n=2), 5, 6, 8 and 9 days.

The AAHL rabies-RFFIT detected seroconversion in only six of 16 ABLV-positive bats, indicating that a negative rabies-RFFIT titre is meaningless with respect to whether or not a bat is infected with ABLV. Titres in ABLV-positive bats were generally low to moderate (0.2 to 1.5 IU/mL). Absent, late or poor development of an ABLV titre in these bats may have contributed to their becoming clinically ill.

Both negative and positive titres have been detected in clinically well, wild-caught flying foxes. A positive titre of 0.9 IU/mL was detected in 1 of 240 flying foxes (Hume Field, ARI, unpublished data), and titres of ~0.2 IU/mL were detected in 3 of 54 (5.5%) Black and Grey-headed flying foxes screened prior to experimental use, this thesis. There is a similar prevalence (3%) of positive titres in apparently well, wild-caught microbats (8 of 264 with titres of 0.4 to 2.0 IU/mL, including 3 positive *S. flaviventris* sera, Hume Field, ARI, unpublished data). As individual bats with ABLV-positive titres have not been serially tested or observed, it remains unclear whether the positive titres in healthy bats indicate exposure, subclinical resolution and protection, a carrier state, or a pre-clinical response and impending disease. As such, while a positive titre presumably indicates prior exposure, it does not indicate whether or not an individual is currently infected or whether it presents a risk of infection to others.

As neither a positive or negative rabies-RFFIT titre in either a clinically ill or healthy bat is predicative for the presence or absence of ABLV-disease or a risk of infection, titres are of no value in the clinical or risk assessment of bats.

2.6.3 Estimating the likelihood of being ABLV-positive

Assessment of likelihood is dependent on taking a detailed and specific history of the circumstances and clinical observations of each case. In particular detailed and specific inquires should be made regarding the three significant factors, species, health status and age. Experienced carers are usually able to identify the species and age. Microbats are comparatively difficult to identify. Fortunately, YBST bats, the only microbat associated with a high risk of ABLV, are distinctive having pale belly fur and dark fur on the back. Most other microbats are brown/black all over.

Considerable care needs to be made to ensure the history consists of what was *seen* and *heard* rather than the submitter's interpretation of the situation. For example, one bat (ABLV-32) was submitted with a history that read:

> "Hand raised. Hanged itself in cage. 2 cortisone shots and Amoxil 5 days".

On getting the ABLV-positive result, further inquires revealed that this orphan, that had been in care for 5 weeks, had had a sudden onset of aggression and vocalizing, deteriorated over 7 days

until unable to hang, with dysphagia, diarrhoea and weight loss, eventually dying on Day 9. No entrapment had been observed but the carer had concluded that the bat *must* have suffered a head injury that *must* have been the result of getting its head caught in the cage. While the actual history readily suggests this bat was likely to be ABLV-positive, the summary assessment submitted did not. The care needed to get an indicative history extends particularly to observations relating to paresis. It is advisable to ask specifically what the bat can and can't do (can hang, can grip with feet, has moved voluntarily around cage, can groom with wings and feet, has inverted to toilet) rather than rely simply on a submitter's assessment of whether the bat was or was not weak.

As shown in Table 2-6 the combination of species, age and health status can be used to differentiate which bats are more likely to be infected. As the table indicates, all Spectacled flying foxes have a low (< 2%) likelihood of being ABLV-positive, while all Black, Grey-headed and Little Red flying foxes showing signs of CNS disease have a high to very high likelihood of infection (8.2 to 58%). Within each species, adult bats are more likely to be infected than juveniles.

Even when a good history is taken and the species, age and health status are known, not all ABLV-positive bats are readily recognized. The clinical signs shown by ABLV-positive bats and an assessment of how easily those bats would be recognized are shown in Table 2-7. Unfortunately none of the circumstances or clinical signs observed in ABLV-positive bats are unique to ABLV, and some bats, in particular the dead, moribund or those showing signs suggesting another disability are clinically indistinguishable from the majority ABLV-negative bats. Comprehensive identification of all ABLV-positive bats would require comprehensive testing of all sick, injured, or orphaned bats, as was essentially the case during late 1996 and 1997. As this is no longer supported by carers or adequate funding, wildlife carers and associated professionals need to make as reasonable an assessment as possible as to which bats are sufficiently likely to be ABLV-positive that they should be tested. Invariably this has and will result in many cases not being identified.

2.6.4 Risk factors for ABLV infection in humans and other animals

The risk of infection with ABLV is related to the risk of exposure to an ABLV-positive bat, the type and extent of exposure to the bat, and the susceptibility of the person or animal to infection. As most humans only come in to contact with sick, injured, or orphaned bats, the risk factors for exposure to ABLV are the same as those discussed for the prevalence of ABLV in these bats. It should be noted that dogs, and presumably other animals *are exposed to* ABLV-positive bats and there is therefore a theoretical if not real risk of infection in these animals. However, as no case of ABLV has been detected in other native or domestic animals, the risk of human exposure to ABLV via indirect transmission from other species appears negligible.

It is presumed that infection with ABLV in humans will occur as a result of the same sort of exposures as those that have led infection with rabies, i.e. principally from bites and scratches, as were reported in the two human cases to date, and possibly by contact with urine or saliva and broken skin or mucus membranes (NHMRC 1997). Members of the public and professionals with no experience of handling bats that attempt to rescue or treat sick, injured, or orphaned flying foxes, are very likely to be bitten and scratched. Experienced wildlife carers can usually handle bats effectively and avoid being bitten, but are almost universally opposed to wearing gloves. They frequently incur minor scratches and abrasions, particularly from the grasping hind limb claws, and are invariably exposed to bat blood, urine, and saliva. ABLV RNA was detected in one of three urine samples from ABLV positive bats, and not in the single saliva sample tested with the pt-ABLV TaqMan® assay. Whether ABLV-positive urine is infective has not been established. The dedication many carers have for bats in their care results in them continuing to care for bats they suspect of having ABLV until they are dead or moribund, increasing the duration of potential exposure to up to 9 days. In addition, within the carer community, some expert carers are more likely to be allocated bats with ABLV-like symptoms, resulting in some carers dealing with multiple ABLV-positive cases a year.

Three rabies vaccines, Verolab (*sic*), a human vaccine produced in the vero cell line, IMRAB a veterinary vaccine, and V-RG vaccinia, a recombinant expressing rabies glycoprotein, were shown to confer cross protection ABLV to intracerebrally inoculated mice challenged 2 months after intraperitoneal immunization (Hooper *et al.* 1997b). The ABLV used in this work was YBST-variant ABLV (Charles Rupprecht, CDC Atlanta *pers comm.*). No specific assessment of the cross protection conferred against pteropid-variant ABLV has been published. In Australia pre-exposure vaccination against rabies and ABLV is done using the human diploid cell rabies vaccine (HDCV, CSL/Pasteur Merieux inactivated rabies vaccine) on the presumption it too confers cross protection (NHMRC 1997). Post-exposure treatment also includes passive immunization with human rabies immunoglobulin (HRIG) (NHMRC 1997). That these vaccines confer protection to humans exposed to ABLV has not been specifically demonstrated *except* that no vaccinated person exposed to either ABLV-positive bats or other ABLV-positive material (e.g. during diagnosis or research) has yet become ill. Confidence in the effectiveness of pre-exposure vaccination with HDCV is generally high in the Australian carer, veterinary and medical communities.

With respect to susceptibility to infection, there are essentially two populations:

- **unvaccinated and susceptible**, which includes virtually all native and domestic animals, the general public, and many veterinarians in general practice
- **effectively vaccinated with very low susceptibility**, which includes most carers and government based professionals that deal with bats, e.g. Department of Environment personnel, DPI, AAHL and Department of Health diagnostic and research scientists.

Fortunately, virtually all wildlife organizations that arrange for bats to be fostered out to carers insist that bat carers receive pre-exposure vaccination with the human diploid cell rabies vaccine. Most were vaccinated during a publicly funded campaign in 1996. Some, as has been reported (Crome *et al.* 1998), may be unaware that they had failed to respond to vaccinations received. Pre-exposure booster vaccinations for bat carers are no longer free through Medicare, and the expense (~$100 /booster) deters carers from requesting regular boosters. Carers that received their original vaccinations in 1996, and have not since required free post-exposure booster vaccinations, may have waning titres. As such the susceptibility of carers may be increasing.

The veterinary community may be uniquely at risk of infection with ABLV. Australian veterinary schools provide free vaccinations for some diseases but not rabies vaccination. The cost of the full course of pre-exposure vaccinations (~$300/ three inoculation course) deters general veterinary practitioners from becoming vaccinated, particularly as most wildlife consultations are done at no charge, most veterinarians see fewer than 3 bats per year, and it appears no veterinarian has been infected. Experienced carers recognize the most common injuries that will respond to supportive care and antibiotics to which they often have access. Veterinarians are likely to only see bats that are unusually sick or require euthanasia and so are particularly likely to be ABLV-positive. In one case a veterinarian was reluctant to get vaccinated because he only saw one bat a year, but two of three bats he killed and submitted for the experienced carer in his area were ABLV-positive. Some practices manage the risk of exposure to ABLV by refusing to see bats, others by directing all bat consultations to particular vaccinated members of staff, but many rely on the assistance of experienced carers to avoid getting bitten and scratched and on the effectiveness of post-exposure vaccinations.

Management of unvaccinated persons potentially exposed to ABLV is by post-exposure vaccination, i.e. when an person reports that they have been bitten or scratched by an ABLV-positive bat, or bat that can not be tested (e.g. flies away), post-exposure vaccinations are offered free by the Department of Health (NHMRC 1997). This protocol will fail if either the person does not appreciate they have been exposed (e.g. unperceived or minor abrasions, or contact with bat blood urine or saliva and broken skin or mucosa), or does not report an exposure, as is the case in most human rabies cases in the USA (Messenger *et al.* 2003; Warrell 1995). Fortunately the high levels of publicity associated with the two human cases of ABLV infection have resulted in many in the general public understanding that bats do pose a risk of infectious disease and that they should seek medical advice after contact with bats.

2.6.5 Non-bat species submissions

During the period June 1996 and March 2002 only 27 non-bat submissions were tested for ABLV with only 1 to 7 submissions of any one species. While all the submissions were ABLV-negative, the numbers submitted are far to low for them to indicate an absence of ABLV (or ABLV-like virus) among non-bat species in Queensland. Even though most of the submissions (19/27) were of

animals with clinical signs suggesting nervous system disease and so had a moderate or high index of suspicion for ABLV, the numbers submitted were so small as to clearly indicate that neither pet owners, veterinarians, wildlife carers, nor the Department of Natural Resources (who through the Queensland Parks and Wildlife Service are responsible for the management of all native animals) genuinely consider the possibility of ABLV as a cause of unexplained aggression or neurological disease.

At this point the apparent absence of ABLV in domestic animals (most notably cats and dogs) and wildlife species (such as possums, dingoes and native rodents) cannot be attributed to a true absence of lyssaviruses in these populations, since the same failure to test appropriate cases persists among non-bat species as had existed for bats prior to the Hendra virus-inspired research. The dogma that lyssaviruses in Australia exist only among bat populations (with rare spill-over to humans), while quite probably true, can simply not yet be justified.

2.7 Conclusions

Prevalence of ABLV in bats and other species

- While ABLV is rare in whole bat populations (<1%) it is common among sick, injured, or orphaned YBST bats (up to 96%) and Black, Grey-headed and Little Red flying foxes (4 to 17%).
- ABLV has been detected in a single Spectacled flying fox.
- The extent to which ABLV or ABLV-like viruses are present in other native or domestic species, either in self-sustaining endemics or in incidental dead end hosts has not yet been adequately tested.

Risk factors for ABLV infection in bats

- There are species (YBST > *P. scapulatus* > *P alecto* ≃ *P poliocephalus* > *P conspicillatus* > other microbats), age (adult > juvenile) and health status (CNS disease > non-CNS disease > clinically well) associations with ABLV-positive bats that can be used to asses the likelihood that a particular bat is ABLV-positive.
- ABLV is present throughout Queensland throughout the year. There is no regional or seasonal association with the proportion of ABLV-positive bats.
- ABLV is rare in captive bats. The single reported case in a captive bat can be attributed to contact with a wild ABLV-positive bat. The risk of ABLV in colony bats, and therefore the risk colony bats pose to humans could be reduced by preventing contact between wild and captive bats, e.g. by housing in ‘double skinned’ enclosures.

Characterization of ABLV infection

- The incubation periods for ABLV in the two flying fox and two human cases, where it is believed to be known, was 36-57 days, 30 days, 6 weeks and 27 months.
- Bats infected by ABLV were usually found on the ground or hanging inappropriately low in trees or man-made structures during the day, and do not appear to be prone to secondary misadventures, such as entanglement in barb wire, or attacks.
- Most ABLV-positive bats have clinical signs dominated by generalized paresis. A small number are overtly aggressive, and others are clinically indistinguishable from ABLV-negative bats.
- Most ABLV-positive bats die or clinically deteriorate within 36 hours of being observed, but some individuals survive as long as 3 to 9 days in care, during which time human exposure to ABLV is likely. All ABLV-positive bats died or became gravely ill or were intractably aggressive leading to euthanasia.
- Rabies-RFFIT titres in clinically ill and normal bats are of no diagnostic significance.

- ABLV-positive bats are statistically no more likely to bite or scratch a human or other animal that any other bat, however a high proportion of the small number of aggressive ABLV-positive bats had bitten or scratched a person or animal.
- Wildlife carers that rehabilitate bats are highly motivated and dedicated to the welfare of bats in their care. Failure to consider the impact of protocols requiring mandatory euthanasia of bats for ABLV testing has resulted in under-reporting and delayed reporting of potential exposure to ABLV through bites and scratches from bats in care.

3 Central nervous system disease in flying foxes

Aims

1. To identify causes of clinical neurological syndromes in flying foxes.
2. To use a targeted approach to evaluate the sensitivity of the fluorescent antibody test performed at the ARI for the detection of Australian bat lyssavirus.

3.1 Introduction

During the study presented in Chapter 2, it became obvious that features of the histories and clinical signs present in many ABLV-positive bats, were similar to those of a number of ABLV-negative bats. Recognition that there was a group of bats with histories consistent with being ABLV-positive that was ABLV-negative by FAT led to the following conclusions. Either some bats infected with ABLV, (or an ABLV like virus) were not being detected in the FAT (false negatives), or/and other conditions were producing ABLV-like clinical signs in bats.

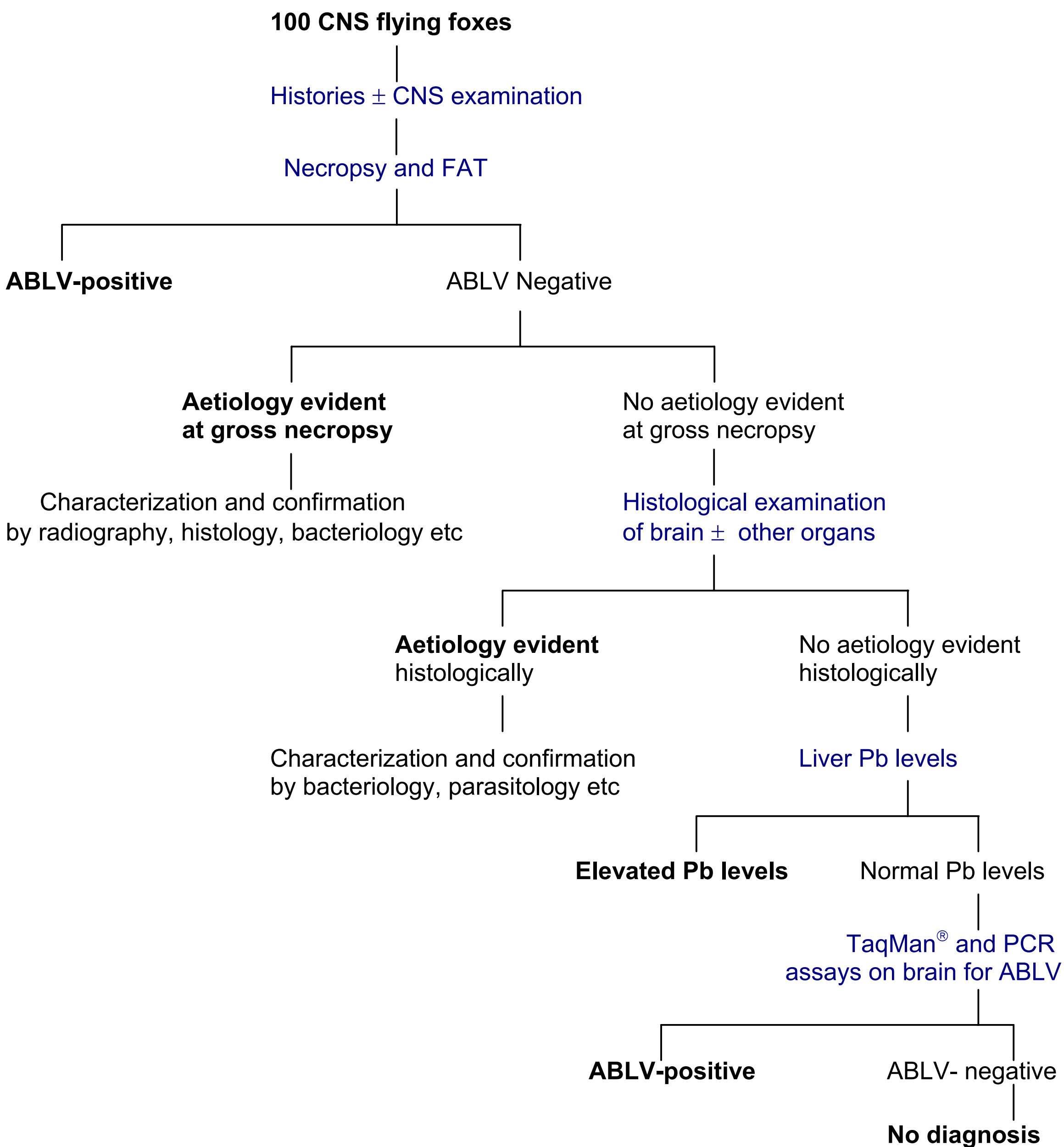
The prospect that the FAT may be failing to recognize all ABLV-positive bats had serious implications for both the research data on ABLV and the post-exposure management of people bitten and scratched by bats. It seemed likely that at least some ABLV-like clinical signs in bats were being produced by other aetiologies that may or may not have been previously reported in bats.

Evidence of lead (Pb) poisoning (plumbism) has been reported as a cause of CNS disease in captive (n= 2 (Zook *et al.* 1970)) and wild (n= 2 (Sutton and Wilson 1983)) flying foxes based on the presence of extremely high (> 500 ppm) or high (15 to 60 ppm) liver or kidney lead levels. However, in clinical trials flying foxes were shown to be highly resistant to clinical plumbism (Hariono 1991), and a Black flying fox with clinical aggression, acid fast eosinophilic inclusion bodies in renal tubular epithelium containing lead, and very high kidney lead levels (370 ppm) was subsequently shown to be infected with ABLV, suggesting that the lead load might have been incidental (Skerratt *et al.* 1998; Speare *et al.* 1997). The extent to which lead toxicity causes clinical disease in ABLV-negative bats remained unclear.

3.2 Materials and Methods

One hundred consecutive diagnostic submissions to the ARI of wild flying foxes from Queensland with a health status classification of CNS disease (see 2.2.1. Case submission and classification), were examined to determine the aetiological diagnosis. Tests and examinations for each bat were done in a sequential manner until a well-supported diagnosis was apparent. Some of the data used in this study was compiled retrospectively and in some cases the full range of tests and examinations were not done because the necessary material had not been collected. The general sequence of tests and examinations is shown in Figure 3-1.

Figure 3-1 Sequence of tests and examinations to determine the aetiological diagnosis for 100 wild flying foxes with CNS disease



3.2.1 Central nervous system examination

Clinical neurological examination was performed on thirteen live flying foxes submitted to the ARI with histories or clinical signs suggesting central nervous system disease.

The examination included assessment of:

- **General demeanour**
- **Cranial nerve functions** examination for evidence of strabismus, nystagmus, head tilt, and facial symmetry, and by eliciting menace, corneal, and gag reflexes, and 'sneeze' reflex on stimulation of nares.
- **Forearm and hind limb reflexes** by observing for voluntary movement, pain and withdrawal responses to 'pinching' carpus (forearm) or interdigital tissue (hind limbs) with forceps, and 'claw clasping' reflex in response to touching the palm of the hind limbs. Flying foxes have a very strong reflex to grasp with their hind limbs, analogous perhaps to the righting reflex of other animals.

3.2.2 Necropsy

Necropsies were done in a Class II biosafety cabinet within the PC2/PC3 Diagnostic Virology Laboratory as described in Section 2.2.1. Of the 100 flying foxes included in this study, 77 necropsies were done by the candidate, the remainder were performed by other members of the Virology Research Group or ARI diagnostic veterinary pathologists.

3.2.3 Fluorescent antibody test

Infection with Australian bat lyssavirus was detected by FAT on fresh brain touch impressions, as described in Section 2.2.3 Fluorescent antibody test.

3.2.4 Radiography

The spinal columns of nearly half of the flying foxes with known or suspected spinal fractures or no known diagnosis were removed and fixed in formalin. Spinal radiographs were taken at the University of Queensland School of Veterinary Science under the direction of Morag Wilson (radiographer) using exposures of 48KVP, 100 ma, and 63 msecs, with a Toshiba DXB-0324CS=A X-ray tube and 0.7 aluminium permanent filtration with single screen cassettes on FUJI UM-MA-HC 9 (soft tissue) film that was automatically processed by a Fuji FPM 3000 X-ray film processor. Interpretation of radiographs was done with the assistance of Annie Rose (radiologist) of the University of Queensland Veterinary Teaching Hospital.

3.2.5 Histology

Brains for histological examination were sectioned in three standard planes.

- ♦ Transversely through the frontal lobe.
- ♦ Transversely through the cerebral cortex and thalamus at the level of the hippocampus
- ♦ Longitudinally through the brainstem and cerebrum, including the caudal colliculus pons, medulla, and proximal spinal cord.

Where available, formalin-fixed lungs from bats with neuro-angiostrongylosis were sectioned through the hilar region so that the major pulmonary artery branches could be examined for nematodes or eggs.

For all other tissues, representative sections were selected.

Tissues were routinely processed overnight, paraffin-embedded and 5 µm sections cut and routinely stained with haematoxylin and eosin by the histology staff of the ARI.

Some sections were reviewed in consultation with senior veterinary pathologists Roger Kelly (University of Queensland), and Ross McKenzie (ARI), and parasitologists Paul Prociv and Peter O'Donahue (University of Queensland).

3.2.6 Bacteriology

Bacterial samples taken by the candidate were submitted to the diagnostic ARI Bacteriology Laboratory for routine aerobic culture.

3.2.7 Parasitology

Nematodes recovered from the brain surface of two flying foxes were fixed in 10% buffered neutral formalin. The frozen half brains of other flying foxes in which nematodes had been found in histological sections were subsequently fixed in 10% buffered neutral formalin, and then gently macerated under stereomicroscopic guidance by Melissa Carlisle (veterinary pathologist, IDEXX, Brisbane).

All worms found were mounted in chorlactophenol on a glass slide under a cover-slip, examined by compound microscopy, drawn using a camera lucida attachment and measured by P. Prociv.

3.2.7.1 Search of archives for evidence of *Angiostrongylus cantonensis* in wild Queensland flying foxes

Histological sections of CNS tissue from flying foxes in the archives of the Veterinary Pathology Laboratory of The University of Queensland Veterinary School were reviewed.

AAHL reports on bat brain specimens that had been forwarded to AAHL from ARI for lyssavirus testing were reviewed.

Dave Spratt of the CSIRO was asked for information on the existence and availability of CNS material or histological sections from unwell flying foxes pre-1997.

3.2.7.2 Case control study for *Angiostrongylus cantonensis*

Queensland National Parks and Wildlife service permit
Scientific purposes permit number – E4/000543/98/SAA

Wild, presumably normal, Black and Grey-headed flying foxes were caught using a mist net at two flying fox colonies in urban Brisbane, namely at Indooroopilly Island, Indooroopilly (GPS South 27.52032, East 152.99458, access courtesy of Indooroopilly Golf Course) and Norman Creek, East Brisbane (GPS South 27.481, East 153.053, access courtesy of Anglican Church Grammar School, 'Churchie'). These bats had been caught as part of a structured surveillance program for ABLV by the ARI Virology Research Group.

Histological sections of brains from 19 wild-caught flying foxes, matched as far as possible for species, sex and age, with bats with *A. cantonensis* in this study, and trapped during months in which cases of neuro-angiostrongylosis in flying foxes were occurring in Southeast Queensland (i.e. matched geographically and temporally), were examined for nematode sections.

Details of the bats examined (species, sex, age, and month, year, and site caught) are given in Appendix 7.

3.2.8 Liver lead (Pb) levels

Wet formalin-fixed livers were submitted to Brian Burren and Suzanne Noon of the DPI Trace Metal Residue Laboratory for Pb determination by inductively-coupled plasma mass spectrometry (ICP-MS) using the ELAN 5000 (Perkin-Elmer). Bovine liver Standard Reference Material (SRM 1577b, National Institute of Standards and Technology) containing 0.129 ± 0.004 µg/g of Pb was digested with each run sequence.

To remove the formalin, livers were washed in ASTM 1193 Type 1 water (18.3 megaohms.cm resistivity), and desiccated in evaporation basins in a 100°C oven overnight. 500 mg $\pm$ 20 mg of

dried liver was prepared by microwave-assisted nitric acid digest (HNO_3, analytical grade). The acid digest was appropriately diluted and introduced into the ICP-MS system, producing results as Pb ppm dry weight.

Approximations of the equivalent wet weight lead concentration (ppm wet weight) were calculated using the average percent dry matter of four clinically normal bats (see Appendix 8), as follows.

$$\text{Approximate ppm wet weight} = \text{ppm dry weight} \ \frac{\times 22.8}{100}$$

One sample (CNS-94) was identified for testing after the DPI Trace Metal Residue Laboratory was decommissioned in ~ April 2001. Washed and dried liver from this case was submitted to the Department of Natural Resources and Mines (DNR) Natural Resource Sciences Laboratories, Meiers Rd Indooroopilly for Pb determination by graphite furnace atomic absorption spectrometry (GF-AAS) using a Perkin-Elmer 4100ZL graphite furnace using Dog-fish muscle Standard Reference Material NRC-CNRC Dorm-2 (National Research Council of Canada) containing 0.064 ± 0.007 μg/g of Pb.

3.2.8.1 Normal liver Pb levels in urban flying foxes

As normal Pb levels in clinically well urban flying foxes are not known, formalin-fixed livers from 50, presumably normal, wild-caught (as described in Section 3.2.7.2), flying foxes from urban Brisbane were submitted to the DPI Trace Metal Residue Laboratory for Pb determination. They included 25 *P. alecto* (14 females, 11 males), and 25 *P. poliocephalus* (11 females, 14 males). Further details of bats examined (species, sex, age, and month, year and site caught) are given in Appendix 8.

Five duplicate liver samples representing low (n=1), mid (n=2) and high (n=2) normal range samples were also submitted to the DNR Natural Resource Sciences Laboratory to verify that the results produced by the different techniques used by the two laboratories were consistent. These duplicate results were not used in any further analysis.

3.2.9 TaqMan® and PCR assays for ABLV

A targeted approach to assessing the sensitivity of the ARI FAT was used presuming that, if the ARI FAT had failed to identify cases of ABLV in fresh brain touch impressions, then of the > 1000 FAT-negative flying fox brains tested, those most likely to have produced a false negative result would be from bats with lyssavirus-like CNS disease with no alternate diagnosis to account for their clinical signs.

Samples of brains from;

- 23 FAT-negative wild flying foxes with CNS signs,
- 3 FAT-positive bats
- 1 FAT-negative control (wild caught, clinically normal, FAT-negative flying fox)
- and an aliquot of Nobivac® Rabies vaccin(*sic*) Lot 79050 (Intervet, Boxmeer, Holland), containing inactivated Pasteur RIVM stain rabies virus

were submitted to QHSS for the detection of pteropid-variant-ABLV-RNA by TaqMan® assay (see Appendix 3) and lyssavirus-RNA by hemi-nested RT-PCR (see Appendix 9). The hemi-nested RT-PCR assay uses a combination of primers designed to amplify all other known lyssaviruses, detects both pteropid and Yellow-bellied sheathtail variants of ABLV, and potentially other unrecognized variants of ABLV.

3.3 Results

The 100 consecutive submissions of wild flying foxes with histories or clinical signs suggesting central nervous system disease occurred during the period August 1997 to July 2001.

A schematic breakdown of the number of flying foxes for each diagnosis, and the level of examination at which diagnosis was reached is shown in Table 3-1 and Figure 3-2. Case details, the aetiological diagnosis, and the results of examinations are shown in Appendix 6.

Subsequent reference to individual cases is by CNS Case Number e.g. CNS-1, CNS-2, as indicated in Appendix 6, or in the case of ABLV-positive bats by ABLV-case number as indicated in Appendix 5 and Appendix 6.

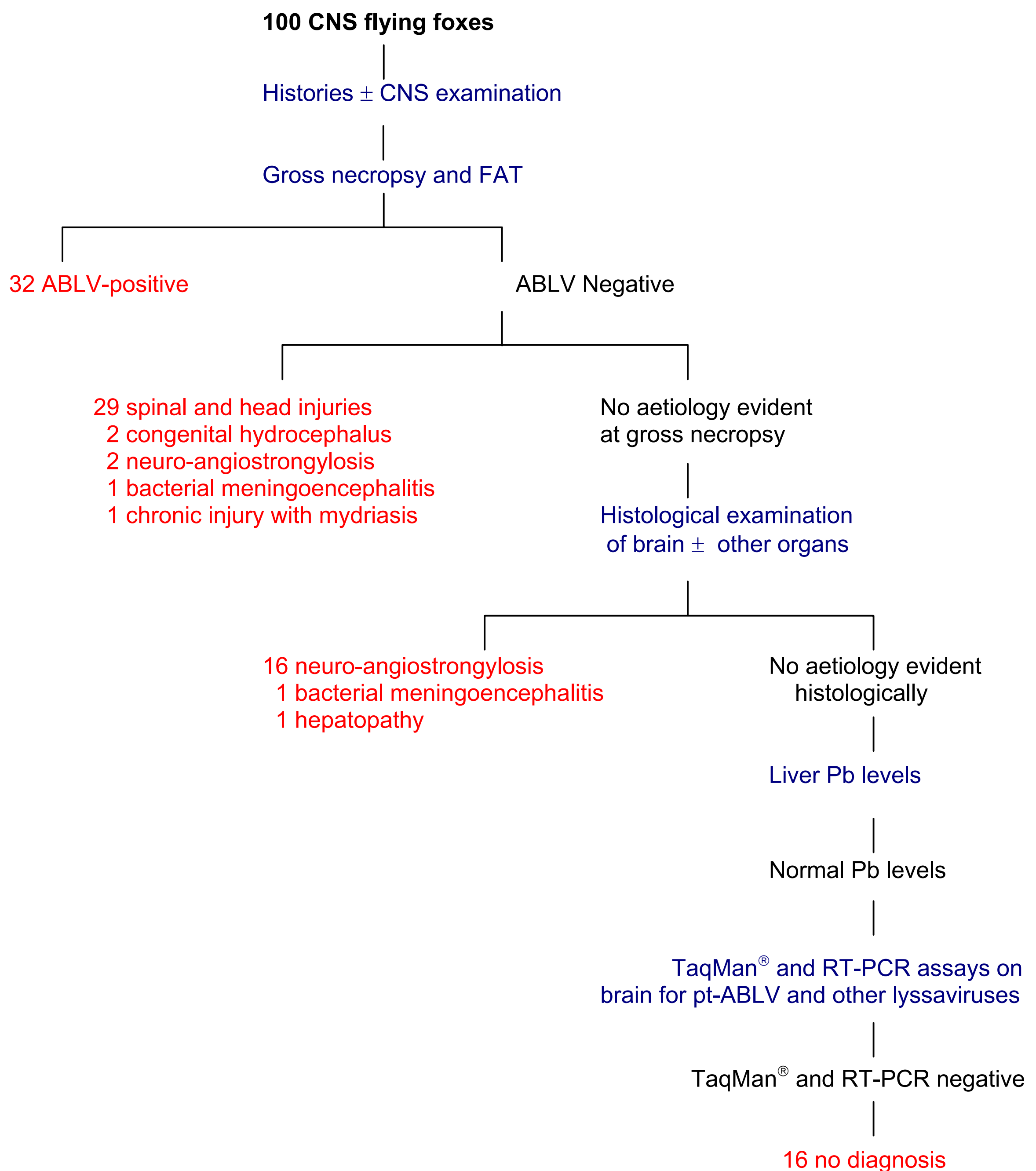
Table 3-1 Aetiology for 100 flying foxes with clinical signs suggesting CNS disease

Diagnosis	Number of cases (equals %)
Australian bat lyssavirus	32
Spinal and head injuries	29
Neuro-angiostrongylosis	18
Congenital hydrocephalus	2
Bacterial meningoencephalitis	2
Vascular hepatopathy	1
Chronic injury with mydriasis	1
Plumbism (lead poisoning)	0
No diagnosis	16

Note: the total number of diagnoses = 101 as one flying fox had both neuro-angiostrongylosis *and* a spinal injury)

Figure 3-2 Aetiology for 100 wild flying foxes with clinical signs suggesting CNS disease

(Note: the total number of diagnoses = 101 as one flying fox had both neuro-angiostrongylosis *and* a spinal injury)



3.3.1 ABLV infection

Thirty-two of the 100 wild flying foxes with clinical signs suggesting central nervous system disease in this study were positive for ABLV by FAT. Case numbers and details are provided in Appendix 5 and Appendix 6 and have been discussed in detail in Chapter 2.

An additional 14 ABLV-positive bats were submitted during this period but were not included in this study because they were:

- **Not a flying fox**, n=1 Yellow-bellied sheathtail bat (ABLV-41)
- **Not recognized as having central nervous system disease** (n=10).
  These bats were either moribund n=5 (ABLV-31, 34, 39, 45, and 48), submitted with inadequate or non-specific clinical histories n=4 (ABLV-46, 47, 56, 61), or submitted with a clinical signs suggesting another specific diagnosis n=1 (ABLV-57 depressed with electrical burns to foot and wing)
- **Not submitted to ARI** n=3 (ABLV-59, 64, and 66, all submitted to QHSS).
  As other QHSS submissions during this period could not be included as a result of the necessary history, clinical signs, and diagnostic test data being unattainable, to have selectively included these 3 QHSS ABLV-cases would have biased this study towards ABLV-positive bats.

3.3.2 Spinal and head injuries

Twenty-nine of the 100 flying foxes in this study had evidence of head or spinal injuries. These injuries included:

- **19 spinal fractures** detected grossly (CNS-2, 13, 18, 29, 31, 33, 34, 35, 40, 41, 42, 44, 45, 49, 58, 60, 66, 70, and 79). Seven of these bats had concurrent head injuries including fractured frontal and nasal bones (CNS-2), a fractured zygomatic arch (CNS-31, 58, and 60), haemorrhage over temporal muscles (CNS-41), fractured skull (CNS-44) and subdural haemorrhage (CNS-44 and 79).
- **2 spinal fractures** not detected grossly were detected radiographically (CNS-9 and 68).
- **1 with evidence of spinal trauma**, extensive haemorrhage of dorsal and pelvic musculature, no fracture identified at post-mortem but radiographs not done (sample not taken, CNS-12).
- **7 with head or neck injuries** that were not associated with spinal injuries including; old skull injury with focal bony proliferation impinging on cranial space (CNS-38), subdural haemorrhage (CNS-61 and 93), fractured zygomatic arch and subdural haemorrhages (CNS-81), skull fractures (CNS-91 and 92), and bilateral areas of petechial haemorrhages over parietal cortex (CNS-96).
  Note: All 7 of these bats were killed by either the candidate or a referring veterinarian and so the head injuries are *not* the result of attempts at euthanasia.

Bats with spinal fractures were often clinically distinguished from other bats in this study by the severity of hind limb paralysis and loss of deep pain and claw clasping reflexes with sparing of forearm (wing) function, and often had a comparatively bright and alert demeanour. Bats with other central nervous system diseases were more often generally weak with reduced but not absent hind limb and forearm function. Four bats with spinal fractures (CNS-18, 41, 49, and 68) were "panicky" or overtly aggressive and to this extent were similar to some flying foxes with ABLV.

Histological sections of brain from one injured bat, CNS-9, showed evidence of neuro-angiostrongylosis and 11 third stage *Angiostrongylus* spp. larvae were recovered from half the brain (see below). It is presumed that the infection with Angiostrongylus predisposed this bat to an accident resulting in the lumbar fracture and that the clinical presentation reflected both the spinal fracture and neuro-angiostrongylosis.

There was no evidence of angiostrongylosis or other predisposing conditions in histological sections of brain from other bats with spinal or head injuries.

Details of the spinal injuries are provided in Table 3-2 and Appendix 6.

Table 3-2 Characterization of spinal fractures in flying foxes (n=21)

CNS case no.	Fracture site	Detected	
		Gross	X-ray
CNS-2	Luxation of T9-10	✓	✓
CNS-9	Fractured caudal endplate of L3	✗	✓
CNS-13	Fractured ~T4-T5	✓	Not done
CNS-18	Thoracic spine (site not specifically recorded)	✓	Not done
CNS-29	Lumbar spine (site not specifically recorded)	✓	Not done
CNS-31	Luxation of L1-L2	✓	✓
CNS-33	Corner fracture of T2	✓	✓
CNS-34	Collapsed T9	✓	✓
CNS-35	Fractured spine (site not specifically recorded)	✓	Not done
CNS-40	Fractured spine (site not specifically recorded)	✓	Not done
CNS-41	Fractured spine (site not specifically recorded)	✓	Not done
CNS-42	Fractured thoracic spine ~ level of base of heart	✓	Not done
CNS-44	"broken neck" (site not specifically recorded)	✓	Not done
CNS-45	Fractured mid-body T3	✓	✓
CNS-49	Fractured T8-9	✓	Not done
CNS-58	Fractured L2-3	✓	✓
CNS-60	Fractured thoraco-lumbar junction	✓	Not done
CNS-66	Fractured thoracic spine ~ level of base of heart	✓	Not done
CNS-68	Subluxation of L2-3	✗	✓
CNS-70	Fractured vertebral body T9	✓	✓
CNS-79	Fractured T7-8 *and* l3-4	✓	✓

3.3.3 Neuro-angiostrongylosis

Nematodes were recovered at necropsy from the brain of two flying foxes. Sections of brain from these and a further 16 of the 100 wild flying foxes in this study showed histological evidence of neuro-angiostrongylosis (synonym neuro-angiostrongyliasis). These bats were found in urban Brisbane (n= 7), the Gold Coast (n= 8), Sunshine Coast Hinterland (n= 2), or Ipswich (n=1). Details of each case are included in Appendix 6 and those of 16 of these 18 cases have been published (Barrett *et al.* 2002). Corresponding case numbers for each case in the paper and this thesis are provided in the keys to Table 3-3 and Appendix 6.

3.3.3.1 Gross pathological findings

Twelve of the 18 bats with neuro-angiostrongylosis were well-nourished, with ample fat reserves. The conditions of CNS-53 and 64 were poor, while those of CNS-7, 8, 9 and 11 were not recorded. CNS-32 and CNS-97, maintained in care for 22 and 28 days respectively, both had considerable amounts of abdominal fat, but wasting of the pectoral muscles and brown discoloration of the meninges. CNS-9 had a retroperitoneal haemorrhage at the level of the left adrenal gland. While no spinal fracture was detected grossly, the spinal column was removed for radiological examination, which revealed displacement of the caudal endplate of the third lumbar vertebra. Petechial haemorrhages were found on the cerebral cortices of Bats 73, 74, 76 and 97, while the meninges of Bat 73 were congested, and the ventral meninges of Bats 76, 87, and 97 were cloudy. One nematode was recovered from the dorsal surface of the cerebellum and brain stem of CNS-87. Three nematodes were recovered from the midline cut surface of CNS-97, near the thalamus.

3.3.3.2 Histopathology

Eosinophilic and granulomatous meningoencephalitis, and one or more sections of nematodes, as shown in Figure 3-3 and Figure 3-4, were found in brain sections from CNS -7, 8, 24, 32, 53, 64, 65, 67, 73, 74, 76, 87, 88, and 97 (n=14). The meningitis was typically most severe around the brain stem and cerebellum, with macrophages predominating in the infiltrate, often with large numbers of lymphocytes and eosinophils, some plasma cells, and occasionally, multinucleated giant cells. In all but one of these cases there were perivascular cuffs of macrophages, lymphocytes and eosinophils. These perivascular cuffs were particularly prominent in CNS-7, 8, 53, and 64. Foci and tracts of tissue disruption, gliosis, and/or haemorrhage, presumably the result of larval migration, were seen in sections from 11 of these cases and were most severe in CNS-24, 32, 73, and 76. Nematode sections were seen most frequently in the subarachnoid space of the cerebral sulci and/or cerebellar folds, but some were located within the cerebral cortex, thalamus, cerebellum, brain stem and lateral ventricles. Bats 9, 11, 77, and 99 (n=4) were distinct in that they displayed virtually no inflammation, and their brain parenchyma appeared unremarkable except for the occasional presence of very small nematodes, as shown in Figure 3-5 (insert).

Sections of lung from all flying foxes with neuro-angiostrongylosis except CNS-53 (sample not taken) were examined histologically and neither nematodes nor their eggs were found.

Figure 3-3 Female fifth stage larvae of *Angiostrongylus cantonensis* from CNS-32
Maximum diameter 102 μm, within a focus of granulomatous meningitis containing macrophages, lymphocytes, eosinophils, and multinucleated giant cells

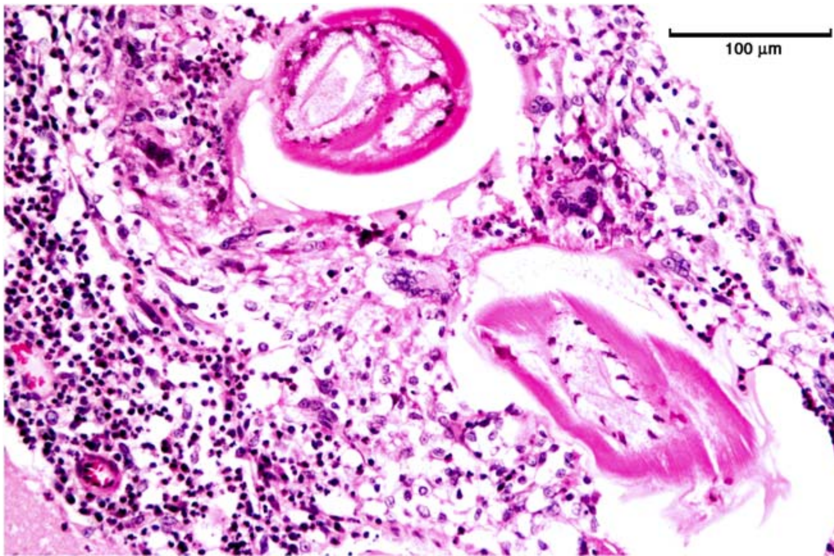
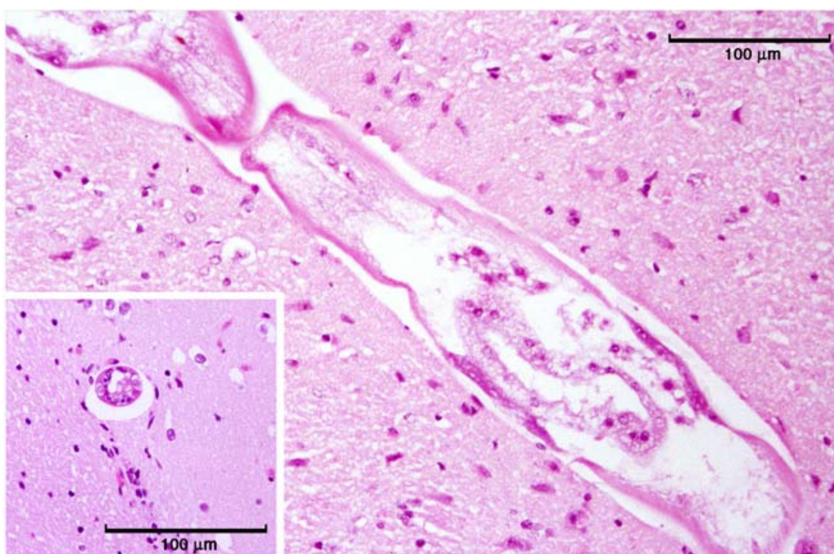


Figure 3-4 *Angiostrongylus cantonensis* (L5) migrating through the thalamus of CNS-73
Maximum diameter 96 μm. Active migration indicated by the absence of an adjacent inflammatory reaction. Severe granulomatous meningitis was seen elsewhere in this section in association with other worms.

Figure 3-5 (insert) *Angiostrongylus* sp. (L3) in the frontal cortex of CNS-77
Maximum diameter 29 μm. Early stage infection indicated by immaturity of larva and lack of inflammatory response in the meninges and brain.



3.3.3.3 Identification of worms

Worms recovered from CNS-8, 24, 32, 65, 67, 73, 74, 76, 87, 88, and 97 (n=11) were identified as fifth-stage (L5) larvae ('immature adults') of *Angiostrongylus cantonensis*, as distinct from *A. mackerrasae,* by the relatively long spicules in the males (0.65 to 1.46 mm) and absence of a caudal mucron in the females. A fragment of an adult worm consistent with *Angiostrongylus* sp. was recovered from CNS-64. Multiple third-stage larvae (L3) 420 to 565 µm in length were recovered from CNS-9 and CNS-11; their identification as *Angiostrongylus* L3 was based on the overall size and shape, internal morphology and dimensions, the buccal details (including a 'double spear') and tail shape (Bhaibulaya 1975; Mackerras and Sandars 1955). No worms were recovered from the brains of CNS-7, 53, or 77. Recovery was not attempted from CNS-99 as the facilities used previously were no longer available. The number and lengths of worms recovered from each bat are shown in Table 3-3. The duration of infection was estimated by comparing the size of nematodes with published growth rates in experimental rats (Mackerras and Sandars 1955). In retrospect, some of these data are known to be derived from *A. mackerrasae*, although the growth of *A. cantonensis* in comparable stages is almost identical (Bhaibulaya 1968, 1975). It was assumed that growth rates in bats, presumably abnormal hosts, equalled the slowest rate found in rats, the natural hosts.

3.3.3.4 Clinical signs of neuro-angiostrongylosis in flying foxes

Of these 18 cases, nine were found on the ground, six were found hanging inappropriately low in a tree and unable to fly away, and three were rescued from a trellis or fence . The clinical picture was dominated by paresis, particularly of the hind limbs, and depression. None were aggressive. CNS-9, which had severe hind limb paralysis and incontinence, was shown radiographically to have a fractured lumbar vertebra and is presumed to have been predisposed to an accident by the *Angiostrongylus* L3 infection, with the clinical presentation reflecting both the spinal fracture and neuro-angiostrongylosis. Whereas seven flying foxes died or were killed within 24 hours of being found (see Table 3-3), 11 of the 18 cases survived in the care of voluntary wildlife carers for variable periods: 3 days (n=3), 7 to 9 days (n=5) and 16, 22, and 28 days.

Table 3-3 Numbers and lengths of *Angiostrongylus cantonensis* recovered from 18 free-living flying foxes and estimated duration of infection

CNS case no.[1]	Male worms		Female worms		Total worms [2] (per ½ brain)	Time in care (days)	Estimated duration of infection [3] (days)
	No.	**Length** (mm)	**No.**	**Length** (mm)			
CNS-7	0	---	0	---	0	7	---
CNS-8	1	3.5	0	---	1	3	15
CNS-9	?	---	?	---	11 [4]	1	2 - 3
CNS-11	?	---	?	---	7 [4]	1	2 - 3
CNS-24	2	4.2 and 6.0	14	4.5 - 15.4	16	7	20 - 30
CNS-32	9	4.4 - 7.2	11	3.9 - 9.7	20	22	24
CNS-53	0	---	0	---	0	1	---
CNS-64	?	---	?	---	1 (piece)	1	---
CNS-65	7	3.9 - 8.9	7	8.6 - 10.1	14	16	25
CNS-67	3	3.9 - 4.3	5	4.4 [5]	8	8	9
CNS-73	8	2.9 - 5.0	12	3.6 - 4	20	1	20
CNS-74	12	2.6 - 5.1	19	2.6 - 4.7	31	3	19
CNS-76	26	4.4 - 9.2	14	6.8 - 11.0	43	7	20
CNS-77	0	---	0	---	0	1	---
CNS-87	---	---	1[6]	8.8	1[6]	9	21 - 24
CNS-88	2	2.9 and 2.3	1	2.2	3	3	12 - 14
CNS-97	Not done		Not done		> 5 [7]	28	Not done
CNS-99	Not done		Not done		Not done [8]	1	Not done

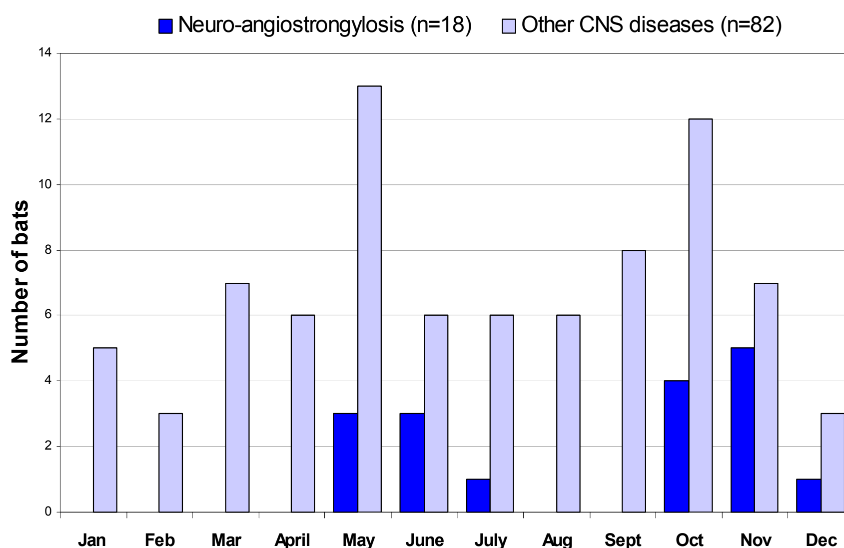
[1] Details of 16 of the 18 cases of neuro-angiostrongylosis referred to here have been published (Barrett *et al.* 2002). The corresponding published case numbers are: CNS-7=Bat 1, CNS-8=Bat 2, CNS-9=Bat 3, CNS-12=Bat 4, CNS-24=Bat 5, CNS-32=Bat 6, CNS-53=Bat 7, CNS-64=Bat 8, CNS-65=Bat 9, CNS-67=Bat 10, CNS-73=Bat 11, CNS-74=Bat 12, CNS-76=Bat 13, CNS-77=Bat 14, CNS-86=Bat 15, CNS-87=Bat 16.
CNS- 97 and CNS-99 were not published. Bat 17 of the publication is an archival case from 1992.
[2] Total number derived from intact specimens plus pieces recovered.
[3] Estimated duration of infection at death calculated from recovered worm lengths and published growth rates (Mackerras and Sandars 1955).
[4] Immature third stage larvae 420-565 µm long, sex differentiation not possible.
[5] Only one could be measured.
[6] Single female worm recovered from brain surface, worms not extracted from fixed brain.
[7] The half brain of CNS-77 was macerated and a moderate number (estimate 5-10) of worms were recovered however, the facilities used to count, sex, and measure these worms ceased to be available and so these worms were not characterized further.
[8] The facilities used to recover, count, sex, and measure worms were not available.
--- Not applicable

3.3.3.5 Temporal clustering of onset of clinical neuro-angiostrongylosis

During the four years of this study (August 1997 to July 2001) flying foxes with neuro-angiostrongylosis were found sick only during the months of May, June and on July 2nd and during October to December.

This clustering of cases does not appear to reflect the distribution for flying foxes with other causes of CNS disease (see Figure 3-6), nor does it appear to be related to the seasons of spring (August, September and October), summer, autumn or winter, nor to periods of high or low rainfall in Southeast Queensland (data not shown).

Figure 3-6 Months in which flying foxes presented with CNS disease (August 1997 to July 2001)



3.3.3.6 Archival evidence of *Angiostrongylus cantonensis* in wild flying foxes of Queensland

Veterinary Pathology Archives, The University of Queensland

Histological sections of brain and lung of an adult male *P. alecto* that died in April 1992 after 15 days in care with clinical signs that included being unable to hang, tremors, failing to respond to antibiotics and treatment for suspected Pb poisoning, depression and terminal respiratory distress, showed eosinophilic and granulomatous meningoencephalitis and worm sections in brain and neither nematodes nor their eggs in lung (UQ accession number 92-570). At necropsy this bat was in good nutritional condition. The location at which this, presumably wild, flying fox was found was not recorded.

Australian Animal Health Laboratory / ARI

"Evidence of a nematode in the brain" was reported by Peter Hooper (veterinary pathologist, AAHL) in sections of brain examined at AAHL from a male adult *P. alecto* submitted to the ARI in June 1996 and forwarded to AAHL from the ARI for lyssavirus testing (ARI accession 96-141694, reference BR149, AAHL SAN 96-1230). This flying fox had been submitted via a veterinary surgery in urban Brisbane with a two word history of "CNS signs".

Other institutions

Dave Spratt (*pers. comm.*) had records from 709 wild animal necropsies done while a postgraduate and post-doctorate fellow of the Department of Parasitology at the University of Queensland between 1967 and 1973. The majority of animals were macropods, with a number of rats, bandicoots, koalas and possums etc, including three ship rats (*Rattus rattus*) from Mount Glorious with pulmonary *A. cantonensis.* This collection included only two flying foxes, a *P. alecto* and a *P. scapulatus* which were shot while free-flying (clinically normal) in Arhnemland in October 1972. The brains of these flying foxes were grossly normal and not examined further.

3.3.3.7 Case control study for *Angiostrongylus cantonensis*

There was no evidence of neuro-angiostrongylosis (neither eosinophilic or granulomatous inflammation nor sections of worms) in sections of brain from 19 wild-caught, presumably normal, flying foxes caught in urban Brisbane.

The difference in the proportion of sick flying foxes with histological evidence of neuro-angiostrongylosis (18 /100) and the proportion of clinically normal flying foxes without neuro-angiostrongylosis (0/19) was tested using a Chi-square test, indicating a significant association between the presence of *Angiostrongylosis* spp. and clinical disease (p= 0.045).

3.3.4 Congenital hydrocephalus

Two orphaned *P. alecto* pups (CNS-16 and 17), both born in late 1997 and submitted in January and February 1998 respectively, were found at gross and histological examination to have congenital internal hydrocephalus (ventricular dilation). Both were submitted as a result of their failure to thrive and physical and social retardation while in the care of experienced bat carers. CNS-16 also had a head tremor and a markedly domed skull.

3.3.5 Bacterial meningoencephalitis

Only 2 of 100 wild flying foxes with clinical signs of central nervous system disease had evidence of bacterial meningoencephalitis.

A female *P. alecto* pup (CNS-57, 6-8 months of age) had extensive abscessation of the neck, with a subcutaneous pocket of blood and pus extending over the cranium and neck and a thinly encapsulated caseous abscess extending from the caudal pharynx ventrally to the epaxial muscles and laterally along the trachea, eroding through the ventral aspect of the atlanto-axial joint and extending proximally and caudally into the spinal canal and caudal cranial cavity.

Swabs of the ventral cerebral surface, cervical lymph node, and subcutaneous pocket grew heavy pure growths of *Pasturella multicida.*

An adult female *P. poliocephalus* (CNS-80) that was found on the ground and died overnight had moderate meningoencephalitis and subarachnoid haemorrhage with infiltrates of predominantly lymphocytes, macrophages and some polymorphonuclear cells and prominent bacterial colonies. Bacteriological culture was not done. This bat also had puncture wounds and extensive subcutaneous haemorrhage to the neck suggestive of a dog or other animal attack.

3.3.6 Hepatopathy

One juvenile female *P. poliocephalus* (CNS-26) that was found on the ground weak and failed to improve during 7 days in care, had multiple 0.1-1.0 cm linear to triangular pale green firm liver lesions at necropsy. Histologically the liver lesions consisted of multiple areas of coagulative necrosis with peripheral zones of fibrosis, with macrophages, and some lymphocytes suggestive of a severe multifocal vascular injury.

3.3.7 Chronic injury and myiasis

A male juvenile *P. alecto* (CNS-59) was rescued as a pup from the ground with wounds on its head, near the right eye, and right shoulder. The pup appeared normal as an infant but once able to hang appeared uncoordinated and held its head to the right. Whilst his carer always considered there was “something amiss with him physically”, it had a healthy appetite, continued to improve and “in due course” was able to fly. As the pup “wasn’t quite right” it was not released in February with its peers. Over Easter, another carer noted it would occasionally shake, did not wrap its wings tightly as was normal, and hung with its head to one side. As the pup was being prepared for release, contact with it was minimized but it appeared bright and was eating well. Unexpectedly the pup was found dead on the cage floor in late May with a flyblown wound to the back of its head.

At necropsy the pup was a small bat with ~ 50 large (1-1.5 cm long) maggots and 100’s of very small maggots in a large open necrotic wound of the back, with sloughing of the skin extending proximally and caudally from the right scapula. There was fibrosis of the subcutaneous tissues and musculature of the back of the head and back such that the neck could not be extended beyond 90 degrees. The right scapula was displaced and fixed medially and caudally. Firm adhesions between some liver lobes suggested a low grade chronic peritonitis. Despite the presence of this obviously chronic injury, the bat was not emaciated but had some peri-salivary and perirenal fat.

It is presumed that the head and neck injuries with which the pup was found, possibly bite wounds, had not resolved but persisted as a chronic subcutaneous abscess of the back, and that subsequent myiasis resulted in its apparently sudden death.

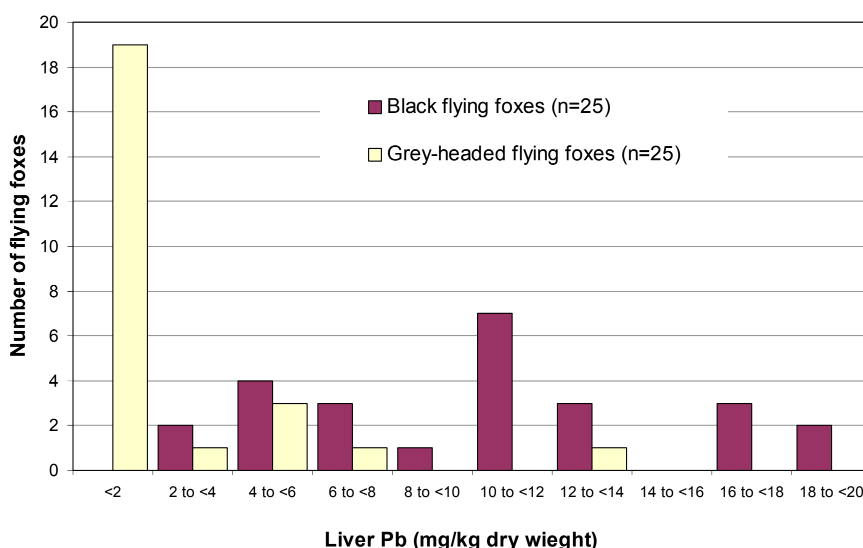
3.3.8 Lead poisoning

3.3.8.1 Liver Pb levels in apparently healthy urban flying foxes

Levels of Pb in the livers of 25 *P. alecto* and 25 *P. poliocephalus* wild-caught in urban Brisbane between November 1997 and July 1999 were in the range 0.1 to 19.77 ppm dry weight (approximately 0.02 to 4.66 ppm wet weight). Details and results for individual flying foxes are shown in Appendix 8. For the purposes of comparison with Pb levels from sick flying foxes, levels of liver Pb of up to ~20 ppm dry matter (~ 4.35 ppm wet weight) do not appear to be associated with clinical disease. The distribution of Pb levels in each species of flying fox is shown in Figure 3-7. Summary statistics are as follows (ppm dry weight).

Mean = 6.24
Median = 5.49
Minimum = 0.10
Maximum = 19.77
Lower quartile = 0.88
Upper quartile = 10.57

Figure 3-7 Normal liver lead (Pb) levels in clinically normal flying foxes captured in urban Brisbane during November 1997 to July 1999 (n=50)



As there appeared to be an unexpected association between species and liver Pb levels, further statistical analysis was done as described in Appendix 8. Levels of Pb in duplicate liver samples (n=5) submitted to the DNR Natural Resource Sciences Laboratory were consistent with those reported by the DPI trace metal Laboratory (see Table A8-1, Appendix 8).

3.3.8.2 Liver Pb levels in bats with CNS disease

Livers from 16 of 100 flying foxes with clinical signs suggesting CNS disease were submitted including 12 of 16 for which no alternative aetiology was diagnosed.

The results were in the range 0.1 to 6.56 ppm dry weight (~0.02 to 1.44 ppm wet weight), well below the median value in clinically normal flying foxes, and not suggestive of lead poisoning as a diagnosis in these cases. Results for specific cases are shown in Table 3-4.

Table 3-4 Liver Pb levels of 16 wild flying foxes with clinical signs suggesting central nervous system disease

CNS case No.	Species	Sex	Age	Diagnosis	Pb ppm dry weight	Approx. Pb ppm wet weight
CNS-9	Black	Male	Adult	Angiostrongylosis and spinal fracture	0.22	0.048
CNS-10	Little Red	Male	Juv	No diagnosis	0.85	0.19
CNS-11	Black	Male	Adult	Angiostrongylosis	1.05	0.23
CNS-19	Black	Male	Juv	No diagnosis	1.62	0.36
CNS-22	Black	Male	Juv	No diagnosis	6.56	1.44
CNS-26	Grey	Female	Juv	Hepatopathy	1.69	0.37
CNS-27	Grey	Male	Adult	No diagnosis	1.6	0.35
CNS-30	Black	Female	Juv	No diagnosis	0.21	0.05
CNS-43	Black	Male	Adult	No diagnosis	0.88	0.19
CNS-47	Black	Male	Adult	No diagnosis	3.09	0.68
CNS-54	Black	Male	Juv	No diagnosis	0.1	0.02
CNS-59	Black	Male	Juv	Chronic injury and mydriasis	1.94	0.43
CNS-72	Grey	Female	Juv	No diagnosis	4.65	1.02
CNS-75	Spectacled	Male	Adult	No diagnosis	0.28	0.06
CNS-78	Spectacled	Female	Juv	No diagnosis	0.82	0.18
CNS-94[1]	Black	Male	Juv	No diagnosis	0.86	0.20[1]

[1] Sample submitted to DNR Natural Resources Sciences Laboratory, Indooroopilly. All other samples submitted to DPI Trace Metal Residue Laboratory, Yeerongpilly.

3.3.9 No diagnosis

No definitive diagnosis was reached for 16 flying foxes submitted with clinical histories suggesting CNS disease (CNS-10, 19, 22, 27, 30, 43, 47, 54, 55, 63, 72, 75, 78, 85, 89, and 94). The level of investigation of these cases varied depending on the availability of relevant samples. In some cases histological lesions of unknown significance were found, and as it was unclear the extent to which they contributed to the presenting clinical signs, no diagnosis was made. Tests performed and comments are provided in Table 3-5, see also Appendix 6.

Table 3-5 Diagnostic investigation of 16 flying foxes with clinical histories suggesting CNS involvement for which no definitive diagnosis was reached

CNS case no.	ABLV FAT	Histology		Spinal X-ray	Liver Pb ppm dry	Comments
		Brain	Other[1]			
CNS-10	Neg.	NSA[2]	LiLgKS	---[3]	0.85	Bacterial colonies in brain and liver suggest peracute septicaemia.
CNS-19	Neg.	NSA	LiLgKS	---	1.62	Fatty degeneration of liver.
CNS-22	Neg.	NSA	LiLgKS and eyes	---	6.56	Pachymeningitis adjacent one optic nerve, bilateral dacryoadenitis.
CNS-27	Neg.	NSA	LiLgKS	NVL[4]	1.6	No significant abnormalities detected.
CNS-30	Neg.	NSA	LiLgKS and skin	---	0.21	Low protein oedema of skin of dorsal head/neck with a mild lymphocytic infiltrate. Gross hyperaemia of ventral neck and oedema of larynx. Suggestive of increased capillary permeability due to neck trauma.
CNS-43	Neg.	NSA	LiLgKS	NVL	0.88	No significant abnormalities detected.
CNS-47	Neg.	NSA	LiLgKS	NVL	3.09	No significant abnormalities detected. Found trapped ‘swimming’ in backyard pool. Clinical signs may simply reflect exhaustion.
CNS-54	Neg.	NSA	LiLgKS	NVL	0.1	No significant abnormalities detected.
CNS-55	Neg.	NSA	Not available	---	ND	No significant abnormalities detected.
CNS-63	Neg.	NSA	Not available	---	ND	No significant abnormalities detected.
CNS-72	Neg.	NSA	LiLgKS	NVL	4.65	Vacuolar change in obex (brain).
CNS-75	Neg.	NSA	LiLgKS	NVL	0.28	No significant abnormalities detected.
CNS-78	Neg.	NSA	LiK	NVL	0.82	No significant abnormalities detected.
CNS-85	Neg.	NSA	Not available	---	ND	No significant abnormalities detected.
CNS-89	Neg.	NSA	Not available	---	ND	No significant abnormalities detected.
CNS-94	Neg.	NSA	LiLgKS and skin	---	ND	4 megaschizonts (synonym merocysts) of *Hepatocystis pteropi*[5] in endothelial cells of kidney. Focal dermatophytosis (‘ringworm’).

[1] Other organs examined: Li = liver, Lg = lung, K =kidney, S = spleen
[2] NSA No significant abnormality detected
[3] --- Not done
[4] NVL No visible lesion
[5] *Hepatocystis pteropi*, malaria-like erythocyte parasite, common in flying foxes (Mackerras 1957). No gametocytes visible in erythrocytes included in tissue sections. Blood unavailable for smear examination. Infection probably incidental.

3.3.10 Targeted evaluation of the sensitivity of the ARI FAT for detection of ABLV

All 23 FAT-negative frozen brain samples from CNS-flying foxes for which either no diagnosis was made (n=16), or diagnosed with spinal and head injuries (n= 5, CNS-2, 38, 66, 68, and 96), hydrocephalus (n=1, CNS-16), or bacterial meningitis (n=1, CNS-80) were negative for ABLV when tested using the TaqMan® assay for pteropid-variant-ABLV and hemi-nested PCR amplification for lyssavirus RNA (see Table 3-6).

This test combination detected pteropid-variant-ABLV, Yellow-bellied sheathtail variant-ABLV and Genotype 1 rabies (representing other known lyssaviruses) in the positive controls.

Table 3-6 Results of TaqMan® and PCR assays for 23 ABLV-FAT-negative flying foxes with CNS signs, 1 FAT-negative clinically normal flying fox (negative control), 3 FAT-ABLV-positive bats, and Nobivac rabies vaccine (positive controls)

Case No.	DPI accession No.	TaqMan® pt-ABLV (C_T)[1]	Lyssavirus PCR	Interpret^n re ABLV	Diagnosis
CNS-2	97-177844	Neg (> 40)	No band	Negative	Spinal fracture
CNS-10	97-203983	Neg (> 40)	No band	Negative	No diagnosis
CNS-16	98-106021	Neg (> 40)	No band	Negative	Hydrocephalus
CNS-19	98-120342	Neg (> 40)	No band	Negative	No diagnosis
CNS-22	98-128821	Neg (> 40)	No band	Negative	No diagnosis
CNS-27	98-140095	Neg (> 40)	No band	Negative	No diagnosis
CNS-30	98-44761	Neg (> 40)	No band	Negative	No diagnosis
CNS-38	98-163314	Neg (> 40)	No band	Negative	Chronic skull/brain injury
CNS-43	98-179890	Neg (> 40)	No band	Negative	No diagnosis
CNS-47	98-183841	Neg (> 40)	No band	Negative	No diagnosis (exhaustion)
CNS-54	99-117491	Neg (> 40)	No band	Negative	No diagnosis
CNS-55	99-126934	Neg (> 40)	No band	Negative	No diagnosis
CNS-63	99-192936	Neg (> 40)	No band	Negative	No diagnosis
CNS-66	99-193425-1	Neg (> 40)	No band	Negative	Spinal fracture
CNS-68	99- 201976	Neg (> 40)	No band	Negative	Spinal fracture
CNS-72	00-133981	Neg (> 40)	No band	Negative	No diagnosis
CNS-75	00-141945	Neg (> 40)	No band	Negative	No diagnosis
CNS-78	00-148284	Neg (> 40)	No band	Negative	No diagnosis
CNS-80	00-171936	Neg (> 40)	No band	Negative	Bacterial meningitis and head trauma
CNS-85	00-180726	Neg (> 40)	No band	Negative	No diagnosis
CNS-89	00-195246	Neg (> 40)	No band	Negative	No diagnosis
CNS-94	01-154853	Neg (> 40)	No band	Negative	No diagnosis
CNS-96	01-158916	Neg (> 40)	No band	Negative	Head trauma
---	98-141060-6	Neg (> 40)	No band	Negative	Clinically normal (negative control)
---	Nobivac rabies vaccine[2]	Neg (> 40)	Appropriate band detected [3]	Negative	Genotype 1 rabies virus (positive ‘other lyssavirus’ control)
ABLV-17	97-122735	19.31	Appropriate band detected	Positive	Pteropid-variant ABLV (positive ABLV control)
ABLV-31	98-102890	19.71	Appropriate band detected	Positive	Pteropid-variant ABLV (positive ABLV control)
ABLV-41	98-144576	Neg (> 40)	Appropriate band detected [4]	Positive	YBST-variant ABLV (positive ABLV control)

[1] C_T > 40 indicates no target RNA detected
[2] Nobivac® Rabies inactivated rabies vaccin (*sic*) Lot 79050 (Intervet, Boxmeer, Holland)
[3] Positive rabies-TaqMan assay (two extractions C_T = 17.7 and 16.3) consistent with genotype 1 rabies virus
[4] Positive YBST-TaqMan assay (two extractions C_T =17.4 and15.9) consistent with YBST-variant ABLV

3.4 Discussion

3.4.1 Central nervous system examination

Flying foxes are reasonably stoic animals and clear assessment of CNS responses required minimal restraint. In cases were the subject was not depressed and retained good wing function (e.g. bats with lower motor neuron paralysis of the hind limbs as a result of thoracic or lumbar spine fractures) minimal restraint allowed flying foxes to attempt to scratch or climb on to the candidate. Given the high likelihood of bats with CNS disease being ABLV-positive, and after a few ‘close calls’, this examination was considered too hazardous and was subsequently abbreviated or discontinued.

It is strongly recommended that physical and central nervous system examination *not be attempted* on conscious bats with clinical histories or neurological signs suggesting CNS disease.

3.4.2 Spinal and head trauma

The presence and extent of head injuries in 7 of 20 bats with spinal fractures, and the absence of these injuries in most other bats found on the ground, suggests that in at least some cases the primary impact had been ‘head on’. Indeed, in two cases (CNS-60 and CNS-85) the bats were heard or seen to fly into (hit) houses. It appears that otherwise healthy flying foxes occasionally ‘crash’ at speed while in flight.

3.4.3 Neuro-angiostrongylosis

The rat lungworm, *Angiostrongylus cantonensis*, is a metastrongylid nematode that inhabits the right ventricle and pulmonary arteries of various rats, being restricted in Australia to the two exotic species, *Rattus norvegicus* and *R. rattus*. Since its lifecycle was elucidated in Brisbane almost 50 years ago (Mackerras and Sandars 1955), infection has been reported in humans, captive wildlife and domestic animals (Prociv *et al.* 2000), almost all presenting with disease of the CNS. Originally, cases were reported in coastal Queensland and north-eastern New South Wales, although, over the last decade, the parasite has been recognized in Sydney in dogs, rats, zoo primates and native mammals (Prociv *et al.* 2000).

Rats, and abnormal definitive hosts, acquire the infection by eating third stage larvae in the tissues of the intermediate hosts, slugs and snails, which have become infected by eating first-stage larvae expelled in rat faeces. Molluscivorous paratenic hosts (such as land planarians, freshwater crustacea) are sometimes infected, although their importance in Australia is unknown. Once in the definitive host, infective larvae undergo an obligatory migration through the CNS, where they grow and moult twice to attain the immature adult stage. They then invade the venous system to return to the right ventricular outflow, where, in suitable hosts, they copulate and

produce eggs. Clinical disease arises from mechanical damage and associated inflammation in nervous tissue, blood vessels and the subarachnoid space (Mackerras and Sandars 1955).

In this study, 18 of 100 flying foxes (18%) with clinical CNS signs had gross or histological evidence of neuro-angiostrongylosis involving the brain. Histological evidence of infection was found in archival material or reports of a further two Black flying foxes from 1992 and 1996. All the flying foxes (n=20) with angiostrongylosis presented in south-east Queensland. Nematodes were recovered from the brains of 11 of the 18 recent cases, and morphologically confirmed as *A. cantonensis.* The adult worm fragment and third stage larvae recovered from a further three cases, and histological sections of nematodes in the remaining four, had features entirely consistent with those of *Angiostrongylus* spp., presumably *A. cantonensis.* To date, only *A. cantonensis*, not *A. mackerrasae*, has been identified as a cause of disease in humans and animals.

The number of recent cases involving Black compared to Grey-headed flying foxes (14 and 4, respectively) reflects the relative numbers of these species in the study (61 and 26, respectively). While the parasite was not identified in the Little Red (*P scapulatus*) or Spectacled flying fox (*P conspicillatus*), this may simply reflect their small numbers in the study (11 and 2, respectively). Histological evidence of *Angiostrongylus* sp. has been reported in *P. scapulatus* from Queensland (Reddacliff *et al.* 1999). The presumptive finding of this parasite in histological sections from 1992 and 1996 suggests that it has been affecting flying foxes for some time. Disease surveillance of flying foxes was virtually non-existent prior to them being linked with Hendra virus (previously equine morbillivirus), and the discovery of Australian bat lyssavirus. Given the scanty material available from flying foxes before 1996, it is not possible to conclude whether or not the apparently high number of cases described here reflects a changing prevalence of angiostrongylosis.

Presumably, flying foxes acquire infection by ingesting intermediate or paratenic hosts, such as slugs, snails and planarians, or their slime (although slime has not been confirmed to be a vehicle of infection)(Prociv *et al.* 2000). These hosts could readily be concealed among fruit. While it might seem unlikely that flying foxes would eat these invertebrates, faecal analysis has shown that flying foxes regularly ingest insects, spiders and mites (Jackson 2000). Slugs and snails do climb high into trees, especially in wet weather (P. Prociv unpublished observation), and might either prove attractive to a hungry flying fox, or be ingested unintentionally.

Continuing habitat destruction in south-east Queensland has been linked to a reduction in flying fox numbers and fragmentation of their colonies, with the establishment of smaller, more numerous colonies in urban areas (Hall and Richards 2000). Habitat destruction, with overgrowth of introduced plant species, could force hungry flying foxes to eat unusual foods, such as molluscs. When food is scarce, flying foxes will retrieve fallen fruit from the ground, thus increasing their risk of ingesting molluscs. However, the good nutritional condition of 13 of the 20 cases suggests these bats had not had difficulty finding food. The circumstances and climatic

conditions under which the flying foxes acquired the parasite are unknown, because infection might have occurred considerable distances from where they were found. Black flying foxes in urban south-east Queensland typically fly up to 7 km per night to feed (Nikki Markus pers. comm.) and also migrate considerable distances (>100 km during 10 weeks) to find native fruit and flowering trees (Nelson 1965). Grey-headed flying foxes from northern NSW have been shown to fly even greater distances each night (up to 50 km) and seasonally (in excess of 600 km) (Eby 1991). During the 4 years of the study, cases were clustered in the months of May, June, and early July or October, November, and December. It is possible that during these months, particular trees that are in flower or fruit attract both flying foxes and molluscs, bringing these species together. Four captive Grey-headed flying foxes with angiostrongylosis from Sydney also presented during May and June (Reddacliff *et al.* 1999), but this coincidence should be interpreted cautiously as the factors leading to infection of captive flying foxes in temperate Sydney might differ from those for free-living flying foxes in subtropical Queensland.

Worm growth rates in flying foxes might differ from those in their normal definitive hosts. Nevertheless, assuming growth rates equal to the slowest found in rats, the estimated duration of infection in some of the cases allows for interesting conclusions. Four flying foxes (CNS-9, 11, 77 and 99) had only very small nematodes in histological sections of brain and virtually no encephalitis, suggesting early infection. Immature L3 only were recovered from Bats 9 and 11, and all four flying foxes were killed within 24 hours of being found. It appears these four flying foxes presented with acute, severe disease just 1 to 3 days after infection. CNS-32 also appears to have been found only 1 to 2 days after infection, but survived in care for as long as 22 days. The remainder from which worms were recovered and measured (n=9) appear to have been found, unable to fly, 9 to 23 days after infection, and died or were killed 16 to 30 days after infection. CNS-73, the captive-born juvenile, appears to have been infected shortly after release. CNS-76 had a particularly intense exposure, with 43 worms recovered from half the brain, yet this bat appears to have been infected for 19 days before being found, and survived a further 7 days in care before being killed. There seems to be no correlation between the number of worms recovered (per half-brain) and either the estimated interval after infection when found, or the duration of infection before death. These estimates suggest that most bats become affected and cannot fly 1 to 3 weeks after infection. This parallels human infections: in one well-documented outbreak in a group of men, several developed neurological symptoms as early as 1 to 2 days after exposure, whereas most were hospitalized within 1 to 2 weeks of the onset of symptoms (Kliks *et al.* 1982).

Ideally, finding worms in the right ventricular outflow would require fresh tissue for careful dissection of the heart and pulmonary arterial tree, but all heart and lung samples had been fixed. Branches of the major pulmonary arteries were considered most likely to harbour adult worms, yet none were found in transverse sections of the pulmonary hilar region, suggesting that *A. cantonensis* had not migrated beyond the CNS. The lungs of infected aberrant hosts are rarely examined specifically for the presence of adult worms, but when this parasite has been found in

human lungs, it has been in cases with heavy and prolonged infections (Cooke-Yarborough *et al.* 1999; Prociv 1999). The ability of *Angiostrongylus* to reach the lungs and develop further probably varies with the host species.

The circumstances in which flying foxes with neuro-angiostrongylosis are found and *initial* clinical signs closely resemble those of flying foxes with the 'paretic' form of Australian bat lyssavirus infection (see Chapter 2). However, whereas most bats with ABLV rapidly deteriorate within 24 to 48 hours of being rescued and die or are killed *in extremis*, most flying foxes with neuro-angiostrongylosis have a comparatively prolonged (> 3 days) and stable clinical course, and are more often killed as a result of failure to improve and the carer's assessment that the bat is unsuitable for release rather than *in extremis*. Also, some flying foxes with neuro-angiostrongylosis had periods of temporary improvement in CNS functions (for example appearing stronger or regaining the ability to hang, CNS-32 and CNS-97) which was never the case with bats with ABLV (n=74).

3.4.4 Hydrocephalus

Two *P. alecto* pups born in late 1997 were found to have gross congenital internal hydrocephalus. In 1990 a similar cluster of pups was investigated by Les Hall and Roger Kelly (*pers. comm.* and review of records) of the School of Veterinary Science at the University of Queensland as described below.

During October and November 1990, carers in Orphan Native Animal Rear and Release Association Inc. (ONARR) reported that 6 of 21 orphaned *P. alecto* pups were found with or developed domed heads and showed retardation while hand-reared. Similarly orphaned *P. poliocephalus* pups did not appear affected. Four live affected pups were submitted to the Department of Veterinary Pathology; all had "increased cranial volumes due principally to expansion of lateral ventricles and compression of cerebral cortex… with some porencephaly and/or hydranencephaly of the cerebellum in 3 pups". Two pups had "irregular asymmetrical defects on one side of the cranium, with some brownish pigmentation", which suggested the pups had been bitten on the head.

There are nutritional and infectious causes of hydrocephalus in other species (DeLahunta 1983) and it is likely that nutritional or infectious factors intermittently cause outbreaks of hydrocephalus in *P. alecto* pups. As only two pups during one season (1997-98) of this study were diagnosed with hydrocephalus and considering the large number of pups examined during the lyssavirus surveillance project in Queensland and the close and supportive relationship between members of ONARR, other carer groups, and the research team at ARI, it appears that this event is uncommon.

3.4.5 Lead poisoning

Evidence of high tissue lead levels have been reported in Black and Grey-headed flying foxes in Queensland (Hariono *et al.* 1993; Skerratt *et al.* 1998; Sutton and Wilson 1983; Sutton and Hariono 1987). The source of the lead for three captive Grey-headed flying foxes in Washington D.C, USA that died with extremely high liver lead levels (> 500 ppm wet tissue), appears to have been peeling lead-based paint in the enclosure (Zook *et al.* 1970). The source of lead for wild bats in Australia remains unknown but is presumed to be either lead arsenate in insecticides applied to fruit or atmospheric lead pollution, principally from petrol vehicle emissions. Arsenic was absent from the liver of one wild bat (Sutton and Wilson 1983), and subsequent research showed that wild urban bats rather than non-urban bats accumulate high levels of lead on their fur that could be ingested when grooming (Hariono *et al.* 1993; Sutton and Hariono 1987). This suggests air pollution is the main source of lead. Lead on the fur may have been the result of primary contamination from the air or secondary to contact while foraging with lead settled on foliage (Halliwell *et al.* 2000; Sutton and Hariono 1987).

In most other species healthy individuals have lead concentrations of < 5 ppm wet tissue (Seawright 1982),(corresponds to < 22 ppm dry flying fox liver). The background level of lead exposure in apparently healthy populations of flying foxes had not previously been surveyed. Liver lead levels from 25 Black and 25 Grey-headed flying foxes (n=50 total) captured in apparent good health from two camp sites in urban Brisbane between November 1997 and July 1999 were low (0.1 to 19.77 ppm dry weight, approximately 0.02 to 4.66 ppm wet weight) and consistent with levels in healthy individuals of other species.

The thresholds above which blood or tissue levels of lead in flying foxes are associated with clinical toxicity are unknown. Data from domestic species indicates that liver levels > 10 ppm wet tissue (extrapolates to 44 ppm dry flying fox liver) are of diagnostic significance (Seawright 1982). The finding of exceptionally high concentrations of Pb (> 500 ppm wet liver) in two captive bats (Zook *et al.* 1970) and two bats dosed with lead over a 13 and 9 week period (540 and 556 ppm liver) (Hariono 1991) suggests flying foxes are resistant to clinical plumbism. The clinical significance of moderate to high tissue lead levels in bats may be over-estimated by extrapolation from toxic lead levels of other species.

In the case reported by Skerratt *et al.* (1998) and Speare *et al.* (1997), a wild female Black flying fox appeared in a backyard with signs of overt aggression that included chasing and biting other bats, including her own pup which sustained fatal skull fractures. A well supported diagnosis of lead poisoning was made on the basis of eosinophilic inclusions in renal tubular epithelium shown to contain lead, and lead tissue concentrations that were very high in kidney (370 ppm) and moderately high in liver (16 ppm). Some 2 years later, a diagnosis of ABLV infection was made from stored tissues. The clinical signs shown by this bat, overt aggression then rapid deterioration and death, are similar to those described for other ABLV bats. It appears that the clinical condition

of this bat was primarily a consequence of ABLV infection and that the accumulation of tissue lead was concurrent and incidental.

It is unclear to what extent other reports may also have attributed undue clinical significance to high tissue lead levels in unwell the bats. Of the two cases reported in Sutton and Wilson (1983), Bat B was found in circumstances very like those of ABLV-positive bats (crawling in a yard with no obvious injuries, unable to fly, alert, and dying later the same day). It is unclear whether samples from this bat were included in a group of archival bat samples sent to the AAHL for ABLV testing, all of which were negative. A subsequent survey of lead concentrations in unwell or dead bats found tissue lead levels suggestive of toxicity (> 10 ppm wet liver, > 25 ppm wet kidney) in 11 of 37 urban bats (Hariono *et al.* 1993)

The study presented here found no evidence of liver lead levels suggestive of toxicosis in either wild caught, apparently normal Black or Grey-headed flying foxes (n=50) or clinically ill bats with evidence of ABLV-like CNS disease (n=16). The apparent absence of elevated liver lead in unwell urban bats in this study, contrasts with earlier findings (n=10 of 37, (Hariono *et al.* 1993)) and may be the result of reductions in atmospheric lead pollution. Lead free petrol was first used Australia in 1975, and in 1987 legislation required all new cars to use lead-free (unleaded) petrol. Decreasing numbers of cars manufactured prior to 1988 continued to use leaded petrol (super) until 2002, when it was replaced by lead replacement petrol (lead free super).

3.4.6 Targeted evaluation of the sensitivity of the ARI FAT to detect ABLV

Epidemiological evidence presented here identified bats with clinical signs suggesting CNS disease as having a significantly higher prevalence of ABLV than unwell bats without CNS clinical signs, and showed that the prevalence of ABLV among bats with CNS signs was high; ~20% (see Chapter 2). By examining 100 flying foxes with CNS signs, this study targeted 21 of 825 FAT-negative wild flying fox samples considered most likely to have given a false-negative FAT result on the basis of

- the bats presenting with CNS signs for which there was no well supported alternate diagnosis (n=16) or
- the bats having spinal or head injuries that they could have been predisposed to by lyssavirus meningoencephalomyelitis (n=5).

The study used the potentially more sensitive TaqMan® and PCR assays to test for the presence of lyssavirus RNA, an indicator of lyssavirus infection independent of the detection of lyssavirus antigen by the FAT. This independent and targeted approach failed to find evidence of false negative FAT results, and did detect pt-ABLV, ybst-ABLV, and non-ABLV lyssavirus (rabies virus) in appropriate controls.

3.5 Conclusions

Causes of clinical neurological syndromes in flying foxes

- ABLV is only one of a number of conditions that cause clinical neurological syndromes in flying foxes.
- ABLV is the most common cause of paresis, paralysis, or overt aggression in flying foxes (32%).
- ABLV, spinal and head injuries (29%), and neuro-angiostrongylosis (18%) combine to account for most (79%) neurological syndromes in flying foxes.
- There was no evidence of elevated liver lead levels that would suggest lead toxicity in 50 apparently healthy urban flying foxes or 16 unwell flying foxes, sugggsting lead poisoning is no longer a cause of CNS disease in bats.
- Severe hind-limb paralysis in a bat with good wing function and bright or aggressive demeanour strongly suggests a diagnosis of spinal fracture.
- A bat with a comparatively long (> 7 days) and stable history of ABLV-like paresis is highly likely to have neuro-angiostrongylosis.
- On initial presentation, bats with the paretic form of ABLV, neuro-angiostrongylosis and head trauma are clinically indistinguishable. Given the difficulty of distinguishing these neurological syndromes, the risks associated with caring for bats infected with Australian bat lyssavirus, and the poor prognosis of all three conditions, wildlife carers should be urged not to care for flying foxes with neurological disease, but to submit them for lyssavirus testing.

Targeted evaluation of the sensitivity of the fluorescent antibody test for ABLV detection

- Targeted evaluation of the sensitivity of the ARI-FAT indicates the FAT is highly sensitive, with no evidence that the test has failed to detect ABLV in high-risk category flying foxes.

4 Molecular diversity of Australian bat lyssavirus

Aims

1. To examine the molecular diversity of ABLV isolated from pteropid and insectivorous bats.
2. To determine if there is host species, geographic or temporal variation among ABLV isolates.
3. To re-examine the phylogenetic evidence for ABLV as a seventh lyssavirus genotype within the *Lyssavirus* genus.

4.1 Introduction

At the time this study was done (2001), the sequences of only two ABLV isolates were available, one from a Black flying fox that died in Ballina NSW in 1996 ((Fraser *et al.* 1996; Gould *et al.* 1998), GenBank AF006497, referred to here as ABLBallina), and one from a 1996 YBST bat (AF081020, referred to here as AAHLYBST, (Gould *et al.* 2002)). At that point all published sequence and phylogenetic analyses of ABLV, and the proposal of ABLV as a separate genotype within the *Lyssavirus* genus, Genotype 7, related to the single ABLBallina sequence (Badrane *et al.* 2001; Gould *et al.* 1998). There was reference to 16 ABLV N gene sequences being highly conserved (Hooper *et al.* 1997b; McColl *et al.* 2000), and the phlyographic depiction of the ABLV genotype was illustrated with two terminal branches (McColl *et al.* 2000), however the sequence details were not published.

Unpublished communications between the principal ABLV research groups, notably the AAHL, QHSS and ARI, strongly suggested that two major variants of ABLV were present, one in flying foxes and the other in YBST bats. However, the extent of sequence diversity among and between ABLV isolates from flying foxes and YBST bats remained unclear. There existed the possibility that there were multiple species-specific, regional, and temporal 'sub-variants' of ABLV. There was also the possibility that other ABLV sequences would be more closely related to rabies virus isolates and that the genetic distinction between ABLV isolates and rabies isolates would be obscured.

The sequence of the N protein was selected for analysis as the N protein has important structural and immunological functions, is involved in the regulation of transcription and replication, and has been proposed as a determinant of host range (Kissi *et al.* 1995). The entire coding sequence was used for phylogenetic analysis, as similar analyses performed on shorter sections of the N sequence or the intergenic N-P region have shown a parallel evolutionary pattern but were less

robust (Kissi *et al.* 1995). In addition the availability of corresponding sequences from other lyssaviruses through GenBank permitted the relationships between the new and available ABLV sequences and those of the other lyssaviruses to be evaluated. Sequencing and analysis of the G gene from the same material was being done simultaneously by other members of the research group (Guyatt *et al.* 2003).

4.2 Materials and Methods

4.2.1 Selection of samples

At the time of sample selection, material was available from 52 ABLV-positive cases submitted to the ARI and stored as frozen tissue (brain and/or salivary glands), as well as inocula prepared from these tissues (see Chapters 2 and 5). Some samples submitted in Queensland, particularly those submitted within the first year of the recognition of ABLV, were not available as they had be forwarded *in toto* to the AAHL, including samples from two Yellow-bellied sheathtail bats, the only Spectacled flying fox sample, and the only sample from a bat submitted from West Queensland (Mount Isa). Limited funds precluded examination of material from every available case. Samples were selected to reflect the range of bat species, geographic locations and years from which they were submitted. The range of available ABLV-positive samples included material from cases with the following characteristics.

- Insectivorous bats (n=3, all Yellow-bellied sheathtail bats) or flying fox (n=49) hosts
- Flying foxes found in Southeast Queensland (n=40), Central Queensland (n=4, two from Rockhampton and two from Gladstone), or Northeast Queensland (n=5, all from Townsville)
- Black flying foxes (n=23), Grey-headed flying foxes (n= 7), or Little Red flying foxes (n=19)
- Samples from 1997 (n=20), 1998 (n=23), 1999 (n=4), or 2000 (n=5).

As the numbers of available samples from insectivorous bats, and from flying foxes from Central and Northeast Queensland were small, all these samples were selected. Inocula 1 to 10, which were used in Experiment 1 of Chapter 5 (Detection of live ABLV by intracerebral inoculation of suckling mice), were also selected. These inocula had produced clinical disease in suckling mice and had been prepared from the tissues of different naturally-infected bats. Other samples were selected in order to have minimum group sizes of 5 and to include cases from each year 1997-2000 for at least one species. Thirty-one samples were selected: samples from three Yellow-bellied sheathtail bats, 15 Black flying foxes, five Grey-headed flying foxes and eight Little Red flying foxes as described in Table 4-1 and Table 4-2.

Table 4-1 Selection of samples for molecular analysis
Six groups permitting comparison of species and geographical diversity.

Groups of samples	Group Abbr.	No. selected/ No. avail.	Comments
Yellow-bellied sheathtail bats (All from Southeast Queensland)	YBST	3/3	---
Central Queensland flying foxes (All from Rockhampton or Gladstone)	Cen	4/4	3 Black flying foxes (Cen-B) 1 Little Red flying fox (Cen-R)
North-eastern Queensland flying foxes (All from Townsville)	Tvl	5/5	2 Black flying foxes (Tvl-B) 3 Little Red flying foxes (Tvl-R)
Southeast Queensland – Black flying foxes	SEQ-B	9/18	---
Southeast Queensland – Grey-headed flying foxes	SEQ-G	5/7	---
Southeast Queensland – Little Red flying foxes	SEQ-R	5/15	---

Genotype 1 rabies virus RNA within the Nobivac® Rabies inactivated rabies vaccin (*sic*) Lot 79050B (Intervet, Boxmeer, Holland) containing an inactivated culture of rabies virus cloned out of strain Pasteur RIVM was also sequenced for comparison with other known rabies virus sequences. As the sequence of the Nobivac Pasteur RIVM was expected to very closely resemble that of other fixed rabies virus strains (e.g. Pasteur virus, SAD-B19 and CVS), and to be clearly distinguishable from the ABLV sequences, it acted as a lyssavirus control sequence during amplification and sequencing.

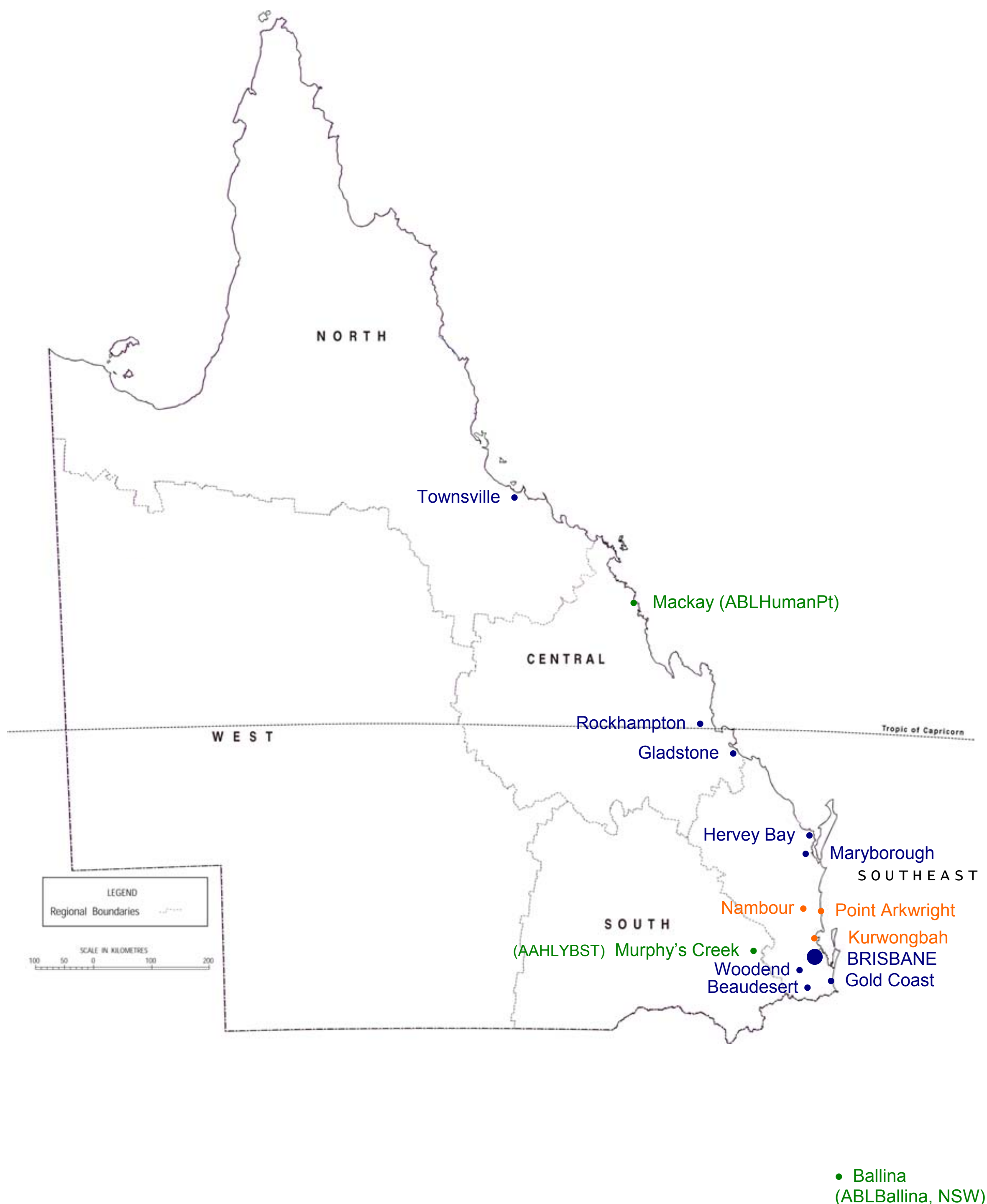
Table 4-2 Details of individual samples used for nucleotide sequencing

Sequence name [1]	GenBank Accession	ABLV case No.	ARI Accession number	Host Species	Month & year host died	Location host found	Sample type
ABL19SEQ-R	AY573951	ABLV-11	97-116174	*P. scapulatus*	Feb 97	Woodend	Brain
ABL11YBST[2]	AY573965	ABLV-12	97-116375	*S. flaviventris*	Feb 97	Kurwongbah	Brain
ABL21SEQ-R[2]	AY573946	ABLV-14	97-121016	*P. scapulatus*	March 97	Brisbane	Brain
ABL05SEQ-B[2]	AY573964	ABLV-18	97-125092	*P. alecto*	March 97	Gold Coast	Inoculum 2 (brain)
ABL12YBST[2]	AY573937	ABLV-22	97-143816	*S. flaviventris*	May 97	Nambour	Brain
ABL22SEQ-B	AY573940	ABLV-23	97-152661	*P. alecto*	June 97	Brisbane	Brain
ABL23SEQ-B	AY573942	ABLV-24	97-155574	*P. alecto*	June 97	Woodend	Inoculum 10 (brain)
ABL07SEQ-G[2]	AY573958	ABLV-26	97-176292	*P. poliocephalus*	Aug 97	Brisbane	Inoculum 3 (brain)
ABL02SEQ-G	AY573948	ABLV-27	97-186955	*P. poliocephalus*	Oct 97	Beaudesert	Inoculum 1 (brain)
ABL08SEQ-G	AY573941	ABLV-28	97-192894	*P. poliocephalus*	Oct 97	Gold Coast	Inoculum 6 (brain)
ABL24SEQ-R[2]	AY573955	ABLV-31	98-102890	*P. scapulatus*	Jan 98	Woodend	Brain
ABL13SEQ-G	AY573950	ABLV-33	98-105331	*P. poliocephalus*	Jan 98	Brisbane	Inoculum 9 (brain)
ABL29Tvl-R	AY573944	ABLV-34	98-40635	*P. scapulatus*	Feb 98	Townsville	Brain
ABL25SEQ-R	AY573956	ABLV-36	98-126853	*P. scapulatus*	April 98	Brisbane	Brain
ABL26SEQ-B	AY573963	ABLV-38	98-137634	*P. alecto*	May 98	Gold Coast	Brain
ABL14Cen-B	AY573943	ABLV-39	98-140331	*P. alecto*	May 98	Rockhampton	Brain
ABL15YBST	AY573949	ABLV-41	98-144576	*S. flaviventris*	June 98	Point Arkwright	Brain
ABL17Tvl-B	AY573939	ABLV-42	98-189710	*P. alecto*	July 98	Townsville	Brain
ABL09SEQ-G	AY573945	ABLV-45	98-167351	*P. poliocephalus*	Aug 98	Gold Coast	Inoculum 4 (brain)
ABL06SEQ-B	AY573959	ABLV-46	98-172971	*P. alecto*	Sept 98	Brisbane	Inoculum 7 (salivary glands)
ABL30Tvl-R	AY573953	ABLV-47	98-50290	*P. scapulatus*	Aug 98	Townsville	Brain
ABL31SEQ-R	AY573952	ABLV-48	98-182934	*P. scapulatus*	Oct 98	Rockhampton	Brain
ABL16Cen-R	AY573960	ABLV-49	98-183454	*P. scapulatus*	Oct 98	Rockhampton	Brain
ABL01SEQ-B	AY573935	ABLV-51	98-188455	*P. alecto*	Nov 98	Maryborough	Inoculum 5 (salivary glands)
ABL03Tvl-R	AY573957	ABLV-52	98-189702	*P. scapulatus*	Nov 98	Townsville	Inoculum 8 (salivary glands)
ABL32Tvl-B	AY573947	ABLV-53	98-189756	*P. alecto*	Nov 98	Townsville	Brain
ABL33SEQ-B	AY573954	ABLV-54	99-128813	*P. alecto*	April 99	Brisbane	Brain
ABL34Cen-B	AY573961	ABLV-55	99-178735	*P. alecto*	Aug 99	Gladstone	Brain
ABL35SEQ-B	AY573938	ABLV-58	99-204785	*P. alecto*	Nov 99	Hervey Bay	Brain
ABL36SEQ-B	AY573936	ABLV-60	00-132366	*P. alecto*	May 2000	Brisbane	Brain
ABL37Cen-B	AY573962	ABLV-63	00-176632	*P. alecto*	Sept 2000	Gladstone	Brain

[1] The sequence name contains a number that relates to the essentially random order in which samples were processed for RNA extraction. This number *does not* imply chronological order or any other characteristic of the sample or sequence. The name also includes a group abbreviation, as described in Table 4-1, indicating the species and regional location of the sample host. Chronology *is implied* by the corresponding ABLV Case No., which was allocated according to the order in which the host died.

[2] Brain from these six cases was forwarded to AAHL for diagnostic confirmation where it was later used for virus isolation and partial N sequences were determined (bases 443-892 of the ABLBallina sequence, corresponding to bases 513 to 962 of Appendix 10) (Gould *et al.* 2002). Corresponding sequence names and AAHL accession no. are: ABL11YBST = 97-034-6375, ABL21SEQ-R = 97-0388-016, ABL05SEQ-B = 97-414-1 and 97-414-2, ABL12YBST = 97-0747, ABL07SEQ-G = 97-1153, ABL24SEQ-R = 98-0126.

Figure 4-1 Locations at which selected ABLV isolates (bats) were found
Flying fox locations are shown in blue. YBST bat locations are shown in orange.
Also shown (green) are the location of other available ABLV sequences (ABLHumanPt, ABLBallina and AAHLYBST)



4.2.2 Primers

All primers were designed and evaluated with the following programs prior to oligonucleotide synthesis.

- Sequencher™ Version 3.1.1 (Gene Codes Corporation, MI, USA)
  Used to align sequences from which primers were to be designed and to identify conserved regions as potential primer binding sites.
- Oligo® Version 4.03 (Natural Biosciences, Inc, Plymouth, USA)
  Used to identify primer dimers, hairpins and calculate the melting temperature.
- Amplify version 1.2 (University of Wisconsin, Madison, Wisconsin, USA)
  Used to identify primer dimers and non-specific binding to target cDNA sequences.

Primers were synthesized commercially by Sigma Genosys (Sigma-Aldrich, Castle Hill, Australia) and were purchased deprotected, desalted and dry. Stock solutions of 1 µg/µL were prepared by resuspension of the oligonucleotides in nuclease free-water and stored at –20°C.

Details of the primers used in this study are shown in Table 4-3 and Table 4-4.

Primers N5'REVERSE, N3'FORWARD and PREVERSE were designed from comparisons of the two ABLV sequences available on GenBank at that time, namely the Ballina isolate, GenBank accession AF006497 (pteropid-ABLV) (Gould *et al.* 1998) and an insectivorous isolate, GenBank accession AF081020 (ybst-ABLV)(Gould *et al.* 2002). Both these sequences begin with the N gene start codon (ATG) that was inferred from the last three bases of the primer used for cDNA sequencing and PCR amplification.

In the absence of any ABLV-specific 5' N leader sequence, Kimberley Guyatt (QABC) designed the Lys-Leader primer from comparisons of available lyssavirus sequences for this region, namely Mokola virus (GenBank S59448), Pasteur rabies virus (X03673), SAD B19 rabies virus (M31046), and RCHLVac rabies virus (AB009663), to correspond to the first 22 nucleotides of the Mokola/rabies genome. Initial attempts by K Guyatt to use the Lys-Leader primer for both cDNA synthesis and as the forward primer for subsequent PCR amplification produced poor results. However, downstream sections of the Lys-Leader cDNA were amplified using other primers. During the study, the 70 nucleotides of the 5' N leader sequence of ABLV isolated from the second human case of ABLV (pteropid-ABLV) that had been cloned and sequenced at QHSS were kindly made available by David Warrilow prior to publication (Warrilow *et al.* 2002), and used to design the primer ABLUTR22. Like the Lys-Leader primer, ABLUTR22 corresponds to the first 22 nucleotides of this untranslated region, but 'corrects' five base mismatches between the Mokola and rabies virus derived Lys-Leader sequence, and the pteropid-ABLV sequence provided by QHSS.

Table 4-3 Forward Primers

Primer name	Length (mer)	Tm °C	Position [a]	Nucleotide sequence 5'-3', + genomic sense	Designed by	cDNA	PCR	Seq.
ABLUTR22	22	64.1	1-22	ACG CTT AAC GAC AAA ACC AGA G	Candidate	✓	✓	✓
Lys-Leader	22	61.9	1-22	ACG CTT AAC AAC CAG ATC AAA G	K Guyatt	✓	✓	✓
N3'FORWARD	21	53.7	634-654	GAC TAC TCA TAA GAT GTG TGC	K Guyatt	✗	✓	✓
YBSTN3'FORW	22	68.4	643-664	CAA AAT GTG TGC GAA TTG GAG C	Candidate	✗	✓	✓
N5'SEQ1F	19	52.0	491-509	GTA GGT CTT CTT CTA AGC C	K Guyatt & Candidate	✗	✗	✓
N3'SEQ2F	21	61.5	1169-1189	CAA GAT TAT GAG GCA GCA GAG	Candidate	✗	✗	✓
NobiVSEQ2F	20	67.2	1093-1112	AGG GGG CTA TTT GGG AGA GG	Candidate	✗	✗	✓

Table 4-4 Reverse Primers

Primer	Length (mer)	Tm °C	Position[a]	Nucleotide sequence 5'-3', - genomic sense	Designed by	PCR	Seq.
N5'REVERSE	22	58.2	932-911	GGA TGA AAT TTG TGT GAG GAA C	K Guyatt	✓	✓
PREVERSE	24	72.7	1584-1561[b]	GTC TCC TCT GCC ATT TCC AGG TCG	K Guyatt	✓	✓
NobiVPRev	23	64.4	1590-1568[b]	TCA ACA GTT TCT TCA GCC ATC TC	Candidate	✓	✓
NobiVSEQ1R	25	67.5	506-482-	TCA GGA GAA GACC GAC TAA AGA TGC	Candidate	✗	✓
N5'SEQ1R	20	54.4	513-494-	TAA AGG CTT AGA AGA AGA CC	K Guyatt & Candidate	✗	✓
N3'SEQ2R	20	52.9	1166-1147-	GTT CTT TCT CAT CTC TGA AG	Candidate	✗	✓

[a] Numbering corresponds to nucleotide positions within the Pasteur virus + sense genome (GenBank X03673).

[b] The positions of reverse primers PREVERSE and NobiVPREV in Appendix 10 are 1589-1566 and 1595-1573 respectively, due to the renumbering of base positions caused by the insertion of 5 additional bases between the N stop codon and intergenic region (CT) of the ABL14CEN-B + sense genome.

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The YBSTN3’FORWARD, N5’SEQ1F, N5’SEQ1R, N3’SEQ2F, and N3’SEQ2R primers were designed from comparisons of the ABLV sequences available on GenBank (AF006497 and AF081020) and the preliminary sequence results of this study. As a number of the primers designed for amplification and sequencing of ABLV did not work with the Nobivac rabies cDNA, some had to be ‘replaced’. The NobiVPRev primer was designed from comparisons of similar rabies virus sequences available on GenBank (Pasteur virus, SAD B19, and RC-HL) and corresponds closely to the binding site for ABLV primer PREVERSE. Primers NobiVSEQ1R, and NobiVSEQ2F were designed from preliminary sequence results of Nobivac vaccine rabies RNA and bind to sites close to the binding sites for ABLV primers N5’SEQ1R and N3’SEQF respectively.

4.2.3 RNA extraction and cDNA synthesis

Extractions from ABLV-positive material were done by K Guyatt in the PC3 facilities of the QHSS laboratory. Total RNA was extracted from either naturally infected brain tissues or 20% weight per volume suspensions of naturally infected brain or salivary gland tissue in Dulbecco’s Modified Eagles Medium that had been prepared for inoculation trials (see Chapter 5). Matchstick-head sized tissue samples or 100 µL of inoculum were prepared using the QIAshredder™ Homogenizer (QIAGEN) and total RNA extracted from 100 µL of the lysate using the RNeasy Mini RNA extraction kit (QIAGEN) in accordance with the manufacturer’s instructions.

Total RNA was extracted by the candidate from 100µL of the Nobivac® Rabies inactivated rabies vaccin (*sic*) Lot 79050B (Intervet, Boxmeer, Holland) using the RNeasy Mini RNA extraction kit at the molecular laboratory of the Queensland Agricultural Biotechnology Centre (QABC).

Initial ABLV cDNA synthesis using the Lys-Leader primer was done by K Guyatt at QHSS. Subsequent synthesis of ABLV cDNA using the Lys-Leader and ABLUTR22 primers and of Nobivac rabies cDNA using the Lys-Leader primer was done by the candidate at QABC.
Four (4) µL of extracted RNA and 10 pmol of primer were heated to 95°C for 3 minutes, and then chilled on ice. The reverse transcription reaction mixture was made up to 10 µL containing the RNA/primer mix, 2.0 µL of 5× First Strand Buffer (GibcoBRL® Life Technologies), 0.5 mM of each dNTP, 0.01 mM dithiotreitol (DTT), 0.5 µL (10-20 units) Recombinant RNasin® (Promega, Madison, Wisconsin, USA), and 100 units of SUPERSCRIPT™ II Reverse Transcriptase (GibcoBRL® Life Technologies). cDNA synthesis was performed using a Gene Amp® System 2400 PCR thermal cycler (Perkin Elmer) at 42°C for 60 minutes followed by inactivation of the SUPERSCRIPT™ Reverse Transcriptase at 95°C for 3 minutes, and the product chilled on ice and stored at -20°C.

4.2.4 PCR amplification, purification and extraction

PCR amplification of cDNA primed by either the Lys-Leader or ABLUTR22 primer was performed in a volume of 50µL containing 2µL of cDNA, 200 pmol of each forward and reverse primer, 200 µmol of each dNTP, 2.5 units of *Taq* DNA polymerase (Roche, Mannheim, Germany) and 5.0 µL of 10× Mg+ Buffer (Roche, Mannheim, Germany). The N gene sequences were amplified as two overlapping PCR products, N5'HALF and N3'HALF in a Gene Amp® System 2400 PCR thermal cycler (Perkin Elmer) programmed for 94°C for 5 minutes, three cycles of 94°C for 30 seconds, annealing 50°C for 45 seconds, and extension 72°C for 90 seconds, followed by 30 cycles where the annealing temperature was increased to 55°C. The program ended with an elongation period of 10 minutes at 72°C, then held the reaction mix at 4°C.

For reactions using the YBSTN3'FORW (Tm= 68.4) or PREVERSE primers (Tm= 72.7), the annealing temperatures were increased to 55°C and 60°C respectively.

PCR products were separated by gel electrophoresis using 1.0% DNA grade agarose (Progen Industries, Darra, Australia) in TAE containing ethidium bromide across 80 V. Bands of product were visualized using Ultra◊llūm electronic UV transilluminator and bands of appropriate length were excised with a sterile scalpel. Products were extracted from the gel using the QIAquick® Gel Extraction Kit (QIAGEN) according to the manufacturer instructions. The eluted purified PCR products were stored at 4°C pending sequencing. The concentrations of the purified PCR products were estimated by gel electrophoresis by comparing aliquots of each product with the known DNA ladder standard. All PCR amplification reactions, purification, sequence reactions, and analyses were done by the candidate.

4.2.5 Automated sequencing

Sequencing reactions were performed in a volume of 20 µL containing 3.2 pmol of primer, 5µL of BigDye Terminator mix Version 2 (Applied Biosystems, Perkin Elmer) and 1-14 µL of purified PCR product as required to provide an estimated 400-800 ng of cDNA template. The sequencing reactions were performed using the Gene Amp® System 2400 PCR thermal cycler (Perkin Elmer) for 25 or 30 cycles of 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes then held at 4 °C. Extraction of unincorporated dye terminators and precipitation of the extension products was done by adding 80µL of 75% isopropanol to the sequence reaction mix for 15-40 minutes and pelleting the extension products at 13 000 G for 20 minutes. The pellet was washed with 250µL of 75% isopropanol, dried under vacuum and stored at 4°C until submitted to the Australian Genome Research Facility at the University of Queensland for EST/cDNA sequencing using the AB377 DNA sequencer (Applied Biosystems, Perkin Elmer) and ABI Prism™ DNA Sequencing Analysis Software (Applied Biosystems, Perkin Elmer).

Table 4-5 Details of 58 GenBank sequences used for sequence comparisons and phylogenetic analysis

Thesis reference	GenBank accession	Isolate reference number	Isolate Host	Year	Location host found	Virus variant[1]	Reference
ABLHumanPt	AF418014	Confidential	Human	1998	Mackay, Qld, Australia	GT-7 Pteropid-ABLV	Warrilow *et al* 2002
ABLBallina	AF006497	96-0591, Ballina	*Pteropus alecto*	1996	Ballina, NSW, Australia	GT-7 Pteropid-ABLV	Gould *et al* 1998
AAHLYBST	AF081020	96-1293[2]	*Saccolaimus flaviventris*	1996	Murphy's Creek, Qld, Australia	GT-7 ybst-ABLV	Gould *et al* 2002
HoaryBat1Canada	AF351845	LC1	*Lasiurus cinereus*	1992	Alberta, Canada	Microbat rabies Group IIa	Nadin-Davis *et al* 2001
HoaryBat8Canada	AF351858	LC8	*Lasiurus cinereus*	1996	Ontaria, Canada	Microbat rabies Group IIa	Nadin-Davis *et al* 2001
SilverHairBatCanada	AF351842	LAN8	*Lasionycteris noctivagans*	1992	Manitoba, Canada	Microbat rabies Group IIb	Nadin-Davis *et al* 2001
LittleBrown4Canada	AF351839	ML4	*Myotis lucifugus*	1992	British Columbia, Canada	Microbat rabies Group 1b	Nadin-Davis *et al* 2001
LittleBrown6Canada	AF351838	ML6	*Myotis lucifugus*	1994	Nova Scotia, Canada	Microbat rabies Group 1a	Nadin-Davis *et al* 2001
RedBatUSA	AF351857	LB7	*Lasiurus borealis*	1998	Connecticut, USA	Microbat rabies Group IIa	Nadin-Davis *et al* 2001
NorthnYellowUSA	AF351843	LI	*Lasiurus intermedius*	1987	Florida, USA	Microbat rabies Group IIa	Nadin-Davis *et al* 2001
BigBrown22Canada	AF351830	EF22	*Eptesicus fuscus*	1988	British Columbia, Canada	Microbat rabies Group III	Nadin-Davis *et al* 2001
BigBrown32Canada	AF351828	EF32	*Eptesicus fuscus*	1993	Ontario, Canada	Microbat rabies Group 1d	Nadin-Davis *et al* 2001
BigBrown57Canada	AF351859	EF57	*Eptesicus fuscus*	1997	Ontario, Canada	Microbat rabies Group 1c	Nadin-Davis *et al* 2001
BigBrown71USA	AF351860	EF71	*Eptesicus fuscus*	1998	Connecticut, USA	Microbat rabies Group 1c	Nadin-Davis *et al* 2001
FreeTailBatUSA	AF351849	TB1, V235	*Tadarida brasiliensis*	?	Texus, USA	Microbat rabies Group IV	Nadin-Davis *et al* 2001
FreeTailBatChile	AF070450	?	*Tadarida brasiliensis*	?	Chile	Microbat rabies	Warner *et al* 1989
InsectivBatChile	AF351850	IB.Ch	Microbat	1998	Chile	Microbat rabies Group IV	Nadin-Davis *et al* 2001
VampireBrasil	U22479	86118BRE	*Desmodus rotuntus*	1985	Brasil	Vampire bat rabies	Kissi *et al* 1995
VampBrasil	AF070449	?	*Desmodus rotuntus*	?	Brazil	Vampire bat rabies	Warner *et al* 1989
VampireHuPeru	AF045166	Pehm3230	Human	1996	Peru	Vampire bat rabies	Warner *et al* 1989
MongooseSthAfrica	U22628	1500AFS	Yellow mongoose	1987	South Africa	Rabies	Kissi *et al* 1995
KuduNamibia	U22632	8708NAM	Kudu (herbivore)	1987	Namibia, Africa	Rabies	Kissi *et al* 1995
DogCentralAfrica	U22651	9229CAF	Dog	1992	Central African Republic	Dog rabies	Kissi *et al* 1995
DogAfrica	U22486	8636HAV	Dog	1986	Burkina Fasso, Africa	Dog rabies	Kissi *et al* 1995, Bourhy *et al* 1999
DogIvoryCoast	U22646	9026CI	Dog	1990	Ivory Coast, Africa	Dog rabies	Kissi *et al* 1995
DogGuinea	U22487	8660GUI	Dog	1986	Guinea, Africa	Dog rabies	Kissi *et al* 1995, Bourhy *et al* 1999
DogAlgeria	U22643	9137ALG	Dog	1982	Algeria, Africa	Dog rabies	Kissi *et al* 1995, Bourhy *et al* 1999
DogIran	U22482	89681IRA	Dog	1986	Iran	Dog rabies	Kissi *et al* 1995, Bourhy *et al* 1999
PV	X03673	Pasteur Virus (PV)	PAS derivative[3]	1882	France ~ Argentina →Paris 1965	Fixed dog rabies	Tordo *et al* 1986
SAD-B19	M31046	SAD-B19 (vaccine)	SAD derivative[4]	1935	Alabama, USA	Attenuated dog rabies	Conzelmann *et al* 1990
CVS	D42112	CVS	PAS derivative[3]	1882	France → USA 1940	Fixed dog rabies	Mannen *et al* 1991

Thesis reference	GenBank accession	Isolate reference number	Isolate Host	Year	Location host found	Virus variant[1]	Reference
Nishigahara	AB044824	Nishigahara	PAS derivative[3]	1882	France → Japan 1915	Fixed dog rabies	Ito *et al* 2001
RCHLVac	AB009663	RC-HL (vaccine)	Nishigahara derivative	1882	France → Japan 1915	Attenuated Nishigahara	Ito *et al* 2001
3AGChina	AF155039	3AG	?	?	China	Fixed laboratory rabies	Unpublished
DogMexico	U22477	9126MEX	Dog	1991	Mexico	Dog rabies	Kissi *et al* 1995
FLARaccoonUSA	U27220	FLA 125	Raccoon	1987	Florida, USA	Raccoon rabies	Nadin-Davis *et al* 1996
PARaccoonUSA	U27221	PA R89-1811	Raccoon	1989	Pennsylvania, USA	Raccoon rabies	Nadin-Davis *et al* 1996
NYRaccoonUSA	U27218	NY516	Raccoon	1992	New York, USA	Raccoon rabies	Nadin-Davis *et al* 1996
SkunkWestCanada	AF344304	WCS2/92RABLV1741	Skunk	1992	Western Canada	Arctic fox rabies	Nadin-Davis *et al* 1997
SKSkunkCanada	L20671	SK (clone)	Skunk	?	Ontario, Canada	Arctic fox rabies	Nadin-Davis *et* al 1993
ONT2SkunkCanada	L20674	ONT2/ 91RABN2756	Skunk	1991	Ontario, Canada	Arctic fox rabies	Nadin-Davis *et al* 1993
ONT3RedFoxCanada	L20675	ONT3/ 91RABN0783	Red Fox	1991	Ontario, Canada	Arctic fox rabies	Nadin-Davis *et al* 1993
ArcticFoxRussia	U22656	9141Rus	Arctic Fox	1988-90	Russia	Arctic fox rabies	Kissi *et* al 1995, Bourhy *et al* 1999
ArcticFoxGreenland	U22654	8684Gro	Arctic Fox	1981	Greenland	Arctic fox rabies	Kissi *et al* 1995, Bourhy *et al* 1999
RaccoonDogEstonia	U42707	9339EST	Raccoon dog	1991	Estonia	Red fox rabies	Bourhy *et al* 1999
RaccoonDogPoland	U22840	8618POL	Raccoon dog	1985	Poland	Red fox rabies	Kissi *et al* 1995, Bourhy *et al* 1999
RedFoxFrance	U43433	9223FRA	Red Fox	1974	France	Red fox rabies	Bourhy *et al* 1999
RedFoxYugoslavia	U42706	86111YOU	Red fox	1986	Bosnia-Herzegovina (Yugoslavia)	Red fox rabies	Bourhy *et al* 1999
RedFoxGermany	U42702	9213ALL	Red fox	1991	Germany	Red fox rabies	Bourhy *et al* 1999
HumanThailand	U22653	8738THA	Human	1983	Thailand	Rabies (dog?)	Kissi *et al* 1995
RabiesIndia	AF374721	?	?	?	India	Rabies (dog?)	Unpublished
EBL-1 Poland	U22844	8615POL	*Eptesicus serotinus*	1985	Poland	GT-5 EBL-1a	Kissi *et al* 1995, Amengual *et al* 1997
EBL-1 France	U22845	8918FRA	*Eptesicus serotinus*	1989	France	GT-5 EBL-1b	Kissi *et al* 1995, Amengual *et al* 1997
EBL-2 Holland	U22847	9018HOL	*Myotis dasycneme.*	1986-87	Holland	GT-6 EBL-2a	Kissi *et al* 1995, Amengual *et al* 1997
EBL-2 Finland	U22846	9007FIN	Human	1986	Finland	GT-6 EBL-2b	Kissi *et al* 1995, Amengual *et al* 1997
Duvenhage	U22848	86132AS (AF)	Human	1970	South Africa	GT-4 Duvenhage	Kissi *et al* 1995, Meredith *et al* 1971
LagosBat	U22842	8619NGA	*Eidolon helvum*	1958	Nigeria	GT-2 Lagos bat	Kissi *et al* 1995, Bourhy *et al* 1993
Mokola	Y09762	?	Cat	1981	Zimbabwe	GT-3 Mokola	Le Mercier *et al* 1997

[1] All viruses are Genotype 1 (GT-1) unless otherwise indicated.
[2] This isolate (AAHL case 96-1293) corresponds to DPI accession 96-33821, described further as ABLV-5 in Chapter 2 of this thesis. The AAHL accession number of this sequenced isolate was erroneously published as 96-1256 in Gould *et al.* (2002)(Allen Gould AAHL *pers. comm.*).
[3] Divergent laboratory strains of rabies virus (PAS) isolated by Louis Pasteur in 1882 from a rabid cow in urban Paris (i.e. dog-variant 'urban' rabies (Sacramento *et al.* 1992).
[4] SAD-B19 is an attenuated strain, which is non-pathogenic when administered orally or intramuscularly, derived from the street virus SAD isolated from a dog in Alabama.
? Unknown or unpublished

4.2.6 Sequence analysis

Nucleotide sequence data obtained by automated sequencing were compiled and edited using Sequencher™ Version 3.1.1 (Gene Codes Corporation, MI, USA). Consensus ABLV sequences, the Nobivac sequence, and other lyssavirus sequences available via GenBank (http://www.ncbi.nih.gov) that represent the range of other lyssaviruses, were uploaded to the Australian National Genomic Information service web site (http://www.angis.org.au) for analysis using the Genetics Group Wisconsin Inc (GCG) suite of programs. Details of the selected GenBank sequences are shown in Table 4-5.

Nucleoprotein amino acid sequences were deduced using eTRANSLATE (GCG Inc, Madison Wisconsin, USA). Multiple sequence alignments of the nucleotide and amino acid sequences were constructed using eCLUSTALW (Thompson *et al.* 1994). Percent amino acid similarities were calculated by the HOMOLOGIES program (Jack Leunissen CAOS/CAMM Centre, University of Nijmegen, The Netherlands). Alignments and the similarity matrix are presented using Excel 2000.

4.2.7 Phylogenetic analysis

Nucleotide sequences of the new ABLV isolates, the Nobivac sequence, and those of the selected lyssavirus sequences available from GenBank were aligned using ClustalX Version 1.83 (a windows interface version of ClustalW, (Jeanmougin *et al.* 1998; Thompson *et al.* 1997) available at http://www.biozentrum.unibas.ch/~biophit/clustal/ClustalX_help.html).

Phylogenetic trees were constructed from the N protein coding sequences using Paup* 4.0b10 (PPC) (Swofford, D.L. 2002 Phylogenetic Analysis Using Parsimony (*and Other Methods) Sinauer Associates, Sunderland, MA) by; (i) neighbour joining using the (Hasegawa *et al.* 1985) distance measure, which estimates a transition/transversion ratio and base frequencies and (ii) maximum parsimony using random stepwise-addition. For all analysis a higher weight was applied to substitutions occurring at first codon positions relative to second and third codon positions (2:1:1) to reflect the higher likelihood of a first codon substitution resulting in an amino acid substitution. Bootstrap resampling analysis (Felsenstein 1985) using 1000 replicates was done to evaluate the reliability of branch groupings. Consensus trees are presented using CorelDRAW Version 7.373.

4.3 Results

An example of the relationship between the N5'HALF and N3'HALF PCR products and the sequence data produced using the relevant suite of 8 primers and the resulting consensus sequence is shown in Figure 4-2.

Each of the 31 ABLV isolates produced a nucleotide sequence of 1,542 (n=30) or 1,543 (n=1) bases that have been submitted to GenBank (for GenBank numbers see Table 4-2). This encoded:

- 35 bases of the leader sequence
- a one base (T) leader to N protein intergenic region
- the complete mRNA N protein sequence, including the complete N protein coding sequence
- a two base (CT) N to P protein intergenic region
- the complete 5' UTR of the P protein mRNA and the first 47 bases of the P protein coding sequence.

The 1,350 base N coding sequences of two isolates (ABL24SEQ-R and ABL25SEQ-R) were identical. The 1,545 bases of the Nobivac vaccine sequence were identical to those of CVS rabies (GenBank D42112). One ABLV sequence (ABL14Cen-B) was one base longer than all other ABLV sequences due to the insertion of an additional 'A' base in the polyadenylation termination signal (AGA_8 rather than AGA_7). This insertion persisted in both sequencing directions despite repeat PCR amplification and sequencing using cDNA synthesized with different primers (Lys-Leader and ABLUTR22) indicating it to be a sequence anomaly of the source extracted RNA population. The aligned nucleotide sequences of this thesis (ABLV n=31 and Nobivac) along with those of the other available ABLV sequences (n=3) are given in Appendix 10, Figure A10-1.

There was an exceptionally high level of nucleotide homology among isolates from pteropid bats (flying foxes 98.4 to 99.9%) and from YBST bats (99.4 to 99.9%), while comparisons between pteropid and YBST isolates demonstrated relatively high divergence (83.5 to 83.9%). This crude analysis suggests that two highly conserved variants of ABLV exist, pteropid-variant ABLV and YBST-variant ABLV.

Many of the base substitutions were synonymous to the extent that the 27 unique pteropid-variant N coding sequences encoded only four unique deduced N amino acid sequences. The deduced N amino acid sequences of ABL30Tvl-R, ABL35SEQ-B and ABL37Cen-B were unique, each as a result of single amino acid substitutions as shown in Table 4-6.

The deduced N protein of all other pteropid variant-ABLV isolates (n=25) were identical to that of ABL01SEQ-B.

Table 4-6 Characterization of low N protein amino acid sequence variability among 28 isolates of Pteropus-variant ABLV

Number of sequences	Isolate	Amino acid position	Amino acid substitution
n=25	ABL01SEQ-B[1]	not applicable	typical pteropid sequence
n=1	ABL35SEQ-B	128	(L) Leu → (F) Phe[2]
n=1	ABL30Tvl-R	181	(I) Ile → (V) Val[3]
n=1	ABL37Cen-B	376	(K) Lys → (R) Arg[3]

[1] Representative of all other pteropid-variant ABLV sequences. Corresponds to flying fox case ABLV-51 and Inoculum 5 as referred to in Chapters 2 and 5 to 7 respectively.
[2] At position 128, Leu, which is typical of pteropid-ABLV is substituted for Phe, typical of ybst-ABLV.
[3] At positions 181 and 376 the amino acids Val and Arg also occur in Lagos bat virus and Mokola virus and not ybst-ABLV or any of the other selected rabies or rabies-like sequences.

Of the three ybst-ABLV isolates, the deduced N protein sequence of ABL11YBST and ABL12YBST were identical. The sequence of ABL15YBST was unique as a result of two amino acid substitutions, Lys →Arg$^{\text{base 34}}$ and Arg →Lys$^{\text{base 290}}$ that did not occur in any other sequence used for comparison.

An alignment of the four unique pteropid-variant ABLV deduced N protein sequences, two unique YBST-variant ABLV sequences, other available ABLV sequences (n=3) and 55 other rabies-like and rabies virus N protein sequences, is shown in Figure 4-3

A percent similarity matrix comparing 33 of these N protein sequences, selected to include those most and least like ABLV in each group, is shown in Figure 4-4. The ABLV N protein amino acid sequences shared the highest similarly with those of rabies virus isolates (88.4 to 93.6%) and were least like Lagos bat virus (81.3 to 82.7%).

Figure 4-2 Alignment of forward and reverse sequence reaction data for ABL01SEQ-B generated from two overlapping RT-PCR products
Numbers correspond to the position on the new sequence. Position 1 corresponds to base 1 of the Pasteur virus + sense genome (GenBank X03673)

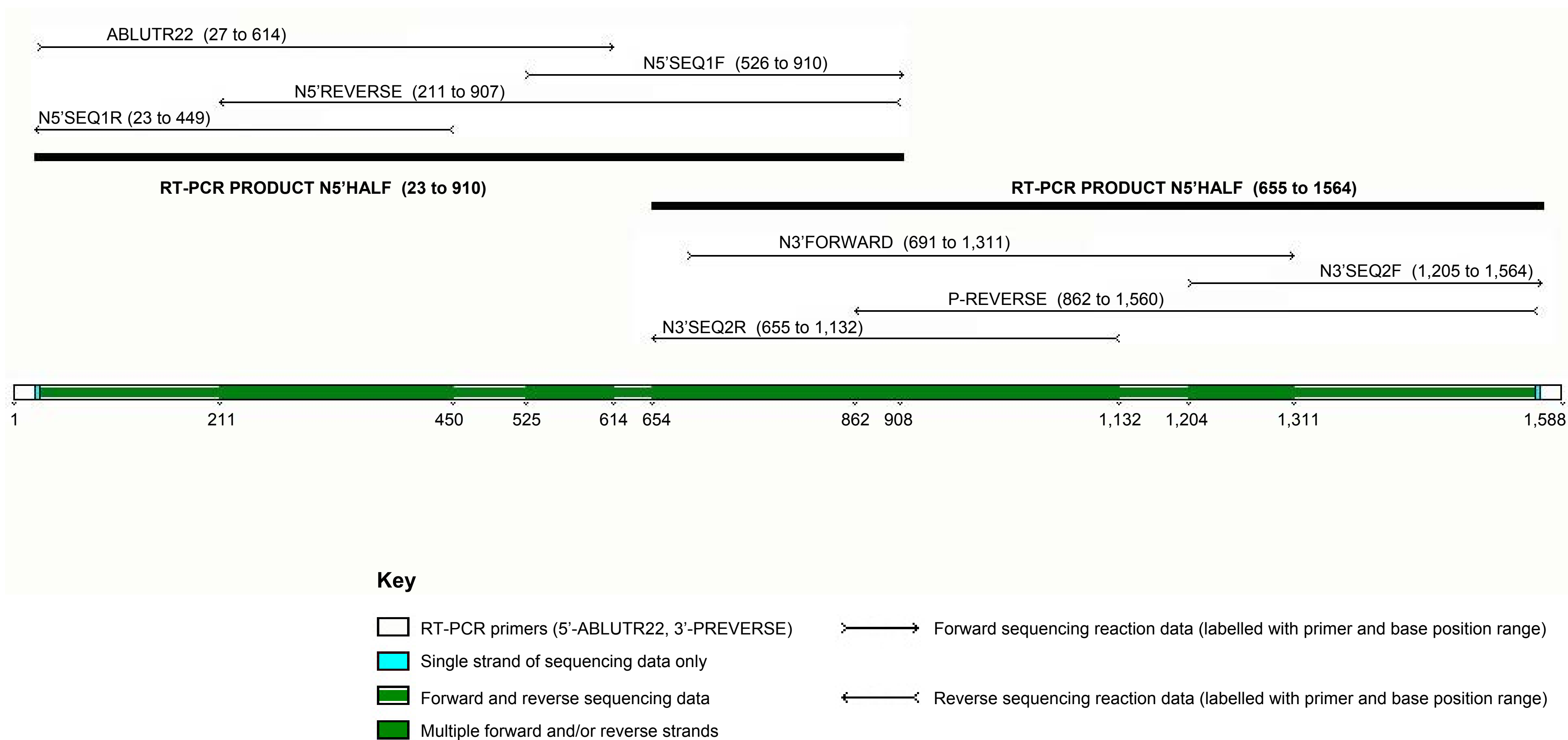
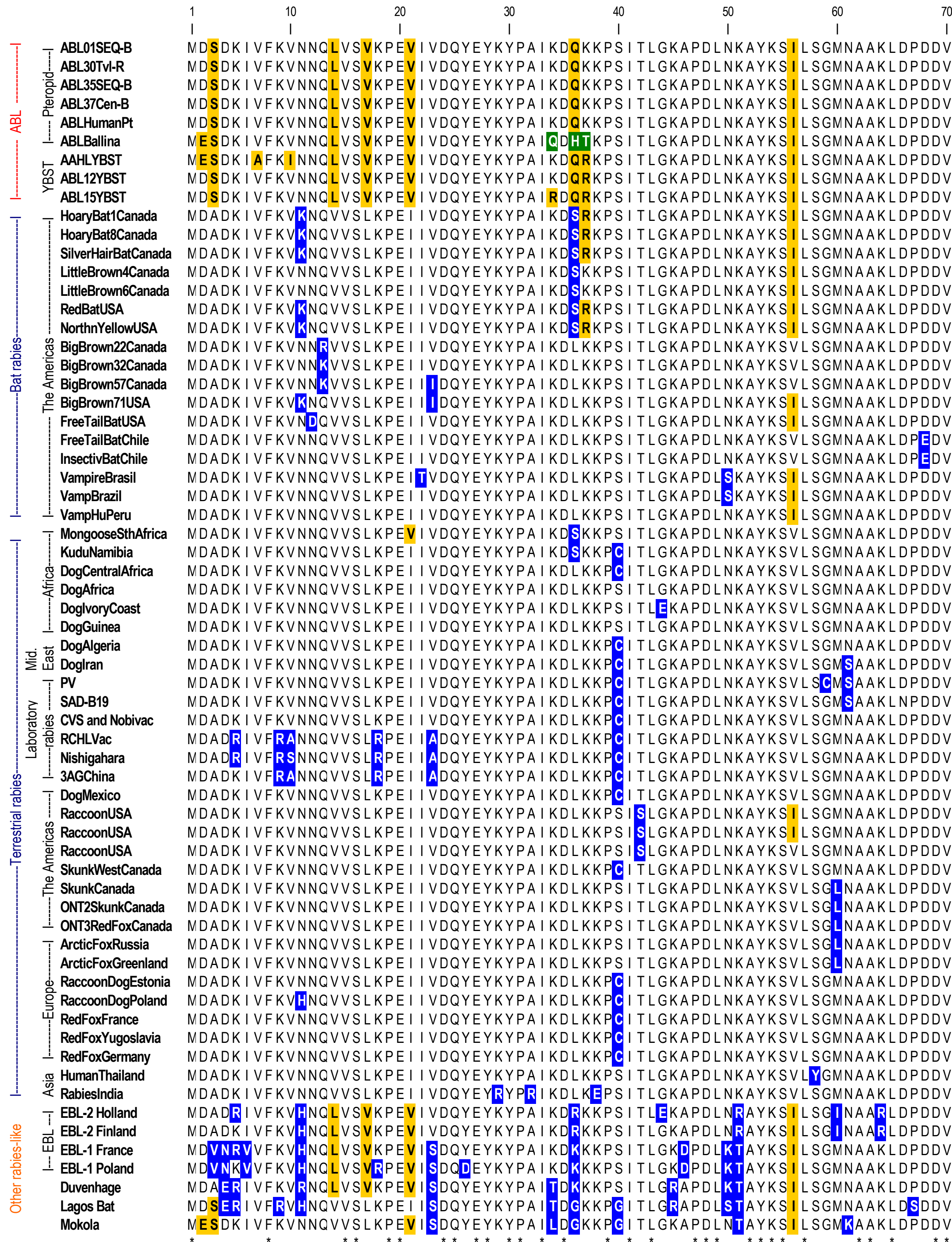


Figure 4-3 N protein amino acid sequence alignment of 4 new deduced pteropid-variant ABLV and 2 new YBST-variant ABLV sequences, with those of 58 other rabies-like and rabies viruses



KEY

ABLV amino acids that differ from the predominant lyssavirus amino acid at each site, and similarly substituted amino acids of other (non-ABLV) lyssaviruses are highlighted in yellow (first substitution e.g. E) or green (second substitution e.g. Q). Other (non-ABLV) lyssavirus amino acids that differ from the predominant lyssavirus amino acid at each site are highlighted in blue (e.g. V). GenBank accession numbers for each sequence are given in Table 4-2 and Table 4-5. Conserved sites are indicated by an asterisk (*), sites at which substitutions with similar amino acids occur are indicated by a stop (.).

Figure 4-3 –continued **N protein alignment (page 2 of 7)**

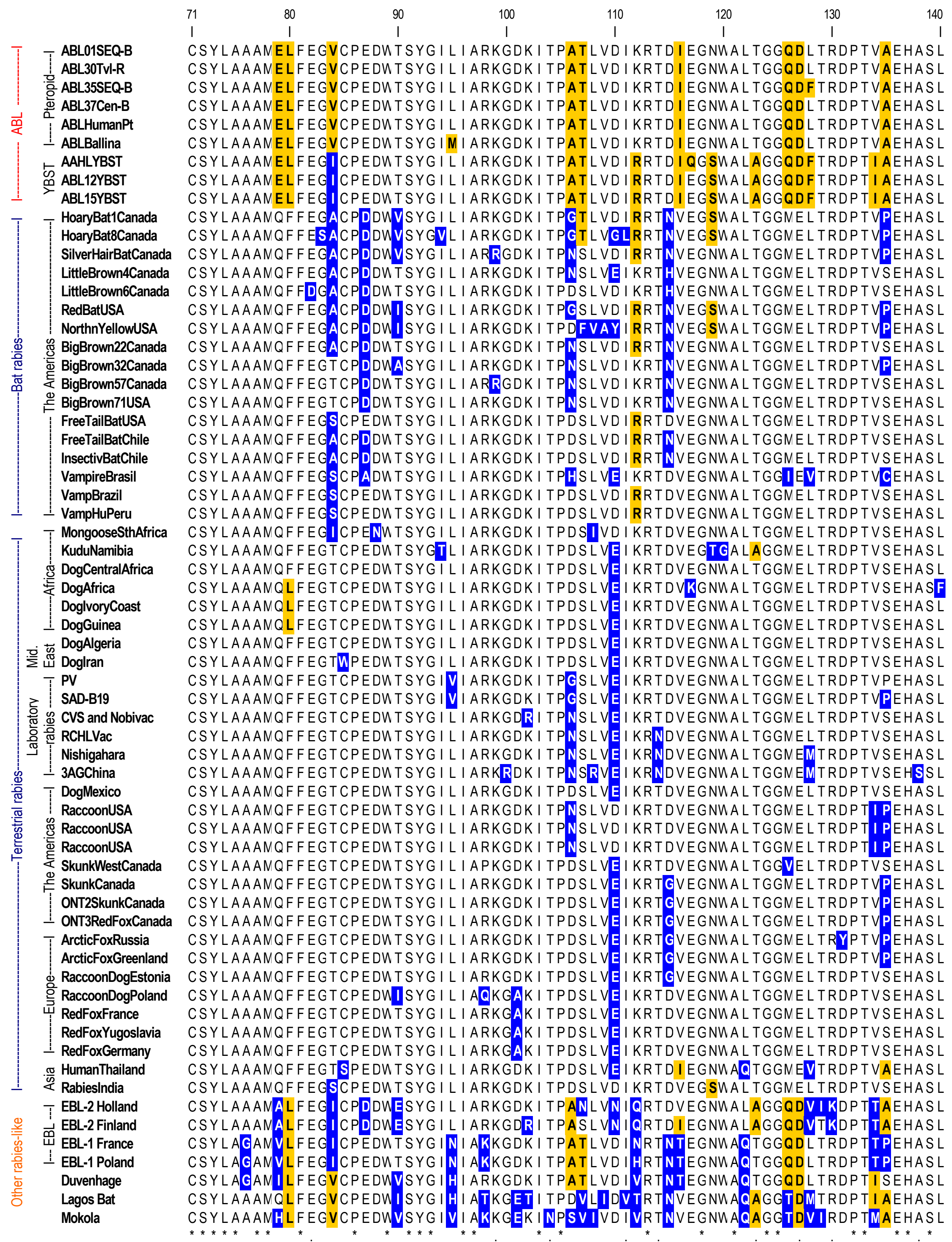
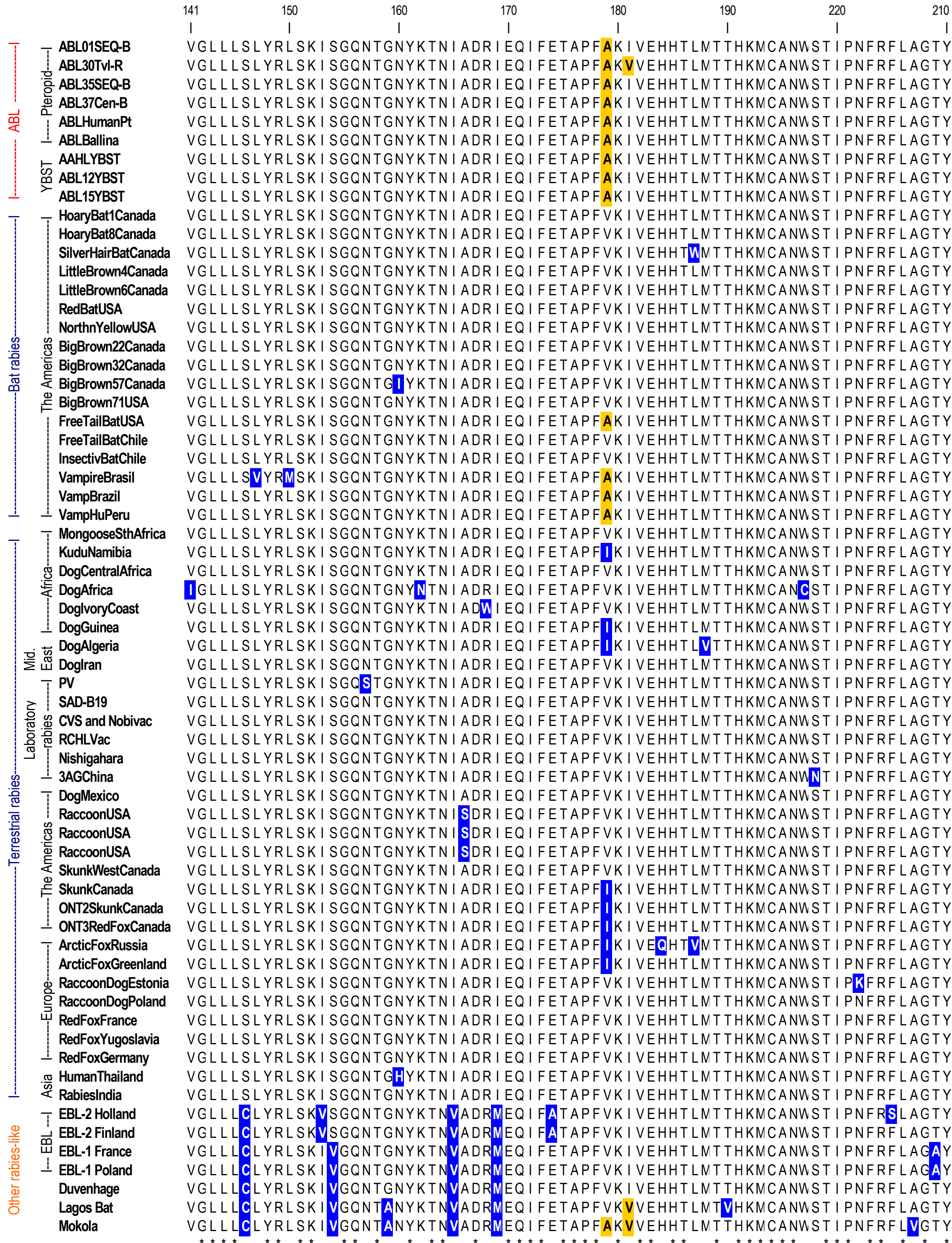


Figure 4-3 –continued N protein alignment (page 3 of 7)



KEY

ABLV amino acids that differ from the predominant lyssavirus amino acid at each site, and similarly substituted amino acids of other (non-ABLV) lyssaviruses are highlighted in yellow (first substitution e.g. E) or green (second substitution e.g. Q). Other (non-ABLV) lyssavirus amino acids that differ from the predominant lyssavirus amino acid at each site are highlighted in blue (e.g. V). GenBank accession numbers for each sequence are given in Table 4-2 and Table 4-5. Conserved sites are indicated by an asterisk (*), sites at which substitutions with similar amino acids occur are indicated by a stop (.).

Figure 4-3 -continued **N protein alignment (page 4 of 7)**

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                    211      220       230       240       250       260       270       280
                    |        |         |         |         |         |         |         |
ABL01SEQ-B          DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ABL30Tvl-R          DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ABL35SEQ-B          DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ABL37Cen-B          DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ABLHumanPt          DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ABLBallina          DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
AAHLYBST            DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ABL12YBST           DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ABL15YBST           DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
HoaryBat1Canada     DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
HoaryBat8Canada     DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEKIRRMFEPGQETA
SilverHairBatCanada DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
LittleBrown4Canada  DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
LittleBrown6Canada  DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
RedBatUSA           DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEKIRRMFEPGQETA
NorthnYellowUSA     DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
BigBrown22Canada    DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
BigBrown32Canada    DMFFSRIEHLYSAIRVGTVVTAYEDCSGLASFTGFIKQTNLTAREAILYFFHKNFEEEIRRMFEPGQETA
BigBrown57Canada    DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAKEAILYFFHKNFEEEIRRMFEPGQETA
BigBrown71USA       DMFFSRVEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAKEAILYFFHKNFEEEIRRMFEPGQETA
FreeTailBatUSA      DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
FreeTailBatChile    DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
InsectivBatChile    DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
VampireBrasil       DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
VampBrazil          DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
VampHuPeru          DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREALLYFFHKNFEEEIRRMFEPGQETA
MongooseSthAfrica   DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAKEAILYFFHKNFEEEIRRMFEPGQETA
KuduNamibia         DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAKEAILYFFHKNFEEEIRRMFEPGQETA
DogCentralAfrica    DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAKEAILYFFHKNFEEEIRRMFEPGQETA
DogAfrica           DMYFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
DogIvoryCoast       DMYFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
DogGuinea           DMYFPRIEHLYSAIRVGTVVTAYEDCSGLASFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPAQETA
DogAlgeria          DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
DogIran             DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRKMFEPGQETA
PV                  DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
SAD-B19             DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
CVS and Nobivac     DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
RCHLVac             DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREATLYFFHKNFEEEIRRMFEPGQETA
Nishigahara         DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
3AGChina            DLFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
DogMexico           DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
RaccoonUSA          DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAKEAILYFFHKNFEGEIRRMFEPGQETA
RaccoonUSA          DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAKEAILYFFHKNFEGEIRRMFEPGQETA
RaccoonUSA          DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAKEAILYFFHKNFEGEIRRMFEPGQETA
SkunkWestCanada     DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAEEAILYFFHKNFEEEIRRMFEPGQETA
SkunkCanada         DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ONT2SkunkCanada     DMFFSRIDHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ONT3RedFoxCanada    DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ArcticFoxRussia     DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
ArcticFoxGreenland  DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
RaccoonDogEstonia   DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
RaccoonDogPoland    DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
RedFoxFrance        DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
RedFoxYugoslavia    DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIKRMFEPGQETA
RedFoxGermany       DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
HumanThailand       DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
RabiesIndia         DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIRRMFEPGQETA
EBL-2 Holland       DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIRQINLTAKEAILYFFHKNFEEEIKRMFEPGQETA
EBL-2 Finland       DMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIRQINLTAKEAILYFFHKNFEEEIKRMFEPGQETA
EBL-1 France        DMFFARIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIKRMFEPGQETA
EBL-1 Poland        DMFFARIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIKRMFEPGQETA
Duvenhage           DMFFSRVDHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQINLTAREAILYFFHKNFEEEIKRMFEPGQETA
Lagos Bat           DMFFARVEHLYSALRVGTVVTAYEDCSGLVSFTGFIKQINLSARDALLYFFHKKFEEEIKRMFEPGQETA
Mokola              DMFFARVEHIYSALRVGTVVTAYEDCSGLVSFTGFIKQINLSPRDALLYFFHKNFEGEIKRMFEPGQETA
                    *..*.*..*.***.***************.******.*.**..*.******.**.*..****.****
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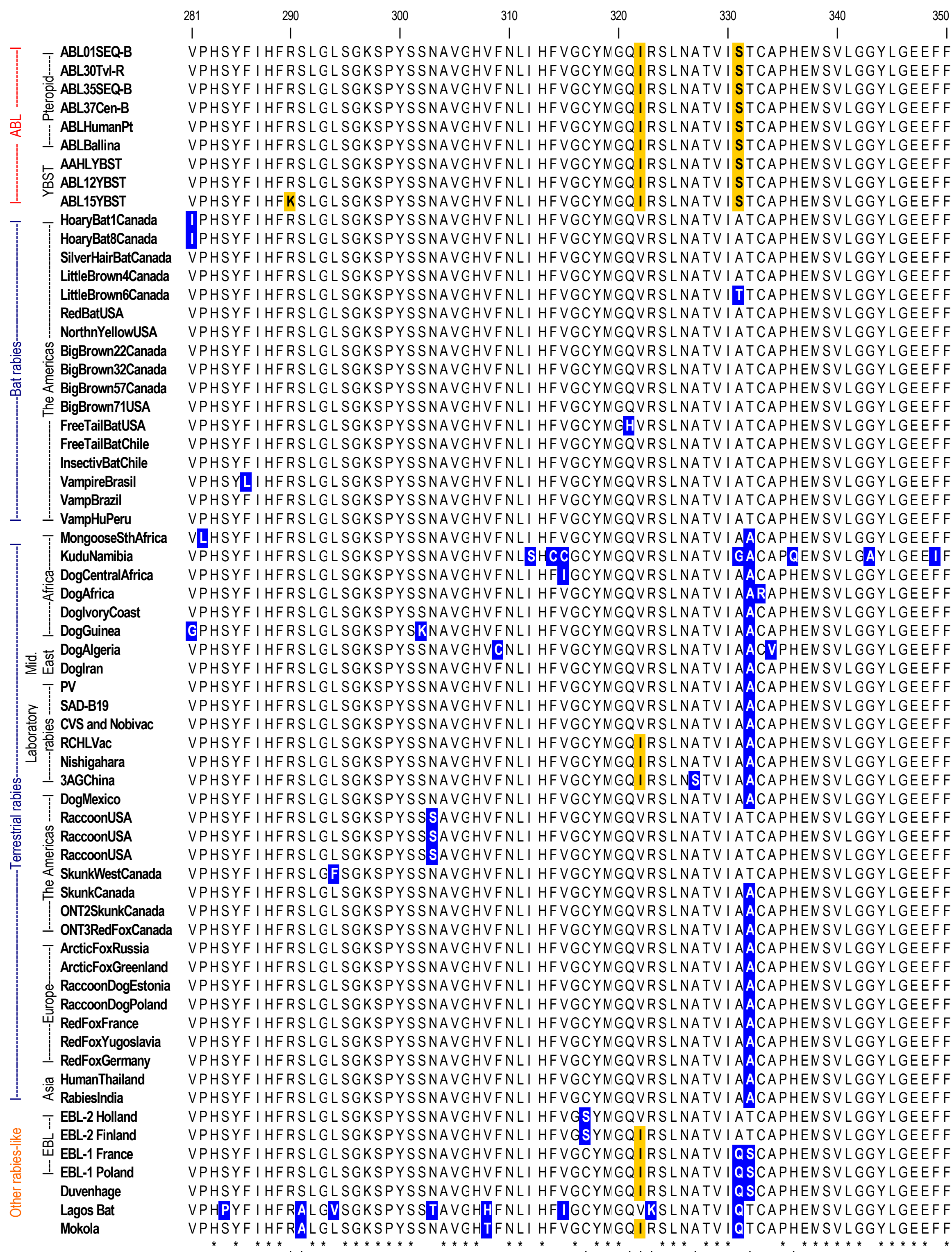
Figure 4-3 -continued N protein alignment (page 5 of 7)



KEY

ABLV amino acids that differ from the predominant lyssavirus amino acid at each site, and similarly substituted amino acids of other (non-ABLV) lyssaviruses are highlighted in yellow (first substitution e.g. E) or green (second substitution e.g. Q). Other (non-ABLV) lyssavirus amino acids that differ from the predominant lyssavirus amino acid at each site are highlighted in blue (e.g. V). GenBank accession numbers for each sequence are given in Table 4-2 and Table 4-5. Conserved sites are indicated by an asterisk (*), sites at which substitutions with similar amino acids occur are indicated by a stop (.).

Figure 4-3 –continued **N protein alignment (page 6 of 7)**

putative phosphorylation site

```
                                   351       360       370       280       390       400       410       420
ABL / Pteropid
ABL01SEQ-B           GKGTFERRFFRDEKELQDYEAAESMKTDIALADDATVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
ABL30Tvl-R           GKGTFERRFFRDEKELQDYEAAESMKTDIALADDATVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
ABL35SEQ-B           GKGTFERRFFRDEKELQDYEAAESMKTDIALADDATVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
ABL37Cen-B           GKGTFERRFFRDEKELQDYEAAESMRTDIALADDATVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
ABLHumanPt           GKGTFERRFFRNEKELQDYEAAESMKTDIALADDATVNSDDEDYFSGETRGPEAVYTRIMMNGGRLKRSH
ABLBallina           GKGTFERRFFRDEKELQDYEAAESMKTDIALADDATVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
ABL / YBST
AAHLYBST             GKGTFERRFFRDEKELQDYEAAEAMKIDLALADDGTVNSYDEDYLSGETRSPEAVYTRIMMNGGRLKKSH
ABL12YBST            GKGTFERRFFRDEKELQDYEAAEAMKIDLALADDGTVNSDDEDYLSGETRSPEAVYTRIMMNGGRLKKSH
ABL15YBST            GKGTFERRFFRDEKELQDYEAAEAMKIDLALADDGTVNSDDEDYLSGETRSPEAVYTRIMMNGGRLKKSH
Bat rabies / The Americas
HoaryBat1Canada      GKGTFERRFFRDEKELQEYEAAELTKTEVALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGSRLKRSH
HoaryBat8Canada      GKGTFERRFFRDEKELQEYEAAELTKTEVALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGSRLKRSH
SilverHairBatCanada  GKGTFERRFFRDEKELQEYEAAELTKTEVAFADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
LittleBrown4Canada   GKGTFERRFFRDEKELQEYEAAELTKTEVALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
LittleBrown6Canada   GKGTFERRFFRDEKELQEYEAAELTKTEVALADDGTVNSDDEDYFSGETRSPESVYTRIMMNGGRLKRSH
RedBatUSA            GKGTFERRFFRDEKELQEYEAAELTKTEVALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
NorthnYellowUSA      GKGTFERRFFRDEKELQEYEAAELTKTEVALADDGTFNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
BigBrown22Canada     GKGTFERRFFRDEKELQEYEAAELTKTEVALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
BigBrown32Canada     GKGTFERRFFRDEKELQEYEAAESTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
BigBrown57Canada     GKGTFERRFFRDEKELQEYEAAESTKTEIALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
BigBrown71USA        GKGTFERRFFRDEKELQEYEAAESTKTEIALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
FreeTailBatUSA       GKGTFERRFFRDEKELQEYEAAELTKADTALADDGTVNSDDEDYFSSETRSPESVYTRIMMNGGRLKRSH
FreeTailBatChile     GKGTFERRFFRDEKELQEYEAAELTKTETALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
InsectivBatChile     GKGTFERRFFRDEKELQEYEAAELTKTETALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
VampireBrasil        GKGTFERRFFRDEKELQEYEAAELTKAETALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
VampBrazil           GKGTFERRFFRDEKELQEYEAAELTKAETALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
VampHuPeru           GKGTFERRFFRDEKELQEYEAAELTKAETALADDGTVNSDDEDYFSSETRSPEAVYTRIMMNGGRLKRSH
Terrestrial rabies / Africa
MongooseSthAfrica    GKGTFERRFFRDEKELQEYEAAELTKTDTNTADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
KuduNamibia          GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTQIMMNGGRLKRSH
DogCentralAfrica     GKGTFERRFFRDEKELQEYEAAELTKTDMALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
DogAfrica            GKATFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
DogIvoryCoast        GKGTFERRFFRDEKELQEYQAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
DogGuinea            GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
Mid. East
DogAlgeria           GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
DogIran              GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDSFSGETRSPEAVYTRIMMNGGRLKRSH
Laboratory rabies
PV                   GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIIMNGGRLKRSH
SAD-B19              GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
CVS and Nobivac      GKGTFERRFFRDEKELQEYEAAELTKSDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
RCHLVac              GRGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVDSDDEDHFPGEARGPEAVYARIMMNGGRLKRSH
Nishigahara          GRGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVDSDDEDYFSGEARGPEAVYARIMMNGGRLKRSH
3AGChina             GRGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRGPEAVYARIMMNGGRLKRSH
The Americas
DogMexico            GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
RaccoonUSA           GKGTFERRFFRDEKELQDYEAAELTKTEVALADDGTVNSDDEDYFSGETRSPEAVYTRIMINGGRLKRSH
RaccoonUSA           GKGTFERRFFRNEKELQDYEAAELTKTEVALADDGTVNSDDEDYFSGETRSPEAVYTRIMINGGRLKRSH
RaccoonUSA           GKGTFERRFFRNEKELQDYEAAELTKTEVALADDGTVNSDDEDYFSGETRSPEAVYTRIMINGGRLKRSH
SkunkWestCanada      GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
SkunkCanada          GKGTFERRFFRDEKELQEYETAELTKTDAALADDGTVNSDDEDYFSGETRSPEAVYARIMMNGGRLKRSH
ONT2SkunkCanada      GKGTFERRFFRDEKELQEYETAELTKTDAALADDGTVNSDDEDYFSGETRSPEAVYARIMMNGGRLKRSH
ONT3RedFoxCanada     GKGTFERRFFRDEKELQEYETAELTKTDAALADDGTVNSDDEDYFSGETRSPEAVYARIMMNGGRLKRSH
Europe
ArcticFoxRussia      GRGTFERRFFRDEKELQEYETAELTKTDTALADDGTVNSDDEDYFSGETRSPEAVYARIMMNGGRLKRSH
ArcticFoxGreenland   GKGTFERRFFRDEKELQEYETAELTKTDTALADDGTVNSDDEDYFSGETRSPEAVYARIMMNGGRLKRSH
RaccoonDogEstonia    GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
RaccoonDogPoland     GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRGPEAVYTRIMMNGGRLKRSH
RedFoxFrance         GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
RedFoxYugoslavia     GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
RedFoxGermany        GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEEYFSGETRSPEAVYTRIMMNGGRLKRSH
Asia
HumanThailand        GKGTFERRFFRDEKELQEYEAAELTKTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
RabiesIndia          GKGTFERRFFRDEKELQEYEAAELTKTDIALADDGTVNSDDEDYFSGETRSPEAVYTRIMMNGGRLKRSH
Other rabies-like / EBL
EBL-2 Holland        GKGTFERRFFRDERELAEHEAIESTKTDVALADDGTVNSDDELYSGGTRTPEAVYTRIMVNGGKLQKSH
EBL-2 Finland        GKGTFERRFFRDERELAEHEAIESTKTDVALADDGTVNSDDELYSGGTRTPESVYTRIMVNGGKLKKSH
EBL-1 France         GKGTFERRFFRDEKELQDYEAAESTKVDVALADDGTVNSDDEDFFSGDTRSPEAVYTRIMMNGGRLKRSH
EBL-1 Poland         GKGTFERRFFRDEKELQDYEAAESTKVDVALADDGTVNSDDEDFFSGDTRSPEAVYTRIMMNGGRLKRSH
Duvenhage            GKGTFERRFFRDEKELQDYEEAEATKIEAALADDGTVNSDDEDFFSGDTRSPEAVYTRIMMNGGRLKGAH
Lagos Bat            GKGTFERRFFRDEKEMQDYADLEGARVEASLADDGTVDSDDEDFFSGETRSPEAVYSRIMMNNGRLKKSH
Mokola               GKGTFERRFFRDEKEMQDYTELEEARVEASLADDGTVDSDEEDFFSGETRSPEAVYSRIMMNNGKLKKVH
```

Figure 4-3 –continued N protein alignment (page 7 of 7)

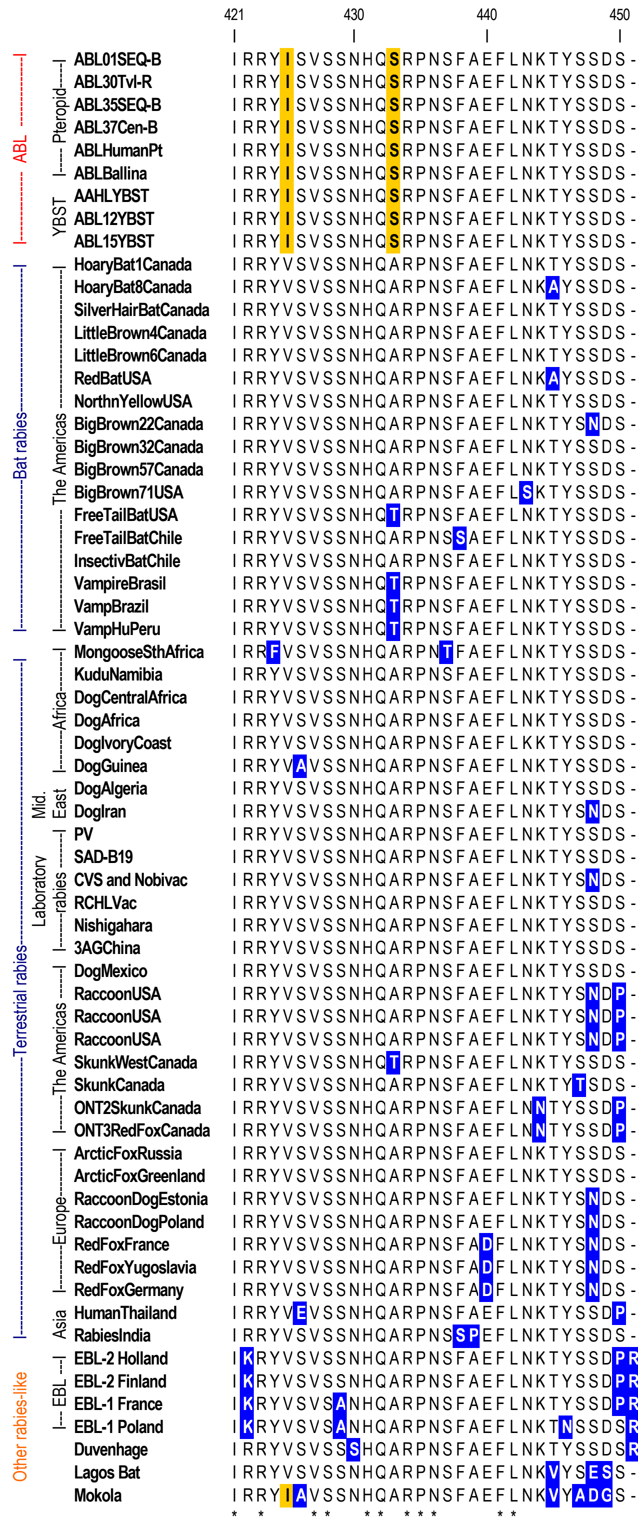
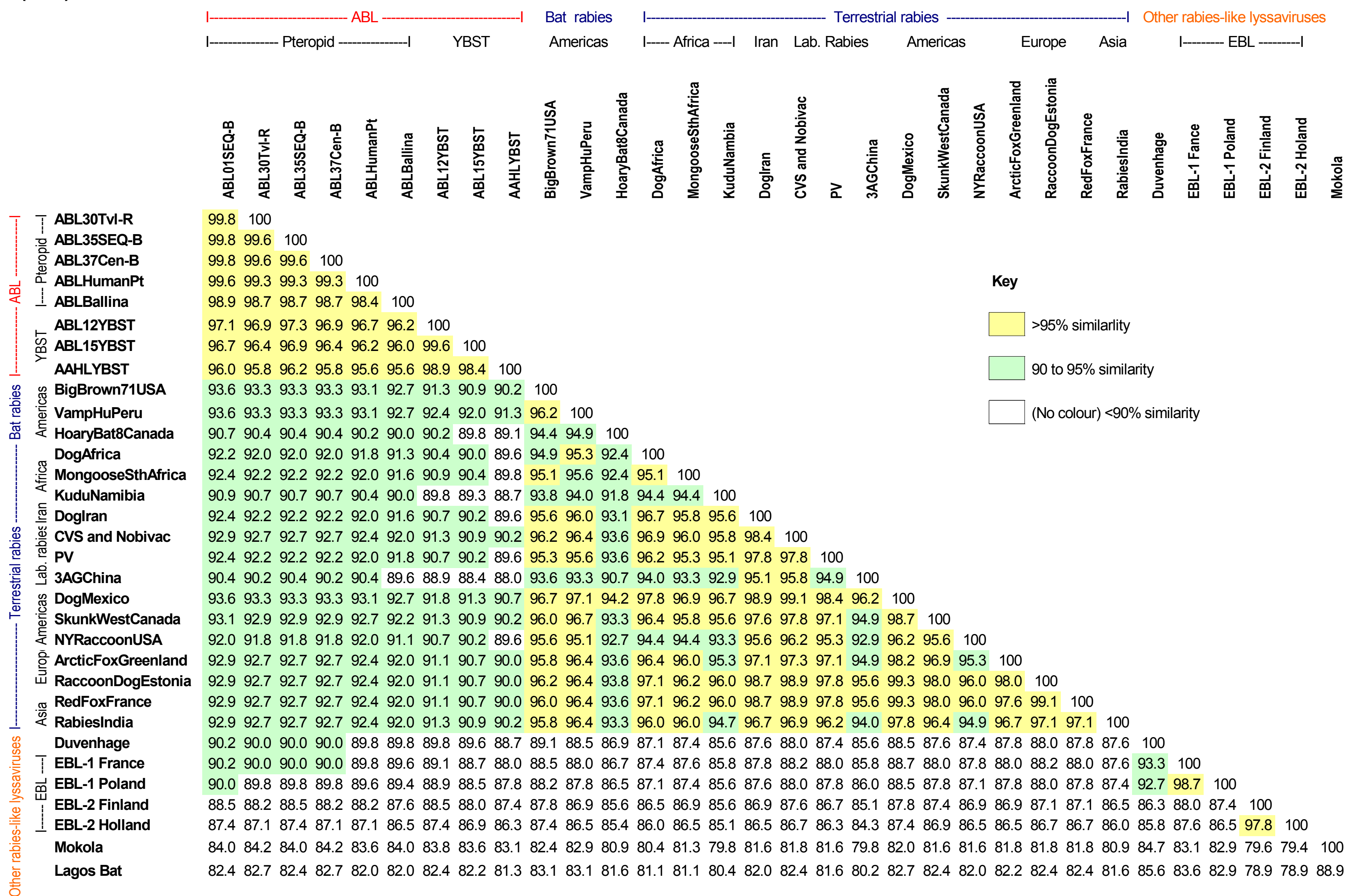


Figure 4-4 Percent similarity matrix comparing the unique N protein amino acid sequences of pteropid-variant ABLV (n=5) and YBST-variant ABLV (n=3) with those of 24 other rabies-like and rabies viruses



I---------------------------- ABL ----------------------------I Bat rabies I------------------------------------ Terrestrial rabies ------------------------------------I Other rabies-like lyssaviruses

I-------------- Pteropid --------------I YBST Americas I----- Africa ----I Iran Lab. Rabies Americas Europe Asia I--------- EBL ---------I

	ABL01SEQ-B	ABL30TvI-R	ABL35SEQ-B	ABL37Cen-B	ABLHumanPt	ABLBallina	ABL12YBST	ABL15YBST	AAHLYBST	BigBrown71USA	VampHuPeru	HoaryBat8Canada	DogAfrica	MongooseSthAfrica	KuduNamibia	DogIran	CVS and Nobivac	PV	3AGChina	DogMexico	SkunkWestCanada	NYRaccoonUSA	ArcticFoxGreenland	RaccoonDogEstonia	RedFoxFrance	RabiesIndia	Duvenhage	EBL-1 Fance	EBL-1 Poland	EBL-2 Finland	EBL-2 Holland	Mokola
ABL30TvI-R	99.8	100																														
ABL35SEQ-B	99.8	99.6	100																													
ABL37Cen-B	99.8	99.6	99.6	100																												
ABLHumanPt	99.6	99.3	99.3	99.3	100																											
ABLBallina	98.9	98.7	98.7	98.7	98.4	100																										
ABL12YBST	97.1	96.9	97.3	96.9	96.7	96.2	100																									
ABL15YBST	96.7	96.4	96.9	96.4	96.2	96.0	99.6	100																								
AAHLYBST	96.0	95.8	96.2	95.8	95.6	95.6	98.9	98.4	100																							
BigBrown71USA	93.6	93.3	93.3	93.3	93.1	92.7	91.3	90.9	90.2	100																						
VampHuPeru	93.6	93.3	93.3	93.3	93.1	92.7	92.4	92.0	91.3	96.2	100																					
HoaryBat8Canada	90.7	90.4	90.4	90.4	90.2	90.0	90.2	89.8	89.1	94.4	94.9	100																				
DogAfrica	92.2	92.0	92.0	92.0	91.8	91.3	90.4	90.0	89.6	94.9	95.3	92.4	100																			
MongooseSthAfrica	92.4	92.2	92.2	92.2	92.0	91.6	90.9	90.4	89.8	95.1	95.6	92.4	95.1	100																		
KuduNamibia	90.9	90.7	90.7	90.7	90.4	90.0	89.8	89.3	88.7	93.8	94.0	91.8	94.4	94.4	100																	
DogIran	92.4	92.2	92.2	92.2	92.0	91.6	90.7	90.2	89.6	95.6	96.0	93.1	96.7	95.8	95.6	100																
CVS and Nobivac	92.9	92.7	92.7	92.7	92.4	92.0	91.3	90.9	90.2	96.2	96.4	93.6	96.9	96.0	95.8	98.4	100															
PV	92.4	92.2	92.2	92.2	92.0	91.8	90.7	90.2	89.6	95.3	95.6	93.6	96.2	95.3	95.1	97.8	97.8	100														
3AGChina	90.4	90.2	90.4	90.2	90.4	89.6	88.9	88.4	88.0	93.6	93.3	90.7	94.0	93.3	92.9	95.1	95.8	94.9	100													
DogMexico	93.6	93.3	93.3	93.3	93.1	92.7	91.8	91.3	90.7	96.7	97.1	94.2	97.8	96.9	96.7	98.9	99.1	98.4	96.2	100												
SkunkWestCanada	93.1	92.9	92.9	92.9	92.7	92.2	91.3	90.9	90.2	96.0	96.7	93.3	96.4	95.8	95.6	97.6	97.8	97.1	94.9	98.7	100											
NYRaccoonUSA	92.0	91.8	91.8	91.8	92.0	91.1	90.7	90.2	89.6	95.6	95.1	92.7	94.4	94.4	93.3	95.6	96.2	95.3	92.9	96.2	95.6	100										
ArcticFoxGreenland	92.9	92.7	92.7	92.7	92.4	92.0	91.1	90.7	90.0	95.8	96.4	93.6	96.4	96.0	95.3	97.1	97.3	97.1	94.9	98.2	96.9	95.3	100									
RaccoonDogEstonia	92.9	92.7	92.7	92.7	92.4	92.0	91.1	90.7	90.0	96.2	96.4	93.8	97.1	96.2	96.0	98.7	98.9	97.8	95.6	99.3	98.0	96.0	98.0	100								
RedFoxFrance	92.9	92.7	92.7	92.7	92.4	92.0	91.1	90.7	90.0	96.0	96.4	93.6	97.1	96.2	96.0	98.7	98.9	97.8	95.6	99.3	98.0	96.0	97.6	99.1	100							
RabiesIndia	92.9	92.7	92.7	92.7	92.4	92.0	91.3	90.9	90.2	95.8	96.4	93.3	96.0	96.0	94.7	96.7	96.9	96.2	94.0	97.8	96.4	94.9	96.7	97.1	97.1	100						
Duvenhage	90.2	90.0	90.0	90.0	89.8	89.8	89.8	89.6	88.7	89.1	88.5	86.9	87.1	87.4	85.6	87.6	88.0	87.4	85.6	88.5	87.6	87.4	87.8	88.0	87.8	87.6	100					
EBL-1 France	90.2	90.0	90.0	90.0	89.8	89.6	89.1	88.7	88.0	88.5	88.0	86.7	87.4	87.6	85.8	87.8	88.2	88.0	85.8	88.7	88.0	87.8	88.0	88.2	88.0	87.6	93.3	100				
EBL-1 Poland	90.0	89.8	89.8	89.8	89.6	89.4	88.9	88.5	87.8	88.2	87.8	86.5	87.1	87.4	85.6	87.6	88.0	87.8	86.0	88.5	87.8	87.1	87.8	88.0	87.8	87.4	92.7	98.7	100			
EBL-2 Finland	88.5	88.2	88.5	88.2	88.2	87.6	88.5	88.0	87.4	87.8	86.9	85.6	86.5	86.9	85.6	86.9	87.6	86.7	85.1	87.8	87.4	86.9	86.9	87.1	87.1	86.5	86.3	88.0	87.4	100		
EBL-2 Holland	87.4	87.1	87.4	87.1	87.1	86.5	87.4	86.9	86.3	87.4	86.5	85.4	86.0	86.5	85.1	86.5	86.7	86.3	84.3	87.4	86.9	86.5	86.5	86.7	86.7	86.0	85.8	87.6	86.5	97.8	100	
Mokola	84.0	84.2	84.0	84.2	83.6	84.0	83.8	83.6	83.1	82.4	82.9	80.9	80.4	81.3	79.8	81.6	81.8	81.6	79.8	82.0	81.6	81.6	81.8	81.8	81.8	80.9	84.7	83.1	82.9	79.6	79.4	100
Lagos Bat	82.4	82.7	82.4	82.7	82.0	82.0	82.4	82.2	81.3	83.1	83.1	81.6	81.1	81.1	80.4	82.0	82.4	81.6	80.2	82.7	82.4	82.0	82.2	82.4	82.4	81.6	85.6	83.6	82.9	78.9	78.9	88.9

Row groups: I---- Pteropid ---I, YBST (ABL); Americas (Bat rabies); Africa, Iran, Lab. rabies, Americas, Europe, Asia (Terrestrial rabies); I---- EBL ---I (Other rabies-like lyssaviruses)

Key

>95% similarity

90 to 95% similarity

(No colour) <90% similarity

4.3.1 Phylogenetic trees

Phylogenetic trees generated from the N protein coding sequences of ABLV and other lyssavirus isolates using either neighbour joining or maximum parsimony show very similar overall topologies that are shown in Figure 4-5 to Figure 4-11.

The ABLV sequences form a discrete clade with strong bootstrap support (100% using neighbour joining and 97% using maximum parsimony) that is separate, but most closely related to, the clade comprised of the rabies virus isolates. Furthermore, the ABLV clade consists of two monophyletic sister groups, each with 100% bootstrap support, one comprised of all 30 ABLV isolates from pteropid bats (pteropid-variant ABLV), and one comprised of all 4 ABLV isolates from YBST bats (YBST-variant ABLV).

Branch lengths differentiating the *Lyssavirus* lineages are in virology taxonomy terms comparatively short, but this is particularly so within the pteropid-variant and YBST-variant ABLV sister groups. The relationships within the ABLV clade were more closely examined using these sequences alone as shown in Figures 4-8 to 4-11. The topology among the YBST-variant isolates was identical using both methods, and the relationships within the pteropid-variant sister group using both methods are more similar than might immediately be apparent. Within the pteropid-variant isolates there is no suggestion of any species specific (i.e. Black, Grey-headed or Little Red flying fox), regional (SEQ, Cen, or Tvl), or temporal (1997 to 2000) sub-variants. Rather the relatively long branch length between the two ABLV variants, their strong bootstrap support, and the very short branch lengths between isolates within each variant suggest that each variant is comparatively stable with only very low levels of genetic divergence between isolates of each variant.

Figure 4-5 ***Lyssavirus*** **phylogeny: unrooted neighbour joining phylogram generated from the complete N protein coding sequences of 30 pteropid-variant ABLV, 4 YBST-variant ABLV, and 55 other rabies-like and rabies virus isolates**
Branch lengths are proportional to evolutionary distances as indicated by the distance bar. Virus isolate and GenBank accession details are shown in Table 4-2 and Table 4-5. Relationships within each shaded group are shown in Figure 4-6 and Figure 4-7.

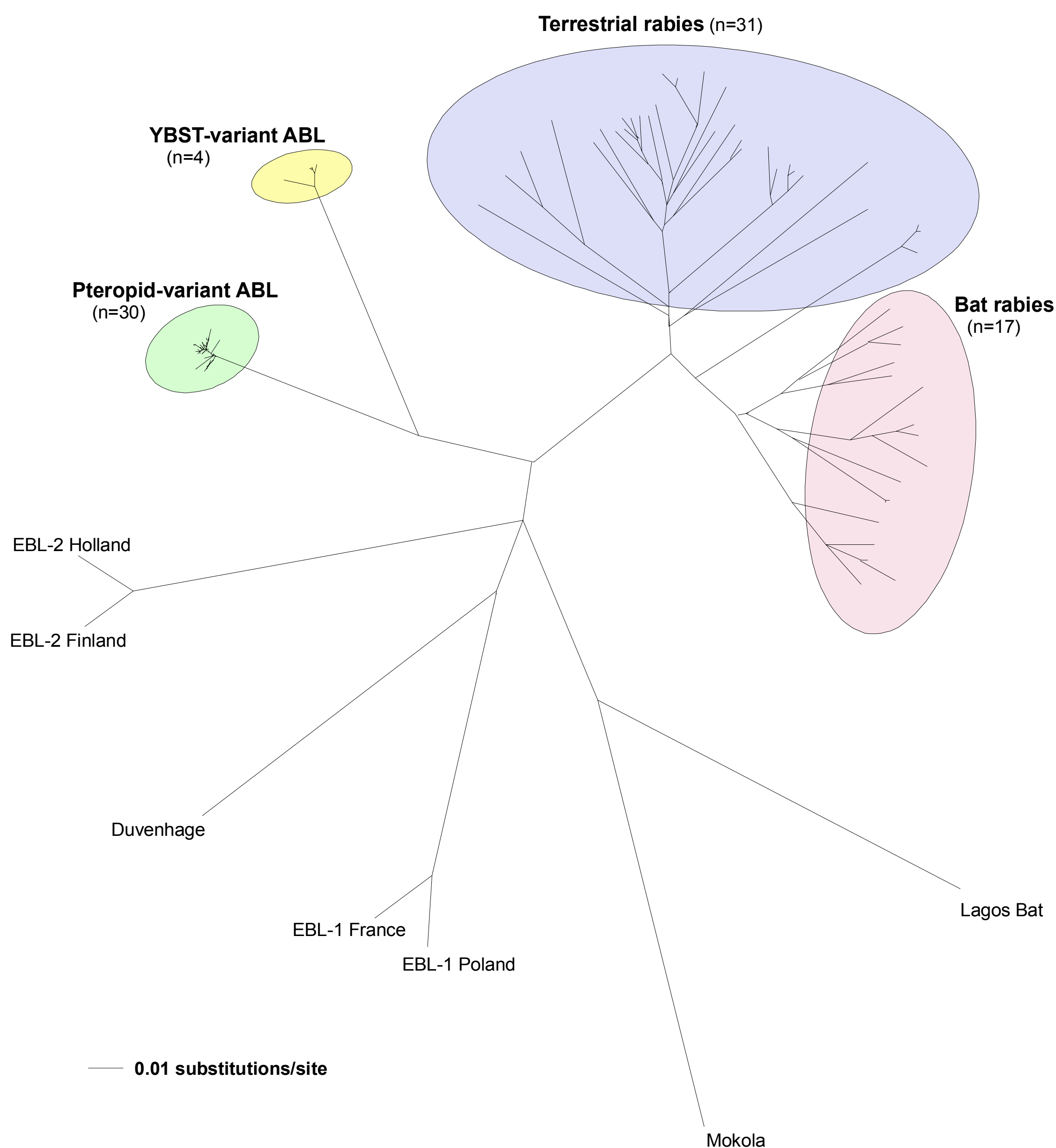


Figure 4-6 ***Lyssavirus*** **phylogeny: rooted neighbour joining phylogram**

Generated from the same complete N protein coding sequences as Figure 4-5. Numbers at major nodes indicate percent bootstrap support (1000 replicates). Horizontal branch lengths are proportional to evolutionary distances as indicated by the distance bar. Mokola virus defined as outgroup. Virus isolate and GenBank accession details are shown in Table 4-2 and Table 4-5.

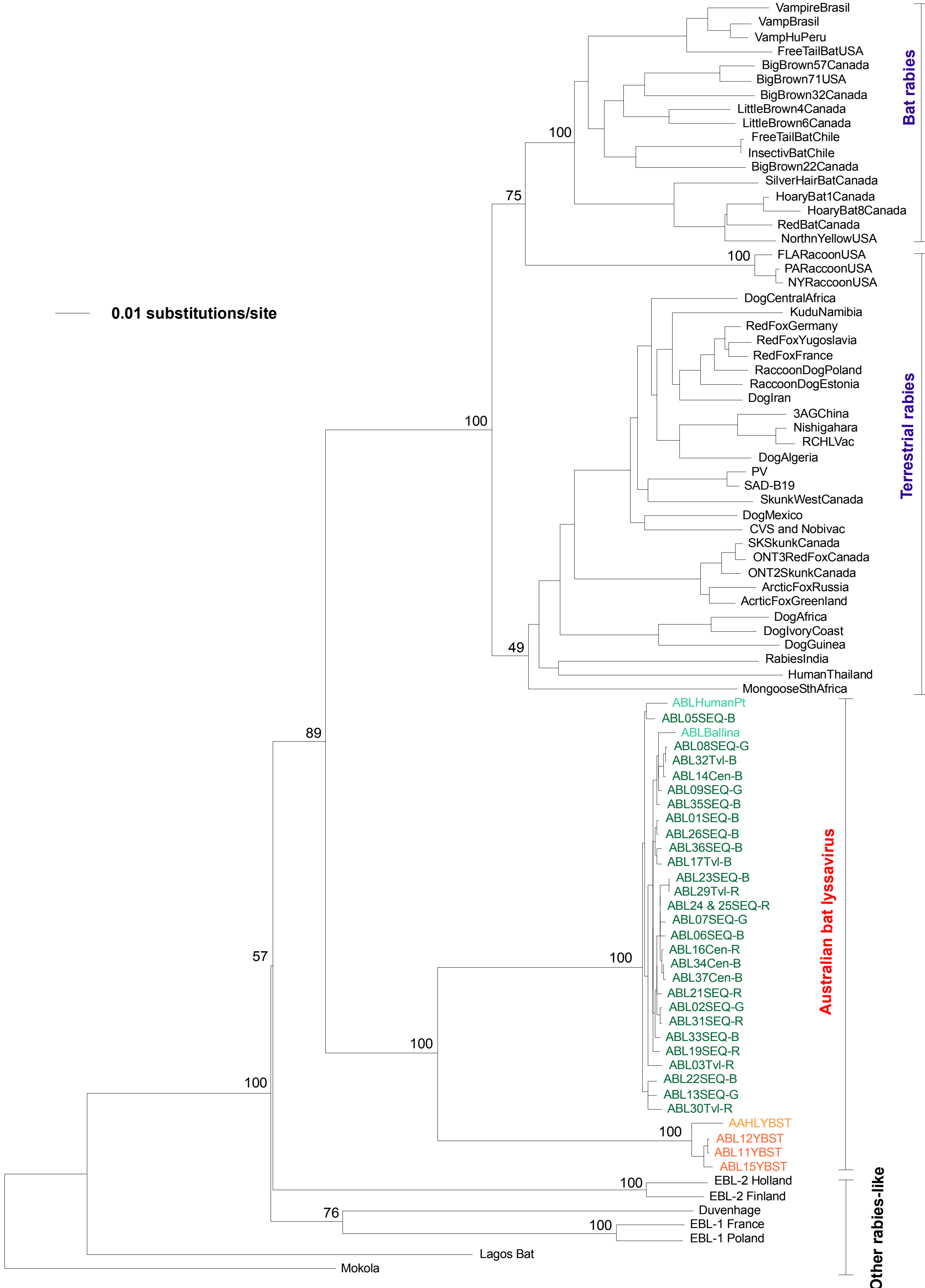


Figure 4-7 *Lyssavirus* phylogeny: rooted maximum parsimony phylogram
Generated by fast stepwise addition search from the same complete N protein coding sequences as Figure 4-5 and Figure 4-6. Numbers at major nodes indicate percent bootstrap support (1000 replicates). Horizontal branch lengths are proportional to the number of changes as indicated by the distance bar. Branches are collapsed (creating polytomies) if maximum branch length is zero. Mokola virus defined as outgroup. Virus isolate and GenBank accession details are shown in Table 4-2 and Table 4-5.

VampireBrasil
VampBrasil
VampHuPeru
FreeTailBatUSA
FreeTailBatChile
InsectivBatChile
BigBrown22Canada
SilverHairBatCanada
HoaryBat1Canada
HoaryBat8Canada
RedBatCanada
NorthnYellowUSA
BigBrown57Canada
BigBrown71USA
BigBrown32Canada
LittleBrown4Canada
LittleBrown6Canada
FLARacoonUSA
PARaccoonUSA
NYRaccoonUSA
DogCentralAfrica
KuduNamibia
RedFoxGermany
RedFoxYugoslavia
RedFoxFrance
RaccoonDogPoland
RaccoonDogEstonia
DogIran
3AGChina
Nishigahara
RCHLVac
DogAlgeria
PV
SAD-B19
SkunkWestCanada
DogMexico
CVS and Nobivac
SKSkunkCanada
ONT3RedFoxCanada
ONT2SkunkCanada
ActricFoxGreenland
ArcticFoxRussia
MongooseSthAfrica
DogAfrica
DogIvoryCoast
DogGuinea
RabiesIndia
HumanThailand
ABLHumanPt
ABL05SEQ-B
ABLBallina
ABL08SEQ-G
ABL32Tvl-B
ABL14Cen-B
ABL09SEQ-G
ABL35SEQ-B
ABL01SEQ-B
ABL26SEQ-B
ABL36SEQ-B
ABL17Tvl-B
ABL07SEQ-G
ABL06SEQ-B
ABL23SEQ-B
ABL29Tvl-R
ABL16Cen-R
ABL34Cen-B
ABL37Cen-B
ABL24SEQ-R
ABL21SEQ-R
ABL02SEQ-G
ABL31SEQ-R
ABL33SEQ-B
ABL19SEQ-R
ABL03Tvl-R
ABL22SEQ-B
ABL13SEQ-G
ABL30Tvl-R
AAHLYBST
ABL12YBST
ABL11YBST
ABL15YBST
EBL-2 Holland
EBL-2 Finland
Duvenhage
EBL-1 France
EBL-1 Poland
Lagos Bat
Mokola

70
62
100
100
44
74
64
100
97
100
100
51
100
100

50 changes

Bat rabies
Terrestrial rabies
Australian bat lyssavirus
Other rabies-like

Figure 4-8 ABLV phylogeny: midpoint rooted neighbour joining phylogram generated from the complete N protein coding sequences of 28 new pteropid-variant ABLV, 3 new YBST-variant ABLV, and 3 other ABLV isolates

Horizontal branch lengths are proportional to evolutionary distances as indicated by the distance bar. The evolutionary distance of the major branch is shown. Virus isolate and GenBank accession details are shown in Table 4-2 and Table 4-5. Due to the short branch lengths, a cladogram showing bootstrap support for this tree is shown separately in Figure 4-9.

Figure 4-9 ABLV phylogeny: neighbour joining cladogram
Generated by bootstrap resampling (1000 replicates) of 34 complete ABLV N protein coding sequences. Shows groups compatible with 50% majority-rule consensus and bootstrap proportions >5%. Virus isolate and GenBank accession details are shown in Table 4-2 and Table 4-5.

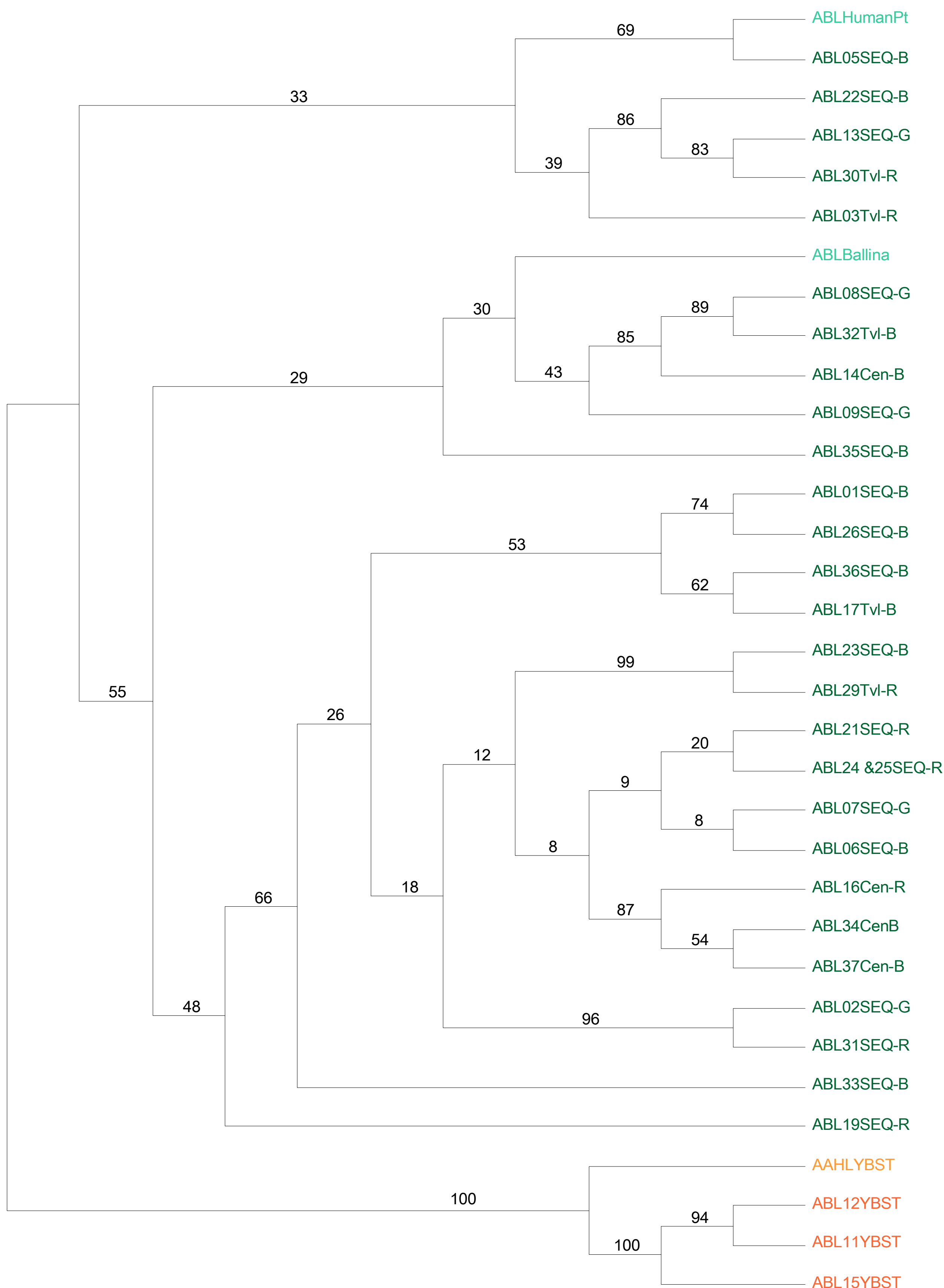
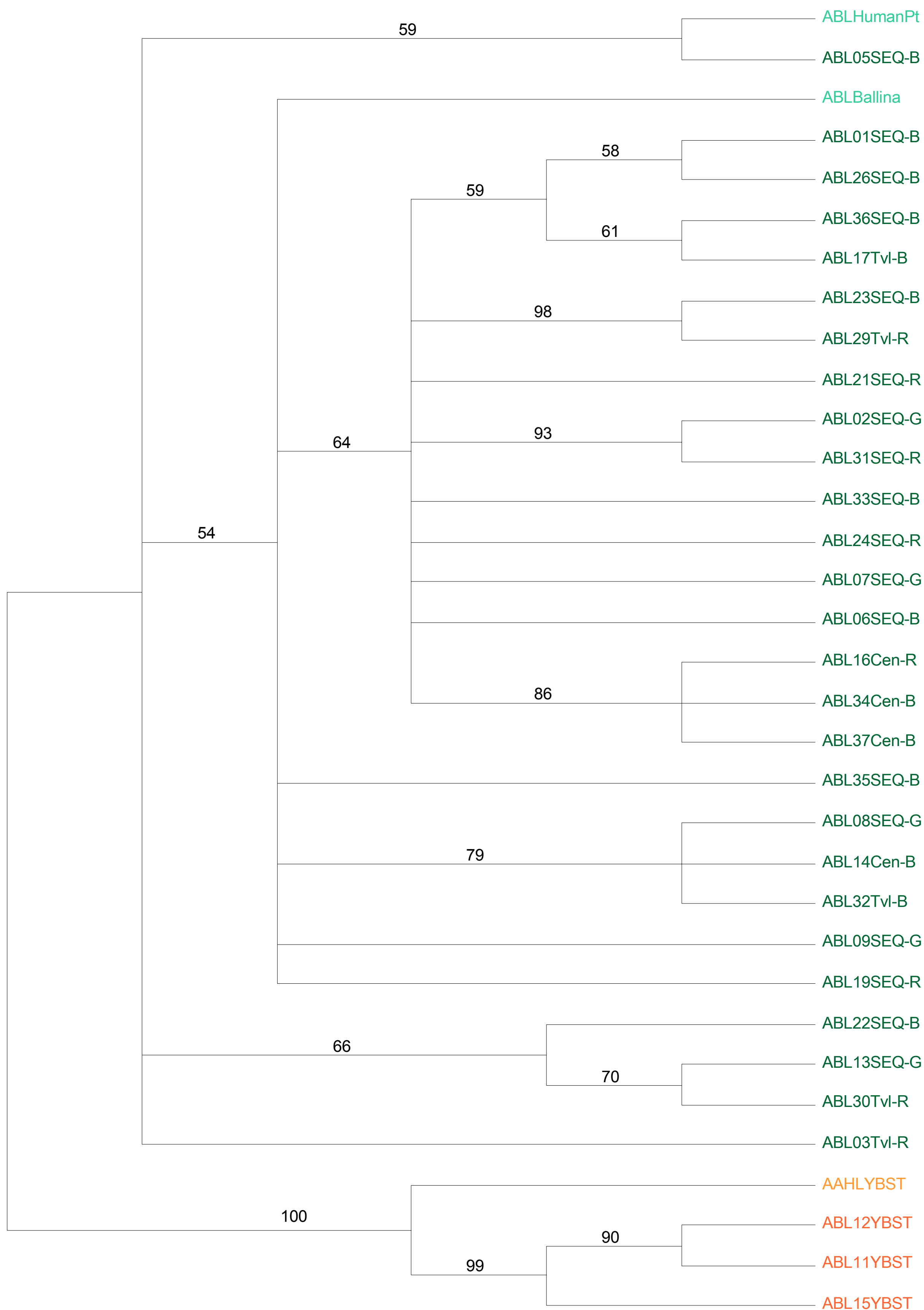


Figure 4-10 ABLV Phylogeny: rooted maximum parsimony phylogram generated from 34 complete ABLV N protein sequences

Horizontal branch lengths are proportional to evolutionary distances as indicated by the distance bar. The evolutionary distance of the major branch is shown. Branches are collapsed (creating polytomies) if maximum branch length equals zero. YBST-variant ABLVs defined as monophyletic sister outgroup. Sequence details are in Table 4-2 and Table 4-5. Due to the short branch lengths, a cladogram showing bootstrap support for this tree is shown separately in Figure 4-11.

Figure 4-11 ABLV phylogeny: maximum parsimony cladogram
Generated by bootstrap resampling (1000 replicates) of 34 complete N protein coding sequences. Shows groups compatible with 50% majority-rule consensus and bootstrap values >5%. Branches are collapsed (creating polytomies) if maximum branch length is zero. Sequence details are in Table 4-2 and Table 4-5.



4.4 Discussion

In this study direct cDNA synthesis, amplification, and sequencing from naturally infected, primary (bat) host tissues was done to preclude the possibility of selection mutations occurring during passage in humans, mice, or *in vitro* cell culture. The sequences were consistent with those of three other ABLV isolates that were (ABLBallina, GenBank AF006497, and AAHLYBST AF081020) or became (ABLHumanPt AF418014) available during this study (Gould *et al.* 1998; Gould *et al.* 2002; Warrilow *et al.* 2002). These other isolates had been passaged in a non-bat species (a human or mice) and amplified in non-bat cell cultures (BHK-21 or mouse neuroblastoma cells) prior to RNA extraction (Gould *et al.* 1998; Gould *et al.* 2002; Warrilow *et al.* 2002).

4.4.1 Functional features of the ABLV and other lyssavirus sequences

The complete ABLV genome is 11,918 nucleotides long (Warrilow *et al.* 2002), with the first ORF encoding the N protein. The functional features of the 5' end of available lyssavirus sequences are shown in Table 4-7. The N gene is preceded by a leader sequence of unknown function which, in the few isolates where it has been completely determined (ABLHumanPt, several rabies isolates, and Mokola virus (Bourhy *et al.* 1993a; Le Mercier *et al.* 1997)), is 57 nucleotides long. The first nucleotide determined in the 31 ABLV and Nobivac RIVM sequences reported here corresponds to position 23 in the leader sequence, and the last corresponds to the 47th (ABLV) or 54th (Nobivac) nucleotides of the phosphoprotein (P) coding sequence.

The leader sequence has an $AAGCA_5TG$ termination signal which is common to all 31 ABLV sequences determined here, the ABLHumanPt sequence, and several isolates of laboratory strain rabies virus (PV, CVS, Nobivac (RIVM), Nishigahara, RCHLVac, and SAD-B19, see Table 4-7). The leader termination signal for PV differs slightly ($GAGCA_5TG$), and is substantially different for Mokola virus (TA_3CA_4TG). For all these lyssaviruses, the leader to N gene intergenic region is a single nucleotide (T). The ABLV N gene transcription initiation signal (A_2CAC_4T) is perfectly conserved among the 31 new ABLV sequences, ABLHumanPt, and three laboratory rabies virus strains (Nobivac RIVM, CVS and SAD-B19), but differs at a single site for some other rabies virus stains (A_2CAC_2TCT, PV Nishigahara and RC-HVL) and for Mokola virus (A_2CACTC_2T). The N protein start codon for all these sequences is ATG, at positions 71 to 73.

The leader sequence, intergenic region, N gene transcription initiation signal, and N protein start codon for Lagos bat virus, Duvenhage virus, EBL-1, EBL-2, ABLBallina and AAHLYBST, have not been determined as all have been sequenced from cDNA synthesized using the PVN7 primer that corresponds to positions 55 to 73 and includes what is presumed to be the ATG start codon.

Table 4-7 Comparison of functional features of the 5' end (leader to P protein) of the lyssavirus sequences
(Differences are highlighted with **red** font)

Virus[1]	Leader sequence		Leader to N intergenic region	N gene						N to P intergenic region	P gene	
	Length (bases)	Termination signal		mRNA transcription initiation	Start codon	Coding length (bases)	Stop codon	3' UTR length (bases)	Polyadenylation termination signal		mRNA transcription initiation	Start codon
ABLBallina	---	---	---	---	---	1350[2]	TAA	66	AGA_6	CT	$A_2CACCCCT$	ATG
AAHLYBST	---	---	---	---	---	1350[2]	TAA	65	AGA_7	CT	$A_2CACCCCT$	ATG
ABLHumanPt	57	$AAGCA_5TG$	T	$A_2CACCCCT$	ATG	1350	TAA	66	AGA_7	CT	$A_2CACCCCT$	ATG
31 new ABLV	>35	$AAGCA_5TG$	T	$A_2CACCCCT$	ATG	1350	TAA	66 or 67[3]	AGA_7 or AGA_8	CT	$A_2CACCCCT$	ATG
Rabies virus	57	$^A/_GAGCA_5TG$	T	$A_2CACC^C/_TCT$	ATG	1350	TAA	62	TAG_7	CT	$A_2CAC^C/_T{}^C/_TCT$	ATG
Mokola	57	TA_3CA_4TG	T	$A_2CACTCCT$	ATG	1350	TAA	71	AGA_7	CTT	$A_2CACCACT$	ATG
Lagos bat	---	---	---	---	---	1350[2]	TAA	68	AGA_7	CTC	$A_2CATCTCT$	ATG
Duvenhage	---	---	---	---	---	1353[2]	TAA	62	TAG_7	CT	$A_2CACCACT$	ATG
EBL1	---	---	---	---	---	1353[2]	TAA	63 or 68	AGA_7	CT	$A_2CACCCCT$	ATG
EBL2	---	---	---	---	---	1353[2]	TAA	73	AGA_7	CT	$A_2CACCCCT$	ATG

--- Not determined, sequence unknown.
1 Virus isolate and GenBank accession details are shown in Table 4-2 and Table 4-5. The rabies isolates referred to are PV, CVS, Nobivac (RIVM), Nishigahara, RCHLVac, and SAD-B19. The N gene 3'UTR and start of the P gene sequence for Lagos bat, Duvenhage, EBL-1 and EBL-2 are from Bourhy *et al.* (1993a).
2 Sequence length based on the presumption that the N protein start codon corresponds with the terminal ATP of the PVN7 primer used for cDNA synthesis and PCR amplification.
3 One of 31 new ABLV sequences (ABL14Cen-B) had a 67 base 5'UTR due to an extra A base in the polyadenylation termination signal (AGA_8).

For all available ABLV, rabies and Mokola virus sequences, the N protein coding sequence is the same length, 1350 nucleotides, encodes a 450 amino acid protein, and terminates with a perfectly conserved TAA stop codon at position 1421 to 1423. The coding sequence for Duvenhage, EBL1 and EBL2 is apparently three bases longer, encoding an extra terminal Arginine (R) before the TAA stop codon (Bourhy *et al.* 1993a). As in all previously published lyssavirus sequences, the putative phosphorylation site at amino acid Ser^{389} (Minamoto *et al.* 1994), is conserved in the pteropid and YBST-variant ABLV sequences reported here.

Regions of rabies virus amino acid sequences have been identified as important linear (RCHLVac antigenic sites I and IV, residues 358-367 and 375-383, (Goto *et al.* 2000), ERA antigenic sites at residues 313-337 and 374-383 (Dietzschold *et al.* 1987a)), or conformational epitopes (RCHLVac antigenic sites II and III). The amino acid sequence at antigenic site I (amino acids 358-367) is perfectly conserved and would be expected to form common epitopes among YBST and pteropid-variant ABLV (except ABLHumanPt, Asp^{362} to Asn^{362}), most rabies viruses (not RaccoonUSA, also Asp^{362} to Asn^{362}), Duvenhage, and EBL-1. This common lyssavirus reactivity has been demonstrated for RCHLVac, HEP-Flury, CVS and ERA rabies virus, Duvenhage, Mokola and Lagos bat viruses using three antigenic site I antibodies (Goto *et al.* 2000). In contrast the YBST-variant and pteropid-variant ABLV sequences at antigenic site IV (amino acids 375-383), while perfectly conserved among sequences of each variant, are very different, not only from those of all other lyssaviruses, but also from each other, so that it is likely that linear site IV epitopes are probably ABLV and variant specific. Within the other linear antigenic site (residues 313-317), all the ABLV sequences are perfectly conserved with a unique combination of I^{321} and S^{331} substitutions that may form epitopes that differentiate them from all other lyssaviruses. There is a very high amino acid homology among isolates of each variant of ABLV over the entire N protein, suggesting that ABLV N protein sequence variation is tightly constrained while circulating in the respective natural pteropid or YBST bat host populations.

In contrast, the sequence and length of the 3' untranslated region (UTR) of the N gene are highly variable between the lyssavirus genotypes, the length varying between 62 bases for rabies and Duvenhage isolates and 73 bases for EBL2 (n=2) (Bourhy *et al.* 1993a). This variability in length and sequence makes alignment and subsequent analysis of this region quite subjective, and contributes to phylogenetic analysis of this region being less robust.

The 3'UTR of the N protein mRNA for all ABLV isolates is 66 bases long, and the lyssaviruses end with a transcription termination polyadenylation signal of either TAG_7 (Duvenhage and rabies viruses) or AGA_7, with the following exceptions. While 30 of the 31 ABLV sequences reported here and that of AAHLYBST and ABLHumanPt end with the AGA_7 signal, in one ABLV sequence (ABLBallina) the polyadenylation signal is one A base short (AGA_6) and in one of the sequences reported here (ABL14Cen-B) the signal is one A base long (AGA_8). Whether the uncharacteristically short signal was present in the host animal virus population or is secondary to *in vivo* or *in vitro* passage prior to cDNA synthesis or an artefact of cDNA synthesis or PCR

amplification is unclear. The long signal reported here occurred in both directions using cDNAs synthesized from RNA extracted directly from the natural host (*P. alecto*) brain and peristed despite use of two separate cDNA primers, and multiple PCR and sequencing reactions. This suggests this anomaly was present in the natural host virus population and that variations in the ABLV polyadenylation signal do occur naturally.

4.4.2 Similarity analysis of ABLV

The large scale of sequence variability between early laboratory rabies virus sequences and those of the other related lyssaviruses, Mokola, Duvenhage, and Lagos bat virus was readily described by percent similarity matrixes, with amino acid sequences within genotypes sharing > 97.1% similarity and sequences from different genotypes having < 93.3% amino acid similarity (Bourhy *et al.* 1993a). As more divergent genotype 1 rabies virus isolates were sequenced and compared with the most closely related EBL-1 and EBL-2 sequences, the greater intragenotype variability and low intergenotype variability meant that clear limits of percentage nucleotide or amino acid similarity enabling classification in or between genotypes was no longer apparent, with some overlap in intra- and inter-group similarity occurring (Kissi *et al.* 1995).

Similarly, percent similarity analysis is unable to differentiate ABLV sequences from those of rabies virus isolates. As shown in Figure 4-4, the similarity between ABLV and the rabies virus sequences (88.0 - 93.6%), was no less than that amongst rabies isolates themselves (90.7-99.3%). At this level of analysis, while the ABLV isolates are clearly unique, they are not *sufficiently* different to be clearly distinguished from the rabies virus genotype.

However, this analysis is comparatively crude as it only counts the *number* of differences between pairs of sequences and does not consider the *characteristics of* and *relationships between* those changes. For example the greater functional significance of non-synonymous rather than synonymous nucleotide changes is not reflected in nucleotide sequence similarity matrixes. While doing amino acid rather than nucleotide similarity matrixes restricts the analysis to only non-synonymous nucleotide changes, it results in the loss of other potentially significant information. The crude similarities between some lyssavirus sequences exceeds the capacity of similarity matrixes to differentiate related virus groups. Proper consideration of the similarities and differences between ABLV and the other lyssaviruses should include not only the number, but the position, type, and the relationships between, the sequence differences. Such consideration is provided by phylogenetic sequence analysis.

4.4.3 Phylogenetic analysis of ABLV

Phylogenetic analysis of all the available ABLV N coding sequences (n=34) alone, and in combination with other sequences representing the range of other lyssaviruses clearly indicate that all the ABLV isolates cluster together as a discrete sister group, with ≥ 97% bootstrap

support, within the genus *Lyssavirus*. The ABLV lineage is most closely related to that of the rabies viruses and then EBL-2. The ABLV lineage consists of two clads, one isolated only from YBST bats (YBST-variant ABLV), and one isolated from the three common species of flying fox (Black, Grey-headed and Little Red flying fox (pteropid-variant ABLV). The distinction between the variants is strongly supported by 100% bootstrap support and a relatively long branch length. Within each variant, the four YBST-variant isolates and 30 pteropid-variant isolates are separated by very short branch lengths, notably shorter than the branch lengths between rabies isolates, indicating that genetic variability within each variant is very low. This analysis supports and strengthens previous analyses using the original pteropid and YBST sequences (ABLBallina, and AAHLYBST) and later pteropid sequence (ABLHumanPt) that proposed that the ABLV isolates consisted a new genotype, Genotype 7, within the *Lyssavirus* genus (Gould *et al.* 1998; Gould *et al.* 2002; Hooper *et al.* 1997b ; McColl *et al.* 2000 ; Warrilow *et al.* 2002).

Among the isolates recovered from pteropid bats, there was no geographical association among the sequences, despite sequences from flying foxes having been collected over a range of more than 700 km (Townsville to the Gold Coast). In particular neither the four isolates from Central Queensland nor the five isolates from Far North Queensland showed unique sequence characteristics compared to those from Southeast Queensland.

This contrasts with what has been demonstrated among terrestrial rabies virus isolates where both global (Arai *et al.* 2001; Kissi *et al.* 1995 ; Nel *et al.* 1993) and local (Bourhy *et al.* 1999 ; Ito *et al.* 1999; Nadin-Davis *et al.* 1993 ; Sacramento *et al.* 1992) associations have been found between sequences and the geographical area in which they occurred. Regional associations within a single rabies reservoir species have been attributed to slow evolution of the virus population in parallel with spatial and temporal progression of an epizootic (Bourhy *et al.* 1999 ; Nadin-Davis *et al.* 1993; Sacramento *et al.* 1992), or to local geographical features such as mountains and rivers producing functional isolation of sub-populations of the host species and independent evolution of the virus within these sub-populations (Bourhy *et al.* 1999; Ito *et al.* 1999; Nadin-Davis *et al.* 1999).

The absence of regional ‘sub-variants’ of pteropid-ABLV can probably be attributed to the fact bats fly and live together in large colonies, with individuals flying considerable distances and interacting with other colonies (Eby 1991; Nelson 1965). As a result, there are probably comparably few degrees of separation between individuals separated by great distance. It appears that with respect to ABLV, the flying foxes of Queensland function as a single population rather than as regionally distinct sub-populations.

More surprisingly, no species-specific associations were found among the pteropid isolates, i.e. no Black, Grey-headed, or Little Red flying fox specific sub-variants. This is not unexpected among the isolates from Black, Grey-headed and Spectacled flying foxes, as these bats are very similar in terms of their reproductive biology and feeding behaviour and frequently camp in mixed colonies of Black and Grey-headed flying foxes in Southeast Queensland, and Black and

Spectacled flying foxes in Far North Queensland. However, Little Red flying foxes differ most from the other species physically, have a reproductive cycle that is offset from other flying foxes by approximately 6 months, disperse to inland Queensland during the winter, and feed principally on blossom and nectar rather that fruit. It was thought possible that these social and biological differences may have been associated with species-specific adaptations of ABLV; however no sequence specificity was found among any of the flying fox species. Little Red flying foxes do camp in mixed colonies with the other flying foxes. It would appear that they, together with the other species, act as a single host population for pteropid-variant ABLV.

The attempt to demonstrate the presence or absence of temporal variation in the ABLV sequences was limited by the relatively brief collection period of 3 ½ years. No temporal associations were found.

All these findings are consistent with those of a closely related study published while this work was underway (Gould *et al.* 2002) that was based on only partial sequencing of the N gene (nucleotides 443 to 892 of the ABLBallina isolate, corresponding to bases 513 to 962 of Appendix 10, Figure A10-1) of ABLV isolates that had been passaged in mice and/or one or more cell culture lines. At least seven of the partial sequences used in that study are derived from material from the same bat ABLV cases as those of this study (see Table 4-2), having been forwarded to the AAHL from the ARI for diagnostic confirmation. Unfortunately the partial sequences themselves have not been published nor provided to GenBank and so direct comparison of the sequence results from the same cases by the two laboratories has not been possible.

The phylogeny of the 31 ABLV N protein coding sequences shown here is also strikingly similar to that of the corresponding G gene sequences determined from 22 of the same RNA extractions by other members of the research group (Guyatt *et al.* 2003). The numbers in the sequence names of these 22 G gene sequences, e.g. G gene ABLPA**01**MB, (Guyatt *et al.* 2003), correspond with the similarly numbered N gene sequence reported here, e.g. ABL**01**SEQ-B. The G gene has been used to examine the phylogeny of ABLV and other lyssaviruses as the glycoprotein is the major antigen involved in eliciting humoral immunity and vaccine efficacy, is important in pathogenesis through its role in binding to host cell receptors and in host cell penetration, and may also play a role in host specificity. As in studies of other lyssaviruses (Nadin-Davis *et al.* 1993; Nadin-Davis *et al.* 1999), the phylogeny of the N and G genes of ABLV are in agreement, with respect to the relationships between individual ABLV isolates, between the YBST and pteropid variants, and the relationship of the ABLV lineages to the other lyssaviruses.

With respect to the absence of temporal variants of ABLV, the capacity to detect variants among the sequences used by Gould *et al.* (2002) and this study is limited by the short period (< 4 years per report) during which sequences were collected. A truer reflection of the temporal stability of ABLV will require isolates from the distant past (none of which have been found), or the future.

With respect to the absence of regional or pteropid species-specific variants of ABLV, the capacity of the partial sequences in Gould *et al.* (2002) to detect potential variants was limited by the well-conserved nature of the section of N gene used. However it is strongly supported by the use in this report of the complete N gene coding sequence, including the more variable 5' and 3' end regions, and the even more variable G gene (Guyatt *et al.* 2003).

The clear phylogenetic evidence for two genus-specific (Pteropus and Saccolaimus) ABLV variants, the exceptionally high sequence homogeneity within each variant, and the lack of regional 'sub-variants' suggests each has been present in their host populations for sufficient time for optimal 'end-point' host specific adaptations to have occurred. This would indicate that ABLV has been present in Australia since long before European colonization (pre 1788) and the apparently fortuitous and incidental recognition of ABLV in 1996.

4.4.4 Two land masses, two lineages of rabies virus

A further finding from the selection of rabies virus (genotype 1) sequences used here in both the neighbour joining and maximum parsimony analyses, was that the rabies virus clade consisted of two monophyletic sister groups, separated by a relatively long branch length, albeit with only modest bootstrap support (75 and 49% using neighbour joining and 62 and 44% using maximum parsimony). While this dichotomy had been noted by Nadin-Davis *et al.* (2001) using fewer complete N sequences, and by Smith *et al.* (1995) using partial N protein sequences from the USA and Mexico only, no interpretation with respect to the evolutionary origins of *lineages* of rabies virus (rather than variants) was made.

There are no pre-colonial references to rabies in the Americas (Smith and Seidel 1993), which may suggest that the multiple species-specific and/or regional variants of rabies in the Americas were all introduced following European colonization. Instead this phyogenetic analysis suggests that there are two ancient lineages of genotype 1 rabies virus, both of which are now present in the Americas. One consists of rabies variants that were initially recognized in Africa, Europe, and Asia during the time that these land mass were made confluent by human activity but remained discrete from the Americas. It is well recognised that some of these 'Old World' rabies variants have since been introduced to the Americas and have established themselves in introduced host populations (e.g. dog rabies in dogs in Mexico) or related native hosts e.g. Arctic fox rabies in Arctic foxes and red foxes in Alaska, in Canadian red foxes and striped skunks (Nadin-Davis *et al.* 1994), and red foxes in north-eastern USA (Smith 1989; Smith *et al.* 1995). The other lineage consists of rabies variants specific to indigenous host populations of the Americas (multiple American bat rabies variants and raccoon-variant rabies). It is proposed here that it represents a separate indigenous lineage of multiple 'New World' variants that were present and evolved in America prior to European colonization and had minimal (unrecorded) impact on indigenous human populations.

Complete N protein sequences for every variant / endemic of rabies within the United States were not found for use in this analysis, however the broad phylogenetic relationships seen using the terminal 320 nucleotides of the N gene (Smith *et al.* 1995), can reasonably be inferred and transposed onto this analysis. Only the rabies variant of striped skunks in Texas would be expected to associate with the bat and raccoon variants in the 'New World' group. Such a clustering was evident in a phylogeneteic study of lyssavirus P gene sequences, in which four Texan skunk isolates clustered with raccoon and American bat isolates (Nadin-Davis *et al.* 2002). The N gene sequences of isolates from the other US endemics not included in this analysis, skunks in California and Iowa-Minnesota-South Dakota, gray foxes in Texas and Arizona, and coyotes in Texas and Mexico would be expected to associate with the 'Old World' group. This was the case for one Texan coyote in the P gene analysis (Nadin-Davis *et al.* 2002). The outbreak in coyotes is known to be a spill-over from domestic dogs in Texas and Mexico (Smith *et al.* 1995).

If the origins of rabies variants along 'New World'/'Old World' lineages is true, then the position of the sequence from the West Canadian skunk in this analysis is interesting. It associates most strongly with two laboratory strains of dog-variant rabies (PV and SAD-B19), then with other dog-variant isolates from Central Africa, Algeria and Iran, other laboratory strains of dog-variant rabies (3AGChina, Nishigahara and RCHLVac) and the red fox and raccoon dog-variants of rabies from Europe that are believed to be dog-variant derivatives (Bourhy *et al.* 1999; Holmes *et al.* 2002). It occurs in the 'Old World' group of rabies viruses that includes the Arctic fox variant rabies virus present in red foxes and skunks in eastern Canada that is believed to have originated in Europe. It is not in the 'New World' group of isolates that affect the other indigenous American host species, the microbats and raccoon. This association with 'Old World' dog-variant rabies suggests that the rabies virus circulating in skunks in West Canada is also a derivative of dog-variant rabies, presumably introduced by European settlers, that has established itself in the endemic skunk population.

Given the phylogenetic relationships shown here and inferred from Smith *et al.* (1995) and Nadin-Davis *et al.* (2002), and what has been previously published about the origins of some of the American endemics, it appears that there were rabies viruses in the Americas that evolved *in* the Americas, prior to European contact. These 'New World' indigenous rabies variants are those of the microbats, raccoons, and skunks in Texas. All the other variants presently in the Americas are either known (Mexican dogs, Arctic foxes, red foxes, coyotes, Ontario skunks, and Caribbean mongooses) or proposed here (West Canadian skunks, skunks of California and Iowa-Minnesota-South Dakota, and grey foxes) to involve exotic dog-derived rabies variants introduced after 1700 (Smith and Seidel 1993) that subsequently adapted to resident host species.

It has been shown that there is no evidence of recombination in lyssavirus evolution, and that point mutation and purifying selection are the major forces in lyssavirus evolution (Badrane and Tordo 2001). In the absence of recombination and presuming evolution at similar rates, Badrane and Tordo (2001) proposed that the phylogeny of the genotype 1 rabies sequences indicates two

successful host switching events from American microbats to carnivores, both estimated to have occurred 888 to 1,459 years ago. They further proposed that one (or more) spillover event produced the native American raccoon and Texan skunk variants, while the other gave rise to the now worldwide 'Old World' terrestrial/carnivore variants. This however is not consistent with historical accounts of rabies.

'Old World' carnivore (dog) rabies is believed to have been present in Mesopotamia at least since 2300 BC and was known to Aristotle in fourth century B.C. Greece (Wilkinson 2002). These accounts indicate carnivore rabies virus evolved far earlier than just 1,459 years ago. No connection between carnivores in Europe and the Middle East, and microbats in the Americas is known for that time. Had 'Old World' rabies in carnivores in Europe and the Middle East arisen from bats, it would surely have been as a result of contact with European, African or Asian bat species; however the relatively distant relationships between carnivore rabies and EBL-1, EBL-2, Duvenhage, Lagos bat and Mokola viruses do not suggest this.

Rather than 'Old World' carnivore rabies having evolved from a spillover from bats, the evolutionary origin of genotype 1 rabies virus that is proposed here is that 'Old World' and 'New World' lineages of rabies virus are each derived from an ancient progenitor virus. Furthermore that these lineages were separated at the time of continental shift. A theory of continental isolation of progenitor lyssaviruses with independent evolution also suggests an explanation for the origins of ABLV: that the ancient progenitor virus was also isolated on the Australian continent and evolved along a third lineage into two variants, pteropid- and ybst-ABLV. The phylogeny of the 'New World' microbat, raccoon and Texan skunk isolates certainly suggests spillover between bat and carnivore hosts, but the direction of the spillover, to or from bats is not indicated.

It is highly relevant to Australia to note that while the establishment of 'new' endemics in 'new' host populations has occurred in Europe and North America on multiple occasions in the past 1000 years, all have involved dog-variant or dog-derived variants of 'Old World' genotype 1 rabies virus (Bourhy *et al.* 1999; Smith *et al.* 1992). Australia has susceptible populations of domestic dogs, red foxes, presumably dingoes, and possibly carnivorous marsupials such as quolls (*Dasyurus* spp.) that would be expected to support the establishment of self sustaining cycles of dog-type rabies infection if adequately introduced. The presence of ABLV in Australia in no way reduces the very real threat genotype 1 dog-type rabies variants pose to these and other potential host populations. This is particularly so given the demonstrated propensity of dog-type rabies to establish itself in new canine and non-canine (e.g. skunk, mongoose, raccoon dog) populations, in contrast to the apparently 'inert' epidemiology of ABLV and other non-rabies bat lyssaviruses. If Australia is to avoid the establishment of multiple dog-type variant endemics, as has occurred in America, it is important our rabies-prevention quarantine measures be upheld, despite the presence of ABLV.

4.5 Conclusions

Examination of molecular diversity of ABLV isolates

- ♦ Sequences for 35 bases of the leader sequence, the entire N gene, and start of the P gene were determined and examined for 28 isolates of ABLV from Black, Grey-headed, and Little Red flying foxes and 3 isolates from YBST bats.
- ♦ Suitable material was not available for determination the ABLV sequence from the only known ABL-positive Spectacled flying fox.
- ♦ All the ABLV isolates cluster together as a discrete sister group within the *Lyssavirus* genus.
- ♦ The ABLV lineage consists of two variants separated by a relatively long branch length, one isolated only from YBST bats (YBST-variant ABLV), and one common to three common species of flying fox; Black, Grey-headed, and Little Red flying fox (pteropid-variant ABLV).
- ♦ Within each variant the level of sequence variability is extremely low, indicating sequence stability and suggesting end-point adaptation to the relevant host genus.

Host species, geographic, and temporal variation among ABL isolates

- ♦ There are no geographical or temporal sub-variants, nor did variants associate with individual flying fox species.
- ♦ A discrete variant of ABLV appears to be associated with one species of insectivorous bat (*Saccolaimus flaviventris*, YBST bat), however as the number of submissions from microbats is small, it is possible this variant is also associated with other microbat species, and/or other variants are also circulating in other microbats.

ABLV as the seventh genotype

- ♦ The ABLV lineage is most closely related to that of the rabies viruses and then EBL-2.
- ♦ The strong bootstrap support (>97%), low inter-variant variability, and the relatively long branch separating all ABLV sequences from all those of rabies virus and EBL-1, provide good phylogenetic evidence for ABLV as a discrete, seventh lyssavirus genotype.

5 Selection and characterization of an inoculum containing ABLV

Aims

1. To evaluate *in vivo* the virulence of a number of inocula prepared from the tissues of naturally ABLV-infected flying foxes.

2. To select and characterize the most virulent inoculum for subsequent use in flying foxes.

3. To evaluate by quantitative-PCR (TaqMan®) the relative amount of viral RNA in each inoculum, relate that to the effects of those inocula in mice, and evaluate the potential role of quantitative PCR in the selection of inocula.

5.1 Introduction

On two previous occasions Grey-headed flying foxes had been inoculated with preparations containing ABLV that had been isolated from a naturally infected bat and had undergone limited passage in mice and cell culture (McColl 1999; McColl *et al.* 2002). Using inocula containing $10^{3.7}$ $TCID_{50}$ and $10^{5.0}$ $TCID_{50}$ respectively, one of seven and three of 10 flying foxes developed clinical disease and were subsequently shown to be infected with ABLV. While the small number of affected bats provided preliminary observations of the disease in flying foxes, further studies of ABLV in bats were indicated. The small proportion of affected animals (23%, n=17) suggested that inducing disease in flying foxes by experimental inoculation would be difficult.

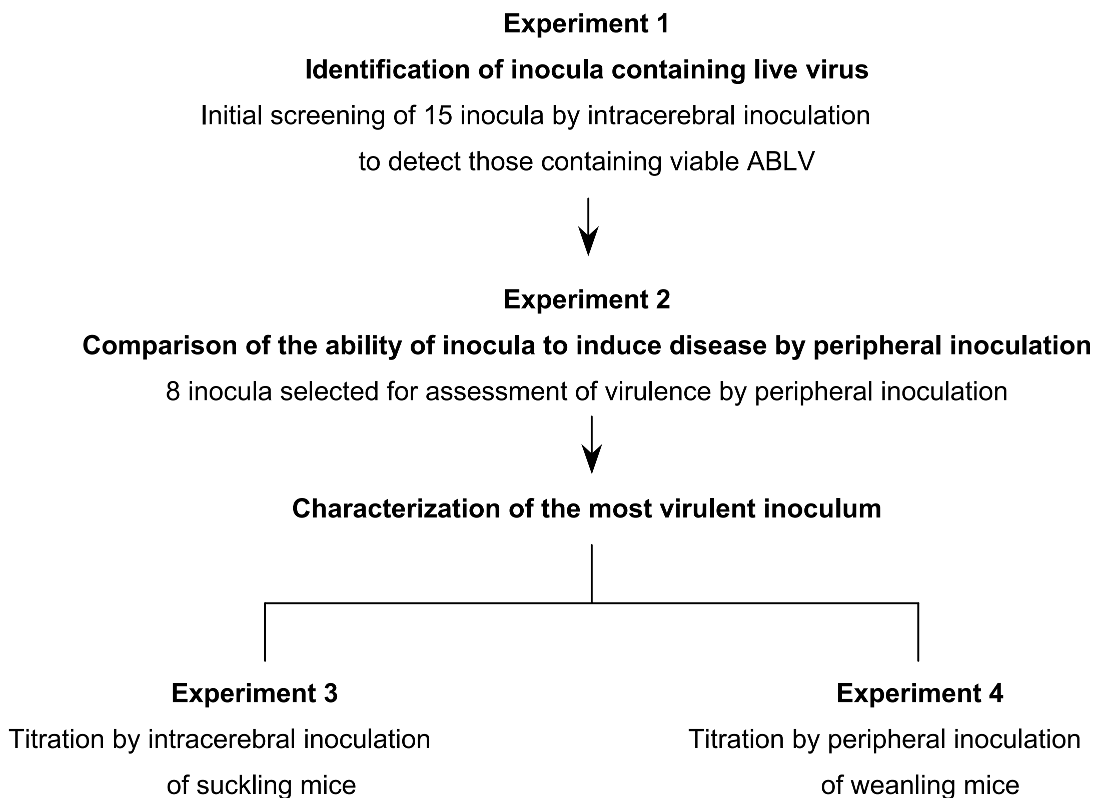
For this study is was decided that inocula would be prepared directly from ABLV-positive tissues recovered from naturally infected bats in order to avoid any alteration in the virulence or pathogenesis of ABLV in experimental animals that might occur due to virus passage and amplification in aberrant species such as mice or cell culture. However, there had been considerable variation in the handling and storage of tissues collected from naturally affected bats, and it was presumed this would affect the viability and virulence of the ABLV in the stored tissues and the inocula prepared from them. Clearly, successful inoculation of flying foxes would require identification and characterization of an inoculum that was highly virulent. Ideally this would be done in the principal host species, however Australian native bats are protected species, some species are declining in numbers, and they are difficult and expensive to keep, making them poor candidates as experimental animals. In order to minimize the number of experimental bats required, and maximize the likelihood that inoculated bats would be affected, aliquots of candidate inocula were first evaluated in mice for their ability to induce disease.

5.2 Experimental Design

Two models of mouse inoculation were used for the evaluation of inocula. Intracerebral inoculation of suckling mice was used as a sensitive method to detect and quantify the presence of live virus in inocula. To ensure selection of an inoculum most likely to induce disease in peripherally inoculated bats, a second model involving peripheral inoculation of weanling mice (~21 days old) was used to compare and quantify the peripheral virulence of inocula.

Animal ethics requirements precluded death as an endpoint. Mice showing clear clinical signs of disease were killed. To reduce the numbers of mice required to identify and characterize an inoculum of high virulence, the following approach was taken.

Figure 5-1 Experimental design to identify an inoculum of high virulence in mice



5.3 Materials and methods

5.3.1 Permits

Animal Ethics Committees

- University of Queensland – approval numberSVASP/263/99/QDPI/PHD
- Animal Research Institute – approval number ARI/015/RES/JUN1999-1

Biosafety Committee

- Animal Research Institute – approval number DPI / IBC / PC3 / 99 / 00

5.3.2 Inocula

Inocula were prepared using tissue stored at –70°C that had been collected from naturally infected flying foxes submitted to the ARI as part of a surveillance program for the detection of Australian bat lyssavirus (see Chapter 2). A systematic evaluation of all available tissue samples was not possible as animal ethics considerations limited the number of mice and hence the number of inocula that could be evaluated. Of 96 available samples from approximately 40 ABLV-positive cases, eight brains and seven salivary gland samples were selected because they satisfied one or more of the following criteria:

- Samples from bats where there had been a short interval between the death of the bat and likely storage of the sample at –70 ^{0}C, such that the tissue sample was likely to be in good condition with presumably less post-mortem degradation of the virus.
- Samples from Grey-headed flying foxes in preference to other species as this was the intended experimental bat species.
- Samples from salivary glands that had fluoresced strongly in the fluorescent antibody test.

Each sample was ground into a 20% weight per volume suspension in a Class II biosafety cabinet within the Diagnostic Virology Laboratory with a sterile mortar and pestle using Dulbecco's Modified Eagles Medium (Gibco BRL®, Life Industries) containing 20% bovine serum albumin (CSL) and an antibiotic/antimycotic preparation containing Penicillin G, streptomycin sulphate and amphotericin B as fungizone (Gibco BRL®, Life Industries). Sterile sand was used to assist in the grinding of the salivary gland tissues. Suspensions were clarified at ~200 G for 5 minutes and the supernatant removed, divided into aliquots, and stored at –70 ^{0}C. All subsequent uses of these inocula, in mice and bats, were done using separate aliquots of the original preparations at the same freeze/thaw generation. Aliquots containing 200 µL of each inoculum were submitted to QHSS for quantitative real-time PCR amplification (TaqMan®, see Appendix 3).

5.3.3 Mice

Specific pathogen-free Quackenbush (outbred) mice were purchased from the barrier-maintained colony at the Central Animal Breeding House of the University of Queensland. Experimental mice were kept in commercial cages with *ad lib* food and water dispensers within the PC3 Animal House at the ARI.

5.3.4 Confirmation of lyssavirus infection

Infection with Australian bat lyssavirus was confirmed by FAT on fresh mouse brain touch impressions, as described in Section 2.2.3 Fluorescent antibody test. The tests were performed and read by the candidate and the results confirmed by Barry Rodwell of the Diagnostic Virology Laboratory of the ARI.

5.3.5 Experiment 1: Detection of viable ABLV by IC inoculation of suckling mice

One to three-day-old suckling mice were inoculated by intracerebral injection by Peter Young (ARI) with 10 to 20 µL of inoculum with a tuberculin syringe and 27 gauge needle. Each of the 15 original inocula were inoculated into litters containing 9 to 11 mice (n=141) on post-inoculation day 0 (PI-day 0). Mice that died or disappeared (presumed eaten by dam) within three days of inoculation were considered to have died of non-specific causes related to their transport to the PC3 Animal House or the trauma of the inoculation. These losses were discounted when evaluating the results, reducing the number of experimental suckling mice to 124. Details of the source of each inoculum (bat accession number, bat species and source tissue) and the number of experimental mice per inoculum are shown in Table 5-1.

Mice were considered to be affected if they died, required euthanasia or disappeared between 3 and 24 days post-inoculation. Affected mice were killed by CO_2 inhalation. All surviving mice were killed on PI-day 24 by intraperitoneal injection of an anaesthetic overdose containing 0.1-0.3 mL of a 1:1 mix of ketamine (100 mg/mL) and xylazine (20 mg/mL).

5.3.6 Experiment 2: Comparison of virulence by peripheral inoculation of weanling mice (3-mouse footpad assay)

Eight inocula from Experiment 1 were selected for further evaluation by peripheral inoculation, for details refer to Table 5-2 . Each inoculum was inoculated into the footpads of three 3-week old weanling mice anaesthetized by intraperitoneal injection of 0.01 mL of a 1:1 mix of ketamine (100 mg/mL) and xylazine (Rompun SA 20 mg/mL). Each mouse was inoculated by the candidate, as

previously demonstrated by Charles Rupprecht, CDC Atlanta Georgia, USA, with 60µL of inoculum divided into the right and left hind footpads, approximately 30µL per foot, using a tuberculin syringe and 27 gauge needle.

Each group of three mice was housed separately. Within each group, mice were individually identified with silver nitrate, creating brown markings on white fur. Mice were killed when clinically ill or at the conclusion of the experiment on PI-day 162 by intraperitoneal injection of an anaesthetic overdose of 0.1-0.3 mL of a 1:1 mix of ketamine (100 mg/mL) and xylazine (20 mg/mL). Blood samples were collected with heparinized syringes peri-mortem by severing the vessels of one axilla. Blood was centrifuged at ~800 G for 10 minutes and the plasma stored at –20 °C and submitted to the AAHL for detection of anti-lyssavirus antibodies using the rabies-RFFIT.

5.3.7 Experiment 3: Titration of selected inoculum by IC inoculation of suckling mice

Based on the results of Experiment 2, one inoculum was selected as the most virulent, for determination of the ABLV-titre by intracerebral inoculation of 2 to 3-day-old suckling mice. Ten fold serial dilutions (10^{-1} to 10^{-8}) were prepared from the neat inoculum (20% w/v suspension) in a Class II biosafety cabinet within the PC3 Diagnostic Virology Laboratory using Dulbecco's Modified Eagles Medium and an antibiotic/antimycotic preparation. One litter containing 6 or 10 mice (see Table 5-2) was inoculated with each dilution of inoculum as described for Experiment 1.

Mice showing clear signs of being clinically ill were killed by CO_2 inhalation. All surviving mice were killed on PI-day 25 by intraperitoneal injection of a ketamine/xylazine overdose as in Experiment 2. Mice were considered positive if they died or required euthanasia and were subsequently shown to be ABLV-positive by FAT on fresh brain touch impressions. All mice that were clinically well on PI-day 25 were considered negative. As clinical disease and a positive FAT result, rather than death, was used as the endpoint, the 50% endpoint dilution, calculated using the Spearman-Kärber Method (Lorenz and Bogel 1973), was determined as the 50% effective dose (ED_{50}) rather than 50% lethal dose (LD_{50}). The titre determined in this assay is expressed as mouse intracerebral 50% effective dose per mL ($MICED_{50}$/mL).

5.3.8 Experiment 4: Titration of selected inoculum by peripheral (footpad) inoculation of weanling mice

The inoculum selected based on the results of Experiment 2 was further characterized for virulence by determination of the ABLV-titre by peripheral inoculation of 21-day-old mice weighing 13-18 g. Ten fold serial dilutions (10^{-1} to 10^{-7}) of the inoculum were prepared from the neat inoculum in a Class II biosafety cabinet within the PC3 Virology Laboratory using Optimax

Modified Eagles Medium (Gibco BRL®, Life Technologies) and an antibiotic/antimycotic preparation. Six unanaesthetised mice were inoculated with 60 µL of each dilution of inoculum as described for Experiment 2. Mice were weighed using digital laboratory scales (UWE SC-1500, increments 0.5 g, Taiwan) when inoculated, when killed, and every second day between PI-days 4 and 40, then every 2nd to 4th day until PI-day 60. Clinically ill mice and all surviving mice on PI-day 91 were killed by ketamine/ xylazine overdose, as in Experiment 2. Mice were considered positive if they died or required euthanasia and were subsequently shown to be ABLV-positive by FAT on fresh brain touch impressions. All mice that were clinically well on PI-day 91 were considered negative. The 90 day mouse footpad 50% effective dose ($MFP_{90}ED_{50}$ /mL) was calculated using the Spearman-Kärber Method (Lorenz and Bogel 1973).

Aliquots of the serial dilutions were submitted to Queensland Health Scientific Services for quantitative real-time PCR assay (TaqMan®).

5.4 Results

5.4.1 Experiment 1: Detection of viable ABLV by IC inoculation of suckling mice

One or more suckling mice were affected in 14 of the 15 inoculated litters; and one to three suckling mice per affected litter were confirmed as ABLV-positive by FAT on fresh brain touch impressions. Details of the bat species and tissue source of the inoculum, the range of days post-inoculation that suckling mice were affected, the number of mice affected of the total alive 3 days post-inoculation, the percent of affected mice per litter, and the number of cycles required for TaqMan® amplification of viral RNA to reach the positive detection threshold, are shown in Table 5-1.

Australian bat lyssavirus was isolated from the three tissue sources used to prepare the inocula: brain, parotid salivary gland, and the submandibular and sublingual salivary glands. Fifty-seven of 67 mice (85%) inoculated with material prepared from the brains of naturally infected flying foxes were affected with 100% of mice affected in six of eight litters. Thirty of 38 mice (79%) inoculated with submandibular and sublingual salivary gland inocula were affected, with 100% of mice affected in three litters and 50% of mice affected in two litters. Only five of 19 mice (26%) inoculated with parotid salivary gland derived inocula were affected. One of these mice disappeared on PI-day 7, midway between the last of the non-specific deaths prior to PI-day 3 and the first of the confirmed ABLV-affected mice on PI-day 11 (see Figure 5-2). The loss of this mouse may have been incidental. The remaining four affected parotid-inoculated mice were among the last mice to be affected.

Table 5-1 Experiment 1: Detection of live ABLV by intracerebral inoculation of 15 inocula into litters of suckling mice

Details of inoculum				-70°C delay[1] (days)	Period of clinical effect[2] (PI-days)	Number affected/total	Percent affected	TaqMan® threshold[3] (cycles)
Bat No. and ARI accession		No.	Bat species and tissue					
ABLV-27	97-186955	1	**Grey[4] brain**	2	11-13	6/6	100	16.9
ABLV-18	97-125092	2	**Black[5] brain**	2	12-13	9/9	100	17
ABLV-26	97-176292	3	**Grey brain**	2	12-13	8/8	100	17.5
ABLV-45	98-167351	4	**Grey brain**	16	12-15	10/10	100	18
ABLV-51	98-188455	5	**Black submandib. and sublingual SG[6]**	13	12	3/3	100	18.6
ABLV-28	97-192894	6	**Grey brain**	2	13-14	8/8	100	19
ABLV-46	98-172971	7	**Black submandib. and sublingual SG**	7	11-13	9/9	100	20
ABLV-52	98-189702	8	**Red[7] submandib. and sublingual SG**	6	12-13	10/10	100	21.2
ABLV-33	98-105331	9	Grey brain	11	14-24	8/8	100	17.8
ABLV-24	97-155574	10	Black brain	1	12-13	8/9	89	18.6
ABLV-45	98-167351	11	Grey submandib. and sublingual SG	16	13-15	5/10	50	26.5
ABLV-33	98-105331	12	Grey submandib. And sublingual SG	11	15-24	3/6	50	26.7
ABLV-18	97-125092	13	Black parotid SG[8]	2	16-20	3/10	33	25
ABLV-27	97-186955	14	Grey parotid SG	2	7 and 15	2/9	22	32.5
ABLV-40	98-140882	15	Red brain	3	Not applicable	0/9	0	26.8

Bold font Inocula selected for further evaluation in Experiment 2.

[1] estimated minimum delay between the death of the flying fox and storage of the source tissue at –70 °C based on diagnostic recording indicating time of death, receipt of bat and testing of sample assuming the sample was transferred to the –70 freezer the day after testing.

[2] period post-inoculation when mice were clinically ill, onset of disease in first affected mouse to death or euthanasia of last affected mouse.

[3] number of cycles required for TaqMan® amplification of viral-RNA from each inoculum to reach the positive detection threshold.

[4] Grey-headed flying fox (*P. poliocephalus*).

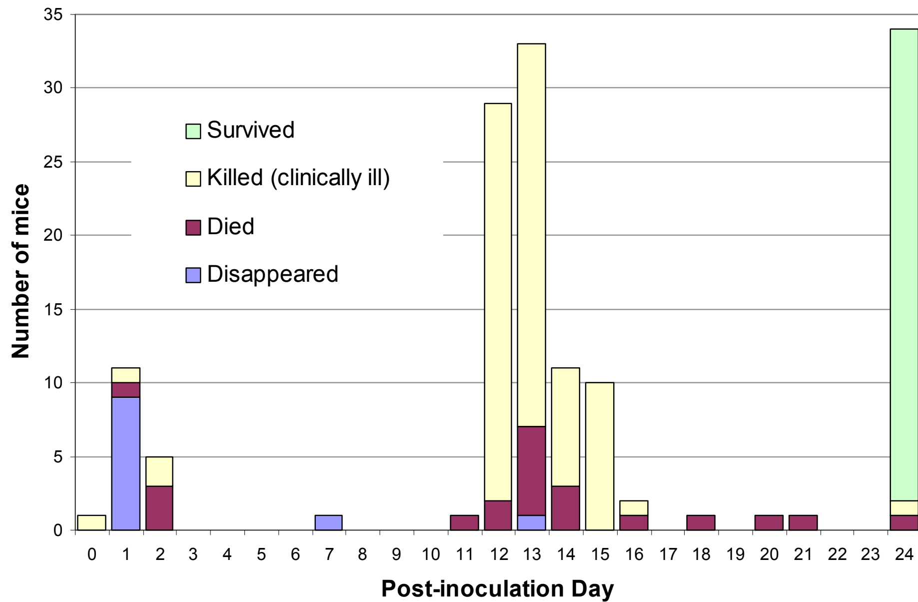
[5] Black flying fox (*P. alecto*).

[6] submandibular and sublingual salivary glands.

[7] Little Red flying fox (*P. scapulatus*).

[8] parotid salivary gland.

Figure 5-2 Cumulative results of Experiment 1: detection of viable ABLV by intracerebral inoculation of suckling mice (n=141) with 15 inocula



Inocula 1 through 8 were selected for further evaluation by peripheral (footpad) inoculation as they contained sufficient live ABLV to affect 100% of mice. Inoculum 9 was not selected for further evaluation, despite 100% of mice being affected and a low qualitative TaqMan® threshold indicating relatively high amounts of viral-RNA, because only sufficient mice for the evaluation of eight inocula had been approved by the animal ethics committees, and the comparatively long incubation periods for the Inoculum 9 mice (PI-days 14 (n=2), 15 (n=3), 16, 21, and 24) suggested this preparation contained a lower titre of live ABLV than Inocula 1-8.

5.4.2 Experiment 2: Comparison of virulence by peripheral (footpad) inoculation of weanling mice

Ten of 24 footpad-inoculated mice developed clinical signs of lyssavirus infection and were subsequently shown to be ABLV-positive by FAT on fresh brain touch impressions. The incubation period of the affected mice ranged from 10 to 39 days as shown in Table 5-2. One clinically normal mouse inoculated with Inoculum 2 was inadvertently killed and discarded on PI-day 61. All other surviving mice remained well until killed on PI-day 162, and were negative for ABLV by FAT on fresh brain touch impressions.

Table 5-2 Experiment 2: Comparison of virulence by 3-mouse footpad assay

Details of Inoculum				Number affected (n=3)	Period of clinical effect[1] (PI-days)	Percent affected (%)	TaqMan® threshold (C_T)[2]	Virulence ranking
ABLV case No. and ARI Bat accession		No.	Bat species and tissue					
ABLV-27	97-186955	1	Grey[3] Brain	3	18-19 21-24 35-39	100	16.9	2nd
ABLV-18	97-125092	2	Black[4] Brain	0	---	0	17.0	Equal last
ABLV-26	97-176292	3	Grey Brain	2	25 39-42	66	17.5	3rd
ABLV-45	98-167351	4	Grey Brain	0	---	0	18.0	Equal last
ABLV-51	98-188455	5	Black submandib. and sublingual SG	3	10-12 10-12 10-12	100	18.6	1st
ABLV-28	97-192894	6	Grey Brain	0	---	0	19.0	Equal last
ABLV-46	98-172971	7	Black submandib. and sublingual SG	1	15-17	33	20.0	4th
ABLV-52	98-189702	8	Red[5] submandib. and sublingual SG	1	27-28	33	21.2	5th

[1] period post-inoculation when each mouse was clinically ill, onset of disease to death or euthanasia of mouse

[2] number of cycles until amplification of viral-RNA reached the positive detection threshold

[3] Grey-headed flying fox (*P. poliocephalus*)

[4] Black flying fox (*P. alecto*)

[5] Little Red flying fox (*P. scapulatus*)

5.4.2.1 Clinical signs

Clinical signs included:

- weight loss
- dehydration
- shaking, tremors and hyperaesthesia (jumped when touched)
- reluctance to move
- proprioceptive deficits (knuckling), ataxia, and paresis, particularly of the hind limbs
- conjunctivitis

Typically, the first indication of disease was the affected mouse appearing smaller than group-mates. Proprioceptive deficits, ataxia and paresis would then become evident in one or both hind limbs, and while use of the forelimbs was initially preserved, peripheral nerve deficits would progress, and unless killed, lead to recumbency. Some mice developed generalized tremors and hyperaesthesia. A number developed conjunctivitis. A clinically ill mouse showing typical signs of ABLV infection is shown in Figure 5-3.

The proportion of weanling mice that became affected following footpad inoculation was more variable than had been observed following intracerebral inoculation of suckling mice with the same inocula (Experiment 1). Whereas 100% of suckling mice given these inocula were affected within 11 to 15 days (n=8 inocula), none (n=3), one (n=2), two (n=1), or three (n=2) mice were affected per footpad-inoculated group within 162 days.

5.4.2.2 Selection of Inoculum 5

Of the two inocula that affected all three mice, one (Inoculum 5) produced disease much more quickly and more consistently than the other (Inoculum 1). All three mice treated with Inoculum 5 were clinically ill by PI-day 10 compared to PI-days 18, 21, and 35 for mice treated with Inoculum 1. The incubation period for Inoculum 5 by footpad inoculation (10 days, n=3) was actually shorter than that observed following intracerebral inoculation (12 days, n=3). Inoculum 5 was considered the most virulent by peripheral inoculation in mice and was selected for further characterization in mice and subsequent use in flying foxes.

Figure 5-3 Clinically ill ABLV-positive mouse showing typical signs of ABLV infection
Three inoculated mice, each identified by brown silver nitrate markings on white fur. One mouse (top left) shows typical signs of ABLV infection including weight loss, dehydration, paresis, ataxia, and proprioceptive deficits, as indicated by its smaller size, gaunt appearance, and abnormal position of the hind limbs. The two companion mice are clinically well, as indicated by their round body condition and normal posture.

5.4.2.3 Prevalence of anti-lyssavirus titres in inoculated mice

Experiment 2 presented the opportunity to compare the prevalence and titres of neutralizing lyssavirus antibodies in mice that were affected or survived experimental inoculation with ABLV. Titres were measured as cross-neutralising titres against rabies virus in a rabies-$RFFIT_{AAHL}$. No quantitative *in vitro* test for titres to ABLV is available in Australia (i.e. no standardized ABLV-RFFIT or ABLV-ELISA).The use of mice for ABLV-serum neutralization tests was not considered justified. The rabies antibody titre for each mouse from Experiment 2 is shown in Table 5-3.

Table 5-3 Experiment 2: rabies-RFFIT titres of peripherally inoculated mice

Accession	Inoculum.mouse[1]	Status of mouse	Died (PI-day)	Rabies-RFFIT titre (IU/mL)
98-188455	5.1	Affected	12	<0.5[2]
98-188455	5.2	Affected	12	0.6
98-188455	5.3	Affected	12	0.7
98-172971	7.1	Affected	17	<0.5
97-186955	1.1	Affected	19	0.8
97-186955	1.2	Affected	23	1.1
97-176292	3.1	Affected	25	<0.5
98-189702	8.1	Affected	28	2.8
97-186955	1.3	Affected	38	<0.5
97-176292	3.2	Affected	42	Not done
97-125092	2.1	Survived	61	Not done
97-125092	2.2	Survived	162	<0.5
97-125092	2.3	Survived	162	<0.5
None	3.uninoc. control	Survived	162	<0.5
97-176292	3.3	Survived	162	<0.5
98-167351	4.1	Survived	162	<0.5
98-167351	4.2	Survived	162	<0.5
98-167351	4.3	Survived	162	<0.5
97-192894	6.1	Survived	162	<0.5
97-192894	6.2	Survived	162	<0.5
97-192894	6.3	Survived	162	<0.5
98-172971	7.2	Survived	162	<0.5
98-172971	7.3	Survived	162	<0.5
98-189702	8.2	Survived	162	**2.8**[3]
98-189702	8.3	Survived	162	<0.5

[1] inoculum number. Mouse number e.g. 5.1 = mouse number 1 inoculated with Inoculum 5.

[2] titres of < 0.5 IU/mL indicate an absence of quantitative serum neutralisation at a serum dilution of 1:20. These results are considered negative.

[3] Aliquots of plasma from mice 8.1, 8.2 and 8.3 were resubmitted to AAHL for repeat testing with consistent results of 2.2, 2.5 and < 0.5 IU/mL respectively.

Plasma samples from nine ABLV-affected mice were tested for rabies antibodies, of which five were positive for rabies antibodies, and four were negative (titres less than < 0.5 IU/mL). There are two instances where plasma was tested from more than one affected mouse per inoculum. Of the three mice inoculated with Inoculum 1, one mouse was negative, and two had low to moderate titres (0.8 and 1.1 IU/mL). Of three mice inoculated with Inoculum 5, again one mouse was negative and two had low titres (0.6 and 0.7 IU/mL). There was no apparent association between the absence of a titre in affected mice and the incubation period, negative titres occurring in affected mice that were killed 12, 17, 25, and 38 days post-inoculation. In affected mice that did seroconvert, there was a slight trend for titres to be higher in mice affected after a longer incubation period.

Twelve of 13 plasma samples collected from mice with no evidence of clinical disease by PI-day 162 were negative (< 0.5 IU/mL, no antibody detected). One inoculated mouse (mouse 8.2) that survived until PI-day 162 had a relatively high antibody titre (2.2-2.8 IU/mL).

5.4.3 Experiment 3: Titration of Inoculum 5 by IC inoculation of suckling mice

There were no non-specific deaths during the first 3 days post-inoculation and no mice disappeared. One mouse inoculated with a 10^{-6} dilution of inoculum was found dead, without preceding clinical signs on PI-day 14 and was negative for ABLV by FAT on three occasions. The death of this mouse was considered incidental. All other mice (n=45) that died or were clinically ill were subsequently confirmed as ABLV-positive by fluorescent antibody test. Mice were attributed as either positive (died or killed *and* FAT-positive) or negative (clinically well when killed on PI-day 24). The outcome for each dilution is shown in Table 5-4.

Table 5-4 Experiment 3: Titration by intracerebral inoculation with 10-fold dilutions of Inoculum 5

Dilution	Positive	Negative	Total
Neat	10	0	10
10^{-1}	6	0	6
10^{-2}	10	0	10
10^{-3}	10	0	10
10^{-4}	7	3	10
10^{-5}	2	8	10
10^{-6}	0	10	10
10^{-7}	0	10	10
10^{-8}	0	10	10

Spearman-Käber formulae

Log_{10} 50% endpoint dilution /0.01-0.02 mL= $-(\chi_0 - {}^{d}/_{2} + d \sum {}^{r}i / n_i)$

Where:

χ_0 = log_{10} of the reciprocal of the highest dilution at which all animals are positive

d = log_{10} of the dilution factor

r_i = number of positive animals at the highest dilution at which all animals are positive and at each higher dilution.

n_i = total number of animals (after discounting accidental deaths) at the highest dilution at which all animals are positive and at each higher dilution.

Such that:

Log_{10} endpoint dilution = – (3 – ½ + 1(10/10 + 7/10 + 2/10 + 0/10)

= – 4.4

The 50% endpoint dilution / 0.01 to 0.02 mL = $10^{-4.4}$

That is there are $10^{4.4}$ mean effective doses per 0.01 to 0.02 mL of neat inoculum

The titre of the neat inoculum = $10^{6.1}$ to $10^{6.4}$ $MICED_{50}$ /mL

5.4.4 Experiment 4: Titration of Inoculum 5 by peripheral (footpad) inoculation of weanling mice

One mouse inoculated with neat inoculum became acutely recumbent, with a right head tilt and persistent rolling to the right-hand side on PI-day 6, was killed, and was subsequently negative for ABLV by FAT on three fresh brain touch impressions, the loss of this mouse was considered incidental. All other clinically ill mice (n=31) were ABLV-positive. The outcome for each dilution is shown in Table 5-5.

Table 5-5 Experiment 4:Titration by footpad inoculation with serial dilutions of Inoculum 5

TaqMan® threshold	Dilution	Positive	Negative	Total
17.5	Neat	5	0	5
21.	10^{-1}	6	0	6
24	10^{-2}	6	0	6
28	10^{-3}	6	0	6
30.5	10^{-4}	6	0	6
34	10^{-5}	2	4	6
50	10^{-6}	0	6	6
50	10^{-7}	0	6	6

$MFP_{90}ED_{50}$ = 90 day mouse footpad 50% effective dose

Spearman-Käber formulae

Log_{10} endpoint dilution /0.06 mL= $-(\chi_0 - {}^{d}/_{2} + d \sum {}^{r}{}_{i} / n_i)$

Such that:

Log_{10} endpoint dilution = $-(4 - ½ + 1({}^{6}/_{6} + {}^{2}/_{6} + {}^{0}/_{6})$

$= -4.8$

Using Spearman-Käber tables or formulae the endpoint dilution /0.06mL = $10^{-4.8}$.
That is there are $10^{4.8}$ mean effective doses per 0.06 mL of neat inoculum

The titre of the neat Inoculum 5 = $10^{6.0}$ $MFP_{90}ED_{50}$ /mL.

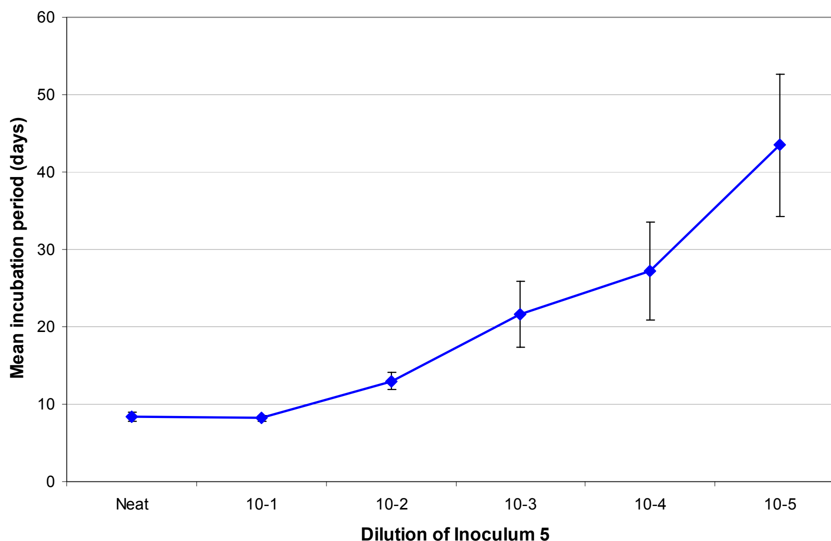
5.4.4.1 Effect of dose on incubation period

Groups of mice inoculated with lower dilutions of Inoculum 5 became clinically ill after shorter and less variable incubation periods than mice inoculated with higher dilutions. The onset of disease was defined as the first day sustained weight loss was detected or the day of onset of more specific clinical signs suggesting ABLV-infection, such as hind limb paresis. In all mice weight loss was the first clinical sign of disease. The relationship between the dose and incubation period is shown in Table 5-6 and illustrated in Figure 5-4.

Table 5-6 Experiment 4: Effect of dose on incubation period

Dilution	TaqMan ® (C_T)	Number Positive	Onset of disease (PI-days)	Mean incubation period (days)
Neat	17.4	5	8, 8, 8, 9, and 9	8.4
10^{-1}	21.1	6	8, 8, 8, 8, 8, and 9	8.2
10^{-2}	24.1	6	12, 12, 12, 14, 14, and 14	13.0
10^{-3}	28.0	6	16, 18, 22, 22, 24, and 28	21.7
10^{-4}	30.4	6	18, 24, 26, 28, 30 and 37	27.2
10^{-5}	34.2	2	37 and 50	43.5

Figure 5-4 Effect of dilution of Inoculum 5 on the mean incubation period in peripherally inoculated mice
(vertical bars indicate ± 1 standard deviation)



5.4.5 Consistency of the effect of Inoculum 5

Aa a result of being chosen for further work, aliquots of neat Inoculum 5 were used on four occasions. The results of all footpad inoculations of weanling mice using Inoculum 5 are presented together here to demonstrate the consistency of the effect in the footpad assay.

Table 5-7 Consistency of the *in vivo* effect of Inoculum 5

Reference	Date	No. affected / No. inoculated	Onset of weight loss	Onset of paresis
This Chapter, Experiment 2 (Screening)	Aug 1999	3/3	not measured	10, 10, and 10
This Chapter, Experiment 4 (Titration)	Sept 1999	5/6*	8, 8, 8, 9, and 9	10, 10, 11, 12, and 12
Chapter 6, Bat inoculation (mouse controls)	Nov 1999	2/2	not measured	9 and 9
Chapter 7, Experiment B (inoculat^n controls)	July 2000	3/3	9, 9, and 9	10, 11, and 11

* One mouse became ill on PI-day 6, was later shown to be ABLV negative, and is considered to have died of incidental causes.

5.4.6 Case history of Inoculum 5

Inoculum 5 was derived from the salivary glands of a young adult female Black flying fox submitted for lyssavirus testing in November 1998 (ARI accession 98-188455, ABLV-51).

The bat had been noticed in a tree in the morning approximately 3 km from the nearest flying fox colony by a groundsman of an aged persons/ retirement complex in a country town in central Queensland. During the afternoon, as the groundsman was raking leaves, the bat flew at him and was hit to the ground by the groundsman. The bat attempted to fly up at the groundsman again and was again hit to the ground. A box was put over the bat and it was collected by the Queensland National Parks and Wildlife Service and transferred to the care of a registered vaccinated wildlife carer. The bat was placed in a cage and as food was put in the cage, it came forward within the cage rather than retreating normally. The bat appeared ravenous, dropping to the floor of the cage to gulp food placed in a dish on the cage floor. Three hours later the bat was found dead, hanging normally within the cage. The dead bat was refrigerated until sample collection 8 days post-mortem. At necropsy small haemorrhages were detected over the right eye and left neck, consistent with the history of minor trauma. The brain and salivary glands were refrigerated until tested by FAT 6 days later and then transferred to the –70°C freezer, a minimum of 13 days post-mortem. The date of transfer to –70°C and the dates of any subsequent thawing and use of the brain or salivary glands (as FAT test controls by ARI staff) prior to preparation as inocula are not recorded.

5.5 Discussion

The principle objective of this study was to identify and characterize an inoculum that was likely to be the most virulent when inoculated peripherally into Grey-headed flying foxes. Mice were selected as the models for disease in bats as they have been used extensively in similar rabies virus studies. So that the mice would as closely as possible represent wild bats, out bred 'ordinary' (Quackenbush) mice were used rather than any of the inbred breeds available.

Only assays that require virus in the sample to replicate detect relative amounts of *viable* virus. Both cell culture assays and intracerebral inoculation of suckling mice do this. However neither differentiates between highly pathogenic, low pathogen, and apathogenic virus under natural conditions, i.e. peripheral infection of immune competent animals. A number of laboratory rabies virus strains, such as SAD-B19 and RV194-2 variants of CVS and ERA, grow in cell culture and cause mortalities in intracerebrally inoculated mice but fail to induce disease in adult mice (Conzelmann *et al.* 1990; Dietzschold *et al.* 1983). Intracerebral inoculation in mice is however the most sensitive method for detecting viable virus and for that purpose it was used to screen samples in Experiment 1. Concurrent attempts to assess the samples by cell culture were unsuccessful (data not shown).

Viable ABLV was detected in samples prepared from all tissue sources used; brain, the combined submandibular and sublingual salivary glands, and the parotid salivary glands. While the numbers of litters and inocula tested are small, the numbers of mice affected per litter, and the periods of clinical effect suggest that the samples derived from brain or submandibular and sublingual salivary glands have similar pathogenicity in mice by intracerebral inoculation, and that preparations derived from the parotid salivary gland are less virulent. Eight of 15 samples screened produced similar results, with 100 percent of suckling mice dying during a brief period (2-4 days) 11 to 14 days post-inoculation. Unexpectedly the better performing samples included Inocula 4 and 5, that had been prepared from material where the minimum post-mortem interval prior to storage at –70 °C was very long (16 and 13 days respectively). It had been presumed that prolonged post-mortem delay prior to –70 °C storage would have led to substantial reduction in virus titre. Clearly samples stored at 5 °C for up to 16 days may continue to contain relatively high quantities of viable virus.

To determine which of the eight best inocula was likely to be most pathogenic in peripherally inoculated bats, a peripheral inoculation model in mice was used in Experiment 2. In this experiment much greater differences in the proportion of mice affected and incubation periods were detected among the inocula. One, Inoculum 5, clearly produced disease in more mice, more quickly, and more consistently than all other inocula, with all three inoculated mice developing clinical signs only 10 days post-inoculation. It was presumed that the incubation periods seen in the intracerebrally inoculated mice would be the minimum possible for each inoculum, because direct inoculation in the brain would bypass the requirement to migrate via spinal axons and avoid

any delays due to immune recognition. That Inoculum 5 produced clinical disease only 10 days after peripheral inoculation, where intracerebrally inoculated mice had not died until PI-day-12, suggested the peripheral virulence of Inoculum 5 was particularly high.

Determination of the rabies-RFFIT titres enabled comparisons to be made between the titres in mice that were ABLV-positive or survived and those of ABLV-positive bats (Chapter 2). As in bats, ABLV-positive mice had both negative (4 of 9) and positive (5 of 9) titres, with the strength of the positive titres in mice (0.6-2.8 IU/mL) being comparable to those of ABLV-positive bats (4 of 15, 0.2-1.5 IU/mL). Surprisingly, only one of 14 inoculated mice that remained clinically well until killed on PI-day 162 had a positive rabies-RFFIT titre. The titre in this mouse (2.5-2.8 IU/mL) equalled the highest seen in any ABLV-positive mouse or bat suggesting that either:

- the mouse had been infected but induction of early and adequate humoral and /or cell-mediated immunity had led to subclinical resolution of the infection,
- this mouse was an asymptomatic carrier,
- or that it was killed during an uncharacteristically long incubation period. If this were the case, the incubation period would have been more than 5 times that of the only other mouse in that group to be affected (mouse 8.1, died PI-day 28) and nearly 4 times longer than the longest incubation period observed (mouse 3.2, died PI-day 42).

The absence of a rabies-RFFIT titre in the vast majority of surviving mice suggests either they had simply not been infected or that subclinical resolution had occurred either without development of a detectable antibody response or that an early antibody response had dropped below the detectable threshold by PI-day 162. As in bats, this study indicates that neither positive nor negative rabies-RFFIT titres in either clinical ill or normal mice can be used to determine whether a mouse is infected or poses a risk of infection to others.

Titration of Inoculum 5 by intracerebral inoculation of mice to determine the 50% effective dose ($MICED_{50}$) was done so that direct comparisons could be made between the virulence of this inoculum and those used in rabies virus studies. Australian codes of practice for the ethical use of experimental animals preclude death as an endpoint. The $MICED_{50}$ assay is the same as that for determining the traditional rabies virus LD_{50}, except that clinically ill mice were killed rather than left to die. Assuming all ABLV-positive clinically ill mice would have died, the $MICED_{50}$ value equals that that would have been determined in a traditional (lethal) LD_{50} assay.

The virus titre of Inoculum 5 ($10^{4.4}$ $MICED_{50}$ / 0.01 to 0.02 mL) was sufficiently high that doses of Inoculum 5 could be prepared that were comparable to those of street rabies virus used to successfully infect more that 30% of peripherally inoculated animals of a relevant host species (for example $10^{2.2}$ to $10^{5.2}$ $MICLD_{50}$ of arctic fox rabies virus in skunks and foxes (Charlton *et al.* 1987b), $10^{1.7 \text{ to } 5.8}$ $MICED_{50}$ of Ethiopian or Mexican origin dog rabies virus in beagles (Fekadu *et al.* 1982a), $10^{2.3}$ and $10^{3.6}$ $MICED_{50}$ of bat rabies virus in Mexican free-tail bats (Baer and Bales

1967; Constantine 1966b)). Consequently Inoculum 5 was considered suitable for further use in bats.

Titration of Inoculum 5 by footpad inoculation was done to determine the mean effective dose in a model that more closely represents natural, peripheral infection ($MFP_{90}ED_{50}$). Just as the incubation periods for Inoculum 5 after intracerebral and footpad suggested the virulence of Inoculum 5 was very similar in both methods, the titre of $10^{6.1 \text{ to } 6.4}$ $MICED_{50}$ / mL is essentially equal to the titre of $10^{6.0}$ $MFP_{90}ED_{50}$/mL, again suggesting this inoculum was as virulent peripherally as by intracerebral inoculation.

The footpad titration assay also demonstrated the effect of dose on incubation period. Mice in groups inoculated with lower doses of Inoculum 5 became ill after longer and more variable periods. At dilutions of 10^{-1} and below (neat) the onset of clinical signs in all mice occurred 8-9 days post-inoculation. At higher dilutions the incubation period increased to 12 to 50 days with the average incubation period increasing with each 10-fold dilution

A secondary objective was to examine the potential use of the *in vitro* TaqMan® assay as a preliminary screening tool to identify inocula suitable for further examination, increasing the numbers of inocula that could be considered and reducing the numbers of animals required for *in vivo* evaluations. The TaqMan® assay detects the relative amounts of viral RNA in a sample, with samples containing higher quantities of target RNA requiring fewer cycles to reach the detection threshold, i.e. having lower C_T values. However, the target RNA can have been in the form of RNA fragments, unincorporated genomic material, within non-viable (non-infective) or non-pathogenic (but infective) viral particles, as well as in the viable virulent virus particles that produce disease. As the proportions of RNA in each of these forms can be affected by post-mortem degradation and the storage history (e.g. variable freeze/thaw durations), RNA assays do not intrinsically indicate the relative viability or virulence of the sample.

There was a crude correlation between lower C_T values and those inocula that killed more intracerebral- or footpad-inoculated mice. In intracerebrally inoculated mice, only inocula with C_T values less than 22 cycles killed 100% of mice, while only 50% or less per litter were killed using inocula with C_T values greater than 25. In footpad-inoculated mice there was no simple correlation between the C_T values and virulence ranking. The three salivary gland inocula, which had relatively high C_T values (18.6, 20 and 21.2), did comparatively well, being three of the five samples that killed at least one mouse. Three brain inocula with lower C_T values (17, 18, and 19) failed to produce disease. The results suggested that the inocula derived from salivary gland material were more virulent than the inocula prepared from brains containing similar or higher quantities of viral RNA. To what extent this could be a property of the distribution of virulent virus in flying foxes, or simply an artefact of variable post-mortem degradation and storage conditions for the source materials remained unclear.

There was however, a clear relationship between the C_T value and number and speed with which mice were affected in the footpad titration assay. Each 10-fold dilution of Inoculum 5 correlated to an approximately 3 cycle increase in the C_T value. This is consistent with the theory of PCR amplification where there is a doubling of RNA in each PCR cycle. Theoretically a 10 fold increase in amplified product occurs approximately every 3.32 cycles. This suggests that, where the variable effects of post-mortem degradation and storage conditions are constant, as in this dilution series of a single inoculum, there is a very strong correlation between C_T values and virulence. A similar strong linear correlation between virus titre ($MICLD_{50}$) and TaqMan C_T value was shown for homogenates of salivary glands (n=5) from experimentally infected skunks (Hughes *et al.* 2004). However, it should be noted that this strong linear relationship was demonstrated on homogenates from a single tissue source (salivary gland) using samples that were presumably collected, stored, and used under controlled and optimised conditions.

5.6 Conclusions

In vivo evaluation of the virulence of inocula prepared from the tissues of naturally infected bats

- Inocula prepared from the brains or submandibular and sublingual salivary glands appear to have similar pathogenicity in mice, while preparations derived from the parotid salivary gland appear to be less virulent. However given the limited number of samples examined, the variation in the tissue handling and storage histories, and the fact that brain and salivary gland preparations from the same animal were not always used, this relationship requires further evaluation.
- Footpad inoculation trials are more sensitive for differentiation of the virulence of inocula than intracerebral inoculation, and require the use of fewer mice to differentiate inocula of interest.
- Prolonged (up to 16 days) post-mortem storage of tissue at 5°C does not preclude the presence of high concentrations of virulent virus in tissues.

Selection and characterisation of the most virulent inoculum for subsequent use

- A combination of intracerebral and footpad inoculation assays in mice identified and characterised the most virulent inoculum (Inoculum 5).
- The titre of Inoculum 5 by intracerebral inoculation of mice ($10^{6.1}$ to $10^{6.4}$ $MICED_{50}$ /mL) was similar to that of rabies virus inocula used to experimentally infect other host species. Consequently Inoculum 5 was considered suitable for use in flying foxes.
- The titre of Inoculum 5 by intracerebral inoculation of mice ($10^{6.1}$ to $10^{6.4}$ $MICED_{50}$ /mL) and by footpad inoculation of mice ($10^{6.0}$ $MFP_{90}ED_{50}$ /mL) were similar, further suggesting Inoculum 5 would be virulent by peripheral inoculation in bats.
- The virulence of the selected inoculum was consistent (number of mice affected and onset of weight loss/paresis) using four aliquots of the neat preparation.

Use of TaqMan® C_T values to determine relative quantities of viral RNA and the potential use of quantitative PCR in the selection of inocula

- There is an association between TaqMan® C_T values and virulence in that inocula with lower C_T values (< 22 cycles) are more virulent than inocula with higher C_T values *when* the variable effects of post-mortem degradation and storage conditions are controlled.
- When inocula are to be compared and factors affecting post-mortem degradation such as storage conditions are unknown or variable, there is a crude association between inocula with low C_T values (< 22 cycles) being more likely to be virulent and inocula with high C_T, values (> 25 cycles) being more likely to be less virulent. This crude relationship could be used for preliminary assessment of large numbers of inocula to identify or prioritize those most suitable for further *in vivo* assessment, leading to a reduction in the number of animals required.

Other conclusions

- As in bats, rabies-RFFIT titres in mice are of no diagnostic value.

6 Experimental infection of Grey-headed flying foxes with ABLV

Aims

1. To develop an experimental model for infection with Australian bat lyssavirus in Australian flying foxes.

2. To characterize the incubation period, clinical disease, histopathology, and distribution of viral antigen as a result of experimental infection.

4. To examine plasma from inoculated bats for evidence of seroconversion.

5. To examine saliva from inoculated bats for evidence of viral excretion.

6.1 Introduction

Characterization of the clinical disease caused by Australian bat lyssavirus in naturally infected Australian bats is limited because;

- the time and route of infection of naturally infected bats are unknown
- naturally infected bats only come to the attention of humans when either they are sufficiently unwell to be found on or near the ground or once their behaviour is grossly altered, and
- Ill bats are generally cared for by lay wildlife carers, with the involvement of veterinary or medical professionals occurring either very late in the progression of the disease or after death.

Consequently the incubation period is unknown, the early stages of the clinical disease are not observed, and most observations are made by persons not trained in clinical evaluation.

In order to characterize the incubation period and the onset and progression of clinical disease, an experimental model of ABLV infection is required. Classically, experimental models of lyssavirus (namely rabies virus) infection involved mice and rats as the experimental hosts. The comparatively recent recognition of relationships between virus variants and their principal host species implies that the pathogenesis and natural history of lyssaviruses is variant-host specific, and that the most appropriate experimental model would involve Australian bat lyssavirus in the principal host, namely pt-ABLV in flying foxes and/or ybst-ABLV in Yellow-bellied sheathtail bats. A model of pteropid-ABLV infection is more pressing as the social, zoonotic, and financial impact of ABLV in flying foxes is considerably greater than that of ABLV in Yellow-bellied sheathtail bats.

Two trials of experimental inoculation of ABLV in Grey headed flying foxes had been already been carried out at the AAHL. In the first, only one of seven flying foxes inoculated intramuscularly with ABLV developed clinical disease on PI-day 27 and in the second, only three of 10 flying foxes were clinically affected on PI-days 15, 23 , and 24 (McColl 1999; McColl *et al.* 2002). The small number of affected animals limits the data from these trials, and further trials were indicated.

It is also possible that research will be directed towards the development of an ABLV vaccine for the wild bat population, much as rabies vaccines have been developed for use in wildlife in Europe and North America (Barrat and Aubert 1993; Charlton *et al.* 1992; Rupprecht *et al.* 1986; Setien *et al.* 1998). Evaluation of a vaccine for use in bats will require an effective experimental model of ABLV infection in bats to demonstrate vaccine efficacy.

6.2 Materials and methods

6.2.1 Permits

Queensland National Parks and Wildlife service
Scientific purposes permit number – E4/001046/99/SAA
Animal Ethics Committees
University of Queensland – approval VET/FAC/111/98/PHD, VPA/411/99/PHD
Animal Research Institute – approval number ARI/032/RES/OCT1998-1
Institutional Biosafety Committee
Animal Research Institute – approval number DPI/IBC/PC3/99/00

6.2.2 Flying foxes

Wild Grey-headed flying foxes (*Pteropus poliocephalus*) were caught with a mist net at two flying fox colonies in urban Brisbane, namely at Indooroopilly Island, Indooroopilly and Norman Creek, East Brisbane. Grey-headed flying foxes were chosen because anecdotal evidence suggested that this species adapted best to captivity. Bats were identified with subcutaneous microchips, their weight, forearm lengths, and general condition noted, and an estimate of age made based on tooth wear, evidence of sexual maturity, forearm length, weight and pectoral, and temporal muscle mass. Plasma was submitted to the AAHL to be tested for naturally occurring anti-lyssavirus antibodies in the modified rabies-RFFIT (see Appendix 2). Captured bats were held as a single colony for an observation period of 19 to 27 days at the University Farm, Pinjarra Hills.

Ten pre-breeding or young adult, seronegative bats in good nutritional condition (four females and six males) were selected for experimental infection and transferred to individual cages within the PC3 Animal House at the ARI, 3 days prior to inoculation. All surplus seronegative bats were released at their site of capture, one seropositive bat was killed.

6.2.3 Cage design and layout

Bats were housed in separate cages so that the incubation period for clinically ill bats could be calculated from the day of inoculation and to prevent infection occurring as a result of bites, scratches or direct contact with infected companions. As inoculated bats posed a risk to health and safety, cages were designed so that bats could be isolated within the cage using a removable sliding panel during routine management procedures such as changing food and waste removal (see Figure 6-1 and Figure 6-2). The sliding panel was made of steel sheeting and could show a reflection. Water and fruit juice were supplied via commercial rabbit drip watering bottles hung on the outside of the cages.

As far as possible, cages were designed to minimize the impact on normal bat behaviour and reduce stress. Factors considered important for their welfare included:

- Being able to extend both wings to groom and flap. Wing dimensions were estimated as approximately 80 cm across.
- Being as high as possible from the ground. Bats usually roost and feed >20 m from the ground. It was assumed that being close to the ground and in particular being lower than a perceived predator would be stressful.
- The cage being tall enough that, when hanging, the bats head was well clear of the bottom of the cage, but not so tall that there was risk of significant injury if an ill bat should fall.
- Being in close proximity to the other bats. Bats usually roost in large colonies, often within <1 m of each other.

Cage design was also limited by the necessity of ensuring that the entire area of the cage was accessible by the candidate while standing on the floor, limiting cage height to 1690 mm. These considerations were met by constructing 900 mm high, 900 mm wide and 600 mm deep cages mounted on stands. The dimensions and features of the cages are shown in Figure 6-1 and Figure 6-2. The cages could be partitioned into two sections by a dividing board, isolating the bat in one or other side, permitting safe access to the other side for cage management. When bats were isolated within the smaller section, the narrow confines prevented bats from opening their wings defensively/aggressively and allowed bats to be given intramuscular anaesthesia. Cages were constructed by the farm staff of the ARI.

Cages containing inoculated bats were arranged in two rows of five cages, such that there was a 10 cm gap between cages, sufficient to prevent direct contact between bats. The arrangement of the cages within the PC3 Animal house is illustrated in Figure 6-3.

Figure 6-1 Cages used to house experimentally inoculated flying foxes
Two cages on stands approximately 10 cm apart. The cage on the left has the dividing board inserted, partitioning the cage as if to isolate a bat in one or other side.

Figure 6-2 Dimensions and features of cages used to house experimentally inoculated flying foxes

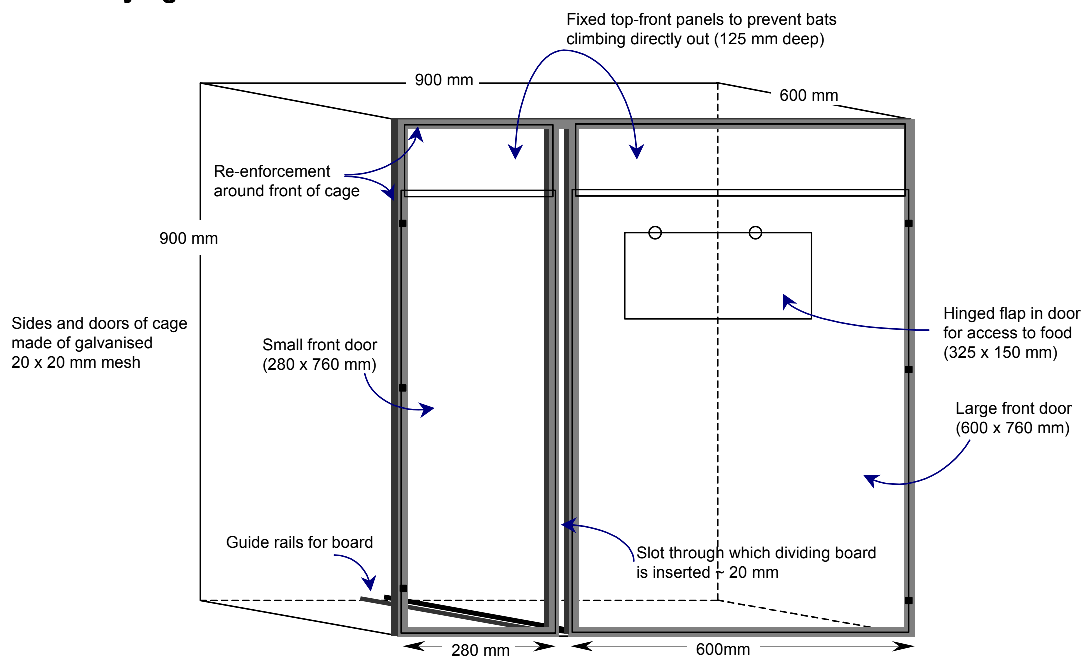
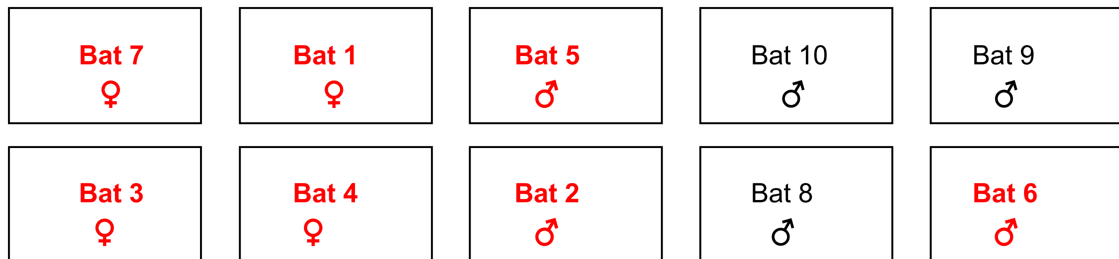


Figure 6-3 Floor plan of cages within the PC3 Animal House
The individuals occupying each cage are identified by their Bat number, which was allocated in the order in which clinical signs were observed. ABLV-positive bats are indicated in **red** font. Cages are separated in both directions by approximately 10 cm.



6.2.4 Daily care and observations

Bats were fed a range of fresh fruit late each afternoon to encourage natural nocturnal feeding patterns, water was available *ad lib* and fruit juice provided intermittently. Their diet included mangos, bananas, papaw (papaya), apples, stone fruit (peaches, plums etc), rockmelon, grapes and other seasonal fruits as were available, the majority of which was kindly donated by the Fresh Foods section of Woolworths at Westfield Shopping Centre, Indooroopilly. Food was provided diced in a dish on the cage wall, and hung to swing freely within the cage. A range of at least three fruits was provided each evening *ad libitum*. The provision of food and removal of waste took approximately 1 to 1.5 hours during which time the condition and behaviour of the bats was observed in detail. Their condition also was monitored by intermittent observation during the day.

6.2.5 Inoculations

6.2.5.1 Mice controls

Two 3-week-old female Quackenbush mice were inoculated into the footpads with 60 μL of Inoculum 5 as described in Chapter 4, Experiment 2. One mouse was inoculated prior to inoculation of the 10 bats, the other after inoculation of the bats. The prepared doses of inoculum were kept at 4°C during the period that the inoculations were taking place.

6.2.5.2 Bat inoculations

Bats were anaesthetized with an intravenous injection of 2.0 to 2.5 mL of a 1:5 dilution of Propofol (10 mg/mL, Rapinovet Schering-Plough Animal Health) in sterile water via the uropatagial vein. *Nine* bats were inoculated with 0.12 mL of neat Inoculum 5, a 20% weight per volume suspension of the submandibular and sublingual salivary gland of a naturally infected Black flying fox (ABLV-51 for details of inoculum preparation, selection and characterization see Chapter 5.).

The 0.12 mL volume, containing $10^{5.2}$ to $10^{5.5}$ $MICED_{50}$ = $10^{5.1}$ $MFP_{90}ED_{50}$, was divided into four inoculation sites (approximately 0.03 mL per site) administered via a single tuberculin syringe and 27 gauge needle. These sites, illustrated in Figure 6-4, were:

- Left 'footpad' (thick dermis over the plantar surface of the metatarsals)
- Left pectoral muscle
- Left temporal muscle
- Left muzzle, under the whiskers

A further bat (Bat 7) was inoculated with only 0.06 mL of Inoculum 5 containing $10^{4.9}$ to $10^{5.2}$ $MICED_{50}$ = $10^{4.8}$ $MFP_{90}ED_{50}$, divided into only two inoculation sites, the left footpad and pectoral muscle.

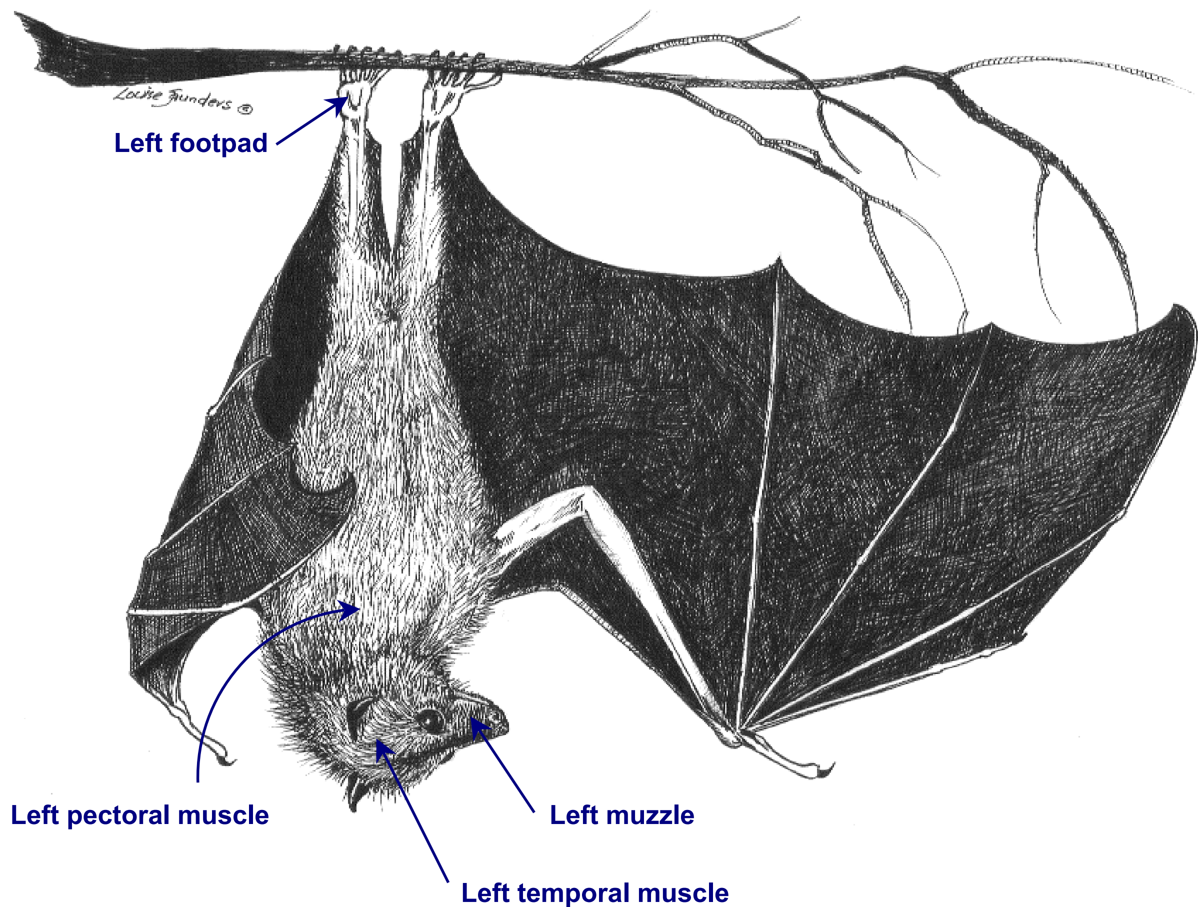
6.2.6 Sample collection

Bats were anaesthetized with an intramuscular injection of 0.1-0.2 mL of a 2:1 mix of Ketamine (100 mg/kg) : Xylazine (20mg/kg), usually in the pectoral muscles, on PI-days 7, 14[1], 21, 28, 35, 42, 49, 56, 63, and PI-day 70. This produced anaesthesia within 1-2 minutes lasting 10-20 minutes during which time the following were recorded/collected.

- general condition
- 1.5 to 2.5 mL plasma samples taken via the uropatagial vein using a heparinized 3 mL syringe and 25 gauge needle.
- a swab of the oral cavity taken using a sterile cotton swab which was then stirred vigorously in SPGA viral transport medium. The virus transport medium sample was stored at -70°C pending submission for quantitative PCR assay.

[1] On PI-day 14, two bats (Bat 3 and Bat 4) were given Reversine (2mg/mL 4-aminopyridine and 1.25 mg/mL yohimbine hydrochloride, Parnell Laboratories) in an attempt to speed recovery. However Bats 3 and 4 had seizures 1 hour 15 minutes and 2 hours 30 minutes later and so the use of Reversine was discontinued.

Figure 6-4 Sites of inoculation with ABLV of Grey-headed flying foxes



6.2.7 Euthanasia and post-mortem sample collection

Animal welfare requirements precluded death of infected bats as an endpoint. When clinically ill, or at the termination of the experiment, bats were killed by intraperitoneal injection of 0.3 mL Lethobarb® (Virbac) diluted to 2.5 to 5 mL in water. This produced a period of anaesthesia (~3 to 10 minutes), during which 10 to 20 mL of whole blood was collected by cardiac puncture using a heparinized 10 mL syringe and 21 gauge needle, followed by death by barbiturate overdose. Weight, general condition and, where appropriate, an oral swab were recorded/collected as described above.

Post-mortem examination was performed in a Class II biosafety cabinet within the PC3 Virology Laboratory, ARI. A range of fresh tissue samples including brain, the parotid salivary glands and the submandibular / sublingual salivary glands were collected using separate sterile instruments. A range of tissues including the brain, trigeminal and coeliaco-mesenteric ganglia, spinal column, salivary glands, adrenal glands, eyes, and skin were collected in 10% buffered neutral formalin.

6.2.8 Confirmation of lyssavirus infection

Infection with Australian bat lyssavirus was confirmed by FAT on fresh brain touch impressions, as described in Section 2.2.3 Fluorescent antibody test. The tests were performed and read by the candidate and the results confirmed by Barry Rodwell of the Diagnostic Virology Laboratory of the ARI.

The presence of ABLV was also confirmed by mouse inoculation and TaqMan® PCR. Details of methods used and results are provided in Chapter 7.

6.2.9 TaqMan® assay for ABLV-RNA in saliva

Virus transport medium containing saliva, cells and other material released from swabs of the oral cavity were submitted to QHSS for the detection of ABLV-RNA in the pteropid-ABLV-TaqMan® assay (see Appendix 3).

6.2.10 Histology and Immunohistochemistry

Brains for histological examination were sectioned in three standard planes.

- Transversely through the frontal lobe.
- Transversely through the cerebral cortex and thalamus at the level of the hippocampus
- Longitudinal section of the brainstem and cerebellum, which included the caudal colliculus, pons, medulla, and cranial end of the spinal cord.

For all other tissues, representative blocks were selected. Tissues were routinely processed, paraffin-embedded and 5 µm sections cut and routinely stained with haematoxylin and eosin by the histology staff of the ARI and examined by light microscopy.

Sections for immunohistological staining were cut at The University of Queensland veterinary histology laboratory to 5 µm onto sialinized slides and dried in a 37°C oven overnight, then dehydrated and cleared. Endogenous peroxidase activity was quenched in 3% H_2O_2 in water for 20 minutes, prior to loading the slides into the Cadenza® (programmable automatic immunostainer). Staining was done with a 1:100 dilution of the HAM monoclonal anti-rabies nucleoprotein antibody (Feiden *et al.* 1988) in Tris buffer containing 8.5 g/l NaCL and 0.1% Triton X-100 for 30 minutes, followed by a Tris-NaCL-Triton X-100 buffer rinse, 30 minute incubation with link (secondary) antibody (DAKO LSAB +/HRP detection system, DAKO, Australia), buffer rinse, 30 minute incubation with streptavidin peroxidase complex (DAKO +), buffer rinse, a 30 minute incubation with AEC chromogen reagent (Immumon, Edward Keller, Australia) followed by two final buffer rinses. Stained slides were then removed from the Cadenza and counterstained with Mayer's haematoxylin for 1-2 minutes, blued in running tap water, dried, and mounted with aqueous mounting media (Faramount, DAKO, Australia) and a clover-slip. Stained sections were examined by light microscopy, with positive immunostaining appearing red (AEC). Duplicate sections stained with a 1:50 dilution of monoclonal mouse IgG1 (anti-*Aspergillus niger* glucose oxidase, DAKO, Australia) were stained and examined as negative antibody controls. Included in each run were sections of rabies-positive brain stained with the HAM and negative control antibodies as positive tissue controls. All immunohistological staining was done by the candidate.

6.2.11 Serology

Plasma was taken by the candidate to the AAHL for the detection of rabies and ABLV antibodies using the modified rabies CVS-RFFIT and a partially developed ABLV-RFFITs using pteropid- and ybst-ABLV (see Appendix 2). The tests were performed by Ross Lunt and Kim Newberry.

Duplicate samples were submitted to the Rabies Laboratory of the Centers for Disease Control and Prevention, Atlanta, USA for confirmation by rabies CVS-RFFIT and an independently developed pteropid-ABLV-RFFIT, see also Appendix 2, (Arguin *et al.* 2002).

6.3 Results

Seven of the 10 inoculated bats developed clinical disease and either died or required euthanasia, including 6 of 9 bats (Bats 1-6) which received 0.12 mL of inoculum divided into four inoculation sites and 1 of 1 bat (Bat 7) that received only 0.06 mL of inoculum divided into the footpad and pectoral muscle only. Infection with Australian bat lyssavirus was confirmed in each case by FAT on fresh brain touch impressions, positive detection of lyssavirus antigen in formalin fixed tissues, virus isolation in mice and detection of pt-ABLV RNA by TaqMan® assay (see also Chapter 7). Three bats, Bats 8, 9, and 10, remained clinically well until killed on either PI-day 80 (Bat 8) or PI-day 82 (Bats 9 and 10). Bats 8, 9, and 10 were negative for ABLV by FAT, immunohistochemical staining, and mouse inoculation.

Both inoculated control mice showed weight loss and a reluctance to move on PI-day 9. Both were recumbent on PI-day 10 at which time they were killed and confirmed ABLV-positive by FAT.

6.3.1 Normal behaviour

6.3.1.1 Pre-clinical period: 3 days pre-inoculation to PI-day 19

Normal behaviour for these bats was to hang quietly from the cage roof in a back corner, typically near an adjacent bat, with wings folded. They would watch attentively as people moved about the room, but would not move themselves unless approached. When the front of the cage was approached bats would move to the furthermost corner, and if the cage were approached closely, for example to remove waste, the bat would attempt to retreat further by crouching up and turning away. They moved across the roof of the cage, not the sidewalls. Bats would rarely approach fresh food when people were in the room or even when observed through glass windows. Bats would evert (hang by the thumbs rather than the feet) to pass faeces and urinate, but rarely while the candidate was in the room. Their coats were normally clean and dry.

6.3.1.2 Long term survivors: 3 days pre-inoculation to PI-day 82

The normal behaviour observed in the first three weeks persisted in the three bats that survived (Bats 8, 9, and 10) until PI-days 80 and 82, except that they appeared to adapt to the presence of the candidate, no-longer crouching up or turning away, and would occasionally groom or start to eat fresh food as the candidate moved around the room. However at no point did they come forward or vocalize at the candidate, rather they would simply hang in the corners that maximized their proximity to each other and distance from the candidate, and watch.

6.3.2 Clinical disease

Seven experimentally inoculated Grey-headed flying foxes (Bats 1-7) developed clinical signs due to ABLV infection. The onset of disease occurred 10 to 19 days post-inoculation, with clinical disease lasting 1 to 4 days. The clinical signs for each bat are described below and are summarized in Table 6-1.

Bat 1 Pre-breeding female, 0.12 mL Inoculum 5

P1-day 10 Vocalizing and scrambling around the cage, appeared to be vocalizing at other bats and the candidate as food was being allocated. Eating well.

PI-day 11-13 Increasing degrees of agitation, loud vocalizing generally and apparently directed at other bats, scrambling up and down the cage wall as if trying to reach food and bats in neighbouring cages yet ignoring fresh food within the cage while the candidate was present. Ate well each evening.

PI-day 14 Had eaten overnight, found hanging over food bowl looking gaunt and depressed with rapid respiration with forced expiration. The coat was unkempt, with food and faeces on the fur suggesting failure to groom/ evert to toilet. Both cheeks were stuffed with food, suggesting dysphagia. On returning four hours later, Bat 1 had died, remained hanging.

Bat 2 Young sexually mature adult male, 0.12 mL Inoculum 5

PI-day 13 Vocalizing, in particular vocalizing at Bat 1 in diagonally opposite cage who was also ill and vocalizing. Moved towards people as they approached the front of the cage rather than moving away and lunging forward rather than retreating as waste was removed, licking penis. Eating well.

PI-day 14 Continuing to vocalize and constantly moving. Repeatedly approaching the front of the cage when approached and climbing up and down the cage front with some clumsiness and knuckling of the feet suggesting ataxia and/or proprioceptive deficits. Appeared to attack his reflection in the cage divider, attempting to bite divider while vocalizing and opening the wings aggressively, attacked inanimate objects such as probes placed in the cage. Licking penis. Unremarkable ketamine/xylazine anaesthesia for data/sample collection, not offered food overnight.

PI-day 15 Appeared calmer but shaky, tongue protruding, suggesting cranial nerve deficits. Ate banana and mango provided in the morning while candidate present. Later voice croaky, mouth contained unswallowed food, coat unkempt with food over face and body and faeces around anus, depressed, killed.

Bat 3 Pre-breeding female, 0.12 mL Inoculum 5

PI-day 12-14 Appeared normal, but eating noticeably less than during preceding week.

PI-day14 16:43 Anaesthetized with ketamine/xylazine for routine data / sample collection.
17:15 given Reversine (2mg/mL 4-aminopyridine and 1.25 mg/mL yohimbine hydrochloride, Parnell Laboratories) to assist recovery.
17:21 Hanging from roof of cage, during next hour moving around cage, apparently normal anaesthetic recovery.
18:30 Dropped to floor of cage, convulsing, copious froth at mouth.
18:35 Dead.

Bat 4 Pre-breeding female, 0.12 mL Inoculum 5

PI-day 12-14 Appeared normal, eating particularly well.

PI-day 14 17:15 Anaesthetized with ketamine/xylazine for routine data / sample collection.
17:35 given Reversine to assist recovery.
17:45 Hanging from roof of cage.
20:05 Convulsing while hanging, copious froth from mouth, remained hanging, seizure lasted approximately 3 minutes.
20:10 Appeared recovered from seizure.
20:45 Continuing to hang from cage roof, not offered food overnight.

PI-day 15 Initially appeared normal, but in the morning ate pear provided while candidate present. Afternoon, scratching body and licking body and wings, responded aggressively towards cage divider/reflection

PI-day 16 Appeared shaky, slight tremors, attacking divider/reflection and newspaper, licking genitals, everted to urinate. Later, observed from adjacent room via window to be hanging half way down cage, and moving around cage walls and heading to the cage floor, appeared ataxic in the hind limbs. On the candidate entering the room, Bat 4 scrambled to cage roof and hung, came to the front of the cage when approached, repeatedly attacked divider/reflection, later moving and hanging low on cage wall again, appeared to be looking for something yet ignoring fresh food in cage.

PI-day 17 Vocalizing, active, coming to front of cage when approached and climbing down cage walls, some faeces on coat, licking genitals, grabbing at food repeatedly but not eating. Later in the day, appeared weaker, less vigorous efforts to attack cage divider/reflection, still vocal and coming to front of cage.

PI-day 18 Found dead, still hanging, food had been knocked / dropped to the floor.

Bat 5 Young sexually mature adult male, 0.12 mL Inoculum 5

PI-day 14 Appeared normal, unremarkable ketamine/xylazine (no Reversine) anaesthesia for sampling, but appeared to be gesticulating at Bat 2 after recovery, not offered food overnight.

PI-day 15 Vocalizing and gesticulating at Bat 2 in opposite cage who was also ill, licking genitals, attacking divider/reflection. Ate mango provided in the morning while candidate present. When at rest, tongue protruding slightly suggesting cranial nerve deficits.

PI-day 16-17 Incessant licking of genitals producing hair loss and excoriation of scrotum, repeatedly attacking divider/reflection, coming forwards when approached and vocalizing and gesticulating aggressively towards candidate.

PI-day 18 Found dead, still hanging, had eaten a large amount of food overnight, mouth packed with food.

Bat 6 Mature adult male, 0.12 mL Inoculum 5

PI-day 14 Appeared normal, uneventful ketamine/xylazine anaesthetic for data /sample collection.

PI-day 15 Stretching wings out, aggressive towards divider/reflection and Bat 8 in adjacent cage. Ate nectarine provided in the morning while candidate present.

PI-day 16 Continuing aggression towards reflection, uncharacteristic hypermetric/violent ‘grabbing’ movement to grasp grapes hanging from the cage roof, biting forcefully at mango.

PI-day 17 Less active, appears shaky, slight tremors, vocalizing and coming to the front of cage aggressively when approached. Tongue protruding at rest, but withdrawn when approached, and both cheeks distended with food, suggesting cranial nerve deficits and mild dysphagia. Coat unkempt with fur wet, presumably by urine and some faeces around anus. There appeared to be paresis and proprioceptive deficits with hypermetric grasping with the right hind foot and right wing with repeated attempts to grasp at each point. Mild excoriation of the penis consistent with incessant licking.

PI-day 18 Very depressed, cheeks distended with food, tongue protruding, less inclined to move to front of cage when approached, very weak attempt to attack divider/reflection, able to evert to urinate, killed.

Bat 7 Pre-breeding female, only 0.06 mL Inoculum 5

PI-day 15-18 Most timid bat of the group, slightly more so during days 15-17, however, this may have been in response to the vocalization and aggressive behaviour of other clinically ill bats. PI-Day 16 noticed to hang with head slightly more extended than other bats, PI-day 18 appeared normal.

PI-day 19 Dramatic incessant movement and licking of genitals, tremors, ataxia, hypermetria and proprioceptive deficits all four limbs, unkempt coat, excoriation around anus due to licking. Had eaten overnight and while being observed, attempted to eat some peach, but not sufficiently coordinated to hold fruit, and dropped it. Not aggressive, not vocalizing. Killed later that day.

Table 6-1 Clinical observations of 10 Grey-headed flying foxes inoculated with pt-ABLV (Inoculum 5)

Name	Incubation period (days)	Clinical duration (days)	Outcome	Diagnosis	Vocalizing	Aggression	Licking	Paresis, ataxia proprioceptive deficits	Protruding tongue	Dysphagia	Seizures assoc. with Reversine
Bat 1	10	3.5-4	Died	ABLV	✓	✓	✗	✗	✗	✓	n/a
Bat 2	13	2-3	Killed	ABLV	✓	✓	✓	✓	✓	✓	n/a
Bat 3	14	<1	Died	ABLV	✗	✗	✗	✗	✗	✗	✓
Bat 4	14	3-3.5	Died	ABLV	✓	✓	✓	✓	✗	✗	✓
Bat 5	14	3-3.5	Died	ABLV	✓	✓	✓	✗	✓	✓	n/a
Bat 6	15	2-2.5	Killed	ABLV	✓	✓	✓	✓	✓	✓	n/a
Bat 7	19	1	Killed	ABLV	✗	✗	✓	✓	✗	✗	n/a
Bat 8	> 80	0	Survived	Negative	✗	✗	✗	✗	✗	✗	n/a
Bat 9	> 82	0	Survived	Negative	✗	✗	✗	✗	✗	✗	n/a
Bat 10	> 82	0	Survived	Negative	✗	✗	✗	✗	✗	✗	n/a

Outcome: Died – found dead in cage, Killed - required euthanasia due to clinical disease, Survived – remained clinically well until killed at end of experiment on PI-day 80 or 82

✓ observed

✗ not observed

n/a not applicable

6.3.3 Gross necropsy

The pectoral muscles of all bats were moderately wasted.

Of the seven ABLV-positive flying foxes, Bat 1 was in poor nutritional condition with very low peri-salivary gland and perirenal fat reserves, Bats 2, 4, 5, and 6 were in moderate nutritional condition with some fat reserves and Bats 3, and 7 were in good condition with ample fat reserves. The meninges of Bats 2 and 4 were slightly congested. The pharynx and trachea of Bat 3, which had died during a post-anaesthetic seizure, contained considerable froth suggesting this bat may have asphyxiated. The skin of the right scrotum of Bat 5 was ulcerated with dehydration and necrosis of exposed underlying fat, the penis of Bat 6 was excoriated, and the perianal skin of Bat 7 was excoriated and hyperaemic, consistent with observed incessant licking by these bats. No other gross abnormalities were detected.

Bats 8, 9, and 10, which had remained clinically normal until killed on days 80 or 82, were in fat to very fat condition with considerable peri-salivary, intra-abdominal and subcutaneous fat reserves. They had similar pectoral muscle wasting, but no other abnormalities were detected.

6.3.4 Histopathology and immunohistochemistry

6.3.4.1 Clinically ill ABLV-positive bats (n=7)

All seven affected flying foxes had very mild to moderate non-suppurative meningoencephalomyelitis, with focal gliosis, peri-vascular cuffs of lymphocytes, and meningeal infiltrates of mononuclear cells, predominantly lymphocytes, in the meninges. In some bats there were occasional cytoplasmic eosinophilic inclusion bodies in neurons of the brain stem (see Figure 6-5A to C). Inflammation of the central nervous system was most obvious in the brain stem. There was also a severe ganglioneuritis in all seven bats involving the dorsal root ganglia, trigeminal (Gasserian, 5th cranial nerve) and coeliaco-mesenteric ganglia with marked gliosis and neuronal necrosis (see Figure 6-5D and E).

Several bats had a very mild to moderate interstitial mononuclear, predominantly lymphocytic, sialoadenitis involving the parotid (Bats 3 and 7) and/or submandibular (Bats 1, 2, 3, 4, 6, and 7, not Bat 5) salivary glands (see Figure 6-5F).

Lyssavirus nucleocapsid antigen, as detected by the HAM monoclonal antibody, was found throughout the central and peripheral nervous systems and in retinal ganglion cells in all seven clinically affected flying foxes (see Figure 6-6 A to D). Antigen was detected in neuronal cell bodies and the neuropil of the:

- cerebral cortex
- brain stem
- cerebellum
- spinal cord
- sensory dorsal root ganglia
- trigeminal and coeliaco-mesenteric ganglia
- parasympathetic ganglia and nerve roots within multiple organs including the salivary glands, lung, heart, stomach, small and large intestine, and kidney
- retinal ganglion cells (of eye)
- and in large nerves such as the brachial and sciatic nerves.

Antigen was also detected in salivary gland epithelial cells of four affected flying foxes (Bats-2, 4, 5, and 6), in each case involving only rare individual or small groups of epithelial cells in the submandibular salivary gland (see Figure 6-6E). While antigen was obvious in the parasympathetic ganglia associated with all three salivary glands (see Figure 6-6C), no antigen was detected in the epithelium of the parotid or sublingual salivary glands.

Small foci of reactivity were detected in rare epithelial cells within the pancreas of two affected bats (Bats-4 and 5, see Figure 6-6F).

Antigen was not detected in the adrenal glands, testes or ovaries of any experimentally infected bats but had been detected in the adrenal glands of at least one naturally infected bat (data not shown).

6.3.4.2 Survivor ABLV-negative bats (n=3)

Neither histological lesions nor lyssavirus antigen was detected in sections of the central or peripheral nervous systems of the three surviving experimentally inoculated bats killed on PI-day 80 or 82. Focal mononuclear sialoadenitis that was indistinguishable from that seen in some clinically ill bats was detected in the parotid (Bats 8, 9, and 10) and submandibular (Bats 8 and 10) salivary glands. No lyssavirus antigen was detected in the salivary glands or other organs.

Figure 6-5 Histology associated with experimental infection of flying foxes with ABLV
Routine haematoxylin and eosin stained sections of formalin fixed, paraffin embedded tissue.

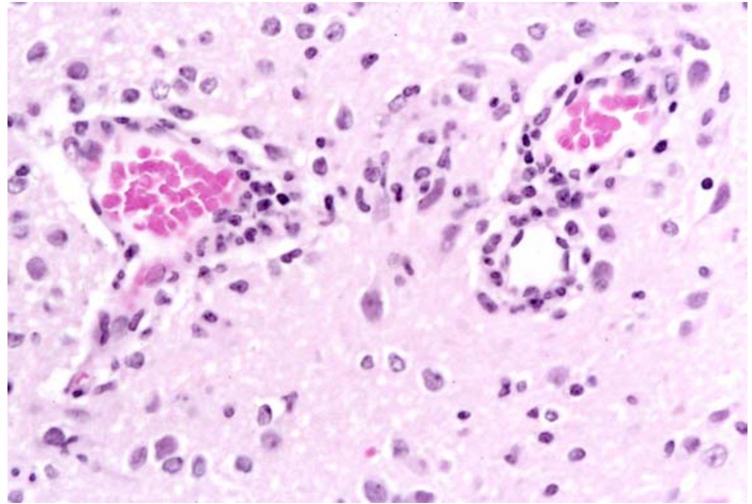


Figure 6-5A Encephalitis Bat 1
Brain grey matter with perivascular cuffs of mononuclear cells, predominantly lymphocytes.
370 x magnification.

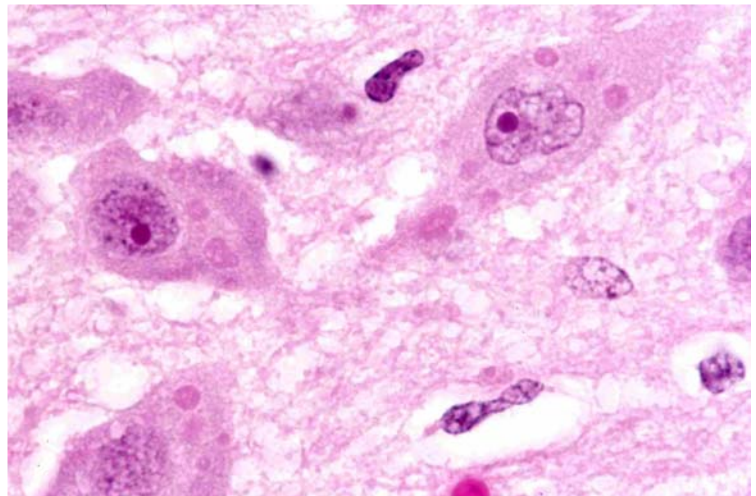


Figure 6-5B Eosinophilic inclusion bodies Bat 1
Small group of brain stem neurons containing multiple cytoplasmic eosinophilic inclusion bodies.
924 x magnification (oil emersion).

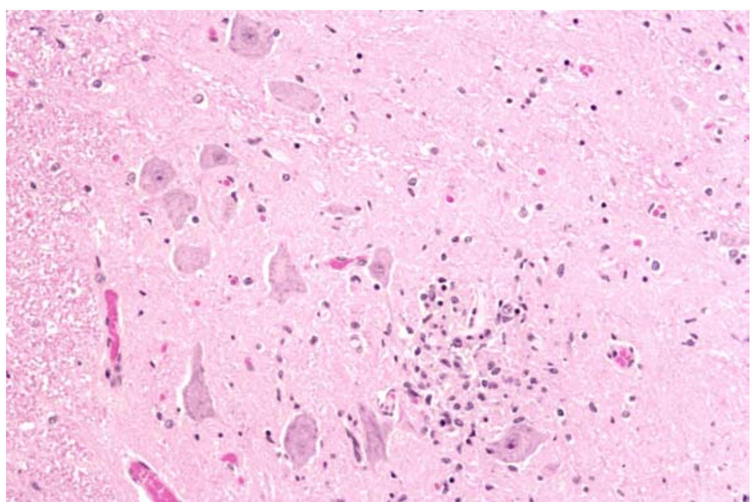


Figure 6-5C Myelitis Bat 2
Focal gliosis in spinal cord grey matter.
185 x magnification.

Figure 6-5 (continued) Histology associated with experimental infection with ABLV

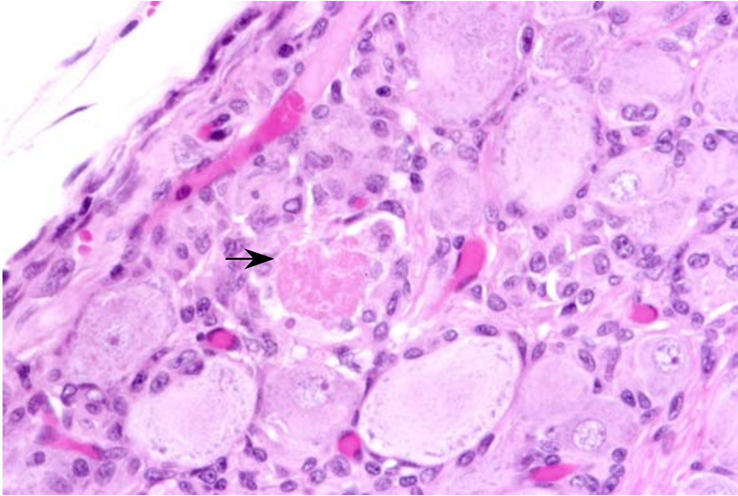


Figure 6-5D Ganglionitis Bat 2
Neuronal necrosis (➔) and gliosis in a lumbar dorsal root ganglion.
370 x magnification.

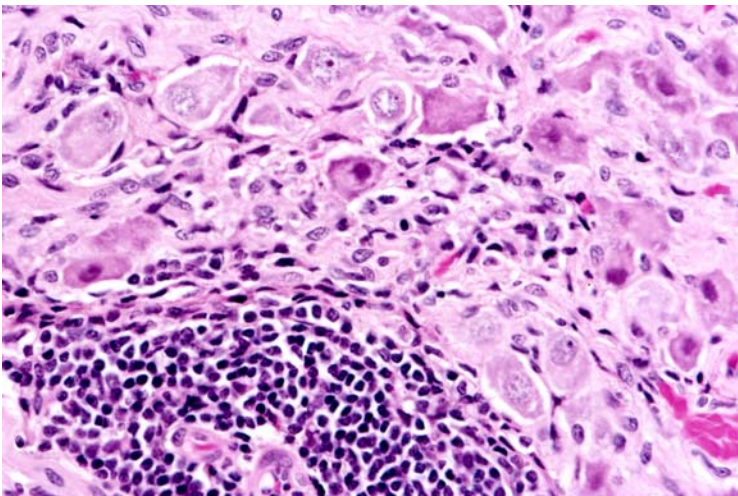


Figure 6-5E Ganglionitis Bat 3
Large perivascular cuff of mononuclear cells, predominantly lymphocytes, and neuronal necrosis in the parasympathetic coeliaco-mesenteric ganglion.
370 x magnification.

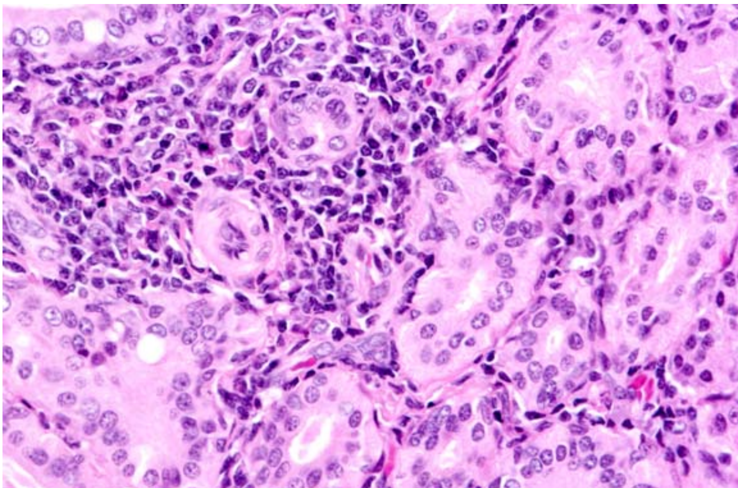


Figure 6-5F Sialoadenitis Bat 2
Focal mononuclear inflammatory infiltrate in the submandibular salivary gland.
370 x magnification.

Figure 6-6 Immunohistological detection of ABLV nucleocapsid antigen in experimentally infected flying foxes
Stained using HAM anti-rabies nucleocapsid monoclonal antibody with AEC chromogen (red) and counterstained with Mayer's haematoxylin (blue).

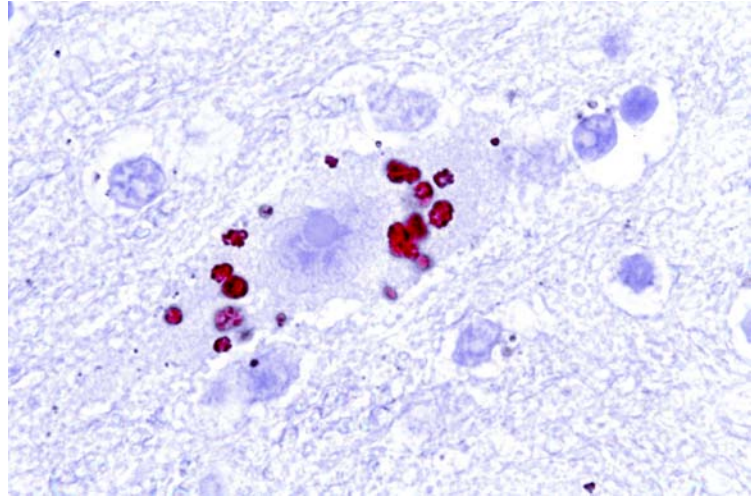


Figure 6-6A ABLV antigen in brain stem neuron Bat 1
924 x magnification (oil emersion).

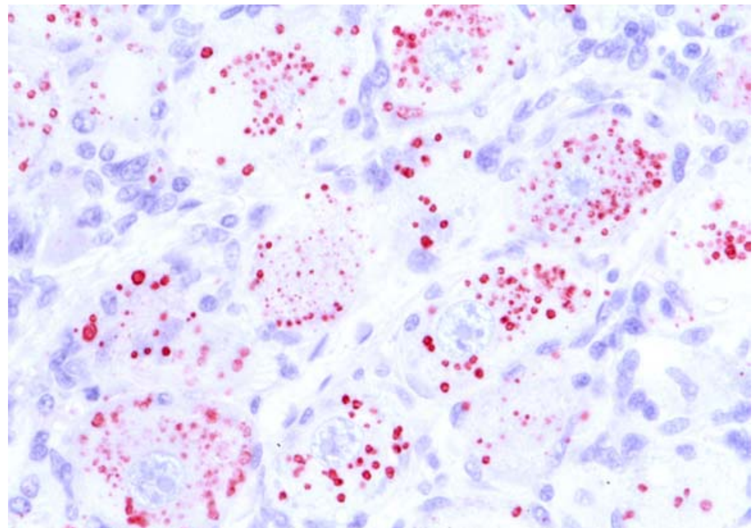


Figure 6-6B ABLV antigen in neurons of a lumbar dorsal root ganglion Bat 2
370 x magnification.

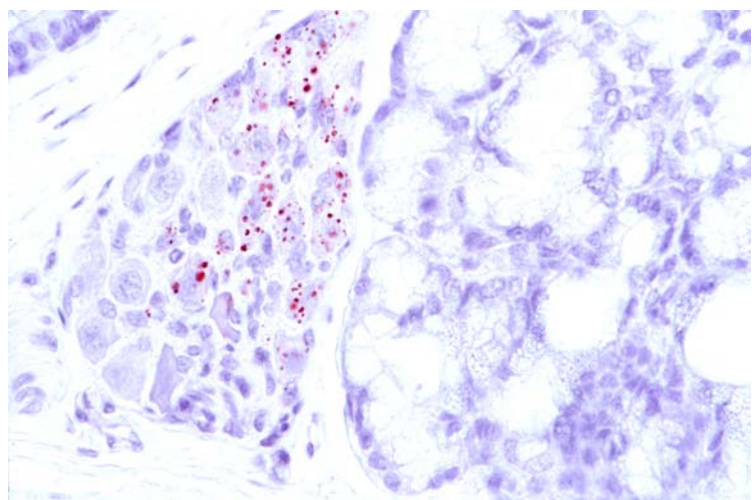


Figure 6-6C Salivary gland Bat 1
Positive (red) staining of ABLV antigen in neurons within a parasympathetic ganglion adjacent to negatively stained (blue) sublingual salivary gland acini.
370 x magnification.

Figure 6-6 (continued) Immunohistological detection of ABLV nucleocapsid antigen

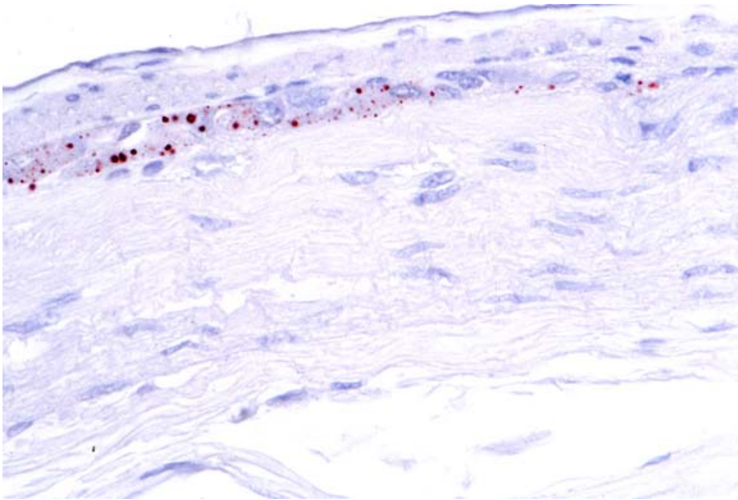


Figure 6-6D ABLV antigen (red) **in myenteric plexus of the stomach Bat 1**
370 x magnification.

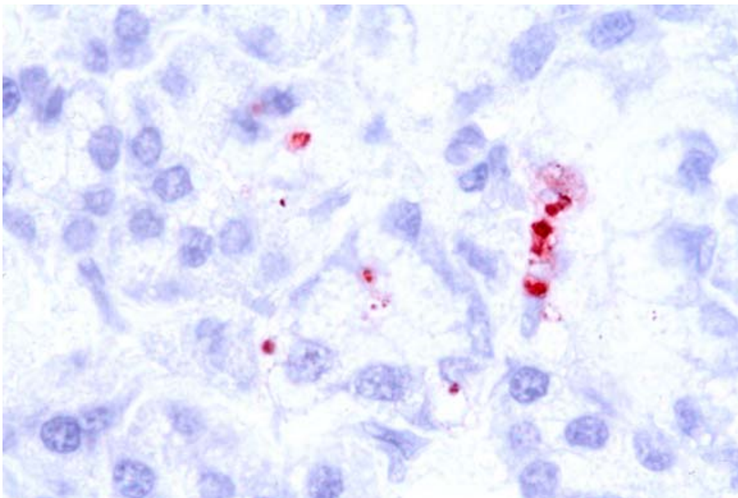


Figure 6-6E Salivary gland Bat 5
ABLV antigen (red) in epithelial cells of the submandibular salivary gland.
924 x magnification (oil emersion).

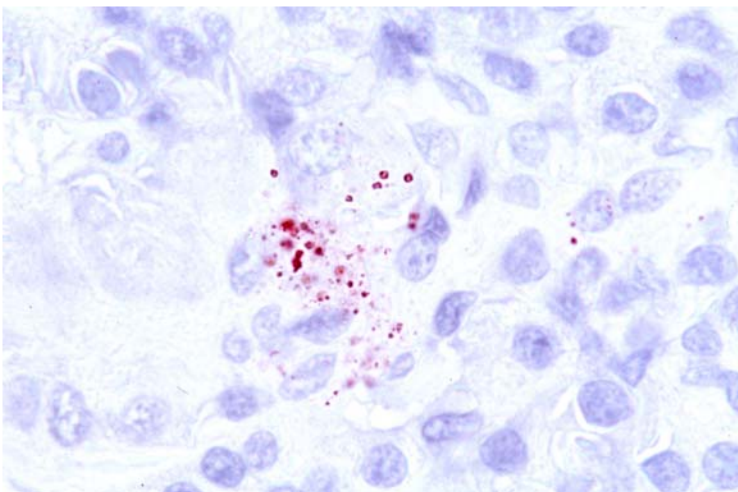


Figure 6-5F Pancreas Bat 5
ABLV antigen (red) in pancreatic epithelial cells.
924 x magnification (oil emersion).

6.3.5 Detection of pt-ABLV RNA in oral swabs

Using the pteropid-ABLV TaqMan® assay, ABLV RNA was detected in the saliva of four affected flying foxes on PI-day 14, and the saliva of a fifth bat on PI-day 19. The results are shown in Table 6-2.

Table 6-2 Detection by TaqMan® assay of pt-ABLV RNA in saliva samples collected from seven ABLV-positive experimentally infected flying foxes

	PI-day 7		PI-day 14		PI-day 19	
	C_T	Result	C_T	Result	C_T	Result
Bat-1	50.0	Neg	No sample	No result	Dead	No result
Bat-2	50.0	Neg	33.6	**Positive**	Dead	No result
Bat-3	50.0	Neg	35.7	**Positive**	Dead	No result
Bat-4	50.0	Neg	50.0	Neg	Dead	No result
Bat-5	45.7	Neg	33.1	**Positive**	Dead	No result
Bat-6	49.1	Neg	31.4	**Positive**	Dead	No result
Bat-7	50.0	Neg	50.0	Neg	35.02	**Positive**

Saliva samples from Bats-8, 9, and 10 (ABLV-negative survivors) collected on PV-days 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70, were all negative (C_T= 50 for all samples, except Bat 8 PV-day 42 C_t=42 and Bat-9 PV-day 49 C_T=38.9)

6.3.6 Serology

6.3.6.1 Clinically ill ABLV-positive bats (n=7)

None of the seven clinically ill ABLV-infected flying foxes showed clear evidence of seroconversion when tested at the AAHL and CDC, Atlanta, using either CVS-11 rabies or ABLV-RFFITs. Two plasma samples showed very weak / equivocal reactivity near the limit of detection of the AAHL modified CVS-11 and ABLV-RFFITs as indicated in Table 6-3.

Table 6-3 Rabies and ABLV serology of seven ABLV-positive experimentally infected bats
Positive results are shown in bold. --- not done

Bat	PI-day	AAHL modified RFFIT			CDC RFFIT (titre)		Interpretation
		CVS-11 (IU/mL)	pt-ABLV	ybst-ABLV	CVS-11	pt-ABLV	
Bat 1	-22 capture	0	---	---	---	<11	Negative
	-3	0	---	---	---	---	Negative
	7	Toxic	---	---	0	0	Negative
	14 died	**< 0.2**	**+**	0	0	0	**Equivocal**
Bat 2	-22 capture	0	---	---	0	<11	Negative
	-3	0	---	---	0	0	Negative
	7	0	---	---	0	< 9	Negative
	14	0	---	---	0	<13	Negative
	15 killed	0	---	---	---	<13	Negative
Bat 3	-23 capture	0	---	---	0	0	Negative
	-3	0	---	---	0	0	Negative
	7	0	---	---	0	<11	Negative
	14 died	**< 0.2**	**+**	0	0	0	**Equivocal**
Bat 4	-22 capture	0	---	---	0	0	Negative
	-3	0	---	---	---	0	Negative
	7	0	---	---	0	<11	Negative
	14	0	---	---	0	<11	Negative
	18 died	0	0	0	---	< 9	Negative
Bat 5	-22 capture	0	---	---	0	<11	Negative
	-3	0	---	---	---	0	Negative
	7	0	---	---	0	0	Negative
	14	0	---	---	0	<11	Negative
	18 died	0	---	---	---	< 9	Negative
Bat 6	-23 capture	0	---	---	0	<11	Negative
	-3	0	---	---	---	0	Negative
	7	0	---	---	0	<11	Negative
	14	0	---	---	0	<11	Negative
	18 killed	0	---	---	---	<10	Negative
Bat 7	-30 capture	0	---	---	0	<11	Negative
	-3	0	---	---	---	0	Negative
	7	0	---	---	0	<11	Negative
	14	0	---	---	0	<11	Negative
	19 killed	0	---	---	0	< 9	Negative

AAHL results: '0' indicates 20 of 20 positive fields contained fluorescent foci at a 1:20 dilution.
Results < 0.2 IU/mL correspond to counts of 14 and 15 of 20 fields.
+ there was subjective evidence of inhibition at a dilution of 1:20 but not 1:50. This test is not standardized. See Appendix 2 for details of the RFFIT procedure and assessment.

CDC results: '0' indicates 20 of 20 positive fields contained fluorescent foci at a 1:5 dilution.
Positive results expressed as calculated 50% endpoint dilutions.
Results expressed as < 9 to <11 had cytotoxicity at the 1:5 dilution and a count of 20 of 20 positive fields at a plasma dilution of 1:25. These results are considered negative.

6.3.6.2 Clinically well, survivor bats (n=3)

Plasma from one of the three survivor flying foxes (Bat-8) showed strong anti-rabies and anti-ABLV activity that peaked around PI-day 7. Results for Bat 8 are shown in Table 6-4.

Plasma from one other survivor (Bat 9) showed weak intermittent responses near the limits of detection for these assays on PI-day 21 and PI-day 70 in the AAHL modified rabies-RFFIT (< 0.2 IU/mL), but no evidence of CVS-rabies or pt-ABLV activity in the CDC RFFITs as shown in Table 6-4. Plasma from all other days was either negative or not done.

Plasma from the other survivor (Bat 10) showed no evidence of anti-rabies or anti-ABLV activity.

Table 6-4 Strong anti-rabies and anti-ABLV response in one of three unaffected experimentally-inoculated flying foxes (Bat 8)

Bat 8 PV-day	AAHL modified RFFIT			CDC RFFIT		Interpretation
	CVS (IU/mL)	**pt-ABLV**	**ybst-ABLV**	**CVS** (titre)	**pt-ABLV** (titre)	
-30 capture	0	---	---	0	**50**	Positive
-3	0	---	---	---	**13**	Weak positive
7	**>> 2**	**++++**	**++++**	---	**480**	Strong Positive
14	**0.54**	**++++**	**++++**	---	**170**	Strong Positive
21	**0.17**	**++++**	**++++**	11	**180**	Strong Positive
28	**0.1**	---	---	0	**50**	Positive
35	**< 0.1**	---	---	9	**125**	Positive
42	0	---	---	---	**54**	Positive
49	0	---	---	0	**60**	Positive
56	0	---	---	---	**54**	Positive
63	0	---	---	7	**70**	Positive
70	0	---	---	7	**70**	Positive
80	0	---	---	---	**25**	Positive
Bat 9	(plasma from all other days either negative or not done)					
21	**<0.2**	---	---	0	<11	Equivocal
70	**<0.2**	---	---	0	<8	Equivocal

--- Not done

AAHL results: '0' indicates 20 of 20 positive fields contained fluorescent foci at a 1:20 dilution.
++++ indicates 0 of 20 low power fields contained fluorescent foci at a dilution of 1:50.

CDC results: '0' indicates 20 of 20 positive fields contained fluorescent foci at a 1:5 dilution.
Results expressed as < 8 and <11 had cytotoxicity at the 1:5 dilution and a count of 20 of 20 positive fields at a plasma dilution of 1:25. These results are considered negative.

6.4 Discussion

An important consideration in the development of this model of ABLV infection was to simulate natural infection as closely as possible. To this end pteropid-variant-ABLV recovered directly from the tissues of a naturally infected bat (ABLV-51), and not passaged in either cell culture or an aberrant species such as mice, was inoculated peripherally, into Grey-headed flying foxes, a natural host. As earlier pathogenesis studies with ABLV in flying foxes had produced clinical disease in only 1 of 7 and 3 of 10 inoculated bats (23.5%) (McColl 1999; McColl *et al.* 2002), two strategies were used to increase the proportion affected. First, candidate inocula were evaluated in a mouse model for peripheral virulence, and the most virulent inoculum (Inoculum 5) selected for use in bats (see Chapter 5). Secondly, multiple inoculation sites, rather than just one, were used in order to reduce the possibility of inadvertent placement of the inoculum into a tissue or site that was not conducive to the establishment of infection.

The use of multiple sites was considered consistent with the presumptive natural route of ABLV transmission by biting. Flying foxes are quarrelsome and do bite and scratch each other while defending territory. One naturally infected rescued bat (ABLV-52, see Chapter 2) was observed attacking and biting another bat in a cage, and was found with multiple small punctures suggesting it had been in prior fights with other bats. An ABLV-positive female Black flying fox was found near a release cage, chasing and biting other flying foxes, including her own pup, which sustained fatal head injuries (Skerratt *et al.* 1998). These observations support biting as a route of natural transmission. Inoculation of the temporal and pectoral muscle was done as these were large, easy to identify and inoculate muscle masses, and were considered to be likely sites of natural bat bite wounds. The muzzle was selected as the face is highly innervated and was considered likely to have a high density of nerve endings that would take up ABLV, and because wounds to the face have been associated with a higher prevalence of clinical rabies and shorter incubation periods in humans (Hattwick 1974; Warrell 1995). The ‘footpad’ was selected because successful footpad models of infection are well established in rodents and it was thought possible that this success may involve suitability of the connective tissue structure for establishing infection.

While the numbers of bats used are small, this appears to be highly successful model for ABLV infection of flying foxes, inducing clinical disease and/or death in 7 of 10 bats (70%). This compares favourably with mortalities of 0 to 31% in six reports of experimental peripheral inoculation of insectivorous bats with bat-variant rabies virus (Baer and Bales 1967; Constantine 1966b; Constantine and Woodall 1966; Kuzmin and Botvinkin 1996; Stamm *et al.* 1956; Sulkin *et al.* 1960), and with the previous ABLV trials in which 4 of 17 were affected (23.5%) (McColl 1999; McColl *et al.* 2000; McColl *et al.* 2002), and is comparable to the 89% mortality of vampire bats given vampire-variant rabies virus (Setien *et al.* 1998). In only one insectivorous-rabies virus study (Baer and Bales 1967) and the vampire bat study were the bats shown to be free of detectable

rabies antibody prior to inoculation. The low mortalities in the other insectivorous bat rabies studies may be secondary to natural immunity resulting from prior exposure.

The incubation period for the seven affected bats was unexpectedly short; only 10 to 15 days (n=6) and 19 days, when compared to the four known incubation periods of naturally occurring ABLV in bats (ABLV-32: 36 to 57 days and ABLV-73: 30 days see Chapter 5), and in humans (6 weeks and 27 months (Allworth *et al.* 1996; Hanna *et al.* 2000)). They were generally shorter but consistent with those of the previous ABLV trials in flying foxes (27 days (McColl 1999) and 15, 23, and 24 days (McColl *et al.* 2002)). The short incubation periods are consistent with those seen using this inoculum in mice (see Chapter 5). The unique inoculation protocol for Bat 7, using only half the dose in only two sites, the footpad and pectoral muscle, was an artefact of there being insufficient inoculum. This bat was the last to become ill, however no significance is attributed to this association.

One bat (Bat 3) died during a seizure that occurred during an otherwise uneventful recovery from ketamine/xylazine/Reversine anaesthesia, without any other clinical signs being detected. The brain of this bat was ABLV-positive by FAT, immunoperoxidase staining, virus isolation in mice and TaqMan® PCR. Bat 4 had a very similar seizure during recovery from the same anaesthetic but recovered and showed other signs of ABLV infection the next day. Bat 2, which was showing clinical signs of aggression and vocalizing at the time, recovered uneventfully from a similar ketamine/xylazine anaesthetic (no Reversine). This suggests there was an interaction with Reversine component of the anaesthetic and pre-clinical CNS ABLV disease, however 'spontaneous' seizures have been recorded in naturally infected bats. No other seizures were seen during the limited time the bats were under direct observation.

Unexpectedly, the clinical manifestation in the six other affected bats was dominated by agitation and/or aggression rather than the depression and paresis seen in most naturally infected bats (see Chapter 2) and the four affected bats of the previous trials (McColl 1999; McColl *et al.* 2002). Why these six inoculated bats should develop a similar but apparently less common 'furious' clinical form is unclear. Clinical signs lasted 3 to 4 days in those that died (n= 3), and three others became very ill and were killed within 1 to 3 days. Clinical signs included vocalizing, 'rushing at' and apparently trying to attack neighbouring bats and the candidate, and incessant licking, particularly of the genitals similar to that noted in at least one naturally occurring case of ABLV infection in a bat (ABLV-73). Involvement of the genitals may involve the same mechanisms that induce increased libido, nymphomania, and spontaneous ejaculation in rabid humans (Warrell 1976). Four bats developed proprioceptive deficits and paresis, most readily seen as knuckling and unsteadiness while scrambling around the cage but none became so weak that they were unable to hang or fell from the cage roof, and the aggression persisted until moribund.

In one naturally infected bat (ABLV-32) the clinical course had progressed from initial signs of aggression (vocalizing, aggressive to cage mate, biting objects) to a prolonged period (2 to 6

days) of typical depression, paresis, and an inability to hang (Field *et al.* 1999). This had suggested that an aggressive phase may have occurred prior to rescue in cases of naturally occurring ABLV that presented alert and calm with unexplained paresis, and led to an expectation that a similar period of calm paresis would develop in the inoculated bats. That three bats died in this trial, rather than being killed, was a consequence of the unexpectedly rapid terminal deterioration of these bats. As was seen in some cases of naturally infected flying foxes (e.g. ABLV-25, ABLV-52, and ABLV-53) ABLV-infected bats showing overt aggression can rapidly change from hyperactive and aggressive to moribund or dead in a matter of hours. This is consistent with observations that humans with 'furious' rabies die after a shorter illness than those with 'paralytic' rabies (Warrell 1995). In general the clinical signs observed fell within the range of clinical signs reported in humans and animals infected with street rabies virus.

The diagnosis of ABLV infection in the seven affected bats was well supported by FAT, immunoperoxidase staining, virus isolation in mice, and TaqMan® PCR (see also Chapter 7). Unlike previous trials (McColl *et al.* 2002), in all cases there was strong and unequivocal staining in the FAT on fresh brain touch impressions, as seen in the vast majority of naturally occurring cases of ABLV infection. In the previous trials, attempts at virus isolation were prohibited by biosecurity concerns in the first trial (n=1) (McColl 1999), and ABLV was isolated from only two of three affected bats in the second trial in mouse neuroblastoma cells (MNA) (McColl *et al.* 2002). Attempts to isolate ABLV in MNA at the time candidate inocula for this study were prepared also failed (data not shown) and isolation of ABLV in cell culture from the second human case required two blind passages (14 day incubation) before detection by fluorescent antibody (Hanna *et al.* 2000). It would appear that wild-type ABLV is more difficult to isolate in cell culture than in mice, however use of the more sensitive *in vivo* models is limited by animal ethics requirements.

The only abnormalities at gross post-mortem examination were wastage of the pectoral muscles, self inflicted skin lesions, and the presence of froth in the trachea of Bat 3 that died during a seizure. The pectoral muscle wastage, seen in both the ABLV-positive and ABLV-negative bats, was considered secondary to confinement, initially in a reasonably large outdoor cage for 19 to 27 days that permitted limited flight, and later in the smaller experimental cages.

Histological lesions were confined to the central and peripheral nervous systems of clinically affected ABLV-positive bats with the exception of a multifocal non-suppurative sialoadenitis involving the parotid and/or submandibular salivary glands of Bats 1, 2, 3, 4, 6, 7, 8, 9 and 10 (not Bat 5). Similar sialoadenitis was observed by the candidate in diagnostic submissions of ABLV-negative flying foxes (data not shown). Sialoadenitis in ABL-negative flying foxes has also been observed by others (Deborah Middleton, AAHL *pers. comm.*) and is considered incidental.

In contrast to the frequently high number of large and easily observed eosinophilic cytoplasmic neuronal inclusions pathognomic for street rabies virus infection, inclusions were absent or small, rare, and very difficult to detect in these bats. This is consistent with the previously reported low

incidence of eosinophilic inclusions in naturally infected ABL-positive flying foxes (Hooper *et al.* 1999b). There was no reference to the presence of eosinophilic neuronal inclusions in reports of the previous ABLV trials, suggesting inclusions were absent (McColl 1999; McColl *et al.* 2002), and inclusions were often absent and in the few reports of natural or experimental infection with Mokola virus or Lagos bat virus (Kemp *et al.* 1973; Percy *et al.* 1973; Shope *et al.* 1970). In general the histological lesions were entirely consistent with those previously reported in naturally infected ABL-positive bats (Hooper *et al.* 1999a), and fall within the range of lesions reported in humans and animals infected with street rabies virus.

The distribution of antigen in the brain, as demonstrated by immunoperoxidase staining with the HAM monoclonal anti-nucleocapsid antibody, did differ from that seen in some naturally infected bats (data not shown) in that antigen occurred in multiple small/fine granules within neurons that were only readily seen at high power. In some naturally affected bats the aggregates of antigen were larger and easily seen at low power. The small size of the aggregates may be a reflection of the short incubation period.

Viral antigen was found throughout the central and peripheral nervous system, including within parasympathetic ganglia in virtually all tissues examined including the heart and gastrointestinal tract as had been reported previously (Hooper *et al.* 1997b). As was noted in the previous ABLV trials (McColl *et al.* 2002), within the nervous system there often appeared to be an inverse correlation between the severity of histological lesions and the quantity of antigen detected by the HAM monoclonal antibody. Antigen was also found in retinal cells and ganglia in the salivary gland, lungs and kidney, and in nerve fibres. Due to the limits of resolution using light microscopy, it remains unclear whether antigen in nerve fibres is within axons and/or dendrites, or in supporting Schwann cells. Detection of antigen in ganglion neurons in the kidney support the speculation that the original isolation of ABLV from kidney samples of the index ‘Ballina’ case was due to the presence of ABLV in parasympathetic neurons within the sample (Fraser *et al.* 1996; Hooper *et al.* 1997b).

The near ubiquitous nature of the virus in organs secondary to parasympathetic innervation means that the presence of antigen and isolation of virus from the salivary glands does not intrinsically indicate saliva plays a significant role in transmission. The presence of antigen in serous epithelial cells of the salivary glands of a YBST bat has been reported previously (Hooper *et al.* 1997b), and small quantities of antigen in rare submandibular (mixed serous and mucous) salivary gland epithelial cells were detected in four flying foxes in this study. This is more suggestive of the salivary glands playing a role in transmission, as is the detection of small quantities of ABLV RNA in the saliva samples of four bats on PI-day 14 and a fifth bat on PI-day 19. However, the quantities of RNA detected were very small (C_T= 30 to 36), and near the positive-negative detection threshold. In retrospect it would have been better to have tested the oral swabs directly rather than transport media in which the swabs had been agitated. A clearer

indication of the potential role of saliva for transmission requires demonstration that the saliva contains *infectious* ABLV rather than simply genomic material.

Interpretation of the serological results of this trial is complicated by a number of factors. Bat plasma is toxic to BHK and neuroblastoma cells at low dilutions. This limits the sensitivity of the RFFIT by limiting the lowest dilution at which plasma that can be tested without interpretation being compromised by poor cell growth. At AAHL, using BHK cells, this is managed by using 1:20 as the lowest plasma dilution, and by reducing the time that plasma is in contact with the cell sheet to 90 minutes (see Appendix 2). The thresholds at which naturally occurring titres are of biological significance, i.e. indicating prior exposure and/or protective immunity to ABLV are unknown. It is possible that low but biologically significant titres below the sensitivity limits of the RFFIT format are not detected.

In Australia there is no standardized RFFIT using ABLV rather than rabies virus, and virtually all Australian research into the seroprevalence of ABLV is based on cross-reactivity to CVS-rabies and expressed as international units (IU/mL) of anti-rabies activity. Prototype pteropid-variant and YBST-variant ABLV RFFITs developed at AHHL suffer from poor growth of the virus isolates in cell culture. Pteropid and YBST-variant RFFITs have been developed at CDC Atlanta, however, the growth characteristics of the virus isolates used in these assays has not been reported. A serological survey of insectivorous and fruit bats of the Philippines using the CDC ybst-ABLV RFFIT showed that there was poor correlation between positive neutralization in the ABLV and CVS-11 RFFITs. Of 231 samples tested, 17 of 22 samples that were positive in the ABLV-RFFIT were negative in the CVS-RFFIT, and one sample was positive in the CVS-RFFIT but negative in the ABLV-RFFIT (Arguin *et al.* 2002). While the lyssavirus responsible for induction of these antibodies was not specifically identified, it is likely to be ABLV or closely related to ABLV, and the results suggest that a negative result in the CVS-RFFIT does not necessarily indicate an absence of neutralizing antibodies for ABLV.

Given these considerations, comparison of the CVS-11 rabies and ABLV RFFIT results from AAHL and CDC points to some interesting conclusions.

None of the clinically affected, ABLV-positive bats developed an early or high antibody response to either CVS-11 or ABLV. Clearly the development of clinical signs in these bats was associated with an absent or insufficient (weak and/or late) antibody response to ABLV infection. The very low or negative CVS-11 RFFIT titres of these experimentally infected bats are consistent with those seen in naturally infected bats (see Chapter 2) and experimentally infected mice (see Chapter 5).

Of the three bats that did not develop clinical disease and were ABLV-negative, Bat 10 showed no evidence of a serological response. Bat 9 showed very weak and intermittent anti-CVS activity on PI-days 21 and 70 in the AAHL RFFIT, which were near or below the tests detection thresholds, and are of questionable significance. Certainly neither bat developed an early or high antibody

response to which their not developing clinical signs could be attributed. It appears that either these bats were not infected with ABLV or they were killed before showing clinical signs that would have developed after an incubation period > 80 or 82 days.

The serological response of Bat 8 is most interesting. The CVS-RFFITs at both AAHL and CDC indicate a brief but strong antibody response that peaked around PI-day 7 and was below the formal test parameters by PI-day 28. This brief spike of cross-reactive anti-rabies activity is similar to that previously reported for three of five survivors of experimental inoculation with ABLV (McColl *et al.* 2002). However, the ABLV-RFFITs at AAHL and CDC indicated a higher and more sustained anti-ABLV response that also peaked around PI-day 7. The time of this peak corresponds to the subclinical development of infection in the seven clinically affected bats, and strongly suggests that failure of this bat to develop clinical signs was not due failure to be infected but was related to an early and adequate immune response.

Of perhaps more significance is the observation that while both the CDC and AAHL CVS-11 RFFITs for Bat 10 were negative prior to ABLV-inoculation, the CDC ABLV-RFFIT detected moderate or low anti-ABLV activity both 30 and 3 days prior to inoculation. These ABLV results suggest this bat had naturally-occurring ABLV antibodies, presumably indicating prior exposure to ABLV, that were not evident in the pre-trial AAHL CVS-11 RFFIT used to screen for susceptible experimental animals. The post-inoculation antibody response and resistance to clinical infection appear to be the result of stimulation of pre-existing ABLV immunity. Similar undetected pre-existing immunity may account for the low number of clinically affected animals and serological responses of survivors in previous ABLV trials (McColl 1999; McColl *et al.* 2002).

The serological results of Bat 8 suggest that:

- While high levels of anti ABLV antibodies may be reflected in CVS-11 cross-reactivity, low levels of ABLV immunity are not detected in CVS-11 RFFITs.
- Levels of ABLV immunity below the detection threshold of the cross-reactive CVS-11 RFFIT may confer protective immunity to ABLV challenge.
- Screening with the CVS-11 RFFIT will not always identify individuals with natural immunity to ABLV infection and resistance to infection in inoculation trials.
- Serological surveys for ABLV done using CVS-RFFITs may underestimate the proportion and level of naturally occurring ABLV immunity in bat populations.

6.5 Conclusions

Development of a successful model of experimental infection of flying foxes with ABLV

- ♦ This experimental model successfully induced clinical disease in 7 of 10 inoculated bats that was consistent with that of naturally occurring ABLV in flying foxes.
- ♦ Diagnosis of ABLV infection in all seven clinically affected bats was unequivocal.
- ♦ No ABLV was detected in any of three bats that remained clinically well until killed at the conclusion of the trial.

Characterization of the clinical disease, pathology, and viral distribution following experimental infection

- ♦ Bats became ill after a comparably short and consistent incubation period of 10 to 19 days, and were ill for 1 to 4 days before dying or requiring euthanasia.
- ♦ One bat (Bat 3) died during a seizure apparently induced by an interaction between pre-clinical ABLV encephalitis and one or more components of the ketamine/xylazine/Reversine anaesthetic.
- ♦ Five Bats (Bats 1,2, 4, 5. and 6) showed clinical signs of overt aggression towards other bats, inanimate objects and/or the candidate, consistent with those described in ~ 14 of 74 (19%) of bats with naturally occurring ABLV infection.
- ♦ One bat (Bat 7) showed intractable agitation, pacing, tremors and ataxia.
- ♦ While 4 of 7 affected bats showed evidence of paresis, ataxia, and proprioceptive deficits, in none were these the dominant clinical signs, and none lost the ability to hang or became recumbent.
- ♦ No significant gross lesion was detected.
- ♦ The histopathology associated with experimental infection was entirely consistent with that reported in naturally infected ABL-positive bats, and within the range of lesions described for rabies virus and other lyssavirus infections.
- ♦ ABLV is only weakly negrigenic (forms few eosinophilic intracytoplasmic neuronal inclusions) compared to terrestrial variants of street rabies virus.
- ♦ The predominant histological lesions were mild to moderate non-suppurative meningoencephalomyelitis and severe ganglioneuritis.
- ♦ ABLV nucleocapsid antigen was detected throughout the central and peripheral nervous systems, including parasympathetic ganglia and nerve fibres within most tissues examined.
- ♦ Antigen was detected in a very small number of submandibular salivary gland epithelial cells from four bats.

Examination of plasma for evidence of seroconversion

- None of seven affected bats showed clear evidence of seroconversion to ABLV in RFFITs using rabies virus or ABLV.
- Two of three unaffected bats showed no or equivocal antibody responses as detected in the CVS and ABLV-RFFITs, suggesting these bats were not infected.
- One of three unaffected bats showed evidence of pre-existing (natural) ABLV immunity in the CDC pt-ABLV RFFIT, and a strong but brief antibody response on PI-days 7, 14 and 21 that waned to pre-inoculation levels by PI-day 28.
- Serological surveys for ABLV and selection of susceptible individuals for experimental infection should be done using ABLV-RFFITs rather than the relying on the cross-reactivity detected in rabies-RFFITs.

Examination of saliva for evidence of viral excretion

- Low levels of ABLV RNA were detected in samples of saliva from four bats that were clinically ill, and the saliva of one bat (Bat 6) that was behaving abnormally within 12 hours. Together with the presence of viral antigen in the parasympathetic ganglia of all three salivary glands and the epithelial cells of the submandibular salivary glands, this suggests that saliva does play a role in the natural transmission of ABLV.

7 Relative virulence in mice of inocula prepared from the brain and salivary gland of experimentally infected flying foxes

Aims

1. To confirm by virus isolation the presence or absence of ABLV in the brains and salivary glands of 10 experimentally inoculated flying foxes.
2. To compare the virulence of inocula prepared from the brain, parotid salivary gland or submandibular and sublingual salivary glands from 10 experimentally inoculated flying foxes.
3. To compare the effects of these inocula in mice with the relative amount of viral-RNA detected by quantitative-PCR (TaqMan) to evaluate the potential role of quantitative-PCR in the selection of inocula.

7.1 Introduction

The results of Chapter 5, where the virulence of 15 inocula were compared to select one for further use, suggested that inocula derived from the submandibular and sublingual salivary glands had higher virulence than either the relative amount of viral RNA in the inocula, or the relative amount of viral antigen detected in some naturally infected bat salivary glands (data not shown) would have implied. However, the source tissues used to prepare the inocula had been collected from naturally affected bats, after variable post-mortem intervals during which they had been stored in a range of different conditions, and the source tissues had subsequently been subjected to variable storage conditions for variable periods and had undergone variable numbers of freeze /thaw cycles prior to inoculum preparation. The extent to which the differences in virulence indicated some specific property of salivary gland-derived inocula or was simply an artefact of the storage and handling histories of the source tissues was unclear.

The experimental infection of bats in Chapter 6, presented the opportunity to re-evaluate the relative virulence of brain and salivary gland inocula using tissues that had been collected after brief post-mortem intervals and stored in similar conditions. It also allowed further evaluation of the potential use of TaqMan® PCR assays in the selection of inocula.

7.2 Materials and methods

7.2.1 Permits

Animal Ethics Committees

University of Queensland – approval number VP/231/00/PHD

Animal Research Institute – approval number ARI/025/2000-1

Biosafety Committee

Animal Research Institute – approval number DPI/IBC/PC3/99/00

7.2.2 Source and preparation of inocula

Ten Grey-headed flying foxes were experimentally inoculated with an inoculum prepared from the submandibular and sublingual salivary glands of a naturally infected ABLV-positive Black flying fox (Inoculum 5, for details see Chapters 5 and 6). Seven of the 10 bats became clinically ill and subsequently died or were killed 14 to 19 days post-inoculation, three remained clinically well until killed on PI-day 80 or 82. Three tissue samples, the brain, the parotid salivary gland and the submandibular and sublingual salivary glands from each bat were removed separately with sterile instruments and stored at –70°C.

Each sample was ground into a 20% w/v suspension with a sterile mortar and pestle in Dulbecco's modified Eagles medium, 20% bovine plasma albumin and an antibiotic/antimycotic preparation as described in Section 5.3.2 Inocula. Suspensions were clarified at ~200 G for 10 minutes and the supernatant removed, divided into aliquots, and stored at –70 ^{0}C. All inoculations were done using separate aliquots of the original preparations at the same freeze/thaw generation.

7.2.3 Experimental design

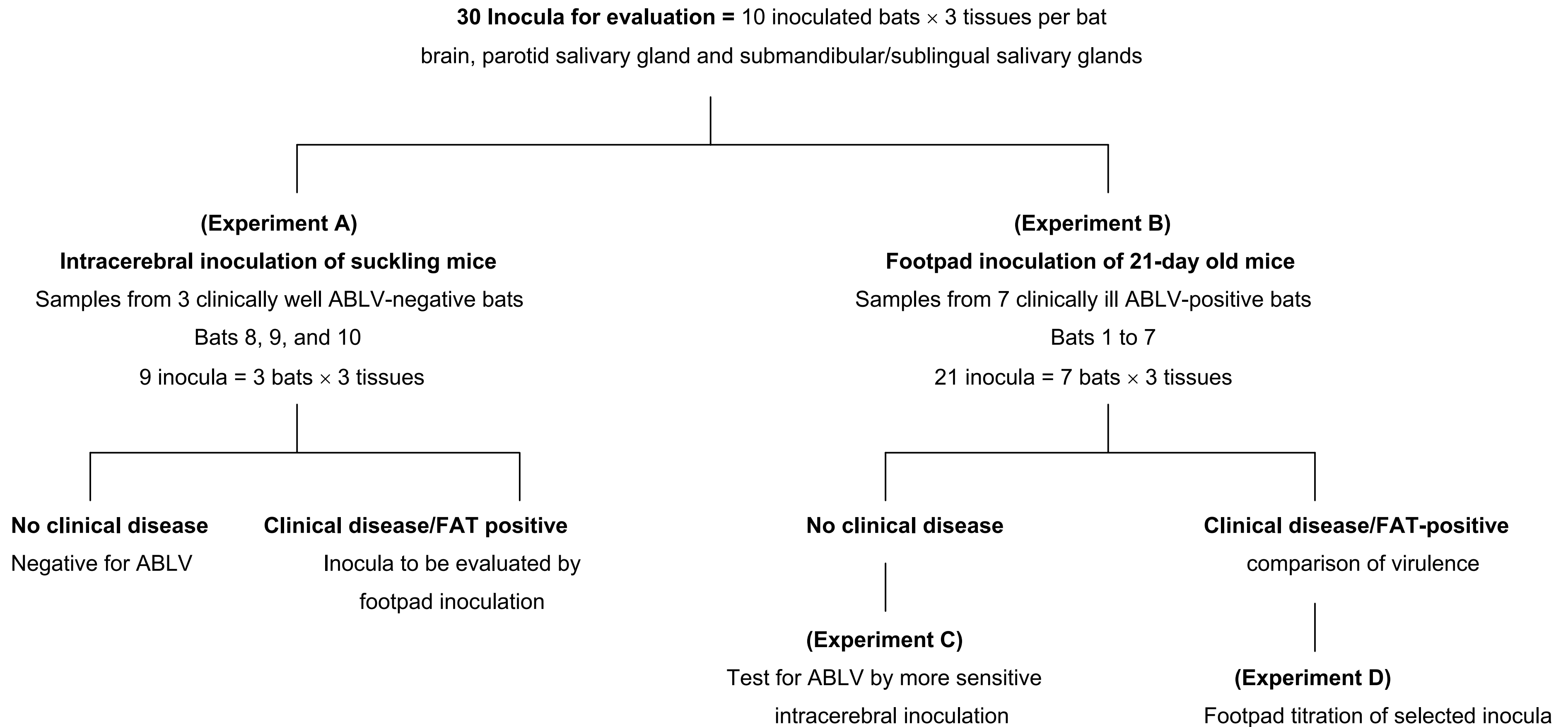
Animal ethics requirements required that the number of mice used be minimized, precluding evaluation by systematic *in vivo* titration of inocula. To reduce the numbers of mice required to evaluate inocula, the following approach was taken.

Inocula prepared from ABLV-positive bats, which were expected to contain viable ABLV, were tested by footpad inoculation of 21-day old weanling mice. Inocula prepared from ABLV-negative bats, which were expected to *not* contain viable ABLV, were tested using the more sensitive method intracerebral inoculation of two to three-day old mice.

Animal ethics requirements precluded death as an endpoint. Mice showing clear clinical signs of disease were killed. All inoculations were performed by the candidate.

The experimental design is illustrated in Figure 7-1.

Figure 7-1 Experimental design for the evaluation of brain, parotid and submandibular/sublingual salivary glands from 10 ABLV-inoculated bats



7.2.3.1 Experiment A and Experiment C: Intracerebral inoculation of suckling mice

Each of the nine inocula prepared from the surviving ABLV-negative bats (3 bats x 3 inocula, Experiment A), and inocula that failed to produce clinical disease in Experiment B by footpad inoculation (Experiment C) were inoculated intracerebrally into litters of two to three-day-old Quackenbush (outbred) mice on PI-day 0. Losses within the first 3 days post-inoculation were considered incidental and not considered in the results.

Inocula were considered positive for ABLV if one or more mice per litter died or required euthanasia 4 to 30 days post-inoculation and were confirmed ABLV-positive by FAT.

Inocula were considered negative for ABLV if all inoculated mice survived with no clinical disease until PI-day 30.

7.2.3.2 Experiment B: Comparison of virulence by footpad inoculation of weanling mice

Each of 21 inocula prepared from the tissues of ABLV-positive bats (7 bats x 3 tissues) were inoculated into the footpads of three 21-day old female Quackenbush mice which were anaesthetized with 0.01 mL of 1:1 mix of ketamine (100 mg/mL) and xylazine (Rompun SA, 20 mg/mL). Anaesthetic deaths reduced the number of mice in four groups to only two mice (see Table 7-2). Each group of two or three mice was housed separately. Within each group, individuals were identified with silver nitrate markings. Mice were weighed with digital laboratory scales every 2 to 3 days until PI-day 30, then once or twice a week until PI-day 90, then intermittently as required. When clinically ill or at the conclusion of the experiment on PI-day 287 (~ 9.5 months) mice were killed and blood collected, as described in Chapter 5, Experiment 2. Plasma was submitted to the AAHL for detection of anti-lyssavirus antibodies using the rabies-RFFIT.

7.2.3.3 Experiment D: Footpad titration of selected inocula

Three inocula were selected from those used in Experiment B for titration by footpad inoculation for quantitative comparison of peripheral virulence. Unanaesthetized mice were inoculated with ten-fold dilutions of the neat inoculum as described in Chapter 5, Experiment 4. Unaffected mice were killed at the conclusion of the experiment on PI-Day 92.

7.2.4 Confirmation of lyssavirus infection by FAT

Infection with Australian bat lyssavirus in clinically ill mice was confirmed by FAT on fresh brain touch impressions, as described in Section 2.2.3 Fluorescent antibody test. The tests were performed and read by the candidate and results confirmed by Barry Rodwell of the Diagnostic Virology Laboratory of the ARI.

7.3 Results

7.3.1 Experiment A: Intracerebral inoculation of suckling mice with inocula from ABLV-negative bats

All inoculated suckling mice survived until killed on PI-day 30 or 31 following inoculation with inocula prepared from the tissues of bats that had themselves survived experimental inoculation (Bats 8, 9, and 10, see Chapter 5) as shown in Table 7-1. These inocula were considered negative for ABLV.

Table 7-1 Experiment A: Intracerebral inoculation of mice with inocula prepared from three clinically well, ABLV-negative bats that survived experimental inoculation

Inoculum source		No. inoculated (PI-day 3)	No. clinically ill & FAT positive	Day killed (PI-day)	Result
Bat	Tissue				
Bat 8	Brain	9	0	31	Negative
Bat 8	Parotid SG	11	0	31	Negative
Bat 8	Submandib./sublingual SG	10	0	31	Negative
Bat 9	Brain	9	0	30	Negative
Bat 9	Parotid SG	9	0	30	Negative
Bat 9	Submandib./sublingual SG	8	0	30	Negative
Bat 10	Brain	9	0	30	Negative
Bat 10	Parotid SG	10	0	30	Negative
Bat 10	Submandib./sublingual SG	10	0	30	Negative

As none of the inocula was positive for ABLV, none required further evaluation by footpad inoculation.

7.3.2 Experiment B: Comparison of inocula from ABLV-positive experimentally-inoculated bats by footpad inoculation of weanling mice

All clinically ill mice were confirmed ABLV-positive by FAT. ABLV was detected in mice inoculated with material from each of the seven clinically ill/FAT-positive bats, and was detected in at least four of the seven inocula from each of the three source tissues; brain, parotid salivary gland and submandibular/sublingual salivary glands. The numbers of ABLV-positive mice per inoculum are shown in Table 7-2, and corresponding TaqMan® PCR values are shown in Table 7-3.

Table 7-2 Experiment B: Footpad inoculation of mice with inocula prepared from seven clinically ill, ABLV-positive, experimentally infected bats
Number of ABLV-positive mice / number inoculated, duration 287 days

	Inoculum source								
Inoculum source tissue	**Bat 1**	**Bat 2**	**Bat 3**	**Bat 4**	**Bat 5**	**Bat 6**	**Bat 7**	**Total positive**	**% positive**
Brain	3/3	3/3	2/2	3/3	3/3	3/3	2/2	19/19[a]	100
Submandib./sublingual SG	1/3	2/3	1/2	0/3	2/2	3/3	0/3	9/19[b]	47
Parotid SG	1/3	0/3	0/3	3/3	3/3	2/3	0/3	9/21[b]	43

Values with the same superscripts are not significantly different ($p < 0.05$) on chi-square test.

Table 7-3 Quantitative PCR (TaqMan®) values for inocula prepared from three different tissues from seven clinically ill, ABLV-positive, experimentally infected bats

	TaqMan® C_T value							
Inoculum source tissue	**Bat 1**	**Bat 2**	**Bat 3**	**Bat 4**	**Bat 5**	**Bat 6**	**Bat 7**	**Mean C_T**
Brain	16.4	15.3	15.5	15.6	15.2	15.4	15.0	15.5
Submandib./sublingual SG	30.5	26.8	27.0	29.0	29.2	23.6	29.0	27.9
Parotid SG	23.0	24.8	25.7	26.0	25.2	27.5	28.6	25.8

The amounts of ABLV RNA detected in the TaqMan® assay and the proportions of mice affected as shown in Table 7-2, suggest that the relative virulence of the 20% w/v inocula produced from these three tissues is

brain > submandibular/sublingual salivary gland ≈ parotid salivary gland.

However, evaluation of the duration of infection (interval from inoculation to day died or killed; that is the incubation *and* clinical period) as shown in Table 7-4 allows further discrimination.

Table 7-4 Experiment B: Durations of infection in footpad inoculated mice

Tissue	Bat 1	Bat 2	Bat 3	Bat 4	Bat 5	Bat 6	Bat 7	Range (days)	Average (days)	Standard deviation
Brain	10, 17, 26	13, 14, 22	16,17	13, 14, 14	15, 20, 21	14, 17, 21	14, 16	10-26	16.5	3.7
Submandib./sublingual SG	52 • •	14, 30 •	39 •	• • •	14, 34	16, 24, 27	• • •	14-52	27.8	12
Parotid SG	253 • •	• • •	• • •	25, 29, 33	40, 55, 156	67, 79 •	• • •	25-253	81.8	71

• Mouse that remained clinically well until killed at conclusion of experiment PI-day 287

Inocula derived from brain tissue produced clinical disease requiring euthanasia in 100% of mice within a relatively narrow period of 10 to 26 days (n=19).

Inoculation with submandibular/sublingual salivary gland inocula resulted in the deaths of only 47% of mice after a more variable period of 14 to 52 days (mean 28 days). A similar proportion of mice inoculated with parotid salivary gland inocula was affected (43%), however the period of infection was generally longer and far more variable at 25-253 days (mean 82 days). Four parotid inocula produced disease in mice. Mice inoculated with the parotid salivary glands of Bats 1, 5 and 6 had long and highly variable incubation times, deaths occurring 40 to 253 days post-inoculation including six of the seven longest incubation periods. The inoculum produced from the parotid salivary gland of Bat 4 produced disease comparatively quickly with low variability, resulting in the deaths of three mice on PI-days 25, 29, and 33. For all inocula produced from bats other than Bat 4, the proportion of mice affected and the duration of infection for the first and last mice affected followed the trend of

brain ≥ submandibular/sublingual salivary gland > parotid salivary gland

As the virulence of the Bat 4 parotid inoculum was uncharacteristically high compared to other parotid inocula and the Bat 4 submandibular/sublingual inoculum, inoculation with these and three other inocula were repeated and mice observed for 60 or 90 days post-inoculation as indicated in Table 7-5

Table 7-5 Experiment B Repeat: Footpad inoculation using five inocula
ABLV-positive/ number inoculated, duration 60 or 90 days

Tissue	Bat 1 60days	Bat 3 60days	Bat 4 90days	Bat 7 60days
Submandib./sublingual SG	0/3	0/3	0/3	0/3
Parotid SG	not done	not done	1/3[1]	not done

[1] one clinically affected/FAT-positive mouse, killed 32 days post-inoculation

7.3.2.1 Prevalence of anti-lyssavirus titres in inoculated mice

All mice that survived until PI-day 287, Experiment B (n=22) or survived until PI-day 60 or PI-day 90 following repeat inoculations, Experiment B Repeat (n=14) were negative for anti-rabies antibodies at a plasma dilution of 1:20 (< 0.5 IU/mL). The rabies antibody titre for each affected mouse from Experiment B is shown in Table 7-6. Both positive and negative anti-rabies titres occurred in ABLV-positive mice inoculated with material from each of the seven bats, from mice inoculated with each of the three tissue sources of inoculum, and from mice which died after short and long durations of infection.

Table 7-6 Titres of cross-reacting anti-rabies antibodies in clinically ill mice infected with ABLV by footpad inoculation

Tissue	Rabies-RFFIT titre (IU/mL) (PI-day died)						
	Bat 1	**Bat 2**	**Bat 3**	**Bat 4**	**Bat 5**	**Bat 6**	**Bat 7**
Brain	Neg., Neg., Neg. (10, 17, 26)	0.8, NS , 0.6 (13, 14, 22)	0.1, Neg. (16, 17)	0.9, 0.7, 0.5 (13, 15, 15)	Neg., Neg., Neg. (15, 20, 21)	Neg., 0.1, 0.3 (14, 17, 21)	0.5, 0.3 (14, 16)
Submandib./sublingual SG	0.2 (52 • •)	0.4, 1.6 (14, 30 •)	Neg. (39 •)	(• • •)	1.2, 1.1 (14, 34)	7.8, Neg., 2.2 (16, 24, 27)	(• • •)
Parotid SG	Neg. (253 • •)	(• • •)	(• • •)	<0.1, Neg., Neg., *0.3** (25, 29, 33, *32**)	0.7, 1.1, Neg. (40, 55, 156)	0.5, 0.2 (67, 79 •)	(• • •)

Neg. no evidence of neutralizing antibodies at a dilution of 1:20 (< 0.5 IU/mL)
NS no sample
• Mouse that remained clinically well until killed at conclusion of experiment PI-day 287 with negative rabies-RIFFT titre
* Clinically ill mouse from Experiment B repeat

7.3.3 Experiment C: Intracerebral inoculation of suckling mice with inocula negative by footpad inoculation

Five inocula failed to produce clinical disease following footpad inoculation of 21 day old mice and required more sensitive testing for ABLV. Four of the five inocula produced clinically ill/ FAT-positive mice following intracerebral inoculation of litters of two to three day old mice.

Table 7-7 Experiment C: Intracerebral inoculation of mice with five inocula that failed to produce disease in footpad-inoculated mice

Inoculum source		Number positive / total	Died (PI-day)	Result
Bat	Tissue			
Bat 2	Parotid SG	6/8	11, 11, 13, 14, 19, 24	Positive
Bat 3	Parotid SG	1/8	14	Positive
Bat 4	Submandib./sublingual SG	5/10	14, 14, 18, 19, 19	Positive
Bat 7	Submandib./sublingual SG	1/8	24	Positive
Bat 7	Parotid SG	0/8	Not applicable	Negative

7.3.4 Experiment D: Comparison of inocula by footpad titration

Limitations by Animal Ethics Committees on the numbers of mice used precluded systematic titration of all inocula. Three inocula used in Experiment B were selected for comparison of peripheral virulence by footpad titration.

- **Bat 4 Brain**, selected as it had produced disease in 3/3 inoculated mice with the shortest average period between inoculation and death.
- **Bat 6 submandibular/sublingual SG**, selected as it was one of two submandibular/sublingual SG inocula that had affected 100% of inoculated mice (3/3) and had the shorter average period between inoculation and death.
- **Bat 6 Brain**, selected for comparison as it was from the same bat as the selected salivary gland inoculum.

All clinically ill mice were FAT-positive. The number of affected mice for each dilution and the calculated endpoint dilutions and titre (90 day mouse footpad 50% effective dose, $MFP_{90}ED_{50}$) are shown in Table 7-8 . Also shown are other indicators of virulence for these inocula such as the durations of infection for mice inoculated with neat inocula in Experiment B (n= 3 mice) and Experiment D (titration, n= 6 mice) and the neat quantitative TaqMan® C_T value.

Table 7-8 Experiment D: Footpad titration of three inocula derived from the tissues of flying foxes infected with Inoculum 5

Dilution		Number positive / total inoculated		
		Bat 4 Brain	Bat 6 Brain	Bat 6 Submandibular/sublingual SG
Neat		6/6	5/6	6/6
10^{-1}		6/6	5/6	6/6
10^{-2}		4/6	3/6	4/6
10^{-3}		2/6	1/6	2/6
10^{-4}		0/6	0/6	1/6
10^{-5}		0/6	0/6	0/6
10^{-6}		Not done	0/6	Not done
50% endpoint dilution /0.06mL[1]		$10^{-2.5}$	$10^{-1.8}$	$10^{-2.7}$
Titre $MFP_{90}ED_{50}$ /mL		$10^{3.7}$	$10^{3.0}$	$10^{3.9}$
Neat inocula (days, n=6)				
Duration of infection	**Mean**	17	21	23
	Median	16	21	23.5
	(Range)	(12 to 23)	(15 to 26)	(13 to 34)
Experiment B (days, n=3)				
Duration of infection	**Mean**	14.3	17.3	22.3
	Median	14	17	24
	(Range)	(13 to 15)	(14-21)	(16 to 27)
TaqMan® C_T (neat inocula)		15.6	15.4	27.6

[1] Calculated using the Spearman-Käber formulae (Lorenz and Bogel 1973), see Section 5.4.3

7.3.4.1 Effect of dose on incubation period

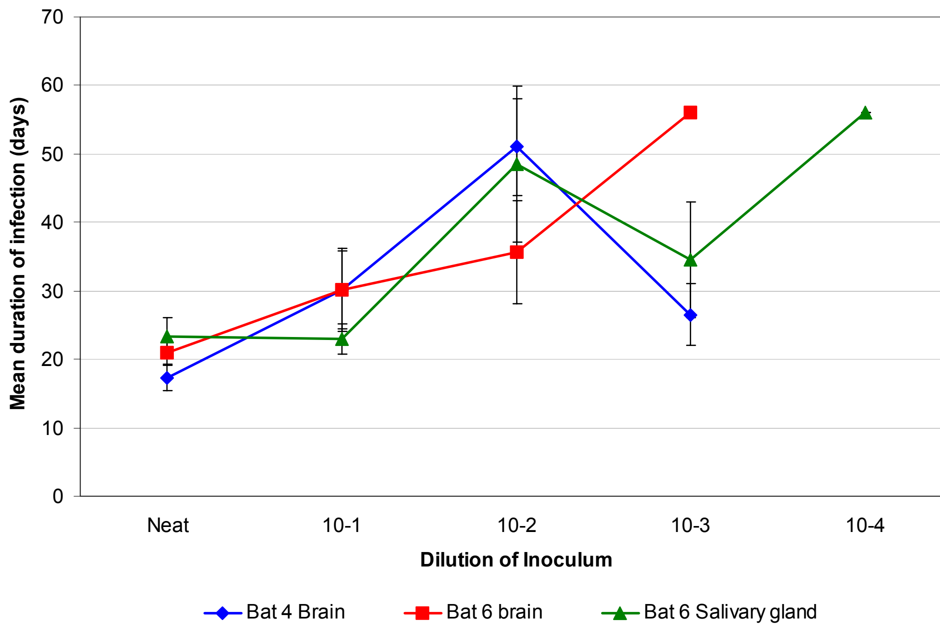
During the 92 days of Experiment D, fewer mice developed clinical disease when inoculated with higher dilutions of each inoculum. There was also a tendency for mice inoculated with lower dilutions to develop clinical disease and require euthanasia after shorter durations compared to those of mice inoculated with higher dilutions of inoculum, as shown in Table 7-9 and Figure 7-2.

Table 7-9 Experiment D: Effect of dose on the duration between inoculation and death following peripheral inoculation of 21-day old mice

Dilution	Bat 4 Brain		Bat 6 Brain		Bat 6 submandib./sublingual SG	
	Died (PI-day)	Mean	Died (PI-day)	Mean	Died (PI-day)	Mean
Neat	12, 14, 14, 18, 23, 23	17.3	15, 20, 21, 23, 26 •	21	13, 21, 22, 25, 25, 34	23.3
10^{-1}	18, 21, 23, 27, 34, 58	30.2	20, 22, 23, 36, 50 •	30.2	15, 20, 21, 24, 28, 30	23.0
10^{-2}	31, 52, 57, 64 • •	51.0	22, 48, 37 • • •	35.7	28, 31, 60, 75 • •	48.5
10^{-3}	22, 31 • • • •	26.5	56 • • • • •	56	26, 43 • • • • •	34.5
10^{-4}	none	n/a	none	n/a	56 • • • • • •	56

• inoculated mouse that remained clinically well until the conclusion of the experiment on PI-day 90

Figure 7-2 Effect of dose on the mean interval between inoculation and death following peripheral inoculation of 21-day old mice
(error bars ± 1 standard error calculated separately for each plotted data point)



7.4 Discussion

These mouse inoculations confirmed the presence of ABLV in the brains and both salivary glands preparations of all seven clinically affected experimentally inoculated bats, with the exception that ABLV was not isolated from the parotid salivary gland of Bat 7. Intracerebral inoculation of suckling mice detected ABLV in four of five inocula that did not produce clinical disease in any of three footpad-inoculated mice, consistent with intracerebral inoculation being a more sensitive method for the detection of ABLV. Using this more sensitive intracerebral assay, no ABLV was detected in the brains or salivary glands of the three inoculated bats that survived. These *in vivo* findings are entirely consistent with the clinical presentations, FAT, and immunoperoxidase staining results of all 10 experimental bats.

The 3-mouse footpad assay (Experiment B) was used to crudely determine the relative virulence of inocula as reflected in the proportion of mice affected and rate at which they were affected. The low number of mice used per inoculum was a result of animal ethics considerations and obviously limits the capacity of the assay to accurately reflect virulence and to differentiate between inocula with similar virulence. Despite these limitations, the considerable variation in numbers of mice affected and durations of infection observed allow for some interesting conclusions.

Comparison of the *number* of mice affected following footpad inoculation clearly indicated that inocula prepared from the brains were most virulent, affecting 100% of mice, while inocula from the submandibular and sublingual or parotid salivary glands affected similar proportions of only 47% and 43% respectively. Further comparison of how *quickly* mice were affected, also indicated that brain derived inocula were most virulent, with all mice being affected within 10 to 26 days, whereas mice inoculation with the salivary glands were generally affected after a longer and more variable period of 14 to 253 days. It also shows that, when considered together, mice inoculated with submandibular and sublingual salivary gland inocula were affected more quickly, (average 28 days, n=9/19, range 14 to 52 days) than mice inoculated with parotid derived inocula (average 82 days, n=9/21, range 25 to 253 days).

Broadly, these *in vivo* results are consistent with the quantitative TaqMan® assay results for viral RNA, where lower C_T values indicate greater amounts of RNA and an increase in C_T value of 3 points indicates an approximately 10 fold decrease in RNA amount. The TaqMan® assays indicated that the brain inocula all contained very similar amounts of RNA (mean C_T = 15.5, range 15.2 to 16.4) and substantially more than either type of salivary gland inocula (submandibular and sublingual mean C_T = 27.9, range 23.6 to 30.5; parotid mean C_T = 25.8, range 23.0 to 28.6). The substantially lower and less variable C_T values of the brain inocula were associated with high and less variable virulence compared to salivary gland inocula. However, among the salivary gland inocula, lower C_T values were not necessarily associated with higher virulence when comparing the two groups of salivary gland inocula, when comparing within groups of salivary gland inocula or when comparing the two salivary glands of individuals.

As a group, compared to the parotid derived inocula, submandibular and sublingual salivary glands had modestly higher C_T values, yet affected a slightly higher proportion of mice after generally shorter and less variable periods.

Among the submandibular and sublingual inocula, that of Bat 6 had the lowest C_T value (23.6) and highest virulence (3/3 mice), and that of Bats 1, 4 and 7 had higher C_T values (30.5, 29.0 and 29.0) and the lowest virulence (0/3 or 1/3 mice), yet that of Bat 5 with a similarly high C_T value (29.2) managed to kill 2 of 2 mice after reasonablly short periods (14 and 34 days). Similarly the most virulent of the parotid salivary gland inocula (Bat 4 C_T = 26.0 and Bat 5 C_T = 25.2) had C_T values that were higher than those of other less effective inocula, and parotid inocula with C_T values as low as 23.0 and 24.8 (Bats 1 and 2) either failed to induce disease or did so after an exceptionally long incubation period (Bat 1, 1/3 mice, 253 days).

While for some individual bats the salivary gland with the lower C_T value had the highest virulence (e.g. Bats 4 and 6), in others (e.g. Bat 2) the relationship was reversed.

Clearly the TaqMan® assay can be used to identify inocula of high virulence by identifying those that contain high amounts of ABLV RNA ($C_T < 16.5$). However, there was a very poor association between the C_T value and virulence among salivary gland inocula with C_T values of 23 to 31. In part this may be due the limited scope of the 3-mouse footpad assay to accurately reflect virulence, and it is also presumed to reflect the indirect relationship between the quantity of RNA and the quantity and characteristics of viable ABLV.

Experiment B and Experiment B Repeat also gave the opportunity to re-examine the prevalence and titres of lyssavirus antibodies in plasma with the AAHL rabies-RFFIT. That none of 36 mice that remained clinically well until the conclusion the experiments had detectable cross-reacting antibodies is consistent with Experiment 2 of Chapter 5 and the results of the bat inoculation study. In Experiment 2, only one of 13 survivor mouse sera was positive, and in the bat study two of three survivor bats had no clear evidence of seroconversion. Apparently survival in mice is usually not the consequence of an adequate humoral response, or at least not of one of sufficient strength or duration to be detected in the rabies-RFFIT 60 to 287 post-inoculation. It suggests that either these mice were not infected by the inoculum, or some other mechanism enabled the mice to remain well. Serial serological tests, preferably using an appropriate ABLV-variant RFFIT would more clearly demonstrate the absence of a humoral response in survivors.

Among clinically affected mice there was no apparent correlation between the presence, absence, or titre of anti-rabies antibodies and either the inoculum source bat or source tissue or duration of infection. The slight trend in Experiment 2 towards affected mice that did seroconvert having higher titres after longer incubation periods was not observed here and is probably coincidental.

To further investigate the results of the 3-mouse footpad assay, in particular to evaluate more precisely the relative virulence of inocula prepared from the brains of Bats 4 and 6 and the

submandibular and sublingual salivary glands of Bat 6, a 90 day mouse footpad titration assay was used (Experiment D) to calculate the clinical titre ($MFP_{90}ED_{50}$). As shown in Table 7-8, while C_T values of brain inocula from Bats 4 and 6 are very similar (C_T = 15.6 and 15.4 respectively) the relative virulence indicated by the number of mice affected and duration of infection in the 3-mouse assay and in the 6 mice inoculated with the neat inoculum as part of the titration, together with the calculated $MFP_{90}ED_{50}$, indicate that the inoculum of Bat 4 brain was moderately more virulent than that of Bat 6 brain ($MFP_{90}ED_{50}$ /mL = $10^{3.7}$ and $10^{3.0}$ respectively). In comparison the C_T value of the Bat 6 salivary inoculum (C_T = 27.6) was up to 12 points lower than that of either brain sample indicating there was ~10^4 times less RNA in the sample, yet the 3-mouse and 6-mouse virulence assays suggested it had similar virulence to the brain inocula and the more precise clinical titre indicated it had a similar or higher virulence ($MFP_{90}ED_{50}$ /mL = $10^{3.9}$).

Clearly this submandibular and sublingual gland-derived inoculum had a much higher virulence than suggested by the relative amount of viral RNA in the gland inoculum compared to that in brain inocula. The same was true for Inoculum 5 prepared from the salivary glands of ABLV-51 and used to inoculate the flying foxes (see Chapter 5, C_T value 18.6, $MFP_{90}ED_{50}$ /mL =$10^{6.0}$). Immunoperoxidase staining had also indicated that the source salivary glands had contained noticeably less visible viral antigen than the brains. It appears that the virulence : viral RNA and virulence : viral antigen ratios for inocula derived from the submandibular and sublingual salivary glands can be very high compared to those of the brain.

Why this should be the case remains unclear. It may be the result of the presence or absence of some ‘contaminating’ factor from the source tissue affecting either the viability of ABLV in the tissues prior to or after preparation of the inoculum, or assisting in the establishment of infection once inoculated. It may be due to differences in the replication strategy in the salivary gland resulting in a higher proportion of replicated RNA and viral proteins being incorporated into viable and virulent virus particles rather than accumulating in non-viable but detectable forms. Or it may be due to some as yet uncharacterized specific and functional difference in the actual virus particles themselves when formed or accumulated in salivary glands. For example there may be subtle differences in the lipoprotein component of the virus capsule that is derived from the host cell in the salivary gland cell that confers greater viability or virulence, such as the capacity to support more or less glycoproteins. It may also be due to differences in the pressures for clonal selection of the virus population within the salivary gland resulting in the salivary gland ABLV population containing quasi-species with higher virulence characteristics. Unfortunately, further investigation of this apparent paradox was beyond the scope of this project.

As the relationship between the C_T value and virulence of inocula prepared from brains and salivary glands appear to differ, it is proposed that different TaqMan® C_T criteria be used to select inocula derived from each tissue source.

In order to select inocula likely to have a $MFP_{90}ED_{50}$ /mL $\geq 10^{3.0}$, priority should be given to

- brain inocula with a C_T value ≤ 16, and
- salivary gland inocula with a C_T value ≤ 24.

Had such a combination of thresholds been used on the candidate inocula prepared in Chapters 5 and 7, it would have led to selection of 12 of the 45 candidates for further characterization, and substantially reduced the amount/number of mice and other resources required. It should be noted that, while all inocula with high virulence would have been selected (notably Inoculum 5, brain inocula of Bats 1 to 7, and Bat 6 submandibular/sublingual gland inoculum), and all the inocula that would have been excluded had low virulence, not all the inocula that would have been selected had high virulence. *In vivo* characterization of TaqMan®-selected candidates is still required to establish which of the selected inocula have high or the highest virulence when inoculated peripherally.

The footpad titration assay also offered the opportunity to observe the effect of dose on incubation period. As seen in the titration of Inoculum 5, higher dilutions were generally associated with either longer incubations times and/or fewer mice being affected. This was particularly true where data for three or more affected mice were available (dilutions Neat, 10^{-1}, and 10^{-2}). At higher dilutions a few mice died after relatively short periods. Had the experiment run longer than 90 days and data from mice that may have gone on to become ill after longer periods been included, continuation of the linear effect of dose on average incubation period at higher dilutions may have been apparent. In general it suggests that hosts dying of ABLV after very long incubation periods, such as in the second human patient who died 27 months after her only reported exposure to a bat (Hanna *et al.* 2000), had been infected with very low doses of virus.

It is of particular interest that two footpad-inoculated mice became sick after very long incubation periods and were killed 156 and 253 days post-inoculation. The mouse that was killed after 253 days was the only mouse in that cage to become ill, precluding the possibility that the long incubation period was the result of re-exposure to an affected cage mate. The data from these mice suggests a model of long-incubation ABLV could be developed. Together with the apparently long incubation period of the second human patient, they indicate it is possible that low dose long-incubation infections with ABLV may play a significant role in the maintenance of ABLV in populations.

It should be noted that, while ABLV can be readily transmitted via salivary gland-derived inocula, and such inocula may have relatively high virulence : viral RNA and virulence : viral antigen ratios, this does not of itself indicate any special role of the submandibular and/or sublingual salivary

glands in natural transmission. Immunoperoxidase staining of the salivary glands detected ABLV in two sites; submandibular salivary gland epithelial cells and the ganglia and nerve fibres of the associated parasympathetic innervation of all three salivary glands. It is unclear what the characteristics and relationships of virus present in these two sites are. It is unclear to what extent virus from either or both locations may contribute to virus excretion in saliva and the role either or both may play in transmission. It is unclear what effect the method of extraction of virus from the salivary glands into the inoculum (post-mortem freezing of glands, defrosting, maceration and clarification in media) has on the quantities of virus from each site in the inocula. It is possible that the high virulence of salivary gland inocula is simply a useful artefact of inoculum preparation and that it is no more an indicator of a role in transmission than the ability to infect others with brain derived inocula indicates ABLV is transmitted via brain tissue. This does not; however detract from the usefulness of using high virulence inocula prepared from the salivary glands in experimental studies.

It is reasonable to assume ABLV is transmitted via saliva contamination of bites and scratches, based on the histories of bat bites in the two human cases and the data available for rabies viruses. However, confirmation that this is the only or most important method of transmission responsible for the maintenance of ABLV in bat populations will require studies on the infectivity of saliva, urine, and other potential modes of transmission and controlled transmission studies between individuals. Given the possible role of low dose, long incubation infections in the population, recognition of important but subtle modes of transmission may be difficult.

7.5 Conclusions

Confirmation by virus isolation of the presence or absence of ABLV in 10 experimentally inoculated flying foxes

- Infection with ABLV in seven experimentally infected, clinically ill flying foxes was confirmed by virus isolation in mice.
- ABLV was not isolated from the brains or salivary glands of three experimentally inoculated flying foxes that remained clinically well until the end of the experiment on PI-day 80 or 82.
- Intracerebral inoculation of suckling mice is a more sensitive method of virus isolation of wild-type ABLV than the 3 mouse footpad assay.
- There is some evidence that intracerebral inoculation of suckling mice is a more sensitive method of virus isolation of wild type ABLV than *in vitro* isolation in neuroblastoma cells.

Comparison of virulence of inocula prepared from the brain, parotid, or combined submandibular and sublingual salivary glands

- The 3-mouse footpad assay appears to be a reasonable method of assessing peripheral virulence using minimal numbers of animals and other resources.
- In the 3-mouse footpad assay, both the number of mice affected and the speed in which they are affected reflect the virulence of the inocula.
- Evaluation of the number *and* speed at which mice were affected indicates that the relative virulence of inocula prepared from the brain and salivary glands are as follows.

  Brain ≥ submandibular/sublingual salivary gland > parotid salivary gland
- There is a more or less linear relationship between dose and incubation period in footpad-inoculated mice, with lower doses associated with longer incubation periods.

Relationship of the relative amount of viral RNA detected by quantitative PCR (TaqMan®) assay, to the virulence of inocula in mice, and the potential for use of quantitative PCR in the selection of inocula

- Using tissues that had been collected and stored in near optimal and very similar conditions, inocula prepared from the brains of seven ABL-positive bats contained substantially more viral RNA (10^2 to 10^5 times) than the salivary glands of the corresponding individual.
- Despite inocula prepared from the submandibular and sublingual salivary glands containing substantially less viral RNA, they may be highly virulent in mice, and more virulent that inocula prepared from the corresponding brain.
- The TaqMan® quantitative PCR assay can be used to select inocula that are more likely to be highly virulent using different selection criteria for inocula prepared from different sources.

- Proposed selection criteria are:
  Brain derived inocula TaqMan® $C_T \leq 16$
  Submandibular and sublingual salivary gland inocula TaqMan® $C_T \leq 24$
- Not all inocula selected using these criteria will be highly virulent and virulence should be evaluated in appropriate *in vivo* models.

Other conclusions

- Despite salivary glands containing much less visible viral antigen by immunoperoxidase staining, inocula prepared from these glands, in particular inocula prepared from the submandibular and sublingual salivary glands may be highly virulent in mice.
- The anti-lyssavirus titre, as detected in the CVS-RFFIT, is of no predictive value in the assessment of potentially exposed individuals, with both positive and negative titres being detected in both clinically ill and clinically well exposed mice and bats.
- There is no apparent relationship between the incubation period and the presence, or absence, or titre of antibodies detected in the CVS-RFFIT.

8 Vaccination of Black and Grey-headed flying foxes with Nobivac rabies vaccine

Aims

1. To demonstrate that flying foxes vaccinated with commercial rabies vaccines would seroconvert and develop titres against rabies virus.

2. To demonstrate that rabies-vaccinated flying foxes developed cross-neutralising titres against ABLV.

3. To co-vaccinate flying foxes with a marker protein that would induce seroconversion that could be used to differentiate rabies-vaccinated flying foxes from unvaccinated flying foxes with naturally acquired ABLV-antibodies.

4. To develop an enzyme linked immunoabsorbant assay to detect seroconversion to the marker protein.

8.1 Introduction

Human exposure to ABLV has occurred through contact with unwell wild bats (see Chapter 2), an orphan in temporary care (ABLV-32), and a long term resident of a wildlife park (ABLV-73). To date management of the risk of ABLV from bats has relied on pre- and post-exposure vaccination of potentially exposed humans and increasing public awareness of the need to avoid contact with bats. The high incidence of urban rabies in humans in developing countries, and the very low but persistent incidence of sylvatic rabies in developed countries such as the USA, demonstrate the limitations of this approach. Vaccination and animal control programs in the United Kingdom, Europe and USA have, by reducing the susceptibility and prevalence of rabies in dogs and foxes further reduced the incidence of rabies in humans. Unfortunately neither control nor vaccination of the general bat population is feasible and no mode of vaccine delivery to wild bats is available. This is not the case for captive bats.

Effective vaccination of captive bats, particularly those in medium term or permanent captivity would have two benefits. It would prevent the development of clinical ABLV in captive bats, such as ABLV-32 and ABLV-73, and so prevent potential human exposure to ABLV from such cases. It would also allow the bat's welfare to be considered in the event of a person being bitten or scratched by a vaccinated captive bat. Current Queensland Health Department policies require any captive bat that bites or scratches a person be killed and submitted for ABLV testing. As a result, at least eight clinically well, ABLV-negative, captive flying foxes have been killed following

reports of them having bitten or scratched humans, causing considerable distress to the staff and keepers (data not shown). Other potential exposures have gone unreported. At some facilities entire colonies of healthy bats were killed as a result of concerns about potential exposure of staff or the public to ABLV. Effective vaccination of captive flying foxes would allow bites or scratches from captive bats to be considered *not* to be potential exposures to ABLV and not requiring surrender of the bat. Staff and the public could again enjoy 'safe' contact with captive bats.

Vaccination trials at CDC demonstrated cross-protection against ABLV in mice challenged after vaccination with commercial rabies vaccine (Hooper *et al.* 1997b). It seemed highly likely that commercial rabies vaccines could provide similar protection to vaccinated bats. A further consideration was that vaccinated bat plasma be distinguishable from unvaccinated bat plasma as the prevalence of naturally occurring lyssavirus antibodies was being studied and had implications for current and ongoing research into the prevalence and distribution of ABLV in Australia. This study evaluated the serological responses of Black and Grey-headed flying foxes to the commercial animal rabies vaccine available in Australia (Nobivac®) and the effectiveness of a serological marker (keyhole limpet hemocyanin) in distinguishing vaccinated from unvaccinated bats.

8.2 Materials and Methods

8.2.1 Permits

Australian Quarantine Inspection Service (AQIS)

- Approval for *in vivo* use of Restricted Imported Biological Material (Nobivac inactivated rabies vaccine) in Non-laboratory Animals Permit # 00/066
- Quarantine approved premises Q1056, Bat Holding Facility at the University of Queensland Farm, Pinjarra Hills.

Queensland National Parks and Wildlife service

- Scientific purposes permit number W4/002537/00/SAA

Animal Ethics Committees

- University of Queensland – approval number VP/232/00/PHD
- Animal Research Institute – approval number ARI/030/2000-1

8.2.2 Flying foxes

Thirteen (13) Black flying foxes (*Pteropus alecto*) and 24 Grey-headed flying foxes (*Pteropus poliocephalus*) were caught from the Indooroopilly Island colony, Brisbane and transferred to the University of Queensland bat facility at Pinjarra Hills, where they were held as a single colony for the trial (see Figure 8-1). Bats were held for observation within the facility for 52 to 60 days prior to vaccination on PV-day 0 (31 July 2000).

8.2.3 Microchips

Bats were individually identified on the day of capture with LIFECHIP™ NDL assembly or TROVAN Passive Transponder microchips, implanted subcutaneously between the scapulae, at the base of the neck. The identifying microchip number was read using a Pocket•Reader *EX*™ (Destron Fearing Corporation). The microchip number for each bat is listed in Appendix 11.

8.2.4 Rabies vaccine

The Nobivac® Rabies inactivated rabies vaccin (*sic*) Lot 79050B (Intervet, Boxmeer, Holland) contains a beta-propriolactone inactivated culture of Pasteur RIVM strain rabies virus, grown on the BHK-21 clone cell line, in an aqueous aluminium phosphate suspension. The vaccine is widely used in rabies-endemic countries for vaccination of dogs, cats and other domestic animals. Its use in Australia is restricted to pre-export vaccination of domestic animals. The vaccine was purchased as individual (1 mL) doses per vial.

8.2.5 Keyhole Limpet Hemocyanin (KLH)

Keyhole limpet hemocyanin (Sigma, Saint Louis, Missouri USA) is a highly immunogenic protein prepared from the hemolymph of the mollusc *Metathura crenulata* (“keyhole limpets”). Two mL of deionised water (dH_2O) was added to each 20 mg vial to prepare a stock solution of 10 mg/mL.

8.2.6 Alhydrogel

Alhydrogel (Superfos a/s, Vedbaek, Denmark) is a stable gel containing 6 mg/mL of aluminium hydroxide with a pH of the order of 6 to 6.5. It is a well-established adjuvant used for vaccine production. The alhydrogel used in this study was kindly provided by Ian Wilkie (The University of Queensland).

Figure 8-1 Flying foxes within the bat holding facility
Black and Grey-headed flying foxes held as a single mixed colony. Each afternoon fresh fruit and water were provided in multiple buckets throughout the cage to reduce the likelihood that dominant or territorial individuals would prevent others from feeding. Hessian bags were hung to provide soft, mobile landing points. Natural tree-limb ‘perches’ were installed to provide alternate ‘footing’ to the cage wire. This was almost universally ignored, bats preferring to hang from the highest possible (wire) position.

8.2.7 Experimental design

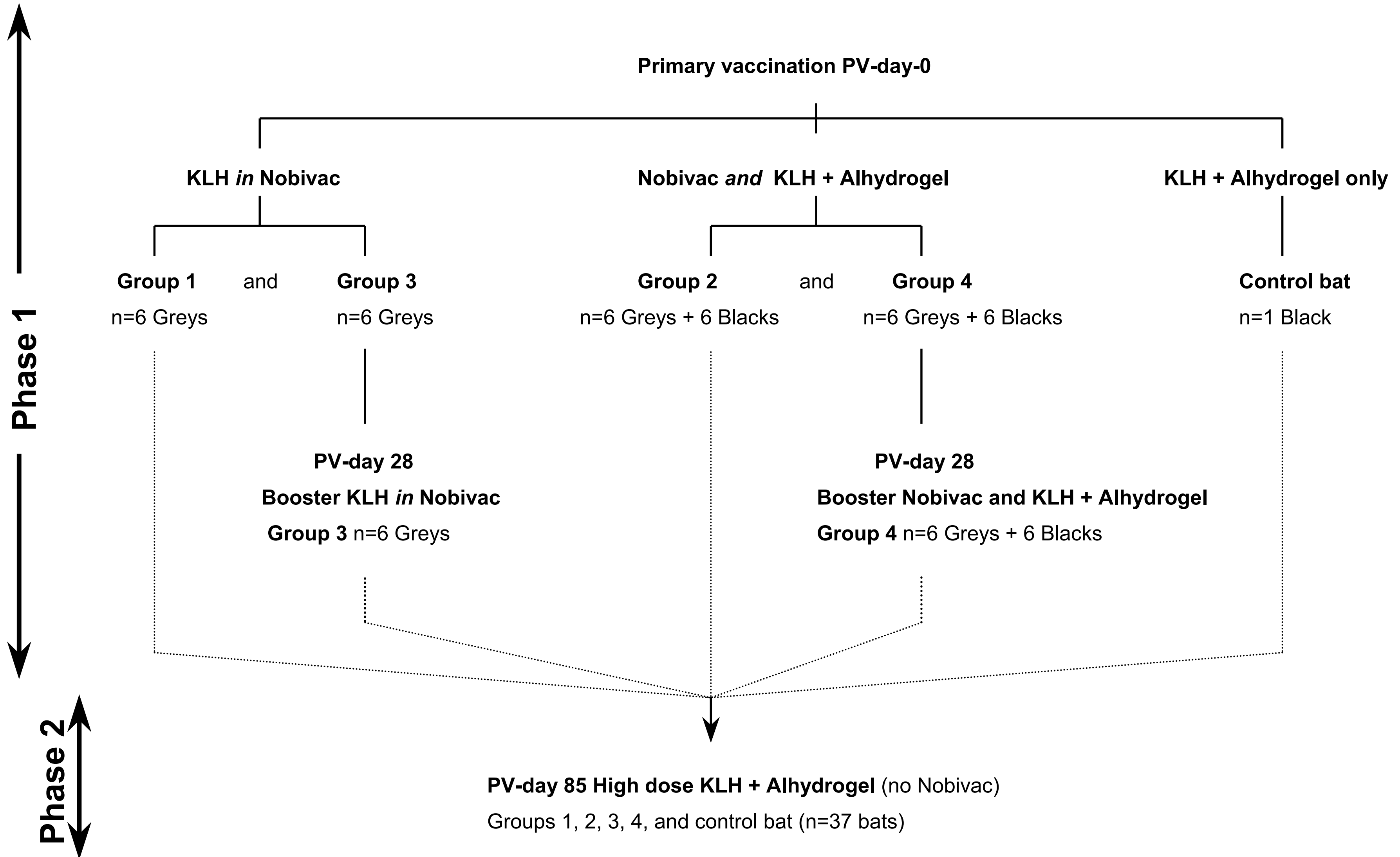
This project was done in two phases.

- **Phase 1**
  Thirty-six bats were allocated in a random block design into one of four treatment groups balanced as far as possible for sex and age (see Table A11-1 in Appendix 11). Bat species were allocated to groups as indicated in Table 8-1. Each bat was vaccinated with one of two rabies vaccine protocols and one of four KLH ± Alhydrogel protocols as described below. One control bat was vaccinated with KLH + Alhydrogel only (no Nobivac).

- **Phase 2**
  Because the response to KLH during Phase 1 was inadequate, all bats received a higher dose KLH + Alhydrogel booster on PV-day 85.

An overview of the experimental design is illustrated in Figure 8-2.

Figure 8-2 Experimental design for the vaccination of Black and Grey-headed flying foxes with Nobivac rabies vaccine and KLH



8.2.8 Rabies vaccination

Thirty-six of the 37 flying foxes were vaccinated intramuscularly in the right pectoral muscle with Nobivac® Rabies inactivated rabies vaccine either;

- on PV-day 0 only (n=18, Groups 1 and 2) or
- on PV-day 0 and a booster on PV-day 28 (n=18, Groups 3 and 4).

One control bat was not vaccinated with rabies vaccine.

The rabies vaccine was administered either mixed with KLH or given separately as described below.

8.2.9 KLH ± Alhydrogel vaccination

8.2.9.1 Phase 1: Rabies and KLH

During Phase 1, four protocols for KLH vaccination, using a dose of 100 μg, were used (see Table 8-1 and previous overview of experimental design in Figure 8-2).

1. **KLH *in* Nobivac – PV-day 0 only (n = 6, Group 1)** 100 μg KLH in 0.1 mL dH_20 added to the vial of Nobivac, administered together as a single intramuscular injection

2. **KLH + Alhydrogel – PV-day 0 only (n = 13, Group 2 and control bat)**
   100 μg KLH in 0.1 mL dH_20 combined with 0.1 mL Alhydrogel, total 0.2 mL vaccine administered as a single intramuscular injection to the left pectoral muscle. Nobivac rabies vaccine administered separately into the right pectoral muscle of 12 bats (Group 2).

3. **KLH *in* Nobivac – PV-day 0 + booster PV-day 28 (n = 6, Group 3)**

4. **KLH + Alhydrogel – PV-day 0 + booster PV-day 28 (n = 12, Group 4)**

Table 8-1 Phase 1 Experimental design: Nobivac rabies vaccine and KLH ± Alhydrogel PV-day 0 to PV-day 85 (n=37)

Bat species	KLH *in* Nobivac		Nobivac and KLH + Alhydrogel		KLH + Alhydrogel *only* (no Nobivac)
	Day 0 only	Day 0 + 28	Day 0 only	Day 0 + 28	Day 0 only
Black flying fox	Nil	Nil	6	6	1 (control)
Grey-headed flying fox	6	6	6	6	Nil

8.2.9.2 Phase 2: High-dose KLH with Alhydrogel

On PV-day 85 all bats (n = 37) received 1 mg KLH in 0.1 mL dH_20 combined with 0.2 mL Alhydrogel, total 0.3 mL vaccine administered intramuscularly i.e. a 10 fold increase in antigen and double the adjuvant compared to the Phase 1 KLH protocols. No additional rabies vaccine was administered.

8.2.10 Sample collection

Bats were captured for blood collection on PV-days 0, 7, 14, 21, 28, 42, 56, 85, 99, 127 and either PV-day 171 or 172. Bats within the enclosure were captured by Craig Smith, wrapped in a towel and held hands-free on the 'bat-board' (flat wooden board, ~34 x 17 cm with thick flexible rubber puncture-proof flap that could be secured over the bat on both sides of board, see Figure 8-3, developed by Hume Field, ARI). They were positioned so that their hind limbs were free, with the body, wings, and head secured, allowing 'hands free' access to the hind limbs while preventing injury to the bat or candidate. Blood (1 to 2.5 mL/ adult, up to 0.5 mL/ pup (baby flying fox)) was collected from the uropatagial vein into heparinised syringes, centrifuged at ~800 G for 10 minutes, and the plasma removed and aliquoted.

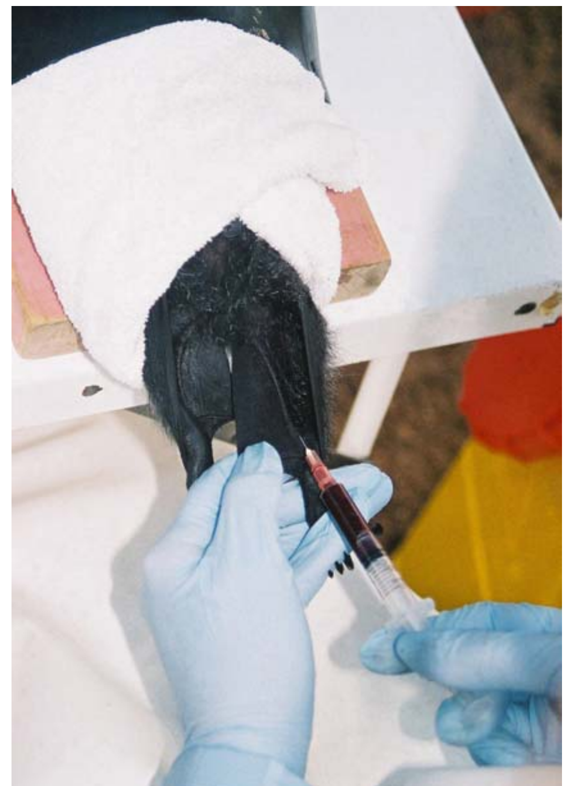
Figure 8-3 Restraint of conscious bat with 'bat-board' for venipuncture

Figure 8-3A Bat caught using protective gloves and clothing and restrained so as to control the head, both wings, and both hind feet.

Figure 8-3B Bat wrapped snugly in a small towel, with the hind feet protruding, being placed in dorsal recumbency on the 'bat board'. The rubber cover of the 'bat board' (black, at right) was then secured over the bat and towel. Without use of the bat board the bat would rapidly escape the towel.

Figure 8-3C (left) Bat secured within the 'bat board', hind limbs protruding.

Figure 8-3D (right) Venipuncture of the uropatagial vein.



8.2.11 Lyssavirus serology

Rabies serology

Plasma stored at 4 °C was submitted to the AAHL for detection of rabies virus titres in the modified rabies (CVS)-$RFFIT_{AAHL}$ (see Appendix 2).

To determine the variability of individual test results, 82 sera with titres in the range of 0.3 to 31.5 IU/mL were submitted and tested on two occasions and the variance between the paired results calculated by Tony Swain (ARI) using GenStat (GenStat 2000). Values were log transformed prior to calculating the variance in order to be consistent with the statistical analysis of the study data.

The back-transformed standard deviation of an individual rabies-RFFIT result was 0.19 IU/mL.

ABLV serology

Twenty-two plasma samples with low (0.2 to 0.7 IU/mL, n=7), moderate (2 to 5 IU/mL, n=8) or high (> 5 IU/mL, n=7) CVS-rabies-$RFFIT_{AAHL}$ titres, were stored at –20°C and later submitted to the Rabies Laboratory, CDC Atlanta, Georgia, USA for CVS-rabies-$RFFIT_{CDC}$ and pt-ABLV-$RFFIT_{CDC}$ (see Appendix 2 for test details).

8.2.12 KLH Serology

Plasma was stored at –20 °C prior to use in the KLH ELISA.

Seroconversion to KLH was detected using an indirect enzyme linked immunoabsorbant assay (ELISA) developed under the direction of John Molloy (ARI). Nunc 96-well microtitre plates (Nunc Maxisorb, Nunc Roskilde, Denmark) were coated with 100 μl/well of KLH antigen (Sigma, Saint Louis, Missouri USA) diluted 1/2,500 in carbonate buffer pH=9.6 at 4 °C overnight. All subsequent incubations were at room temperature. Excess antigen was discarded and the plate blocked with 200 μl/well of 2% casein in carbonate buffer pH = 9.6 for a minimum of 1 hour. After a brief rinse (3 × 200 μl/well) with phosphate buffered saline containing 0.1% Tween 20 (PBST20), plates where incubated with 100μl/well of plasma diluted 1/200 in PBST20 containing 2% skim milk powder for 30 minutes with continuous gentle shaking. Plates were washed with PBST20 (3 × rinse, 1×5-min soak with shaking, 3 × rinse) then incubated with 100 μl/well of peroxidase-conjugated recombinant Protein G (Zymed, San Francisco, California USA) diluted 1/ 500,000 in PBST20 containing 2% skim milk powder for 30 minutes with continuous gentle shaking. After washing, 100 μl of TMB peroxidase substrate (KPL, Gaithersburg, Maryland USA) was added to each well and the plates incubated for 8 minutes, at which point the absorbance of the positive control standard wells approximated an optical density of ≥1. The reaction was stopped by the

addition of 50 μl of 2M phosphoric acid and the absorbance read at 450 nm. Positive and negative control plasma and blanks (nil plasma) were included on each test plate and all samples were tested in duplicate/plate. The percent absorbance (PA) for test samples was calculated relative to the positive control standard plasma where:

$$\text{Test PA} = \frac{\text{mean test absorbance} - \text{mean blank absorbance}}{\text{mean positive control absorbance} - \text{mean blank absorbance}} \times 100$$

8.2.12.1 Variability of the KLH-ELISA

Sixteen plasma samples with low (PA <20 n=4), intermediate (20 < PA >60, n=8) or high (PA >80, n=4) KLH-titres were tested on four separate occasions. The variability of each result was determined as the sum of the estimated between-plate and within-plate components of variance. The components were estimated by analysis of variance using a residual maximal likelihood model with GenStat 2000.

The standard deviation of an individual test result was:

- Low titre (PA < 20) standard deviation = 1.6
- Intermediate titre (PA= 20 to 60) standard deviation = 4.3
- High titre (PA > 80) standard deviation = 4.8

8.2.12.2 KLH-ELISA positive / negative result threshold

To determine the positive/negative result threshold for KLH titres in the ELISA, 200 sera from wild, presumed unvaccinated and KLH-negative, Black and Grey-headed flying foxes were tested. They included pre-vaccination sera (PV-day 0) from the bats used in this vaccination experiment (n=37) and 163 other stored (-20°C) wild-caught bat sera collected in Southeast Queensland by Hume Field (ARI) and Craig Smith (ARI) for other serological studies, see Table 8-2.

Table 8-2 Wild-caught unvaccinated-bat sera used to determine the positive/negative result threshold for KLH-ELISA titres

Source of Sera	Grey-headed flying foxes	Black flying foxes
Expt. bats PV-day 0	24	13
Other bats (stored sera)	76	87
Total	100	100

8.2.13 Statistical analysis

The statistical package GenStat 2000 was used for all analyses under the direction of Tony Swain (ARI).

Response to rabies (Nobivac) and KLH vaccination

The effects of vaccination method (KLH *in* Nobivac versus Nobivac *and* KLH + Alhydrogel), booster vaccination on PV-day 28, age (juvenile versus adult) and their interactions on the rabies-RFFIT or KLH-ELISA titres at each sample day were tested by analysis of variance using a general linear model. Species differences (within the KLH + Alhydrogel, adult bat category), the interaction with booster vaccination and the main effect of sex (female versus male), were also included in the model.

Values for rabies-RFFIT titres were log transformed prior to analysis after examination of the residuals from analysis of the original test values showed increasing variation with increasing means.

Values for KLH titres were analysed untransformed.

Estimated duration of positive KLH titres

The length of time that KLH-titres in individual flying foxes could be expected to remain positive (KLH ELISA titre >20 PA) following either Phase 1 vaccinations alone or following the subsequent PV-day 85 high-dose KLH + Alhydrogel booster was estimated by Tony Swain (ARI) by mathematical extrapolation (Tukey 1977) from the known titres using GenStat 2000.

The duration of positive titres following Phase 1 vaccination alone was estimated by fitting an exponential regression line to the declining titres from PV-days 21, 28, 42, 56 and 85 for bats in Groups 1 and 3, which had received a single primary vaccination, and by linear extrapolation of the declining titres following the PV-day 28 booster (titres from PV-days 42, 56, and 85) for Groups 2 and 4. These calculations estimated how long titres could have been expected to have remained positive had the Phase 2 high dose KLH + Alhydrogel booster *not* been given on PV-day 85.

The estimated duration of positive titres following the high-dose KLH + Alhydrogel booster on PV-day 85 was estimated by linear extrapolation of titres from PV-days 99, 127 and 171.

8.3 Results

All bats responded to the Nobivac rabies vaccine and the KLH immunogen, however the extent and duration of seroconversion varied between individuals and with the vaccination protocol.

8.3.1 Response to vaccination with Nobivac

Details of each bat (microchip number, species, sex, age, capture weight, treatment group, n=37) and the rabies-RFFIT titres are shown in Appendix 11. Plots of the individual responses within each group are also shown in Appendix 11.

8.3.1.1 Nobivac response: Crude summary

The mean responses of treatment groups 1, 2, 3 and 4, for each species are summarized in Figure 8-4 and Figure 8-5. There were no treatment differences between Groups 1 and 3 (KLH *in* Nobivac) or Groups 2 and 4 (Nobivac and KLH + Alhydrogel) until PV-day 28, at which time Groups 3 and 4 received their respective booster vaccinations, so data for these pairs of groups have been combined for PV-days 0 to 28.

Figure 8-4 Mean response to Nobivac vaccination in Grey-headed flying foxes
(vertical bars ± 1 standard error of mean calculated separately for each plotted data point)

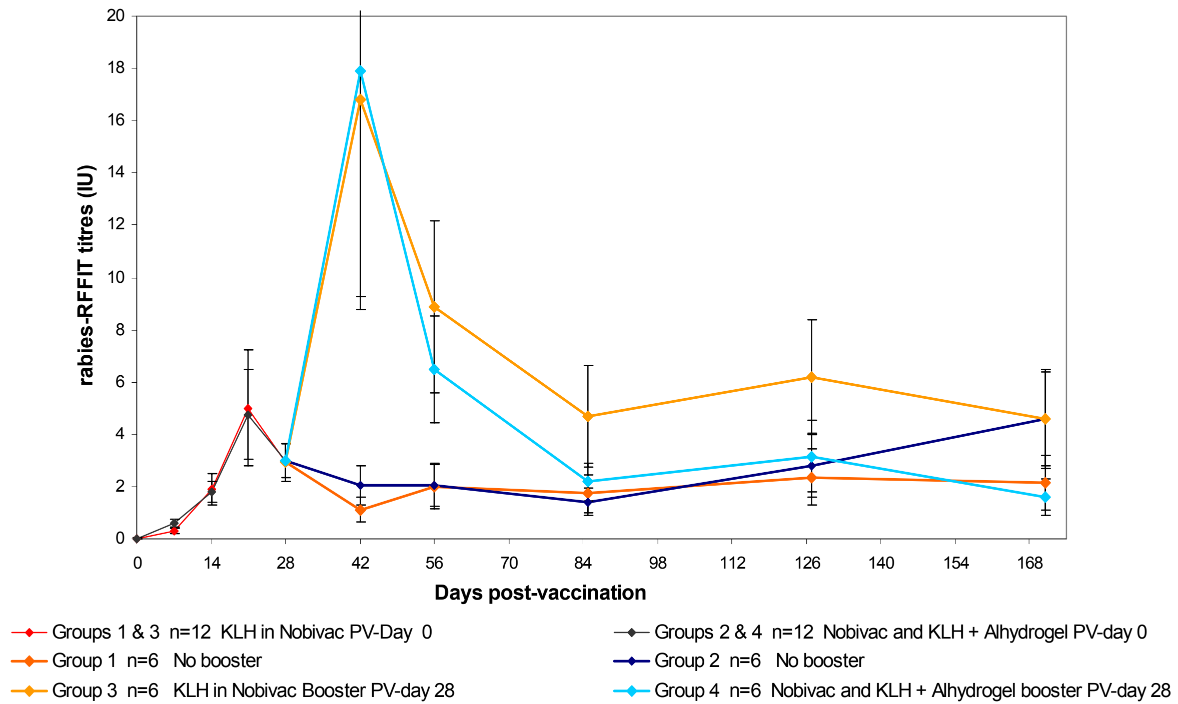
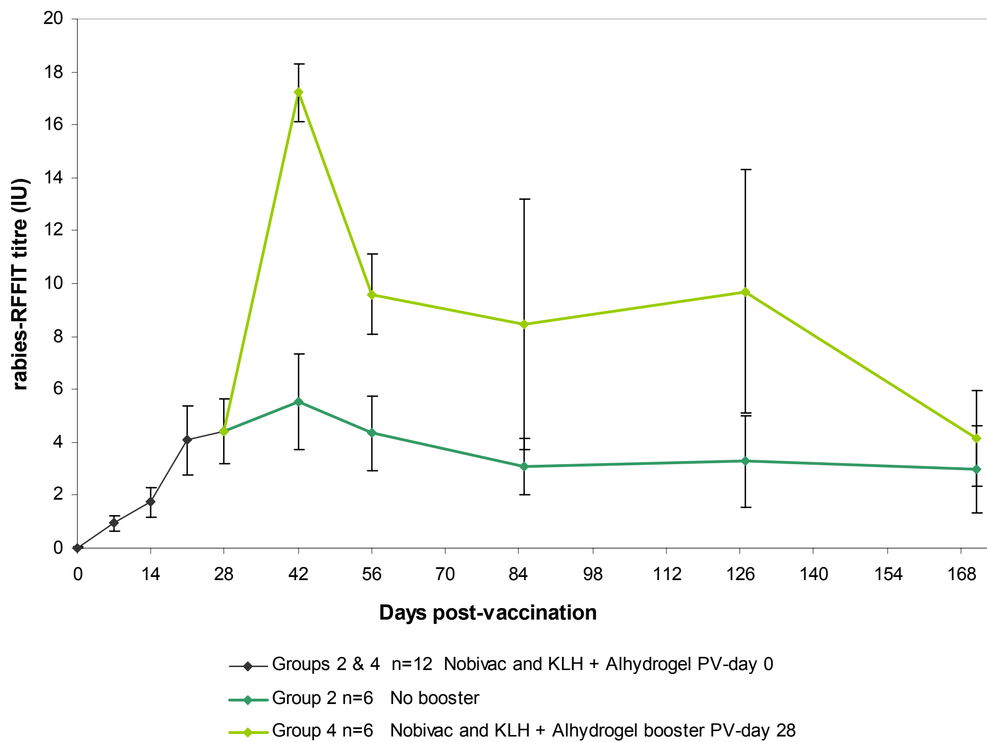


Figure 8-5 Mean response to Nobivac vaccination in Black flying foxes
(vertical bars ± 1 standard error of mean calculated separately for each plotted data point)



8.3.1.2 Nobivac response: Analysis of variance

Analysis of variance indicated that five factors; age, sex, species, booster vaccination, and method had statistically significant effects on rabies-RFFIT titres (conditional probability < 0.05).

- **Age: Juveniles > Adults**
  Titres were higher in juveniles compared to adult flying foxes on PV-days 14, 21 and 171.
- **Sex: Females > Males**
  Titres were higher in females compared to male flying foxes on PV-days 14, 85 and 171
- **Species: Blacks > Grey-headed flying foxes**
  Species comparisons could only be made among adult flying foxes vaccinated with Nobivac *and* KLH + Alhydrogel, as no juvenile Blacks or Blacks vaccinated with KLH *in* Nobivac were included in the study. Within this class, titres were higher in Black flying foxes compared to Grey-headed flying foxes on PV-days 14 and 85.
- **Booster on PV-day 28 > No booster**
  Booster vaccination produced higher rabies titres on PV-days 42 and 56 only.
- **Method: Nobivac and KLH + Alhydrogel > KLH *in* Nobivac**
  Concurrent vaccination with KLH + Alhydrogel as a separate injection produced a higher rabies-RFFIT titre on PV-days 7 and 42 compared with vaccination with KLH *in* Nobivac.

Estimated mean titres for statistically significant factors (conditional probability < 0.5), adjusted with respect to other factors, are shown in Table 8-3.

Table 8-3 Nobivac response: significant factors
Back-transformed[1] fitted mean rabies-RFFIT$_{AAHL}$ titres (IU/mL) for statistically significant factors as predicted by the regression model (conditional probability < 0.5)

Significant factors		PV-day 7	PV-day 14	PV-day 21	PV-day 42	PV-day 56	PV-day 85	PV-day 171
Age	**Adult**	---	0.8**	1.8	---	---	---	1.3
	Juvenile	---	3.0**	6.5	---	---	---	2.9
Sex	**Male**	---	0.6**	---	---	---	1.1	1.4
	Female	---	1.7**	---	---	---	1.8	1.8
Species[2]	**Grey-headed**	---	0.5	---	---	---	0.7**	---
	Black	---	1.4	---	---	---	3.6**	---
Booster	**None**	n/a	n/a	n/a	1.2**	1.3	---	---
	PV-day 28	n/a	n/a	n/a	11.2**	4.7	---	---
Method	**KLH *in* Nobivac**	0.2	---	---	1.5	---	---	---
	KLH + Alhydrogel	0.4	---	---	3.6	---	---	---

[1] anti-log 10
[2] species comparisons only available among adult bats vaccinated with Nobivac *and* KLH + Alhydrogel (Groups 2 and 4)
--- not statistically significant
n/a not applicable as booster factor not applied until PV-day 28
** conditional probability < 0.01

In general, the interactions between these factors were not statistically significant ($p > 0.5$). However on PV-days 56, 85 and 171 there was a significant interaction between vaccination method and age, with the response associated with the two methods (KLH *in* Nobivac compared with Nobivac *and* KLH + Alhydrogel) being dependant on age. In juveniles the response was statistically higher using the KLH *in* Nobivac method and the response in adults was statistically higher using the Nobivac *and* KLH + Alhydrogel method (see Table 8-4).

Table 8-4 Nobivac response: significant interactions
Back-transformed[1] fitted mean rabies-RFFIT titres (IU/mL) for statistically significant interactions of factors as predicted by the regression model (conditional probability < 0.5)

PV-day	Method	Juvenile	Adult
56	**KLH *in* Nobivac**	9.4	0.6
	Nobivac and KLH + Alhydrogel	3.6	2.4
85	**KLH *in* Nobivac**	4.9	0.8
	Nobivac and KLH + Alhydrogel	1.6	1.4
171	**KLH *in* Nobivac**	4.6	0.9
	Nobivac and KLH + Alhydrogel	2.0	1.8

[1] anti-log 10

8.3.1.3 Comparison of titres against rabies and ABLV in rabies vaccinated bats

There was a good correlation between the CVS-RFFIT results for the same sera when done at AAHL and CDC, Atlanta.

There was good evidence for cross-reactive neutralisation of ABL in the pt-ABL-$RFFIT_{CDC}$. In fact, for all sera with moderate (> 2 to < 5 IU/mL) or high (> 5 IU/mL) CVS-RFFIT titres, the 50% endpoint dilution against pt-ABL_{CDC} was higher than that for CVS-11_{CDC} rabies virus. However, this may simply reflect poorer cell-adaptation and growth characteristics of the pt-ABL strain used. In the absence of a pt-ABL plasma standard, and without the results being expressed relative to the 50% endpoint dilution of the rabies 2 IU/mL standard (control) plasma (results not provided by CDC), it is difficult to assess the relative neutralising cross reactivity neutralization of this rabies–vaccinated plasma against pt-ABL. The CVS-RFFIT results from both AAHL and CDC, Atlanta, as well as the pt-ABL-RFFIT results from CDC are shown in Table 8-5.

Table 8-5 Comparison of titres against rabies and ABLV in response to Nobivac

Bat No. PV-Day	Species. Sex. Age	Booster PI-day 28		CVS rabies $RFFIT_{AAHL}$ (IU/mL)	CVS rabies $RFFIT_{CDC}$ (titre)	pt-ABLV $RFFIT_{CDC}$ (titre)
CIB.D56	Black.Male.Adult	No	Low AAHL titre (<1 IU/mL)	0.2	7	NR
CIB.D85	Black.Male.Adult	No		0.5	11	NR
361.D85	Grey.Male.Juvenile	No		0.5	11	<11
A24.D85	Grey.Female.Adult	Yes		0.5	9	NR
11A.D85	Grey.Female.Adult	No		0.6	11	<11
51D.D85	Grey.Female.Adult	Yes		0.6	9	<11
E64.D85	Grey.Female.Adult	Yes		0.7	9	NR
256.D85	Grey.Male.Adult	No	Moderate AAHL titre (>2.0 to <5 IU/mL)	2.5	25	NR
E64.D56	Grey.Female.Adult	Yes		2.8	13	NR
565.D85	Black.Female.Adult	Yes		2.9	56	280
10B.D85	Black.Male.Adult	Yes		2.9	16	NR
F43.D56	Black.Male.Adult	Yes		3.0	42	NR
B32.D85	Black.Male.Adult	No		3.1	33	NR
84D.D85	Grey.Female.Adult	No		3.1	60	224
179.D85	Grey.Female.Juvenile	No		3.1	70	353
E4B.D56	Black.Female.Adult	No	High AAHL titre (>5.0 IU/mL)	5.5	56	214
778.D56	Grey.Male.Juvenile	No		5.7	40	154
179.D56	Grey.Female.Juvenile	No		5.9	50	665
D03.D56	Grey.Male.Juvenile	Yes		5.9	50	418
B43.D56	Grey.Female.Adult	Yes		6.1	125	771
565.D56	Black.Female.Adult	Yes		7.3	70	270
119.D56	Grey.Female.Juvenile	Yes		12.5	>390	1100

Bat No.PV-Day samples of plasma are identified by the unique last three digits of the bat's microchip number and the day post-primary vaccination on PV-day 0 that the sample was taken e.g. CIB.D56 is the sample from the bat with the microchip having the last three digits of CIB taken on PV-day 56.

AAHL results: expressed as international units, i.e. relative to 2 IU/mL standard (control) plasma. In the CVS rabies $RFFIT_{AAHL}$ a result of 0.5 IU/mL ≃ 50% endpoint dilution of 10.

CDC results: expressed as calculated 50% endpoint titres. See Appendix 2.

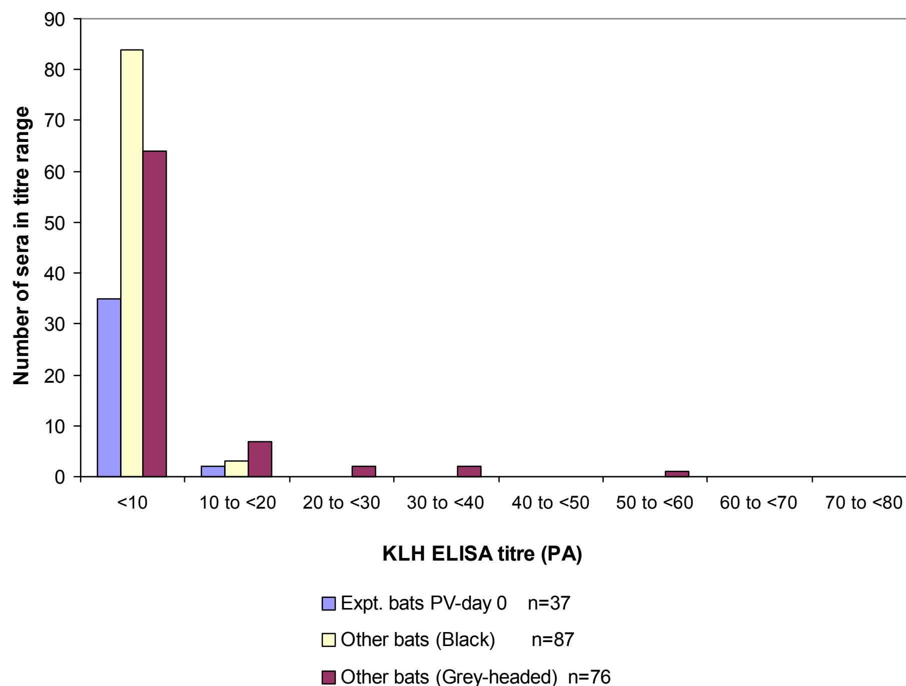
NR No result Cytotoxicity at the 1:5 and 1:25 dilutions precluded test evaluation. The higher incidence of cytotoxicity in the ABL-$RFFIT_{CDC}$ compared to the CVS-$RFFIT_{CDC}$ is probably due to the cell sheet remaining in contact with the bat serum for a longer test incubation time (48 hours rather than 24 hours).

8.3.2 Result threshold for KLH-ELISA titres

KLH-ELISA titres for 200 wild-caught (negative) bats are represented in Figure 8-6.

Only five stored sera from Grey-headed flying foxes had titres > 20 PA. Four of these 'high-negative' sera were tested on four occasions, giving consistent results. A moderate number of both Black (n=4) and Grey-headed (n=8) flying foxes had titres between 10 and 20 PA, the remainder (n=183) had titres <10 PA.

Figure 8-6 KLH-ELISA titres of 200 wild-caught (KLH-negative) flying foxes



These titres suggest that > 95% of unvaccinated Black and Grey-headed flying foxes have KLH titres < 20 PA. Accordingly, titres < 20 PA were considered negative for KLH, and titres > 20 PA were considered positive, indicating previous exposure (vaccination) to KLH. The possible result outcomes and types of errors made when interpreting titres with a result threshold of 20 PA are shown in Table 8-6.

Table 8-6 Interpreting KLH titres: Results and types of errors

	Unvaccinated bat	Vaccinated bat
Titre < 20 PA	True negative (>95%)	False negative
Titre > 20 PA	False positive (<5%)	True positive

8.3.3 Response to the KLH immunogen

Details of each bat (microchip number, species, sex, age, capture weight, treatment group, n=37) and their KLH titres are shown in Appendix 11. Plots of the individual responses within each group are also shown in Appendix 11.

8.3.3.1 KLH Phase 1 (PV-day 0 to 85): Crude summary

The mean responses of treatment groups 1, 2, 3 and 4, for each species ars summarized in Figure 8-7 and Figure 8-8. There were no treatment differences between Groups 1 and 3 (KLH *in* Nobivac) or Groups 2 and 4 (Nobivac and KLH + Alhydrogel) until PV-day 28, at which time Groups 3 and 4 received their respective booster vaccinations, so data for these pairs of groups have been combined for PV-days 0 to 28.

Figure 8-7 Mean response to Phase 1 KLH vaccination in Grey-headed flying foxes
(vertical bars indicate ± 1 standard error of mean)

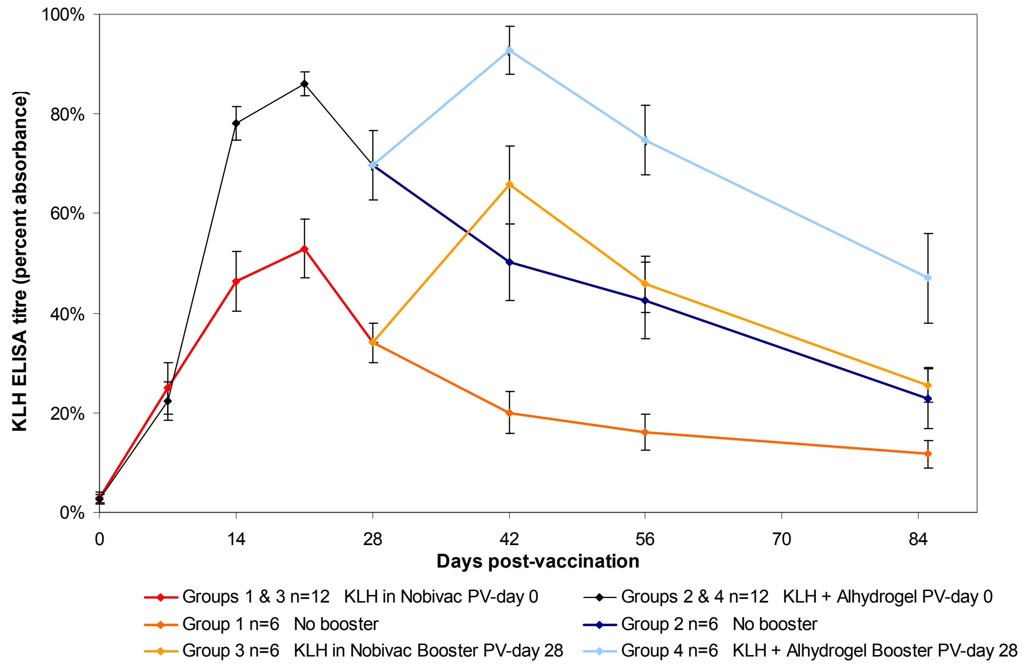
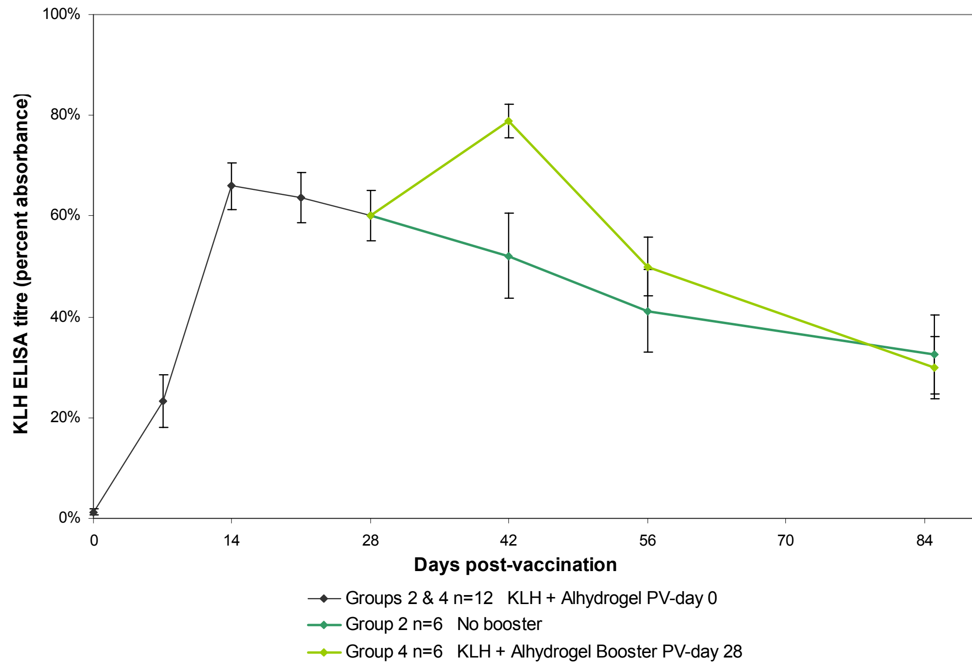


Figure 8-8 Mean response to Phase 1 KLH vaccination in Black flying foxes
(vertical bars indicate ± 1 standard error of mean)



8.3.3.2 KLH Phase 1 (PV-days 0 to 85): Analysis of variance

Analysis of variance indicated that three factors; vaccination method, booster vaccination and age, had statistically significant effects on KLH titres (conditional probability < 0.05).

- **Method: KLH + Alhydrogel > KLH *in* Nobivac**
  Vaccination using KLH + Alhydrogel as a separate injection produced higher KLH titres between PV-day 14 to 85 compared to those following vaccination with KLH *in* the Nobivac vaccine.
- **Booster PV-day 28 > No booster**
  Booster vaccination using either method produced higher KLH titres between PV-day 42 to 85
- **Age: Juveniles > Adults**
  There was a higher KLH response in juveniles compared to adult flying foxes on PV-day 21, and again between PV-day 42 and 85.

Two of these factors, method and age, had similarly significant effects on the concurrent rabies-RFFIT titres during this period.

Estimated mean values of statistically significant factors (conditional probability < 0.05), adjusted with respect to other factors, and appropriate standard errors are shown in Table 8-7.

Table 8-7 Phase 1 KLH response: significant factors.
Fitted mean values and standard errors for statistically significant factors predicted from regression model (conditional probability < 0.05)

Significant factors		PV-day 14		PV-day 21		PV-day 28		PV-day 42		PV-day 56		PV-day 85	
		fM%	s.e	fM%	s.e	fM%	s.e	fM%	s.e	fM%	s.e	fM%	s.e
Method	**KLH *in* Nobivac**	47.6**	4.7	52.6**	4.9	34.7**	5.8	37.9**	4.1	27.2**	4.5	17.0**	4.2
	KLH + Alhydrogel	73.6**	3.6	78.4**	3.7	65.7**	4.5	62.0**	3.1	48.4**	3.4	28.3**	3.2
Booster	**None**	n/a	n/a	n/a	n/a	n/a	n/a	40.6**	3.6	34.2**	4.0	20.6	3.7
	PV-day 28	n/a	n/a	n/a	n/a	n/a	n/a	79.2**	3.4	56.6**	3.8	32.7	3.5
Age	**Adult**	---	---	69.1	3.8	---	---	41.5**	2.9	39.5**	3.2	22.1	3.0
	Juvenile	---	---	79.7	5.6	---	---	72.8**	5.0	56.6**	5.5	38.0	5.1

fM% fitted mean percent absorbence
s.e standard error of fM%
--- not statistically significant
n/a not applicable as booster factor not applied until PV-day 28
** conditional probability < 0.01

In general, the interactions between these factors were not statistically significant (conditional probability > 0.05). However, during the period of peak response to the primary vaccination, i.e. PV-days 14 and 21, there was a significant interaction between vaccination method and age, with a stronger effect of method for the juveniles than the adults (see Table 8-8)

Table 8-8 Phase 1 KLH response: significant interactions.
Fitted mean values and standard errors for statistically significant interactions of factors predicted from regression model (conditional probability < 0.05)

	Method	Juvenile		Adult	
		fM%	s.e	fM%	s.e
PV-day 14	**KHL *in* Nobivac**	37.0	7.1	51.7	6.2
	KLH + Alhydrogel	84.5	7.1	70.8	4.3
PV-day 21	**KLH *in* Nobivac**	50.3	7.3	53.5	6.4
	KLH + Alhydrogel	94.3	7.3	74.3	4.4

fM% fitted mean percent absorbence
s.e standard error of fM%

8.3.3.3 Estimated duration of positive KLH-titres following Phase 1 vaccination on PV-days 0 and 28

The estimated duration of positive KLH-titre resulting from vaccination on PV-day 0 ± PV-day 28 (i.e. Phase 1 only) for each flying fox is shown in Table A12-2 in Appendix 11 and summarized in Figure 8-9 , Table 8-9, and Figure 8-10. They indicate how long titres could have been expected to have remained positive (KLH ELISA PA > 20) had the Phase 2 high-dose KLH + Alhydrogel booster *not* been given on PV-day 85.

Figure 8-9 Estimated median duration of positive KLH titres after Phase 1 vaccinations on PV-day 0 and 28

Unbroken lines indicate *means* of actual titres, dashed lines indicate *median* duration until KLH titre= 20 PA. Error bars omitted for clarity.

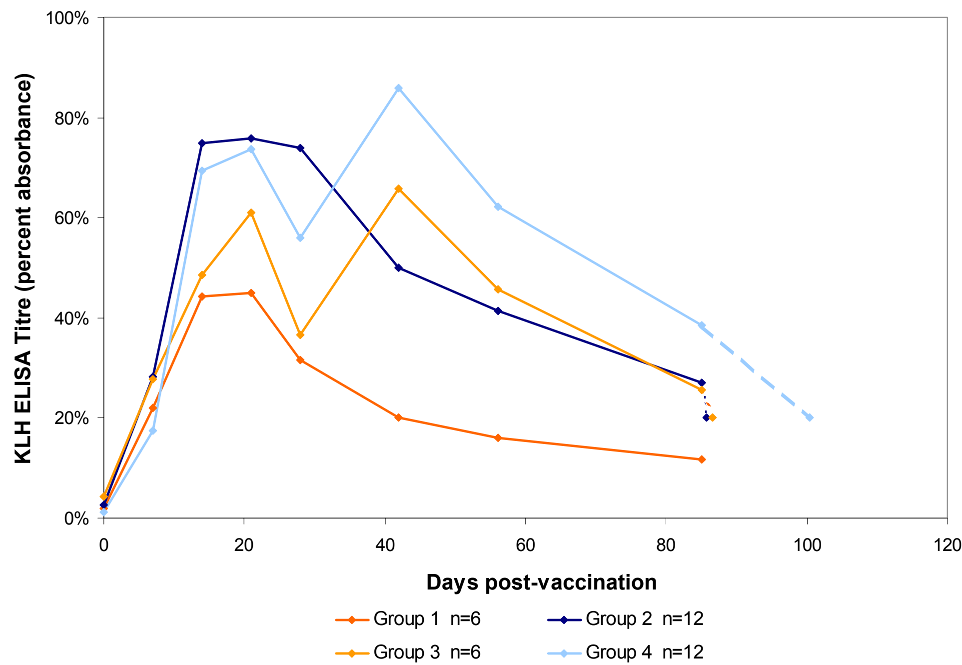
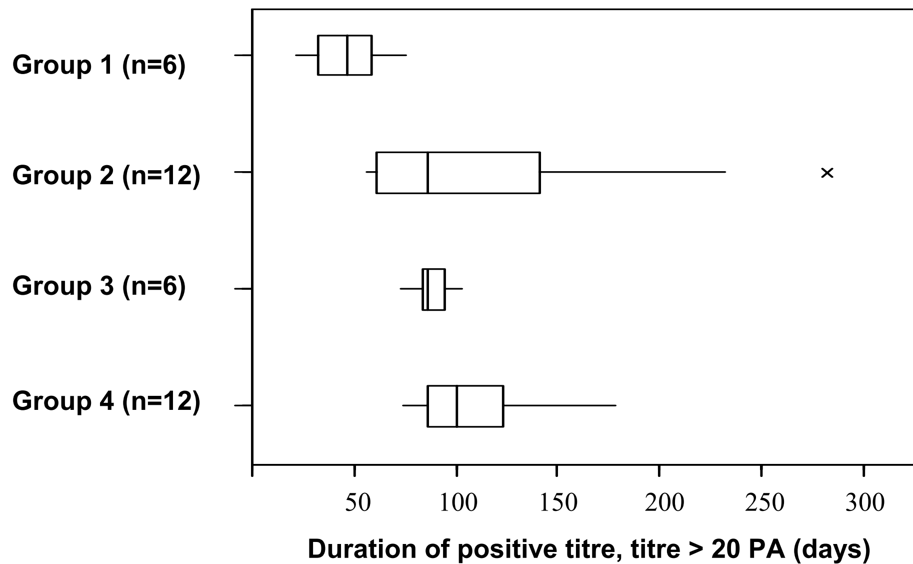


Table 8-9 Summary statistics of estimated duration of positive KLH titres after Phase 1 vaccinations

Group	Estimated duration of positive KLH response (days)		
	Median	**Minimum**	**Maximum**
Group 1	47	21	75
Group 2	86	56	282
Group 3	87	72	102
Group 4	100	75	178

Figure 8-10 Schematic plots of estimated duration of positive KLH titres after Phase 1 vaccinations



As the median duration of positive KLH response is only 45 to 100 days and the maximum estimated duration is only 282 days, i.e. considerably less than one year, all four of the original Phase 1 KLH vaccination protocols failed to produce positive KLH titres for sufficient duration to be a used to indicate a history of rabies vaccination.

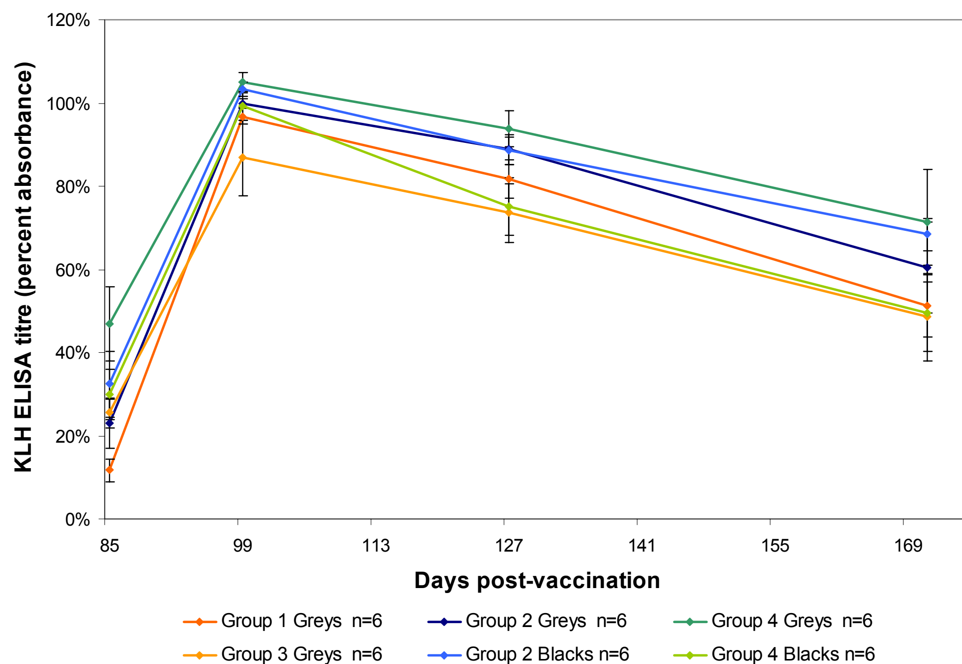
In an attempt to increase the strength and duration of KLH titres, bats in all four groups and the control bat (n=37) were vaccinated with the 10 times the quantity of antigen (1 mg KLH) and twice the Alhydrogel adjuvant (0.2 mL) on PV-day 85 (i.e. Phase 2 KLH trial).

8.3.3.4 KLH Phase 2 high-dose KLH booster: Crude summary

On PV-day 85 all flying foxes (n=37), having previously been vaccinated according to one four different KLH protocols, received the *same* high-dose KLH+ Alhydrogel booster.

The mean response of bats in Group 1, 2, 3 and 4, for each species is summarized in Figure 8-11.

Figure 8-11 Mean response to Phase 2 PV-day 85 High-dose KLH + Alhydrogel booster
(n=37, vertical bars indicate ± 1 standard error of mean)



8.3.3.5 KLH Phase 2 (PV-days 85 to 171): Analysis of variance

Analysis of variance indicated that four factors, previous vaccination method (during Phase 1), age, species, and sex had statistically significant effects on the response to the high-dose KLH + Alhydrogel booster given on PV-day 85 (conditional probability < 0.05).

♦ **Previous vaccination method: KLH +Alhydrogel > KLH *in* Nobivac**

Flying foxes that had previously been vaccinated using KLH + Alhydrogel had higher titres in response to the high-dose KLH between PV-days 99 and 171 than flying foxes previously vaccinated with KLH in the Nobivac vaccine.

♦ **Age: Juveniles > Adults**

Juveniles had higher titres in response to the high-dose KLH than adult flying foxes on PV-days 127 and 171.

♦ **Species: Black flying foxes > Grey-headed flying foxes**

Species comparisons could only be made between adults that had previously been vaccinated with KLH + Alhydrogel (no juvenile Blacks or Blacks vaccinated with KLH *in* Nobivac were included in the study). Within this class, Black flying foxes had significantly higher titres in response to the high-dose KLH than Grey-headed flying foxes on PV-day 171 only.

♦ **Sex: Females > Males**

Females had significantly higher titres than males on PV-day 171 only.

Two of these factors, prior vaccination method and age had had similarly significant effects on rabies-RFFIT and KLH –ELISA titres during Phase 1.

Prior Phase 1 booster vaccination on PV-day 28 did not have a significant effect on the response to the subsequent PV-day 85 high-dose booster.

Estimated mean values of statistically significant factors (conditional probability < 0.05), adjusted with respect to other factors, and appropriate standard errors are shown in Table 8-10.

Table 8-10 Phase 2 High-dose KLH response: Significant factors
Fitted mean values and standard errors for statistically significant factors (conditional probability < 0.05)

Significant factor		PV-day 99		PV-day 127		PV-day 171	
		fM%	s.e	fM%	s.e	fM%	s.e
Method	KLH *in* Nobivac	91.8**	3.0	76.5**	3.5	46.4**	5.0
	KLH + Alhydrogel	101.4**	2.2	86.0**	2.7	56.4**	3.8
Age	Adult	---	---	80.8**	2.6	46.5**	3.6
	Juvenile	---	---	93.9**	4.3	81.4**	6.0
Species†	Grey	---	---	---	---	37.5**	6.1
	Black	---	---	---	---	61.5**	4.8
Sex	Male	---	---	---	---	52.8	4.7
	Female	---	---	---	---	54.7	4.0

fM% fitted mean percent absorbence
s.e standard error of fM%
** conditional probability < 0.01
--- not statistically significant
† species comparisons only available among adult bats vaccinated with Nobivac *and* KLH + Alhydrogel (Groups 2 and 4)

In general, the interactions between these factors were statistically not significant ($p > 0.05$). However, during the period of peak response to the high-dose KLH, i.e. PV-day 99, there was a significant negative interaction between previous vaccination method and previous booster on PV-day 28 (conditional probability = 0.045), with bats that had previously received two doses of KLH *in* Nobivac having lower titres in response to the high-dose KLH (see Table 8-11).

Table 8-11 Phase 2 High-dose KLH response: Significant interactions
Fitted means and standard errors for statistically significant interactions of factors (conditional probability < 0.05) predicted from regression model

PV-day	Method	No PV-day 28 Booster		Booster PV-day 28	
		fM%	s.e	fM%	s.e
99	KLH *in* Nobivac	96.6	4.3	85.2	4.0
99	KLH + Alhydrogel	100.9	3.1	102.1	3.0

fM% fitted mean percent absorbence
s.e standard error of fM%

8.3.3.6 Estimated duration of positive KLH titres following Phase 2 vaccination

The estimated duration of positive KLH titres following the high-dose KLH + Alhydrogel booster for each flying fox is shown in Table A12-2 in Appendix 11. Titres from two juvenile Grey-headed flying foxes (Bats 179 and 402, Groups 2 and 4 respectively) which failed to cross the KLH titre = 20 PA threshold (i.e. infinity positive) were nominally extrapolated to 1000 days.
The results are summarized in Figure 8-12, Table 8-12 and Figure 8-13.

As the median estimated duration of KLH positive titres is only 217 to 264 days, i.e. less than one year, even the high-dose KLH booster failed to produce positive titres of sufficient duration for the intended purpose.

Figure 8-12 Estimated median duration of positive KLH titres following Phase 2
Bold lines indicate mean titres, dashed lines indicate extrapolation to estimated median duration until KLH titre = 20 PA. Error bars omitted for clarity
Vaccinations:
PV-day 0 KLH *in* Nobivac (Groups 1 and 3) or Nobivac and KLH + Alhydrogel (Groups 2 and 4)
PV-day 28 booster KLH *in* Nobivac (Group 3) or Nobivac and KLH + Alhydrogel (Group 4)
PV-day 85 high-dose KLH + Alhydrogel (Groups 1, 2, 3, and 4)

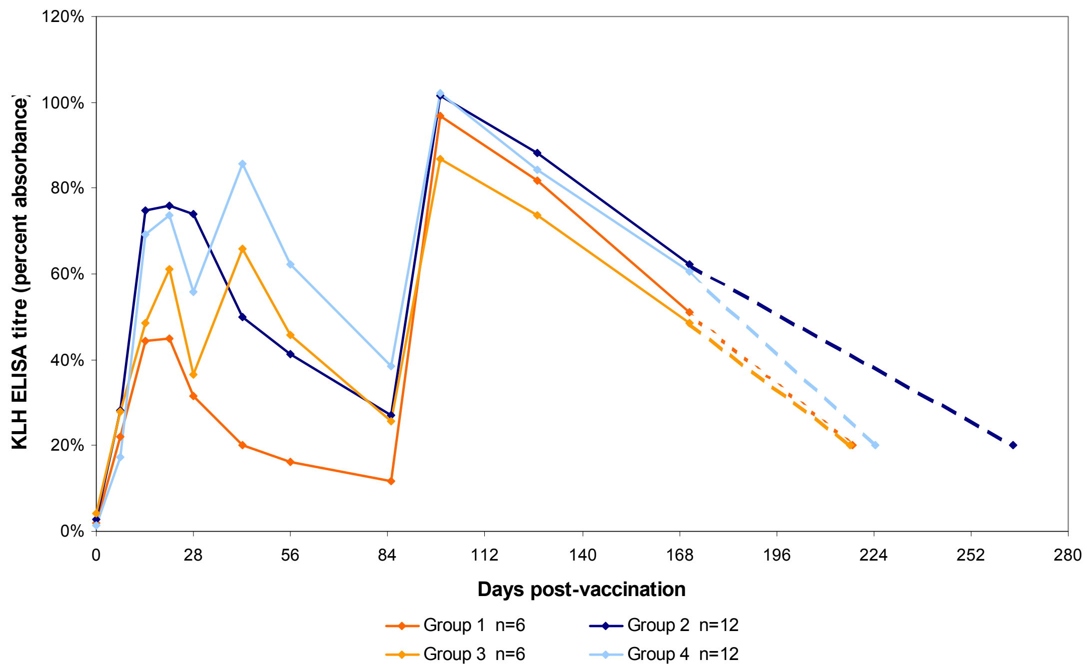
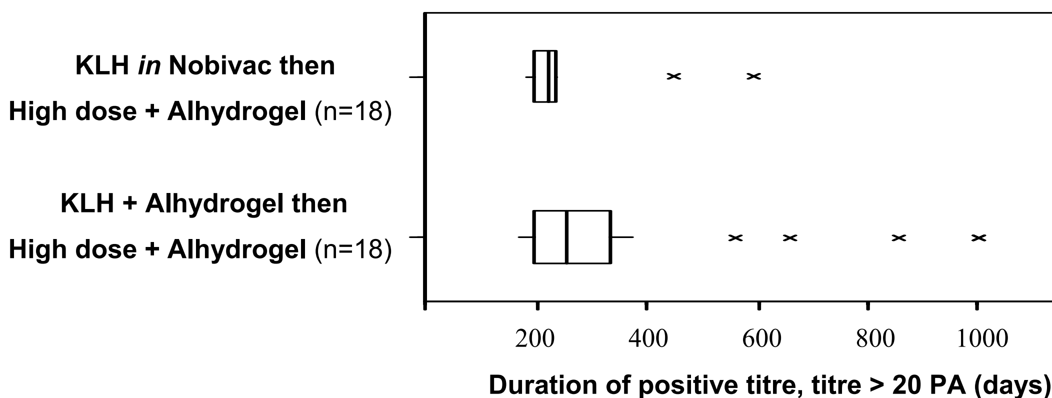
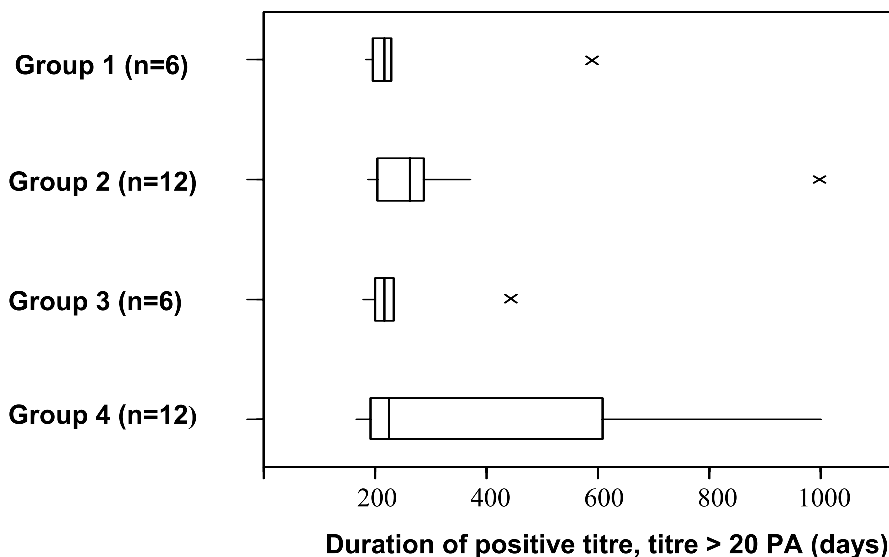


Table 8-12 Summary statistics for the estimated duration of positive KLH titres following Phase 2 vaccination

Method	Estimated duration of positive KLH-response (days)		
	Median	Minimum	Maximum
KLH *in* Nobivac / High dose (n=12)	218	181	592
KLH + Alhydrogel / High dose (n=24)	255	166	1000+
Group			
Group 1 (n=6)	218	182	592
Group 2 (n=12)	264	187	1000+
Group 3 (n=6)	217	181	445
Group 4 (n=12)	224	166	1000+

Figure 8-13 Schematic plot summaries of the estimated durations of positive KLH titres





8.3.4 Relationship between Nobivac and KLH responses

The relationship between responses to the Nobivac and KLH vaccinations within individuals was examined using the transformed rabies-RFFIT titres and the untransformed KLH titres. The time points examined were:

- PV-days 14, 21, and 28, the points of maximum response to the primary vaccination on PV-day 0
- PV-day 42, the point of maximum response to the Phase 1 booster vaccination of PV-day 28 and
- PV-day 85, the point of maximum duration following the Phase 1 vaccinations

Titres from PV-day 171 were not considered as these titres reflect a response to the high-dose KLH + Alhydrogel booster *without* a concurrent Nobivac booster.

The correlation co-efficients and corresponding F probabilities indicating the level of significance of these relationships are shown in Table 8-13. The relationship between the Nobivac and KLH responses are illustrated in Figure 8-14, Figure 8-15, and Figure 8-16.

Table 8-13 Summary statistics of the relationship between the responses to Nobivac and KLH vaccination.

	Correlation co-efficient	F probability	Interpretation
PV-day 14	0.20	0.23	No correlation
PV-day 21	0.25	0.14	No correlation
Pv-day-28	0.31	0.06	No correlation
PV-day 42	0.83	< 0.001	Highly significant correlation
PV-day 85	0.40	0.02	Significant correlation

Figure 8-14 PV-day 21: Relationship of responses to Nobivac and KLH vaccination

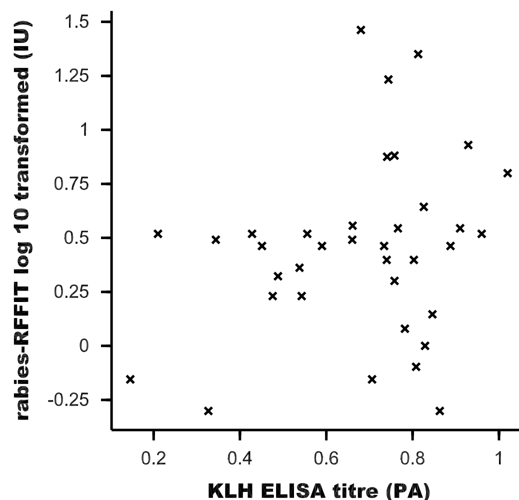


Figure 8-15 PV-day 42: Relationship of responses to Nobivac and KLH vaccination
x = Groups 1 and 2, no boosters **o** = Groups 3 and 4, boosters on PV-day 28

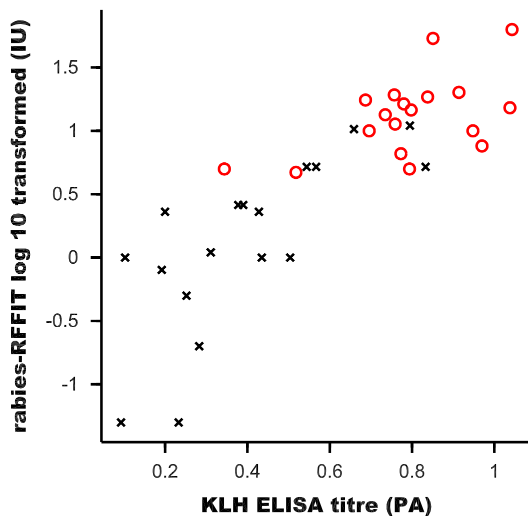
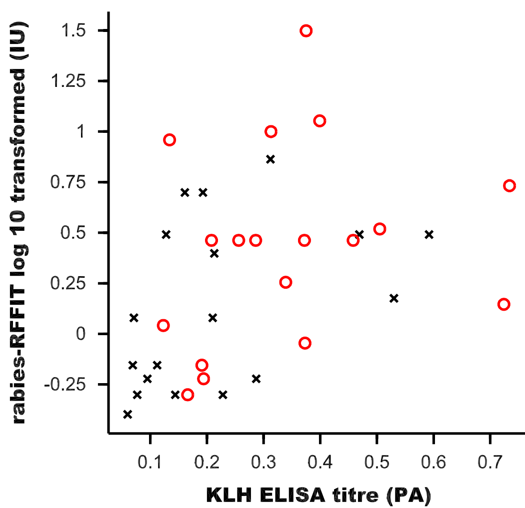


Figure 8-16 PV-day 85: Relationship of responses to Nobivac and KLH vaccination
x = Groups 1 and 2, no boosters **o** = Groups 3 and 4, boosters on PV-day 28



8.3.5 Effect on pups of pre- and post-natal vaccination of dams

Of the 18 female flying foxes captured for the trial, two were pre-breeding juveniles, one adult showed no evidence of being pregnant (no weight gain or abdominal distension) and 15 developed clear evidence of pregnancies that were the result of matings prior to capture.

Eleven healthy pups, three abortions and one stillborn pup were born to flying foxes during the trial. The cause of the stillbirth remains unclear.

The three abortions and the death of one of the apparently healthy pups occurred within 24 hours of the colony being disturbed by:

- capturing flying foxes for blood sampling (n=2)
- the installation of 'tree limb' perches in the cage in an attempt to provide environmental enrichment and more natural 'footing' than cage wire (n=1)
- and by feeding bats uncharacteristically late (after dark), at which time they were flying around the cage where the food was stationed rather than hanging calmly as a 'colony' at the far end (n=1).

The loss of these pups was attributed to the stress of these procedures rather than as a specific interaction with any components of the vaccine/s. Extreme susceptibility to stress-induced abortion (20 to 100%) has been demonstrated among Little Red flying foxes (Dukelow *et al.* 1990).

Details of these losses are shown in Table 8-14.

Table 8-14 Details of pup losses during the vaccination trial

	Details of dam		Circumstances
	Species (Bat no.)[1]	**Group, vaccination protocol**	
PV-day 30	Black (E64)	Group 4 Nobivac and KLH + Alhydrogel PV-day 0 + 28	Pre-term foetus found 48 hours after sampling and vaccination on PV-day 28, which was also 24 hours after the colony was disturbed by feeding after dark on PV-day 29
PV-day 38	Grey (B7A)	Group 4 Nobivac and KLH + Alhydrogel PV-day 0 + 28	Pre-term foetus found next day following disturbance of colony on PV-day 37 to install tree-limb 'perches' in cage
PV-day 43	Grey (B43)	Group 3 KLH *in* Nobivac PV-day 0 + 28	Pre-term foetus found next day following sampling on PV-day 42
PV-day 64	Grey (11A)	Group 2 Nobivac and KLH + Alhydrogel PV-day 0	Stillborn full term pup
PV-day 128	Grey (326)	Group 1 KLH *in* Nobivac PV-day 0	Pup 610. Apparently healthy 65-day old pup found hanging dead in cage next day following sampling on PV-day 127

[1] Bat no. = the unique last three digits of the female flying fox's microchip number

8.3.5.1 Rabies-RFFIT and KLH ELISA titres in pups

Bat details, rabies-RFFIT, and KLH ELISA titres for the 11 healthy pups and their dams are shown in Appendix 12. Pups were born between PV-days 48 and 82, 28 days (Bat 51D) to 82 days (Bat 95B) after the dams last Phase 1 vaccination, and 2 to 37 days prior to the High-dose KLH booster on PV-day 85.

Sampling of the colony was minimized during late pregnancy and while pups were maternally dependant and samples were not taken from pups < 4 weeks of age, to reduce the likelihood of pup losses/abortions. Consequently, the number of samples from pups is small and no statistical analysis was attempted. Pup titres are summarized in Figure 8-17 and Figure 8-18. The age of pups when sampled was calculated using their known or nominal birth dates, which were determined as described in Appendix 14.

Figure 8-17 Positive rabies-RFFIT titres detected in six of 11 pups born to female flying foxes vaccinated with Nobivac rabies vaccine 28 to 82 days before birth
Horizontal error bars indicate possible error in age for pups with positive titres.

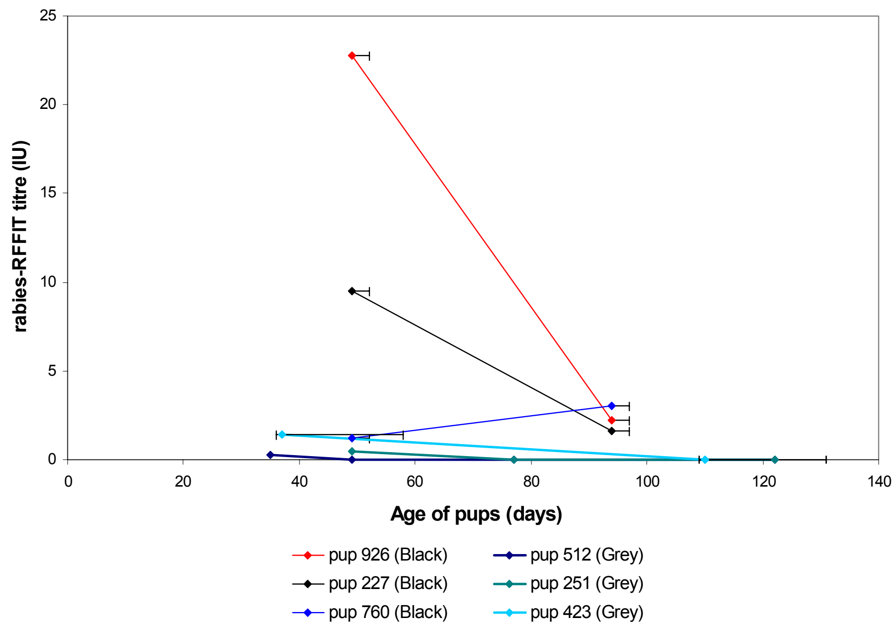
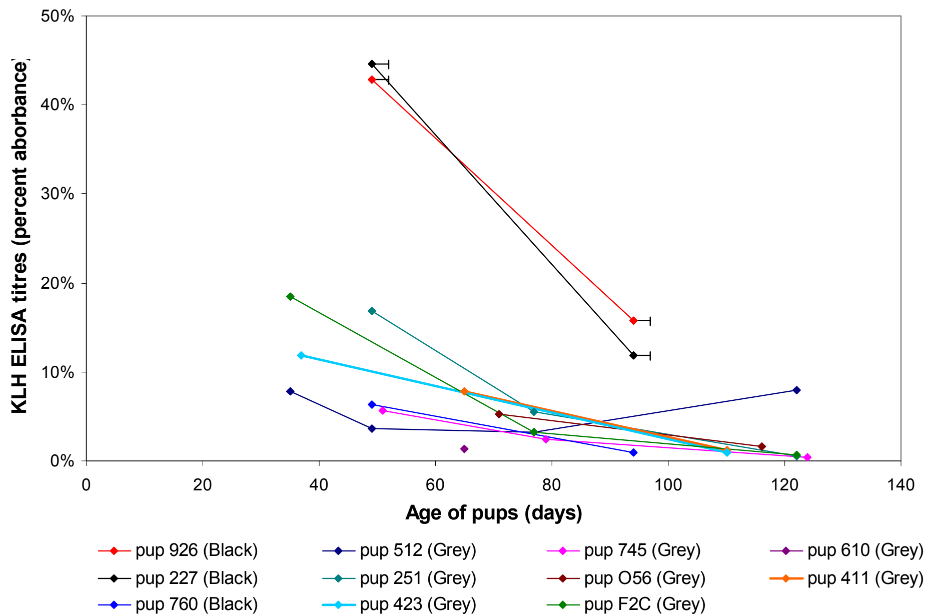


Figure 8-18 KLH ELISA titres in 11 pups born to females vaccinated with KLH during (Phase 1) and after pregnancy (Phase 2)
Positive threshold = 20 PA. Horizontal error bars indicate possible error in age for pups with positive titres.



8.4 Discussion

No vaccination trials or protocols exist for *Pteropus* species (flying foxes) in Australia or elsewhere using either rabies virus or other vaccines. This trial demonstrated induction and quantification of neutralizing serological responses to rabies virus and ABLV in Black and Grey-headed flying foxes using a commercial animal rabies virus vaccine (Nobivac). This discussion includes consideration of the use of vaccination in the pre- and/or post-exposure management of captive flying foxes.

8.4.1 Response to vaccination with Nobivac

The prime objective of vaccinating captive flying foxes with the animal rabies vaccine commercially available in Australia (Nobivac®, Intervet) is to confer protective immunity against ABLV infection and so reduce the incidence of ABLV in captive flying foxes and the risk infected captive flying foxes pose to humans. To have demonstrated that vaccination was cross-protective would have required post-vaccination ABLV challenge experiments that were unfortunately beyond the available resources. In lieu of experimental challenge, modified rabies-RFFITs were done at AAHL to asses the serological response to the rabies antigen.

All the Black and Grey-headed flying foxes developed peak neutralising antibody titres of 0.8 to 29 IU/mL (mean = 6.0, median = 3.8, n= 36) typically 21 or 28 days (n=31 of 36) days after vaccination with a single dose of Nobivac vaccine, and those given a booster vaccination on PI-day 28 developed peak neutralizing titres of 4.7 to 53.6 IU/mL (mean = 17.6, median = 14.0, n= 18) on PI-days 42 or 56.

A nominal rabies-RRFIT titre of ≥ 0.5 IU/mL is considered indicative of protective immunity for rabies virus in humans (QHSS) and a titre ≥ 0.5 IU/mL is considered indicative of protective immunity for domestic animals being assessed for export/import by Australian authorities (AQIS). While the minimum rabies titre associated with protective immunity for flying foxes is not known, extrapolation from other species suggests that vaccination with the Nobivac vaccine using either the single or booster vaccination protocol conferred protective immunity to rabies virus challenge, as all the flying foxes developed titres ≥ 0.5 IU/mL.

Analysis of variance indicated that age, sex, species, and method (Nobivac and KLH + Alhydrogel versus KLH *in* Nobivac) had statistically significant effects on the rabies-RFFIT titres at various time points post-vaccination. However, these effects were typically transient or intermittent, and did not involve large or functionally relevant differences in mean titre values (e.g. titres substantially more or less than the nominal 0.5 IU/mL). These effects are not considered to be sufficiently important to be considered in the development of Nobivac vaccination protocols for flying foxes, except that the trial demonstrated that juvenile flying foxes (estimated age 6 months

to <3 years of age) are suitable vaccination candidates, producing titres as high or higher than those in adults. Interestingly while booster vaccination on PI-day 28 had highly significant effect on rabies titres 14 and 28 days after the booster (PI-days 42 and 56), this strong effect was only transient, with titres rapidly returning to levels that were no longer significantly higher than those of bats that received only a single vaccination by PI-day 85. This would suggest that booster vaccination, while potentially useful in inducing rapid and high titres, as would be desirable for post-exposure management of situations such as occurred with a captive colony in Rockhampton (case nos. ABLV-66 and ABLV-73, see Appendix 5), may not provide better long-term immunity.

There was an intriguing significant interaction between vaccination method and age on longer term rabies titres (PI-days 56, 85 and 171), with juveniles having a higher response when vaccinated with KLH *in* Nobivac, while adults had higher responses to Nobivac and KLH + Alhydrogel. Again the differences in titres were not consistent, nor large, nor likely to be functionally relevant, and so are not considered sufficiently important to recommend different vaccination methods for different age classes of flying foxes.

Because the purpose of the vaccination was to provide immunity to ABLV, not rabies virus, duplicate sera were sent to CDC Atlanta for determination of the cross-neutralisation response to ABLV as assessed in a pt-ABLV-RFFIT. Twenty two sera were selected from PI-days 56 and 85, (being the longest PI-days for which rabies-$RFFIT_{AAHL}$ results were known at the time) to represent low (~0.5 IU/mL, n=7), moderate (>2 to <5 IU/mL n=8), and high (> 5 IU/mL, n=7) persistent titres from flying foxes that either had (n=11) or had not (n=11) received a booster on PI-day 28. Unfortunately all sera with low (~0.5 IU/mL) rabies-$RFFIT_{AAHL}$ and rabies $RFFIT_{CDC}$ titres (n= 7) demonstrated cytotoxicity at the 1:5 plasma dilution, precluding detection of positive neutralizing ABLV endpoint titres < 1:11. Four of these sera were also toxic at the 1:25 dilution, precluding demonstration of positive ABLV titres <1:25. Consequently it remains unclear to what extent sera with low rabies titres in response to Nobivac rabies vaccination, have low, potentially protective cross-neutralising activity against ABLV.

All sera with rabies-$RFFIT_{AAHL}$ titres of ≥ 2 IU/mL for which results were obtained (n=10) demonstrated neutralising titres against ABLV of 1:154 to 1:1100. While the minimum pt-ABLV $RFFIT_{CDC}$ titre associated with protective immunity for flying foxes is not known, the results for Bat-8 in the experimental inoculation trial (see Chapter 6) suggest that naturally occurring pt-ABLV $RFFIT_{CDC}$ titres as low as 1:13 to 1:50 may be associated with immunity to ABLV challenge, and that challenge can stimulate a rapid increase in pt-ABLV-titre to 1:480 within 7 days. In combination, these results suggest that flying foxes that develop rabies titres ≥ 2 IU/mL in response to vaccination with Nobivac, do develop cross-protective ABL immunity and that flying foxes with rabies titres as low as 0.5 IU/mL may also be ABLV immune.

In the absence of post-vaccination challenge experiments, the duration for which Nobivac rabies vaccination confers immunity to rabies virus or ABL remains unclear. Individual flying foxes with titres that had waned below 0.5 to 2.0 IU/mL may still have protective immunity to naturally

occurring ABLV exposure, depending on the exposure dose, given the potential for a strong and rapid anamnestic response such as that seen in Bat-8. Rabies virus challenge experiments after a single dose of Nobivac in domestic animals indicate that the duration of protection is at least; dogs and cats 3 years, cattle and horses 2 years, foxes, ferrets, sheep and goats 1 year (Intervet 1996). By PI-day 171, flying foxes that had or had not received a Nobivac booster on PI-day 28 had rabies-$RFFIT_{AAHL}$ titres of 0.2 to 11.7 IU/mL (n= 18) and 0.7 to 10.2 IU/mL (n= 18) respectively. Mean rabies-$RFFIT_{AAHL}$ for each group of Black or Grey-headed flying foxes on PI-day 171 ranged from 1.6 to 4.6 IU/mL, which is comparable to the mean rabies-RFFIT titres of 3.0 to 3.5 IU/mL in groups of dogs and cats 6 months after single primary vaccination that persisted as mean titres of 2.0 to 3.0 IU/mL after 36 months (Intervet 1996). Pending the results of challenge experiments with flying foxes more than 6 months post-vaccination, it appears that Nobivac/ABLV vaccination protocols for flying foxes should include an ongoing, at least yearly, booster vaccination schedule.

8.4.2 Use of KLH as an immunological marker

KLH is frequently used in medical research as a strongly immunogenic carrier protein to which haptens such as short peptides are conjugated to elicit specific antibodies to the hapten of interest. It is also used as a non-specific immune stimulant to investigate the role of the immune system in disease. No reports of the strength or duration of antibody responses to KLH itself, in bats or other species, were found.

As the objective of the KLH vaccination was to induce positive antibody titres that could be used to differentiate rabies-vaccinated from unvaccinated flying foxes, the purpose of the KLH vaccination was not to produce high KLH titres *per se* but rather to produce positive titres (KLH ELISA >20 percent absorbance, PA) for as long a period as possible, a persistent moderate response of 30 or 40 PA being more desirable than an initially high response that rapidly falls to < 20 PA. For KLH to be an effective serological marker, a KLH vaccination protocol would need to reliably produce positive KLH titres that lasted either:

- for a minimum of one year, such that annual boosters would sustain a positive KLH titre
- or preferably substantially longer than the concurrent positive rabies-RFFIT response, so that any positive rabies-RFFIT titres could be recognized as due to vaccination
- or ideally for the lifespan of the flying fox (~10 years), ‘permanently’ indicating a history of vaccination.

Most flying foxes (n= 35 of 37) responded to the primary KLH vaccination with titres of > 40 to 96 PA which were clearly distinguishable from >95% of unvaccinated flying foxes using the 20 PA positive threshold. Two bats (B66 and F19, both juvenile Grey-headed flying foxes) failed to develop positive titres in response to primary vaccination (maximum titres of 14 and 21 PA respectively), however Bat F19 subsequently responded well (75 PA) to booster vaccination on PI-day 28. There was a consistent (PV-day 14 to PV-day 85) and highly significant association

between the KLH + Alhydrogel method and higher KLH titres, and both booster vaccination on PI-day 28 and age (juveniles) were associated with significantly higher KLH titres on days 42, 56 and 85, i.e. in the medium term, towards the end of Phase 1.

Unfortunately, the KLH titres declined sharply and by PI-day 85, 19 of 37 flying foxes (52%) had titres near or below the positive threshold (<23 PA), including 3 of 12 (25%) that had benefited from the optimal method and protocol combination of KLH + Alhydrogel and PV-day 28 booster. As such it was clear that none of the trialled KLH vaccination strategies would induce KLH titres >20 PA for the minimum duration requirement of 1 year. Indeed mathematical extrapolations of the declining titres predicted that the median duration for which the KLH response was expected to remain positive was as short as ≤ 100 days, and the maximum expected duration was only 282 days.

At this point, the KLH component of the vaccination trial was deemed to have failed. A second attempt to induce sustained positive KLH responses was made using a higher dose of KLH antigen (10 X) and the most effective method (KLH + Alhydrogel), using twice the adjuvant, on PV-day 85 (Phase 2). All bats (n= 37) responded very well to the high dose, Phase 2 booster, with titres of 82 to 111 PA 14 days later. Again however, titres fell rapidly. By the termination of the experiment on PV-day 171 (86 days after Phase 2 booster), the response of one bat (F43) had fallen from 102 to 16 PA (negative), and the titres of three others were less than 30 PA. Mathematical extrapolations of the titres indicated that median date to which KLH titres could have been expected to have remained positive was between PV-days 217 to 264 days, i.e. only 132 to 179 days after the Phase 2 booster. While it is likely that the titres of at least some flying foxes would plateau and remained positive for longer than that calculated by linear extrapolation, it is clear that in some or most cases, the positive KLH response would not persist for the minimum I year required, and certainly not for the potential lifespan (~10 years) of the bat.

When vaccinated with the rabies virus and/or KLH antigen, a substantial proportion of bats responded with a transiently high serological titre, with a tendency towards low or nominally negative titres in the mid to long term. Given the apparent failure of KLH as a serological marker and the often strong but transient nature of the serological response to antigens in flying foxes, differentiation of vaccinated from unvaccinated bats might more reliably be done with the use of traditional identification methods, such as thumb bands and microchips.

8.4.3 Effect on pups of vaccination of pregnant and/or lactating dams

A secondary objective of the trial was to evaluate the serological response in pups born to flying foxes that were vaccinated when pregnant. Black and Grey-headed flying foxes were caught during May, i.e. shortly after the principal mating season for these species in southeast Queensland, when most breeding age females were expected to be in early pregnancy. Selection

of females for the trial was biased towards those of breeding age, and 14 of 15 subsequently showed evidence of pregnancy. Three females aborted in late pregnancy; in each case this followed stressful interactions with the colony. While these losses were attributed to stress rather than to the vaccinations *per se,* the stage of possible pregnancies should be considered when instituting a vaccination program for 'untamed' captive bats in order to avoid stress-induced abortions.

Six of 11 pups born to vaccinated dams had rabies-$RFFIT_{AAHL}$ titres of 0.3 to 22.5 IU/mL at ~ 30 to 60 days of age, which dropped to 0 to 3.0 IU/mL by ~100 days of age. Similarly all 11 pups had KLH titres of 5.2 to 44.6 PA at ~30 to 70 days of age, which dropped to 0.4 to 15.8 PA at 80 to 120 days of age. This antibody profile is highly suggestive of maternally-derived antibody, and it appears that the maternal antibodies in most pups would be very low or absent by 3 to 4 months of age.

The extent to which maternally derived antibodies could have been acquired via milk is unclear as no studies have been done to determine whether or not flying foxes produce colostrum, or the capacity of pups to absorb antibody sized macromolecules. In a detailed study of the placenta of the Indian flying fox (*Pteropus giganteus*) using both light and electron microscopy, the placenta was shown to be endotheliodichorial at late limb bud stage and by advanced pregnancy showed a tendency to be haemochorial (Karim and Bhatnagar 1996). The placentation of Black and Grey headed flying foxes has not been so thoroughly examined. The aborted terminal placentas of Grey-headed and Little Red flying foxes were shown to be discoidal labyrinthine endotheliochorial (Hughes 1989), however the use of aborted placentas rather than 'fresh' placentae *in utero,* may have precluded detection of a tendency towards a haemochorial placenta in these species. Trans-placental transfer of maternal antibodies would be expected to occur across a haemochorial placenta and is also likely, at least to some extent, across an endotheliochorial placenta. It is likely that the positive rabies and KLH titres detected in the pups of this trial are the result of maternal antibody transfer *in utero*, and possibly also via milk. The extent to which maternally-derived rabies antibodies could be protective in pups is unknown. By 3 to 4 months of age titres had dropped to levels that would not be expected to interfere with subsequent vaccination. While the capacity of pups of this age to respond to vaccination has not been demonstrated, flying fox pups are comparatively precocious when born (fully furred, eyes open, able to cling to dam during flight), and are capable of flight and living independently by 3 to 4 months of age. Given this and the excellent response of young bats in this trial that were possibly only 6 months of age when vaccinated, it is presumed that the immune system of 3 to 6 month old pups would be sufficiently mature to respond adequately. This should however be established by determining the post-vaccination rabies-RFFIT titre of pups of known age.

8.4.4 Consideration of an ABLV vaccination protocol for captive flying foxes

Both pre-and post-exposure rabies vaccination protocols are regularly used to manage human exposure to rabies worldwide. Pre-exposure management of domestic animals in the USA consists of vaccination with an approved veterinary rabies vaccine and appropriate regular booster vaccinations, with immediate re-vaccination following any potential exposure to rabies. It is recommended that post-exposure management of *unvaccinated* animals consist of euthanasia to preclude subsequent transmission or placing the animal in isolation for 6 months with vaccination 1 month before release should no clinical rabies develop (Jenkins *et al.* 2003). Protocols for the post-exposure vaccination of unvaccinated domestic animals have not been validated and are controversial. However, successful post-exposure vaccination of naturally exposed pigs and experimentally infected sheep with commercial animal vaccine ± equine rabies immunoglobulin, suggests that post-exposure vaccination can be used safely and effectively to manage exposure to rabies in valuable farm (captive) animals (Blancou *et al.* 1991; Mitmoonpitak *et al.* 2002). In Australia, use of animal rabies vaccines is strictly controlled by the Chief Veterinary Officer of each state and the Australian Quarantine Inspection Service (AQIS). Its use is currently limited to pre-export vaccination and management of rabies outbreaks.

Captive flying foxes generally fall into one of two categories: those in permanent or long term captivity, such as those in zoological parks and sanctuaries, and those in temporary care, typically bats that are sick, injured, or orphaned, and being cared for pending re-release. Captive flying foxes may be considered ‘valuable’ animals, if not in terms of monetary value, then because they are protected native species, often induce strong feelings of attachment in those that care for them, and in some cases because they are trained and used for educational display.

Management of potential exposure of captive flying foxes to ABLV could take two forms:

- Maintenance of colonies in enclosures that preclude direct contact with wild flying foxes
- Pre-exposure vaccination

Currently very few captive colonies are kept in ‘contact-proof’ facilities and use of vaccines in wildlife, including captive bats is not authorized. Use of a pre-exposure vaccination program in permanent captive residents could act as a primary strategy to reduce the risk of ABLV infection in bats kept in single layer facilities, and as a secondary level of protection in bats in double layer facilities. Zoos and research institutions may establish rabies vaccination programs for wildlife in the USA (Jenkins *et al.* 2003). While the incidence of ABLV in permanent captive residents is particularly low (a single case, ABLV-73), the close and on ongoing nature of their contact with staff, and in some cases the use or display of these animals with the unvaccinated public warrants consideration of this approach. The need to differentiate vaccinated from non-vaccinated bats by serological means would be essentially eliminated because these bats would not be released into

the wild population and veterinary records and individual identification (thumb bands, microchips) would indicate their vaccination status.

The two ABLV cases involving the Rockhampton colony also raise the prospect of *post*-exposure vaccination of captive flying foxes. The colony was known to have been exposed to ABL on two occasions: 1. when wild ABLV-66 landed on the enclosure and 2. as a consequence of contact with colony resident ABLV-73. In response to the ABLV-positive diagnosis of ABL-66, there was a reluctance to slaughter the entire colony to preclude further transmission and bats were retained on public display (albeit separated from the public by a pre-existing double fence) and normal management practices continued. Following the development of ABLV disease within the colony (ABLV-73), the remaining bats were removed to an outdoor facility at the home of a volunteer where they were observed for 3 months (Warrilow *et al.* 2003). In the absence of any clinical or serological evidence of infection they were then returned to public display, and the cage upgraded to double layer mesh (contact proof). Post-exposure vaccination of the colony following the diagnosis of either case would potentially have reduced the risk of ABLV infection for both the colony bats and staff. While post-exposure treatment of humans ideally uses both vaccines and immune serum, the cost of immune serum is high and it is not known if commercial equine or human immune serum would be tolerated in flying foxes. Given the good immune response demonstrated in this trial to the Nobivac vaccine alone in comparison to those of pigs receiving a similar vaccine ± immune serum (Mitmoonpitak *et al.* 2002), the strong booster effect, and known incubation periods of naturally occurring ABLV infection being ≥ 30 days (n=4, see Chapter 2), a vaccine only protocol for flying foxes may be effective. However, post-exposure prophylaxis with rabies vaccine only was universally unsuccessful in dogs experimentally infected with a high dose of street rabies virus. All vaccinated dogs died earlier (days 9 and 10, n=4) than unvaccinated controls (days 11 to 13, n=5), suggesting an 'early death' effect (Hanlon *et al.* 2002). Because the high rabies dose used may not represent natural ABLV exposures, these results in dogs do not preclude a protective effect for post-exposure vaccination in naturally infected bats. Should post-exposure vaccination of bats fail, it may well be associated with an also advantageous effect of early death.

8.4.4.1 Proposals for *pre*-exposure vaccination protocol for flying foxes in captivity

- Vaccination of long-term permanent residents only. Eligible bats to have been held in captivity for at least 6 months (or since birth to long term resident), to demonstrate probable ABLV negative status.
- Vaccination only of bats with (or given) suitable permanent identification, preferably a combination of a microchip (permanent, but requiring scanner to read and not externally visible) and a thumb band (externally visible and so readily identifying the bat as one of interest, but can be lost).

- Vaccination schedule to consist of one initial vaccination with Nobivac® rabies vaccin (*sic*), followed by yearly boosters.
- Consideration to be given to not vaccinating bats susceptible to the stress of handling during late pregnancy, and to vaccinating captive born pups at 3 to 6 months of age, e.g. yearly vaccination of Black and Grey-headed colonies during March to May.
- Formal records of bat identification and vaccination history to be kept at the colony, and potentially on a centralised register (such as the Australian Bird and Bat Banding Scheme register kept by the federal environment department, http://www.enviroment.gov.au/bg/abbs).
- Vaccinated bats would be exempt from a demand for surrender for FAT in the event of a bite or scratch of a human.

While the optimum protocol to induce rapid and high titres is not known some suggestions are offered.

8.4.4.2 Proposals for *post*-exposure vaccination protocol for flying foxes in captivity

- Vaccination to be given as soon after confirmation of an ABLV-positive result in bat (or other animal) to which flying foxes were exposed. Use of vaccine in post-exposure management to be authorized by the Chief Veterinary Officer on a case by case basis.
- Exposed flying foxes to be isolated from other animals and contact strictly restricted to vaccinated staff.
- Vaccination with two (or three) Nobivac inoculations, e.g. PV-Day 0 and 14, or PV-Day 0, 10, and 20.
- Identification and record requirements should apply as for pre-exposure vaccination.
- Response validated by determination of plasma titres in rabies-RFFIT on PV-day 28.
- Flying foxes that respond with rabies-RFFIT titres ≥ 2 IU/mL and remain clinically well for 3 months to be returned to normal status.
- Bats that respond with titres < 2 IU/mL to be housed separately and given a further booster, and observed for a further 4 months before reverting to normal status.

Sick, injured, and orphaned bats temporarily in captivity are typically in the care of volunteer wildlife carers and the facilities in which they are kept vary. Often unwell bats are kept within the carer's home in small 'cat basket/carrier' type cages. Some carers also have larger walk-in style cages in their yards, which are invariably single barrier type and permit contact with wild bats. Most bats require care for only days or weeks. While bats in this category have the highest incidence of clinical ABLV disease, or are most likely to have had recent exposure to ABLV, pre- and/or post-exposure vaccination of this group is precluded by the expense of vaccination and permanent identification, the unfunded volunteer nature of wildlife care, the high number bats treated each year (>1,000, Helen Luckoff ONARR, *pers. comm.*), and the undesirability of releasing vaccinated animals into the wild population where the outcome and impact of the

animals would be unknown. Consideration could be given to providing pre-exposure vaccination to the few bats that are retained by carers permanently due to injuries that preclude their release.

8.5 Conclusions

Demonstration that flying foxes vaccinated with a commercial rabies vaccine will seroconvert and develop detectable titres against rabies virus.

- All flying foxes vaccinated with the commercial Nobivac rabies vaccine did seroconvert to the rabies virus antigen and developed detectable rabies titres.
- All vaccinated Black and Grey-headed flying foxes responded to a single inoculation of Nobivac® rabies vaccine developed titres greater than or equal to 0.5 IU/mL, which is nominally indicative if protective immunity, within 28 days.
- Booster vaccination on PV-day 28 induced a rapid and very strong serological response in most flying foxes that waned in the medium (< 6 months) term to levels comparable to those that did not receive a booster.
- Young bats (possibly as young as 6 months of age) responded to Nobivac vaccination as well or better than adults.

Demonstration that rabies-vaccinated flying foxes developed cross-neutralising titres against ABLV

- Demonstration that rabies-vaccinated flying foxes were cross protected against ABLV required post-vaccination challenge experiments that were beyond the resources of this trial.
- Bats with rabies-RFFIT titres of ≥ 2 IU/mL were shown to have cross-reactive neutralising ABL-titres ≥ 1:154. The cross reactive titres of seven samples with rabies-RFFIT titres of ~ 0.5 IU/mL could not be determined due to the cytotoxic effect of the plasma at low dilutions.
- Extrapolation from Bat 8 (Chapter 6) suggests residual ABLV-titres as low as 1:13 and 1:50 can protect against subsequent challenge.

Differentiation of rabies-vaccinated from unvaccinated flying foxes by inducing concurrent seroconversion to a marker (KLH) protein

- Attempts to reliably differentiate Nobivac-vaccinated bats from naturally immune bats by inducing a concurrent serological response to a marker protein (KLH) failed. While there was a strong immune response that was readily detectable in a specifically developed ELISA, titres had a tendency to fall to levels (≤ 20 PA) similar to those seen in up to 5% of unvaccinated bats, after an unacceptably short (<<1 year) duration.
- Permanent recognition of the vaccination status of captive bats can most reliably be done by suitable individual identification (banding and microchipping) and the maintenance of adequate records.

Development of an enzyme linked immunoabsorbant assay for the detection of seroconversion to the marker (KLH) protein.

- An enzyme linked immunoabsorbant assay was successfully developed to detect seroconversion to the KLH marker protein, with fewer than 5% of wild-caught presumably unvaccinated flying foxes having titres > 20 PA.

Other conclusions are:

- There was evidence of passive maternal antibody transfer to pups born to vaccinated dams; however the level of protection provided is unknown.
- Use of commercial rabies vaccines such as Nobivac for pre-or post-exposure management of captive flying foxes is likely to reduce risk of ABLV infection in captive bats and the people with whom they have contact.
- Use of the vaccine for this purpose requires approval by State Veterinary Officers and AQIS, which would be subject to other considerations such as the impact of such use on Australia's rabies-free status.

9 General Discussion

The overriding objectives of this project were to characterise ABLV and ABLV infection, to compare those characteristics with those of other lyssaviruses, in particular rabies virus, and to identify risk factors for consideration in the management of potential exposure to ABLV.

As described in this study and in reports from Australia's WHO Rabies Laboratory, AAHL (Fraser *et al.* 1996; Gould *et al.* 1998; Gould *et al.* 2002; Hanna *et al.* 2000; Hooper *et al.* 1997b; Hooper *et al.* 1999a; Speare *et al.* 1997), Australian bat lyssavirus has only been identified in each of the four common species of Australian flying fox (Black, Grey-headed, Little Red and Spectacled), Yellow-bellied sheathtail (YBST) bats, and two humans.

The complete coding sequence of the nucleoprotein of 31 isolates of ABLV were determined in this study and provided to GenBank (accession numbers AY573935 to AY573665). They include sequences of ABLV isolated from all affected bat species except for the single isolate from a Spectacled flying fox. Phylogenetic analysis presented here of these sequences together with other ABLV, rabies, and rabies-like virus sequences indicated that ABLV occurs as two variants. One was only isolated from YBST bats and the first human case, the other was isolated from flying foxes and the second human case. The results of this study confirm those of a similar analysis done using only partial coding sequences of a conserved region of the N gene from fewer samples (Gould *et al.* 2002), and complements analysis of the complete G protein coding sequence done on a subset of the isolates used here that was done by other members of our research group (Guyatt *et al.* 2003). This molecular epidemiological pattern, where infection with a particular virus-variant is found primarily in one host with occasional spill-over to dead end hosts, is very similar to that of the other lyssaviruses, including rabies virus. In the Americas, there are many rabies virus variants, each of which is most frequently recognised in a single (primary) host species in a region (e.g. dogs, foxes, raccoons). The primary host is believed to be responsible for enzootic maintenance, with sporadic disease occurring in many mammalian species (Smith *et al.* 1990; Smith *et al.* 1995). The basis of maintenance host-virus specificity is unknown. Unlike dog- and fox-variant rabies virus (Holmes *et al.* 2002; Nadin-Davis *et al.* 1993), ABLV does not appear to have regional sub-variants. This is presumably because bats fly, thus precluding regional isolation of hosts and virus that could give rise to genetic drift. In general a similar lack of regional sub-variants is seen for rabies virus isolates from insectivorous bats in the USA and Canada (Nadin-Davis *et al.* 2001; Nadin-Davis *et al.* 2002; Smith 1988; Smith *et al.* 1995).

In the case of ABLV it appears YBST-variant ABLV is maintained among YBST bats. Phylogenetic analysis of isolates from flying foxes (pteropid-variant ABL) indicated that only a single ABLV variant occurs among Black, Grey-headed, and Little Red flying foxes. The level of sequence homology among isolates from the three flying fox species throughout Queensland was very low, and there was no evidence of species specific-sub-variants. That pteropid-ABLV

infection is common in three, albeit closely related, bat host species is similar to the situation in Canada where Arctic foxes (in the north) and Red foxes and skunks (in the south) are vectors for Arctic fox-variant rabies (Nadin-Davis *et al.* 1993). For ABLV it is not clear whether one or all three flying fox species function as maintenance hosts. The populations of these bat species are in close proximity, with Black, Little Red and Spectacled flying foxes sharing camps in Queensland's north; Black, Grey-headed, and Little Red flying foxes sharing camps in Queensland's south, and considerable migration of Black and Little Red flying foxes occurring throughout Queensland (Eby 1991; Hall and Richards 1979; Hall and Richards 2000). It is possible that ABLV is maintained in only one or two flying fox species with frequent 'incidental' dead-end spill-over affecting the other species as a consequence of their close proximity and social interactions. A similar situation affects thousands of cattle each year in South America. There a variant of rabies maintained in vampire bats kills humans and cattle as dead-end hosts because infected vampire bats transmit rabies when feeding on the blood of living animals (Martinez-Burnes *et al.* 1997; Warner *et al.* 1999; World Health Organization 1992). The extent to which other Australian native or domestic animal species are infected with ABLV remains unclear as adequate numbers of non-bat species with ABLV-like clinical signs have not been submitted for ABLV exclusion.

This study is the first to describe and analyse the epidemiology of ABLV in the population of unwell bats with which most humans have contact and from whom the there is a risk of human infection with ABLV that is of public health significance. The results reported here identify risk factors for ABLV infection and characterise the clinical histories and progression of ABLV in wild bats. These data provide a basis on which assessment of the likelihood of ABLV infection in individual bats can be made by carers and veterinarians.

Statistical analysis of 1,143 bat submissions indicated there was a highly significant association with species and prevalence of ABLV among sick, injured, and orphaned bats. The rate of ABLV infection in the few YBST bat submissions (5 of 7, 71%) was exceptionally high, and ABLV was significantly more prevalent among Little Red flying foxes (16.9%) than Black and Grey-headed flying foxes (7.8 and 4.6%), and significantly less prevalent in Spectacled flying foxes (1%). These data are based on bats submitted as a consequence of them having come in to contact with humans, and indicates that species is a highly significant risk factor for human exposure to ABLV. This data does not, however, necessarily indicate that the same species-specific differences in the prevalence of ABLV occur in the whole bat population as species-specific factors other than the true population prevalence may be reflected in this data. Factors such as proximity of camps to human activity, feeding, migration and behavioural patterns, prevalence of other diseases or injuries, and the enthusiasm and experience of local wildlife carers, may have contributed to the proportion of each species submitted that was ABLV-positive. Similarly this data does not indicate which flying fox species is/are important in the maintenance of pteropid-ABLV. Differentiation of maintenance as against incidental hosts will be important should a method or ABLV-control in wild bats become available.

Both age and health status were also associated with the prevalence of ABLV. Infection was higher in adult rather than juvenile bats. However even orphan pups, hundreds of which are raised by volunteers each year, pose a risk of ABLV infection to humans (Field *et al.* 1999). Infection with ABLV was very common (21%) in bats showing clinical signs suggesting CNS disease, significantly less common (3%) among unwell bats *not* showing signs of CNS disease, and virtually absent (0%) among apparently healthy bats. In the absence of any reported bite or scratch of a human by a bat, veterinarians presented with bats need to make assessments as to whether or not to treat an individual and whether or not to submit individuals for testing. Criteria for making this assessment have not been published elsewhere. Knowledge about the species, age, and health status of a presented bat, based on careful inquires in to the clinical history, should allow veterinarians to make rational choices using the information presented in Table 2-6 and Table 2-7. For example, the very high predicted prevalence of ABLV among unwell adult Little Red flying foxes with CNS signs (>55%) suggests all carers presenting with these bats should be strongly advised to kill the bat and submit it for testing.

The incubation periods, clinical signs, clinical duration and outcome, serological responses, gross and histological lesions, and distribution of viral antigen of bats naturally and experimentally infected with ABLV are described in this report. They are indistinguishable from those of humans and animals infected with other lyssaviruses, notably rabies (Allworth *et al.* 1996; Fraser *et al.* 1996; Hanna *et al.* 2000; Hooper *et al.* 1999a; Hooper *et al.* 1999b; McColl *et al.* 2002; Samaratunga *et al.* 1998). The few known incubation periods for natural ABLV infection in bats (36 to 57 days and 30 days), humans (4.5-6 weeks, and 27 months), and experimentally infected bats (10 to 19 days, this study, and up to 27 days McColl (1999) and McColl *et al.* (2002)), fall within the wide range of incubation periods for rabies of 7 days to 6 years. As described here, the clinical picture of natural ABLV infection, like rabies, occurs as two forms; furious, characterised by uncharacteristic aggression including flying out of trees to bite humans and dogs, and the more common paralytic form characterised by progressive hind limb or generalised paresis. As in rabies, bats with furious ABLV infection often die after a short clinical period (within 1 day of rescue) with rapid terminal deterioration. Those with paralytic ABLV infection can linger for up to 9 days in care. As in rabies, bats infected with ABLV have absent or low antibody titres at death and lyssavirus titres in clinically ill or normal potentially exposed bats are of no diagnostic significance and should not be used to determine post-exposure management procedures. Street (wild-type) ABLV has a tendency to form less obvious and less numerous eosinophilic neuronal inclusions (poorly negrigenic) than street rabies virus, however this characteristic is not distinctive as the presence of inclusions is highly variable in rabies.

The mode of transmission of ABLV among bats is not known, but anecdotal evidence from the two human cases suggests that like rabies it is transmitted by biting, presumably via saliva. A role for saliva in the transmission of ABLV is supported by detection in this study of ABLV RNA in the saliva of experimentally infected bats, and by the detection of ABLV antigen in the salivary gland parasympathetic ganglia and rare epithelial cells of some submandibular glands. The extent to

which other modes of transmission play a role in the maintenance of ABLV is unknown but is suggested by the detection during this study of ABLV RNA in the urine of one of three naturally infected bats. A potential role for urine and other body fluids in the transmission of rabies virus via atmospheric contamination has been suggested for cave dwelling insectivorous bats. However as flying foxes roost in open tree canopies, there is substantially less potential for ABLV to accumulate in the atmosphere.

The question of how long ABLV has been present in Australia remains unclear due a lack of suitable archival material. ABLV was first recognised in 1996 as a consequence of bats becoming the focus of research for the natural reservoir of Hendra virus (Fraser *et al.* 1996). ABLV had not been recognised in bats or humans prior to that, despite Australia having good standards of veterinary care, suggesting ABLV may have been recently introduced. However, the presence of ABLV in all flying fox populations from which sufficient numbers of appropriate samples have been taken, and the presence of two discrete, genetically stable variants in two host genera (YBST bats and flying foxes), suggest ABLV has been present for sufficient time to allow evolution and distribution into these two ecological niches. The diagnostic investigations of 100 consecutive flying fox submissions with signs suggesting CNS disease indicated that (1) CNS disease was common among flying foxes (100 cases in a 4 year period), (2) ABLV infection in bats was not unique in its clinical presentation, and (3) ABLV was not the only cause of infectious CNS disease in wild flying foxes that had not be previously reported e.g. neuro-angiostrongylosis. It is proposed here that our apparent failure to have recognised the presence of ABLV prior to 1996 is not an indication that ABLV is new in Australia. It more probably reflects (1) that flying foxes had not previously been viewed as being of veterinary or economic importance and so appropriate material had not previously been examined (2) that most Australians, including veterinarians, expected 'rabies' to present with overt aggression, (3) that most flying foxes present with paralytic (dumb) clinical signs and (4) spill-over into domestic animals or humans is apparently rare.

Recognition of lyssaviruses in bats was first made in vampire bats as a consequence of relating human rabies virus infection to a history of vampire bite. Similarly recognition of Duvenhage virus was a consequence of human infection following a bat bite (Meredith *et al.* 1971). The discovery of all other bat lyssaviruses occurred only as a result of surveillance of bat populations prompted by recognition of rabies in vampires and the resulting recognition of other lyssaviruses in bats. It was not as a consequence other bat lyssavirus infections having caused economic losses, human deaths, or bat health concern (Amengual *et al.* 1997; Boulger and Porterfield 1958; King and Crick 1988; Lumio *et al.* 1986; Shope *et al.* 1970). In the case of ABLV, its recognition in flying foxes was 'incidental' to a separate investigation for the natural host of Hendra virus (Fraser *et al.* 1996). With the notable exceptions of rabies in vampire bats and the cost of post-exposure treatment, the presence of lyssaviruses in bats is of comparatively little consequence to humans, causing only rare human fatalities and having virtually no impact on domestic or commercially significant animals. As this also appears to be the case for ABLV, and given the previous lack of professional interest in Australian bat diseases, it is not surprising ABLV was not detected earlier.

In this study a targeted approach and TaqMan® PCR assay were used to demonstrate that detection of ABLV by FAT on fresh brain touch impressions at ARI was highly sensitive. The results reported here showed that ABLV is relatively easy to find *if* sufficient numbers of *appropriate* tissues are examined, being the brains of unwell adult bats, particularly those showing signs of CNS disease. With the exception of samples from Spectacled flying foxes, the predicted ABLV prevalence in such samples is > 20%, forecasting a 99% detection rate from targeted surveillance of as few as 21 samples. Targeted surveillance of unwell/CNS bats in southeast Asia is likely to reveal that the geographical and host ranges of ABLV extend north of Australia and may also detect other related lyssaviruses. Attempts to detect ABLV (and presumably other lyssaviruses) through surveillance of apparently normal animals is comparatively wasteful, but has been successful (Botvinkin *et al.* 2003; Boulger and Porterfield 1958). In Australia collection/submission of appropriate samples was relatively easy due to the extensive network of volunteer carers that rescue and treat unwell wildlife. This resource has the potential to reveal any number of other pathogens of Australian wildlife. The establishment and development of relationships with local wildlife carers and members of the public interested in animal welfare is likely to allow this targeted approach to be used for more effective surveillance of other bat populations in Australia and elsewhere.

Isolation of ABLV in mice (Fraser *et al.* 1996), demonstration of cross-protection of rabies vaccines by mouse neutralisation tests (Hooper *et al.* 1997b), and the experimental mice infections described here demonstrate that the mouse is a suitable, convenient, and affordable model for ABLV research. As for other lyssaviruses there is evidence that isolation of ABLV in mice is more sensitive than by cell culture, however the use of mice, very common in rabies virus research, is limited in Australia by animal welfare concerns.

A core objective of this study was to successfully demonstrate experimental infection of flying foxes, the primary host species, with pt-ABLV in a manner that would as closely as possible simulate natural infection in the wild. It was considered important to maximise the likelihood that inoculated bats would develop clinical disease while minimising the extent to which the experimental model differed from what was presumed to be the typical exposure to ABLV in wild bats; transfer of ABLV via saliva through the bite of an infected bat. To do this the following strategies were used.

- Preparation of the inoculum directly from tissue of a naturally infected bat in order to inoculate a virus population with the same characteristics as occurs naturally. There was no prior virus isolation in cell culture or by mouse inoculation to avoid selection effects that may have altered the virulence and pathogenesis of the inoculum in the experimental host.
- Matching the experimental host species (pteropid bat) with ABLV variant (pteropid-ABLV). Phylogenetic analysis of the sequence from an aliquot of the inoculum used (Inoculum 5, ABL01SEQ-B, GenBank AY573935) indicated that while the inoculum was derived from a

Black flying fox, the inoculum was of the same ABLV-variant present in Grey-headed flying foxes, the species used as the experimental host.

- Storing the initial preparation of all candidate inocula in multiple aliquots in the -70 freezer so that all uses of the inoculum would be of the same freeze/thaw generation, and the virus titre, virulence, and pathogenesis resulting from multiple use would be consistent.
- Selection of an inoculum not only based on a demonstrated ability to affect IC inoculated mice or cell culture, but by characterizing and titrating the virulence of multiple (n=15) candidate inocula in IC and footpad inoculated mice. In particular the selection procedure used a model of peripheral inoculation of weanling, immune competent, outbred (ordinary) mice in a new 3-mouse footpad assay. This assay considered both the number and rate at which inoculated mice were affected to rank the relative peripheral virulence of candidate inocula and select the most virulent. This 3-mouse footpad assay used fewer mice than classic titration methods (Reed and Muench 1938) and successfully identified a consistent and highly virulent inoculum (Inoculum 5) from 15 candidate inocula.
- Selection of bats without demonstrable anti-lyssavirus titres for experimental inoculation using the modified rabies-$RFFIT_{AAHL}$. Subsequent testing in a pt-ABLV-RFFIT at CDC suggests the rabies-RFFITS at both AAHL and CDC are not sufficiently sensitive to identify all flying foxes with naturally occurring immunity to pt-ABLV and that such a selection process should be based on serology specifically to ABLV.
- Use of a moderate dose of virus (compared to other lyssavirus infection studies) to maximise the likelihood of clinical disease without excessively influencing the pathogenesis by using a very high virus dose.
- Peripheral inoculation of the inoculum in multiple sites on the body (2 or 4) that were considered likely to be places a bat might be bitten and/or were likely to support establishment and progression of infection. It was expected that this would avoid inadvertent inoculation into an inappropriate site.

Many experimental rabies virus studies use either well characterised, stable ‘fixed’ laboratory strains, or isolates of street (wild type) virus that have been amplified by limited mouse brain or cell culture passage. The only previous trials of ABLV infection in flying foxes used ABLV isolated from the brain of a naturally infected Grey-headed flying fox (AAHL 96-1432-1) passaged four times in mice and four times in cell culture (McColl *et al.* 2002). Inoculation of primary host species with rabies virus inocula prepared directly from the salivary glands of the same naturally infected host species was done for a large body of work with Canadian skunks by Charlton and co-workers (Balachandran and Charlton 1994; Charlton and Casey 1979a, 1981; Charlton *et al.* 1983, 1984a, 1987a; Charlton *et al.* 1987b; Charlton 1994; Charlton *et al.* 1996; Charlton *et al.* 1997; Smart and Charlton 1992). A similar approach was used here. Fortunately the intrinsic virulence of Inoculum 5 was sufficiently high that further amplification was not required.

The use of a selection procedure to identify virulent lyssavirus inocula for subsequent experimental use is rarely described in the literature. The titres of inocula are usually simply described as the LD_{50} of intracerebrally infected mice ($MICLD_{50}$) or TCID's. Neither of these intrinsically reflect peripheral virulence as the titres of some rabies virus strains that have low pathogenicity or are apathogenic in peripherally inoculated adult mice (e.g. HEP-flury, SAD-B19) can be high when expressed with these assays. The use of the 3-mouse footpad assay to select, characterise and/or quantify the virulence of inocula is proposed here as a more appropriate means of expressing the dose of virus used in experimental research.

Use of these strategies resulted in the demonstration of a highly successful model for ABLV infection of experimentally inoculated bats, with clinical disease occurring within 10 to 19 days in seven of 10 bats. The clinical, gross, histological and immunological findings of this study (Chapter 6) were consistent with those of naturally infected bats (Chapter 1), allowed further observations to be made regarding the incubation period, early clinical signs and clinical progression, and provided relatively controlled/standardized tissue samples for subsequent comparative studies in mice. Use of this model may contribute to further successful investigation into the characterisation of ABLV and ABLV infection.

Comparison of virulence of inocula from the brain and salivary glands of the experimentally infected flying foxes indicated that while the relative virulence of inocula from the brain was always high, inocula from the combined submandibular and sublingual salivary glands was often as high or higher. This was despite substantially less ABLV RNA being present in salivary gland inoculum and substantially less ABLV antigen being detected in salivary gland tissue. The relative virulence of inocula prepared from the brains and salivary glands of naturally infected flying foxes is likely to be variably impacted by post-mortem degradation, storage, and transport conditions, and so the virulence of a particular inoculum requires *in vivo* assessment. This study demonstrated a crude correlation between the quantitative detection of ABLV-RNA using the TaqMan assay and the peripheral virulence of multiple inocula determined in the 3-mouse footpad assay. It is proposed that the TaqMan assay could be useful screening tool to identify inocula of interest reducing the number of mice, time, and other resources required to assess large numbers of inocula.

As expected from the close serological and antigenic relationship between ABLV and rabies virus, commercial rabies vaccines conferred protection to mice challenged with YBST-variant ABLV (Hooper *et al.* 1997b)(Charles Rupprecht CDC *pers. comm.*). No person known or suspected to have been exposed to ABLV that has received pre- or post-exposure rabies vaccination with the human diploid cell vaccine ± human rabies immune immunoglobulin has developed clinical ABLV infection. It is presumed that rabies vaccination confers cross-protection against both pteropid-variant and YBST-variant ABLV because of their high antigenic and G gene sequence similarities to rabies virus. Rabies vaccination is not cross-protective for the more divergent Lagos bat or Mokola viruses and is protective at higher titres for Duvenhage, EBL-1 and EBL-2.

This study reports for the first time use of an animal rabies vaccine in pteropid bats. Black and Grey-headed flying foxes were vaccinated with the only commercial animal rabies vaccine available for use in Australia (Nobivac® Rabies inactivated vaccin (*sic*)). Vaccination resulted in the development of neutralizing antibody titres to rabies virus (CVS-11) and ABLV that were detected in RFFITs done at AAHL and CDC, Atlanta. All bats developed rabies titres > 0.5 IU/mL, which are indicative of protective immunity in other species, as calibrated against international standard control serum. In the absence of post-vaccination challenge experiments or an international reference standard for protection against ABLV, it is unclear to what extent the detected cross-reactive neutralizing titres to ABLV indicate protection to subsequent ABLV challenge. However, one of three experimentally infected bats to survive ABLV inoculation had a fluctuating low (1:13 and 1:50) neutralizing ABLV titre prior to inoculation and developed a strong (up to 1:480) but transient ABLV titre that coincided with the onset and progression of clinical disease in seven other bats. The protective ABLV immunity apparently present in this bat was not detected in pre-inoculation samples tested in the rabies-RFFIT at either AAHL or CDC. These results suggest that while the serological relationship between ABLV and rabies virus in bats is close, low but biologically significant ABLV titres occur below the cross-reactive rabies-RFFITs detection threshold. Clearly meaningful surveys of the prevalence and titres of naturally occurring ABLV immunity will require development of a reliable and sensitive ABLV-RFFIT in Australia.

9.1 ABLV: a rose by any other name

The recognition and classification of a lyssavirus in Australia is not just an academic exercise as it has substantial cultural, trade, financial, legal, and veterinary/medical implications with respect to Australia's WHO rabies-free status. The same is true of the presence of EBL-1 and EBL-2 in parts of Europe, which are also otherwise lyssavirus free. There are essentially two schools of thought regarding the classification of the EBLs, Duvenhage, and ABLV. One, that these rabies-like viruses are sufficiently different from the comparatively diverse rabies-virus variants that in addition to being unique and distinguishable variants/species/genotypes of the *Lyssavirus* genus that they warrant classification completely distinct from the multiple variants of rabies virus. The other, that these differences, while discernable and useful as a basis for nomenclature, do not justify classification of these viruses as other than simply the most divergent variants/genotypes within a broader functional rabies virus group. The classification of ABLV has been the subject of considerable private if not published international debate.

The morphology, chemical composition, and replication strategies of ABLV are indistinguishable from those of rabies virus or the other lyssaviruses (Hooper *et al.* 1997b). As discussed, all aspects of clinical ABLV disease are indistinguishable from rabies virus and the other lyssaviruses. Both rabies virus and ABLV are serotype 1 lyssaviruses and consequently diagnostic reagents for rabies (Centocor, HAM) and rabies vaccines are effective in the diagnosis and management of ABLV. The ecological niche in which ABLV is maintained (Australian bats) is

unique among lyssaviruses. However, it is identical in its characteristics; maintenance in primary bat species with occasional 'dead-end' spill-over into humans and potentially other mammalian species, to other unique ecological niches occupied by other bat lyssaviruses including rabies virus. In the absence of monoclonal antibody typing, molecular sequence and phylogenetic analysis, ABLV is simply indistinguishable from rabies virus, and would presumably have been called such had it been recognised prior to 1970. Indeed this may already have been the case for two isolates of 'bat rabies' in fruit bats from India and Thailand (Pal *et al.* 1980; Smith *et al.* 1967).

Antigenic typing of multiple ABLV isolates indicates ABLV has a unique antigenic profile that differentiates it from all other known lyssaviruses, including multiple variants of rabies virus in bats and terrestrial animals (Gould *et al.* 1998; Hooper *et al.* 1997b). However this uniqueness is dependant on a negative reaction to only one of a panel of monoclonal antibodies (62-15-2) of the ERA series (Smith *et al.* 1984a), having 18 other reactions in common with raccoon variant rabies virus (Smith 1989). When compared to those of rabies virus variants and the clearly more distantly related Lagos bat and Mokola viruses, the antigenic profile of ABLV, while unique, is simply not unique *enough* for ABLV to be considered other than another unique variant of rabies virus.

The most distinctive feature of ABLV is its molecular sequence. Based on sequence similarity of either the N or G genes, even this is not *sufficiently* different (maximum ABLV N protein amino acid similarity to rabies virus 93.6%, Figure 4-4) from all other rabies virus variants to be recognised as a new type of virus rather than a new rabies virus variant (minimum intra-rabies genotype N protein similarity 92.2% (Kissi *et al.* 1995)). Phylogenetic analysis alone, a powerful and very sensitive tool, suggests ABLV warrants classification as its own 'type' of virus. Both maximum parsimony and neighbour joining methods when used to analyse the N, P or G genes or amino acid sequences place both ybst-variant and pteropid-variant ABLV together, with very strong (>97%) bootstrap support. ABLV lies between EBL-2 and all rabies virus isolates, separated from each by a relatively long branch length (Gould *et al.* 2002; Guyatt *et al.* 2003; Nadin-Davis *et al.* 2002; Warrilow *et al.* 2002). There are however no objective criteria for defining a lyssavirus genotype based on phylogenetic trees, and this is problematic for Duvenhage and EBL-1, ABLV and rabies virus, and the new Asian bat isolates; Aravan, Khujand, Irkut and West Caucasian bat viruses (Botvinkin *et al.* 2003; Kuzmin *et al.* 2003). A high bootstrap value is supportive of a genotype, indicating the level of confidence that all sequences on that branch belong on that branch and not elsewhere on the tree. However, it in no way indicates that the *level* of similarity within the branch and the level of divergence between that and the adjacent branch are sufficiently high that the branch defines a genotype. Controversy surrounding virus taxonomy, both in terms of how it is expressed and what criteria should be used to group and differentiate related isolates is ongoing (Eberhard 2004; van Regenmortel and Mahy 2004).

Of greater practical significance is the distinction between ABLV and rabies virus, notably terrestrial rabies variants and vampire bat rabies, in terms of the threat posed to human and

domestic/economically important animal health. Unlike rabies in vampire bats and carnivores, especially dogs, ABLV, like the other bat lyssaviruses, is an insignificant cause of rabies or rabies-like disease in humans. The standard of medical care in Australia is sufficiently high that were ABLV a frequent cause of disease in Australians, even without prior recognition of the virus in bats, it would have been recognised and isolated from humans. The same is also true, to a lesser extent, of the level of veterinary surveillance for rabies-like disease in domestic animals. That ABLV has not been detected in other animals, particularly dogs and cats since the deaths of two women received widespread media attention, suggests that ABLV is not a frequent cause of non-bat disease. However, it is certainly possible, even likely, that occasional cases of ABLV infection in humans and other animals have gone undetected given the often unspectacular clinical signs associated with the paralytic form, the indistinct or absent gross and histological lesions, and the low level of specific surveillance testing for lyssaviruses by FAT in non-bat species. The presence of ABLV in Australia in no way decreases the threat to human and domestic animal health posed by the introduction of dog-variant or dog-derived variants of ABLV present in rabies endemic countries. These variants have a history of establishing new enzootic cycles in dogs and related carnivore species (Bourhy *et al.* 1999; Holmes *et al.* 2002). Domestic dogs, the Australian dingo, red foxes, and carnivorous marsupials such as quolls are expected to be suitable maintenance hosts into which sylvatic cycles of dog- or dog-derived rabies variants could adapt and become established. Presuming ABLV has been present for the past 200 years, the risk to our export markets has not increased and the threat to Australia from imported animals and virus remains high. Consequently there is no justification for changing Australia’s rabies-free status.

9.2 General conclusions and future directions

General conclusions

- ABLV is a unique virus of the genus *Lyssavirus.*
- Two variants of ABLV have been recognised; YBST-variant ABLV apparently prevalent in YBST bats and pteropid-variant ABLV prevalent in four species of Australian flying fox (*Pteropus alecto*, *P. poliocephalus*, *P. scapulatus*, and *P. conspicillatus*).
- ABLV infection is associated with a rabies like-disease in bats and humans that like rabies occurs in two clinical forms, furious and paralytic.
- Species, age and health status are risk factors associated with the prevalence of ABLV in bats.
- The prevalence of ABLV in healthy bats is <1%, is 5 to 8% among unwell bats, and is as high as 20 to 30% in bats with clinical signs suggesting central nervous system disease, particularly paresis or uncharacteristic aggression.
- Members of the public, wildlife carers, and veterinarians that have contact with unwell bats are at moderate to high risk of exposure to ABLV.
- Histories of bites from uncharacteristically aggressive bats in both human cases of ABLV infection, a history of direct contact with an ABLV-positive bat in a bat case of ABLV (ABLV-73), the high virulence of inocula prepared from the salivary glands in bats, and demonstration of ABLV RNA in the saliva of experimentally infected bats support transfer of saliva in a bite site/other lesion by infected bats as a mode of transmission for ABLV.
- The cross-reactive rabies-RFFIT is not sufficiently sensitive for the detection of natural ABLV immunity in bats.
- The serological response in captive flying foxes to vaccination with a commercial rabies vaccine (Nobivac) is likely to confer cross-protection against ABLV challenge.

Future directions

- While transmission by bites is probably *a* mode of ABLV transmission, it is unclear to what extent this and/or other modes of transmission are important in the maintenance of ABLV in bats. Further research into modes of natural ABL transmission is indicated.
- While it is clear that ABLV infection is common in Black, Grey-headed, and Little Red flying foxes, and significantly less common in Spectacled flying foxes, it unclear whether one or all of these species is responsible for maintenance of ABLV infection. Further research into the epidemiological role of each species is indicated.
- Development and standardisation of ABLV-RFFITs in Australia, particularly for pteropid variant ABLV, but also YBST-variant ABLV, is required if meaningful interpretations of bat immune responses to natural and experimental exposure to ABLV are to be made. This should preferably involve development of the test in a cell line not subject to the cytotoxic

effects of bat serum, or identification and 'treatment' of the cause of this toxic effect on BHK and neuroblastoma cells.

- The cross-protective effect of rabies vaccination in bats against ABLV challenge needs to be established by post-vaccination challenge experiments. The duration of this effect and the effectiveness of immunity in different age classes of bats should be established if vaccination of captive bats is to be used to reduce the risk of infection in captive bats and the risk of exposure to ABLV in the humans that care for them.
- Detection of 'rabies virus' in fruit bats in India and Thailand, extension of the range of Black flying foxes into islands north of Australia (Craig Smith and Hume Field, unpublished data by satellite tracking), and the detection of serum with neutralising activity for rabies virus and ABLV (Arguin *et al.* 2002) suggest ABLV and/or another lyssavirus is present in Asia. Targeted surveillance of sick bats in Asia, particularly those with signs of paresis or aggression is indicated.
- Development of a suitable means of vaccine delivery is necessary if control programs for bat lyssavirus such as ABLV are to be implemented.

Appendix 1 Map: DPI regions and centres of Queensland

Figure A1-1 Map of Queensland: Department of Primary Industries Regions and Centres

Appendix 2 Rapid fluorescent focus inhibition test

The rapid fluorescent focus inhibition test (RFFIT) is an *in vitro* assay for neutralizing antibodies to rabies virus. The test is essentially a 24 or 48 hour serum neutralization test that uses immunofluorescent staining to detect viral growth. In Australia all lyssaviruses other than ABLV, which includes all Genotype 1 rabies variants, are classified as PC3 Exotic animal viruses (AS/NZS 2243.3:1995) and so can only be used at the AAHL, precluding the performance of this test at the ARI. Unless otherwise specified, all RFFITs referred to in this thesis were performed by Ross Lunt and Kim Newberry of the AAHL Rabies Laboratory. The RFFIT can be adapted for use with any of the lyssaviruses. For the purposes of this thesis the various RFFITs referred to are:

rabies-RFFIT	RFFIT using genotype 1 rabies virus (CVS-11), synonym CVS-RFFIT
modified rabies-RFFIT$_{AAHL}$	rabies-RFFIT modified for bat plasma to reduce toxic effects of bat plasma on BHK cells at AHHL
pt-ABLV-RFFIT	RFFIT using pteropid-variant ABLV
ybst-ABLV-RFFIT	RFFIT using Yellow-bellied sheathtail bat-variant ABLV

Rabies-RFFIT at AAHL

Within the AHHL all lyssaviruses are classified as Biosafety Level 3Z (PC3 Zoonotic).

The test

- The challenge virus is rabies challenge virus standard (CVS–11).
- BHK 21 BVT vaccine strain cells were used to propagate and grow stock quantities of virus, and in the rabies-RFFIT assays.
- The tests were conducted in Lab-Tek 8-Well Chamber Slides (Permanox).
- Plasma samples heat inactivated at 56 °C for 30 minutes to inactivate complement.
- Each test sample is normally screened at final dilutions (after the addition of virus) of 1:20 and 1:200, and the 2 IU/mL WHO international standard serum is used at dilutions of 1:8, 1:16, 1:32, and 1:64. For endpoint titration of test sera, further test sera dilutions may be required.
- CVS–11 and plasma samples are diluted in Basal Medium Eagle (BME, Gibco) with 5% TPB, hepes, glutamine, pen/strep and Fungizone.
- 100µL of diluted plasma and 100µL of diluted CVS–11 containing 50 50% Fluorescing Focus Doses (i.e. 50 FFD_{50}/0.1mL) are added to each well and incubated at 35°C in 3.5% CO_2 for 90 minutes.
- Healthy BHK vaccine cells that had been passed 2 days earlier are prepared as a suspension containing 0.5×10^6 cells/mL in BME - 5% TPB with 10% heat inactivated foetal calf serum, and treated with 1µL/mL of cells of 1% DEAE dextran (final concentration 10µg/mL).
- 200µL of treated cells (100,000 cells) are added to each well and incubated at 35°C in 3.5% CO_2 for 24 hours.

- After incubation, culture medium is removed and discarded and wells washed with 200-400µL of PBS with Mg and Ca, fixed with acetone, air dried, and stained for rabies virus with a 1:100 dilution of Centocor anti-rabies monoclonal FITC conjugate containing Evans Blue (counterstain) at 35°C in 3.5% CO_2 for 30 minutes. Slides are cover-slipped with glycerol immersion fluid and read under low power (20×) magnification lens by epifluorescence.
- Twenty low-power microscopic fields are examined per well and the number of fields containing fluorescent cells recorded. Wells that show poor cell coverage are noted, poor cell growth being attributed to toxic effects of the test plasma. Sera were considered negative if 20 of 20 low power fields contained fluorescent foci at a dilution of 1:20.
- Endpoint titres were calculated in international units per mL (IU/mL) by using the calculated 50% endpoint dilutions (titres) of the test plasma with that of the 2 IU/mL WHO international standard as follows:

$$\text{Test plasma (IU/mL)} = 2 \times \frac{\text{test plasma 50\% endpoint titre}}{\text{2 IU/mL International standard 50\% endpoint titre}}$$

Cell and virus controls

A control virus back-titration is run for each test that comprises two wells each with 50, 5 and 0.5 FFD of CVS-11 and diluent (no plasma), and two wells containing diluent only (no virus and no plasma) during the initial serum neutralization phase. The wells containing CVS-11 indicate the ability of the virus to grow in the absence of plasma. The diluent only wells indicate the capacity for growth of the BKH cells in the absence of virus and plasma. Only when the virus growth and cell growth in these controls are sufficient, are the results from test wells recorded.

Quality assurance

The quality of the rabies-RFFIT at AAHL is monitored by

- *Progressive monitoring* Assay performance is monitored by progressive monitoring of the cell and virus control titres and by plotting the titre of the international standard control serum.
- *External proficiency testing* AAHL participates in a program of external proficiency testing of the RFFIT by the UK Pet Travel Scheme.

Additional notes on the rabies-RFFIT at AAHL

The 2 IU/mL WHO international standard was the operating standard for the AAHL rabies RFFIT until May 2001, at which time it was replaced with the 0.5 IU/mL OIE international standard. The WHO standard remains a valid and recognized standard. The vast majority of tests performed by AAHL for this research were done before May 2001, using the WHO standard. The CVS rabies virus was obtained in 1986 from the Commonwealth Serum Laboratories (Melbourne, Australia) and has been determined to be antigenically indistinguishable from CVS-31. The designation of this virus as CVS-11 in prior records does not appear to have any recorded basis. The virus has been passaged once in mice and five times in BHK cells at AAHL.

Bat plasma modification of rabies-RFFIT at AAHL (modified rabies-RFFIT$_{AAHL}$)

Samples from bats were collected as plasma, through the use of heparinized syringes, because the small size of bat peripheral blood vessels meant that sample collection was frequently slow enough to cause blood to clot in plain syringes before acquiring adequate volumes. It became obvious early in the use of the rabies-RFFIT at AAHL, that flying fox plasma often had a strong toxic effect on the BHK cells. The basis of this toxicity remains unclear. It may be related to the variable quantities of heparin in the samples, although heparinized samples are usually considered suitable for other neutralization based tests (Ross Lunt, AAHL *pers comm.*). It is therefore presumed that the toxic effect is due to a specific interaction between the cells used and some unknown component of the bat plasma. Using the standard RFFIT protocol cell growth was often so poor the test could not be read at low plasma dilutions (e.g. 1:20) reducing the sensitivity and reliability of the test. The toxic effect appeared to be random within batches of plasma submitted and there was no obvious correlation with method of sample collection, preparation, or storage. To reduce these toxic effects, the standard rabies-RFFIT protocol was modified to reduce exposure of the BHK cells to flying fox plasma by discarding the virus/plasma/culture medium supernatant 90 minutes after addition of the treated cells and completing the 24 hour cell incubation with replacement supernatant. The relatively short virus/plasma/cell incubation was sufficient to allow adequate adherence of the BHK cells to the wells and for un-neutralized virus to infect the cells. The modified cell, virus and international standard controls continued to perform within acceptable parameters, and the incidence and severity of cell toxicity was reduced. Occasional individual bat sera still had persistent toxic effects on cells.

pt-ABLV-RFFIT and ybst-ABLV-RFFIT at AAHL

Variations on the rabies-RFFIT using two isolates of ABLV were used for research purposes only.

The pt-ABLV-RFFIT at AAHL uses an isolate of ABLV from a pteropid bat, (AAHL SAN 96-0648), a juvenile female Black flying fox found under a tree at Ballina (Fraser *et al.* 1996). The virus growth of the pt-ABLV isolate is comparatively poor despite extending the cell incubation time from 24 to 48 hours and back titration controls of this isolate indicate 48 hour viral growth is below that which would be acceptable for the rabies-RFFIT. Nevertheless, virus growth is sufficient to differentiate sera that inhibit/ neutralize virus. Results for each well were recorded as either none (0), some (+), moderate (++) or high (+++) numbers of fluorescent cells per well rather than by counting the number of low-power microscopic fields containing fluorescent cells.

The ybst-ABLV-RFFIT uses an isolate of from the first human case of ABLV (SAN 96-1256) that was characterised as being YBST-variant ABLV (Gould *et al.* 2002). This ABLV isolate is more vigorous and performs better in the assay, but still does not meet the virus back titration standards.

Rabies-RFFIT and pt-ABLV-RFFIT at CDC

Sera were sent to Charles Rupprecht of the Rabies Laboratory at the Centers for Disease Control and Prevention in Atlanta, Georgia, USA for testing in their rabies-RFFIT and independently developed pteropid-variant ABLV-RFFIT.

The CDC rabies-RFFIT is very similar to that used at the AAHL, also using CVS-11 as the challenge virus as described in (Arguin *et al.* 2002). Differences include:

- Use of 75,000 murine neuroblastoma (MNA) cells/well rather than BHK cells
- Growth of MNA cells, and dilution of viruses and bat plasma in Eagle minimum essential medium containing 10% foetal calf serum and antibiotics (Smith *et al.* 1996)
- Use of Lab-Tek 8-well glass slides with covers (Nalgen Nunc International, Naperville, IL) rather than plastic chamber slides.
- 100 infectious units of CVS-11 per well, rather than 50 FFD_{50}.
- Incubation of the MNA cells with the virus/ plasma/ culture medium for 20 hours (CVS) or 40 hours (pt-ABLV).
- Sera were considered negative if 20 of 20 microscope fields contained fluorescent foci at a dilution of 1:5. For positive sera, the 50% endpoint titre was calculated using Reed-Muench values (Reed and Muench 1938).

Sera were screened at dilutions of 1:5 and 1:25, and when necessary retested at higher dilutions to calculate the endpoint dilution titres. Standard immune globulin (HRIG) diluted to contain 2 IU/mL anti-rabies antibodies was used as a positive control. The standard reference serum dilution of 1:90 ≈ 1.0 IU/mL.

The CDC pt-ABLV-RFFIT uses isolate sm4476, first isolated from the second human case (Mackay 1998), subsequently characterised as pteropid-variant ABLV (Warrilow *et al.* 2002), and forwarded to CDC by QHSS (Charles Rupprecht, CDC, and Ina Smith, QHSS, *pers. comm*.).

The CDC also has a ybst-ABLV-RFFIT that uses isolate sm4068, first isolated from the first human case (Rockhampton 1996, AAHL SAN 95-1256), subsequently characterised as YBST-variant ABLV (Gould *et al.* 2002), and forwarded to CDC by AAHL (Charles Rupprecht , CDC *pers comm*.). This test was not used due to insufficient plasma volumes.

Acknowledgement

Parts of this Appendix have been adapted from or quote directly the CSIRO Livestock Industries Quality Assurance Manual QA/13/4/61 Rabies Fluorescent Focus Inhibition Test (RFFIT) for the Measurement of Serum Neutralizing Antibody to Rabies Virus (serotype 1 lyssavirus), kindly provided by Ross Lunt of the Rabies Laboratory, AAHL.

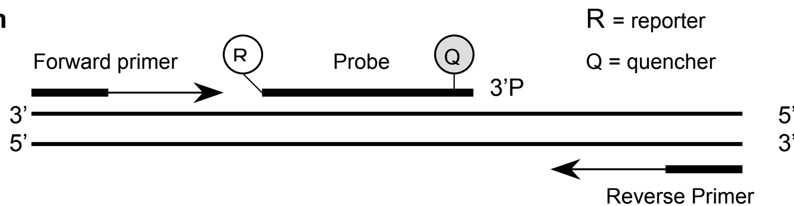
Appendix 3 Quantitative real-time PCR: TaqMan®

The TaqMan® assay (Perkin Elmer Applied Biosystems) is a PCR-based molecular genetic assay that amplifies target regions of DNA that is detected as a fluorescent signal produced as a result of simultaneous cleavage of a fluorogenic probe.

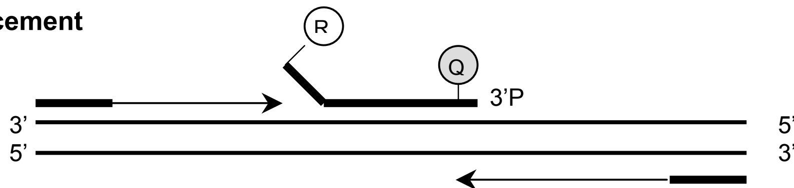
The probe, a 25 to 35 mer oligonucleotide that is labelled with a fluorescent reporter (6-carboxy-fluorescein, FAM) and quencher dye (6-carboxy-tetramethyl-rhodamine, TAMRA), is added to the PCR cocktail containing forward and reverse primers, and anneals between these primers. The quencher dye reduces emission intensity of the reporter dye when the probe is intact and phosphorylation of the '3 end of the probe prevents probe extension during amplification. The assay utilises the 5'-3' endonuclease activity of AmplTaqGold® DNA polymerase to cleave the probe, during primer extension. Cleavage of the probe releases the reporter dye from the activity of the quencher. With successive PCR cycles there is both exponential amplification of PCR product and cleavage of the probe, resulting in increased fluorescence intensity that is detected and displayed in real time on the ABI Prism 7700 sequence detection system (PE, Applied Biosystems, USA).

Figure A3-1 Principles of the TaqMan® Assay
Adapted from TaqMan® One-Step RT-PCR Master Mix Reagents Kit Protocol, Applied Biosystems.

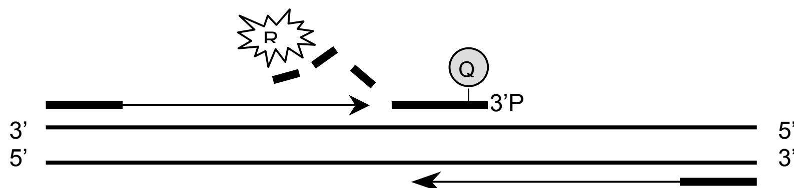
Polymerization



Strand Displacement



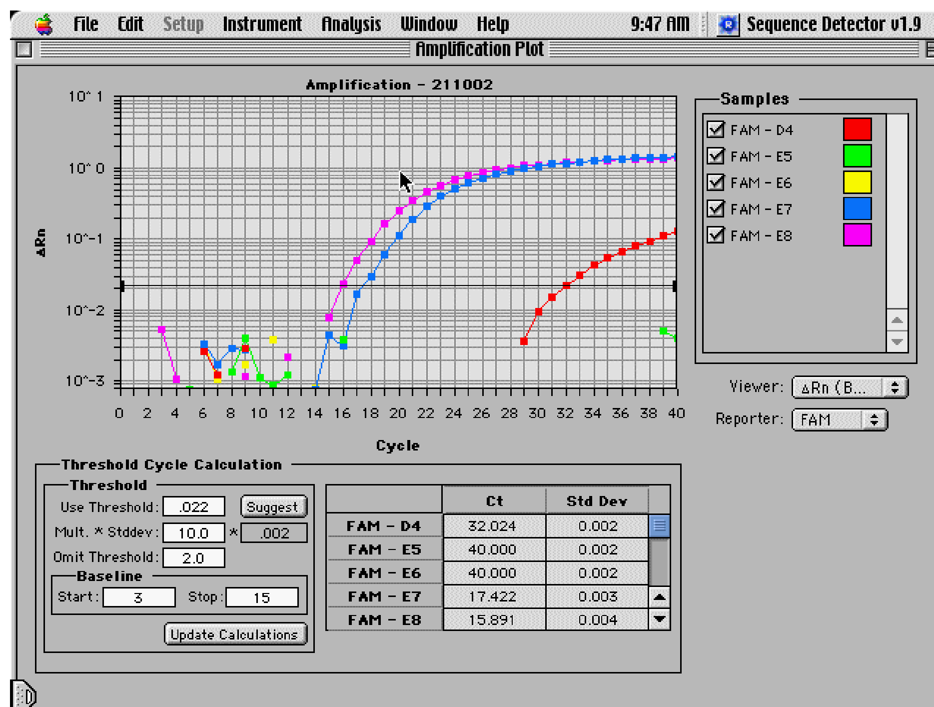
Cleavage



The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, non-specific amplification is not detected.

The change in fluorescence that occurs during PCR amplification is plotted by the sequence detection software while the PCR reaction is under way, hence the term “real-time PCR”. The threshold cycle value (C_T) is the PCR cycle number at which a statistically significant increase in the normalized reporter emission (ΔRn) is first detected (see Figure A3-2). The C_T value is predictive of the quantity of target genome initially present, hence the term “quantitative PCR”.

Figure A3-2 Example of plotted output of TaqMan® assay fluorescence detection
The output below shows the results for five samples (see designations D4 to E8 in ‘Samples’ window), three of which are positive (D4, E7 and E8). The fluorescent signals of the three positive samples cross the significant (positive) threshold after 32.0, 17.4, and 15.9 amplification cycles; as illustrated in the graph and indicated in the ‘Threshold Cycle Calculation’ window. Other samples that failed to cross the threshold have a C_T value of 40 and are negative. The threshold cycle value (C_T) reflects the amount of template RNA or DNA in the original sample, with samples initially containing higher quantities of RNA/DNA crossing the threshold after fewer cycles. In this example, sample E8 had a low C_T value (15.9) indicating a large amount of target template in the original sample, whereas sample D4 had a high C_T value (32.0) indicating only a small amount of template in the sample.



	Ct	Std Dev
FAM - D4	32.024	0.002
FAM - E5	40.000	0.002
FAM - E6	40.000	0.002
FAM - E7	17.422	0.003
FAM - E8	15.891	0.004

As lyssaviruses are -ssRNA viruses, a One-Step RT-PCR version of the TaqMan® assay (Applied Biosystems) allows for reverse transcription as well as PCR in a single buffer system using total RNA. The TaqMan® assays referred to in this thesis were developed and performed by Ina Smith, Greg Smith and staff of the QHSS (Smith *et al.* 2002). The ABLV specific primers and probes used are shown in Table A3-1.

Table A3-1 Primers and probes for pt-ABLV and ybst-ABLV TaqMan® assays

Primer/Probe	Sequence
pt-ABLV forward primer (LYSF-FF)	5' ^{175}GGA ATG AAT GCT GCA AAG CTG^{195} 3'
ybst-ABLV forward primer (LYSF-YB)	5' ^{180} GAA CGC CGC GAA GTT GG^{195} 3'
common reverse primer for pt-ABLV and ybst ABLV (LYSR)	5' ^{256}GGC AGA YCC CCT CAA ATA ACT C^{235} 3'
pt-ABLV probe (FF-FAM)	5' 6FAM-^{197}ACC CCG ATG ATG TAT GTT CTT ACT TAG CTG CAG^{229}-TAMRA 3'
ybst-ABLV probe (YB-FAM)	5' 6FAM-^{200}CGG ACG ATG TTT GCT CCT ACC TAG CTG C^{227}-TAMRA 3'

Sequence numbers indicate corresponding nucleotide base position in the lyssavirus N protein coding sequence, where 1 = A of ATG start codon.

Advantages of the TaqMan® PCR assay over other detection methods include:

- Simple and quick allowing rapid diagnosis (3-4 hours, similar to fluorescent antibody test, same day results).
- Highly sensitive as relatively low levels of dye fluorescence can be detected compared to those required for gel electrophoresis. Sensitivity comparable to nested RT-PCR assays.
- Highly specific as a result of requiring adequate annealing of not only the two primers (43 mer pt-ABLV or 39 mer ybst-ABLV) but also the probe between them (33 mer pt-ABLV or 28 mer ybst-ABLV). This high specificity has been utilized to develop pt-ABLV and ybst-ABLV specific assays, but limits its use where the sequence of the target genome is unknown or has changed.
- Greatly reduced risk of contamination as a result of the one-step, closed tube, single buffer system, particularly when compared to nested or hemi-nested PCR and gel electrophoresis.
- Large numbers of samples (up to 96) can be done simultaneously.

Appendix 4 Non-bat submissions for ABLV testing

Table A4-1 Non-bat animal submissions for ABLV-testing June 1996 to April 2002 (n=27)
All ABLV-Negative by FAT

NR not recorded
Rx treatment
Dx diagnosis
PME post-mortem examination

Accession number	Month/Year	Status	Genus. species (common name)	Sex/Age	Health status	Contact status	Comments
97-138340	April 1997	Domestic	*Canis familiaris* (Labrador dog)	Male/NR	CNS	None	Sudden change in behaviour, previously friendly/good with children, became highly aggressive, "manic'" foaming at mouth.
97-150074	June 1997	Domestic	*Canis familiaris* (Rottweiler dog)	Male/Adult	CNS	None	Dog missing several days and found on island in middle of creek. 2 days later sudden onset of, twitching, constricted pupils, died overnight. Cerebral vasculitis, suggested *Toxoplasma* or *Neospora* sp.
98-157960	July 1998	Domestic	*Canis familiaris* (Australian Terrier dog)	Male/Adult	CNS	Human	Abnormal aggression, bit 2 people. Wandering aimlessly, ataxic, disoriented, going to water but not drinking, killed. As and Pb negative. Mild vacuolation of cerebral white matter, congenital status spongiosis.
98-174714-1	Sept 1998	Domestic	*Canis familiaris* (Miniature Poodle dog)	Male/Adult	Normal	None	2 companion miniature poodles in a back yard in which an ABLV-positive flying fox was found stuck in fence (Case no. ABLV-46). Dogs had access to and 'sniffed' bat, 1 dog thought to have bitten bat about head. Owner requested euthanasia of both dogs.
98-174714-2	Sept 1998	Domestic	*Canis familiaris* (Miniature Poodle dog)	Male/Adult	Normal	None	
96-204224	Dec 1996	Domestic	*Felis catus* (domestic cat)	Male/Adult	CNS	None	Change in behaviour, violent seizures, biting body and cage, and abnormal posture, for 2 ½ days, killed.
97-151174	June 1997	Domestic	*Felis catus* (Burman cat)	Male/Adult	CNS	Human	Dribbling saliva, frothing at mouth, eating, R_x antibiotics. 2 days later aggressive, growling, barking, circling, hydrophobic, then paralysis, incontinent, spasms, killed. Malacia and encephalitis of both grey and white matter with intralesional organisms consistent with *Cryptococcus neoformans*.
97-186746	Oct 1997	Domestic	*Felis catus* (domestic shorthair cat)	Male/NR	CNS	Human	1-2 week illness, hind and forelimb paresis/paralysis, erratic behaviour. PME: 2 cm fungal granuloma in right rostral cerebrum with coning of the cerebellum. Dx phaeohyphomycosis due to *Xylohypa bantianum*.

Accession number	Month/Year	Status	Genus. species (common name)	Sex/Age	Health status	Contact status	Comments
97-50290	Oct 1997	Domestic	*Felis catus* (domestic cat)	Male/Juv	CNS	Human	3 day history of progressive neurological signs. Initially protruding nictitating membranes, slight depression, went “berserk” when restrained, “exhausted” with froth in mouth when released. Next day, less mobile, “jumpy” and hyper-responsive to movements and noise, protruding tongue and dilated pupils. Third day, paralysis of tongue, recumbent, able to move head in response to stimuli, killed. No diagnosis following haematology, biochemistry, bacteriological and mycology cultures of brain and histological examination of brain and other tissues.
99-100175	Jan 1999	Domestic	*Felis catus* (Persian cat)	F/Adult	CNS	Human	4 week history of ataxia and bumping into things, though apparently not blind. 1 week history of reluctance to eat, difficulty swallowing. Became unable to stand and constantly growling, killed. Some months earlier cat had been found in yard next to a bat that crawled across the ground and up a tree, bat gone next day (bat not tested).
97-43846	June 1997	Domestic	*Equine caballus* (Thoroughbred horse)	Male/Adult	CNS	None	Grinding teeth, generalized ataxia, bright, alert, eating. PME: unilateral focal haemorrhages in lumbosacral, posterior and mid-thoracic spinal cord grey matter, non-suppurative encephalomyelitis, possibly an arbovirus.
99-101936	Jan 1999	Domestic	*Equine caballus* (Stockhorse)	F/Adult	CNS	Human	Acute onset apparent blindness, circling, head pressing, muscle tremors, frothing at mouth, and photosensitisation (no jaundice) worsening to mania and biting of legs when sedated with xylazine and acepromazine, killed. History of chronic lantana/weed ingestion, suspect hepatoencephalopathy.
98-179723-1	Oct 1998	Domestic	*Mustela putorius furo* (ferret)	Male/NR	Normal	None	3 ferrets seized and destroyed as illegal pets (Qld) by Department of Natural Resources. Vet inspection found no clinical abnormalities, killed. Origin of ferrets in doubt, possible illegal import from rabies-endemic country, hence lyssa-FAT.
98-179723-2	Oct 1998	Domestic	*Mustela putorius furo* (ferret)	Male/NR	Normal	None	
98-179723-3	Oct 1998	Domestic	*Mustela putorius furo* (ferret)	F/NR	Normal	None	
97-116464	Feb 1997	Wild	*Pseudocheirus peregrinus* (Ringtail possum)	F/Juv	CNS	None	Rescued from power lines, unable to balance, fit-like episodes of waving paws and jumping about, died overnight.
97-147033	May 1997	Wild	*Pseudocheirus peregrinus* (Ringtail possum)	Male/NR	CNS	None	Fell from tree, unable to use hind limbs, positive withdrawal reflex, no voluntary movement, deteriorated overnight, cold, disoriented, killed. PME revealed spinal fracture T7-T8.
97-46655	July 1997	Wild	Possum	NR	CNS	None	Salivating, vague neurological signs, two in-contact possums died in past week. PME: one lung partly collapsed, focal interstitial pneumonia and non-suppurative epicarditis.

Accession number	Month/Year	Status	Genus. species (common name)	Sex/Age	Health status	Contact status	Comments
98-199790	Dec 1998	Wild	*Pseudocheirus peregrinus* (Ringtail possum)	F/Adult	CNS	None	Fell out of tree. Hind limb paralysis, head arched back, nystagmus.
99-187301	Oct 1999	Wild	*Pseudocheirus peregrinus* (Ringtail possum)	F/Adult	CNS	None	Found recumbent in yard with dead joey in pouch. No external injuries, R_X antibiotics and fluids. Next day cyanotic, frothy salivation, twitching of face and ears, R_X atropine and dexamethasone, died 2 hours later. D_X Neuro-angiostrongylosis due to *Angiostrongylus cantonensis* (Rat lung worms recovered from fresh brain).
2000-132656	April 2000	Wild	*Trichosurus vulpecula* (Brushtail possum)	F/Juv	CNS	None	Acute onset CNS signs in previously healthy hand-reared possum. Died within 48 hours.
2001-198515	Oct 2001	Wild	*Pseudocheirus peregrinus* (Ringtail possum)	F/Adult	CNS	None	Found on ground, salivating, in lateral recumbency with periods of frantic behaviour, biting cage bars and towel.
97-183992	Aug 1997	Wild	*Vulpes vulpes* (Red fox)	Male/Juv	Non-CNS	None	3-4 wk old orphan with scarcoptic mange and seborrheic dermatitis, depressed, killed.
97-191481	Oct 1997	Wild	*Vulpes vulpes* (Red fox)	NR	Non-CNS	None	Found in extremis with respiratory distress near railway, suspected poisoning. Mild heartworm disease (*Dirofilaria immitis*) and mild segmental nephropathy considered incidental. Stomach contents negative for 1080.
97-201616	Nov 1997	Wild	*Vulpes vulpes* (Red fox)	Male/Juv	Non-CNS	None	Found near road with fractured hind leg.
97-117595	Feb 1997	Captive colony	*Sarcophilus harrissi* (Tasmanian devil)	F/Adult	CNS	None	Captive > 6 yrs. Sudden onset 'rabid' behaviour, furious and fixative biting inanimate objects, hind limb paresis, progressed to coma, died.
98-108116	Feb 1998	Captive colony	*Macaca sp.* (Macaque monkey)	Male/Adult	CNS	None	Tethered on grassy area. Became drowsy and almost drunk/'high'. R_x fluids for 2 days, still not himself, died during transport to 2nd vet. PME Acute necrotising enteritis and acute suppurative (possibly aspiration) pneumonia with peracute septicaemia.

Appendix 5 Case details of 74 ABLV-postive bat submissions

Table A5-1 Case details of 74 ABLV-positive bat submissions, Queensland June 1996 to March 2002

Key

Case No. Positive cases listed in the order in which bats died, not the order in which they were submitted, as some bats were not submitted until 4 or 6 months later.
DPI regions SEQ = South East Queensland, Central = Central Queensland, North = North Queensland, West = West Queensland, see map, Appendix 1.
Health status CNS = unwell with signs suggesting CNS involvement, Non-CNS = unwell without signs suggesting CNS involvement, as defined in Materials and methods, Chapter 2.
Contact status None = no reported contact with animals or humans, Animal = reported to have had contact with another animal, Human = reported to have bitten or scratched human/s, Dual = reported contact with humans *and* animals, as defined in Materials and methods, Chapter 2
NR = not recorded, F = Female, M = Male, A = Adult, Juv = Juvenile, PME = Post-mortem examination, NVL = No visible lesions, NC = nutritional condition

Case No.	Laboratory accession number	Month/ Year died	DPI region/ Location found	Species/ Sex/Age	Health / Contact status	Days in care	Summary of history, post-mortem examination and other comments
ABLV-1	96-198384	Aug 1996	SEQ / Mt Coolum	YBST / NR	Non-CNS / None	8	Found sick on ground, would not eat or drink, died 8 days later Note: frozen until submitted 4 months later.
ABLV-2	96-48186	Sept 1996	North / Townsville	Little Red / NR	NR / None	NR	Records held at regional DPI lab. could not be found.
ABLV-3	96-168934	Sept 1996	SEQ / Robina	Black / F/A	Non-CNS / None	< 1	Found on ground “sick”, put back in tree, fell down, put in box and hung briefly, very weak, drank a lot, then choking, became unable to hang with laboured breathing. Dead on arrival to vet. PME: Pregnant (foetus not tested).
ABLV-4	96-185461	Nov 1996	SEQ / Tally Valley	Black / M/A	CNS / Animal	< 1	Hanging low in tree all day, tooth through right lip, killed. May have bitten or scratched a dog, as dog had blood on nose.
ABLV-5	96-33821	Nov 1996	South / Murphy's Creek	YBST / F/A	Non-CNS / None	< 1	Found alive on ground in cleared bushland. Aborted still born full term foetus in box during transport, killed.
ABLV-6	96-199751	Dec 1996	SEQ / Bald Hills	Black / F/A	CNS / None	< 1	Seen hanging low in tree (~1m from ground), had fallen to ground when rescued, gravely ill, tremors and hind limb paralysis, died within an hour, appeared to have bump on head, suspected head injury. PME: NVL.
ABLV-7	97-109143	Jan 1997	SEQ / Woodend	Black / M/NR	CNS / None	< 1	Found on ground, “nervous signs”.
ABLV-8	97-109700	Jan 1997	SEQ / Woodend	Black / F/A	CNS / None	< 1	Found on ground debilitated, uncoordinated, hypersalivation. Rabies-RFFIT< 0.5 IU/mL.
ABLV-9	97-40585	Feb 1997	West / Mt. Isa	Black / NR	CNS / None	NR	Had difficulty (paresis) using legs, killed.
ABLV-10	97-114442	Feb 1997	SEQ / Pialba	Little Red / F/A	CNS / None	3	Found on ropes of children school play area, “fitting” shaking, tremors, docile, not eating, killed. Brain FAT only weakly positive at ARI and AAHL, positive by immunoperoxidase test on paraffin tissues and virus isolation at AAHL. PME: pregnant (foetus not tested).

Case No.	Laboratory accession number	Month/ Year died	DPI region/ Location found	Species/ Sex/Age	Health / Contact status	Days in care	Summary of history, post-mortem examination and other comments
ABLV-11	97-116174	Feb 1997	SEQ / Woodend	Little Red / M/A	Non-CNS / None	NR	Suspected electric shock or electrocution?
ABLV-12	97-116375	Feb 1997	SEQ / Kurwongbah	YBST / NR	Non-CNS / None	NR	Ill bat found, taken to vet, died 2 to 3 days later.
ABLV-13	97-117003	Feb 1997	SEQ / Hervey Bay	Little Red / F/NR	CNS / None	1	Hanging in tree on wire, seen to fly into path of oncoming car, rescued from verge. Able to hang, vocalizing, tremors, aggressive, would not calm down when placed in covered cage, persistently agitated/distressed, killed next morning PME: well nourished, haemothorax with bruising of head, thorax, lung, and olfactory lobe of brain.
ABLV-14	97-121016	Mar 1997	SEQ / Sandgate	Little Red / M/A	CNS / None	NR	Paralysed legs.
ABLV-15	97-121024	Mar 1997	SEQ / Ipswich	Little Red / F/A	NR / None	NR	No history supplied, no submitter identified.
ABLV-16	97-122333	Mar 1997	SEQ / Toan Toan Ck	Little Red / M/NR	Non-CNS / None	< 1	Ill, died. PME: lean, NVL.
ABLV-17	97-122735	Mar 1997	SEQ / Hervey Bay	Little Red / M/A	Non-CNS / None	< 1	Found in canal, weak, died soon after. PME: Fat, moderate haemoperitoneum.
ABLV-18	97-125092	Mar 1997	SEQ / Nth Tamburine	Black / F/A	CNS / None	< 1	Found under tree bright and alert but weak and unable to move, paralysed, killed. PME: distended haemorrhagic urinary bladder. Rabies-RFFIT< 0.5 IU/mL.
ABLV-19	97-127470	Mar 1997	SEQ / Ipswich	Little Red / M/A	Non-CNS / None	0	Found dead under fig tree.
ABLV-20	97-130104	April 1997	SEQ / Hervey Bay	Little Red / F/A	Non-CNS / None	< 1	Found in weak state, died overnight. PME: poor body condition.
ABLV-21	97-72873	April 1997	Central / Rockhampton	Black / NR	Non-CNS / None	NR	Original records at regional laboratory could not be found.
ABLV-22	97-143816	May 1997	SEQ / Nambour	YBST / F/A	CNS / None	1	Presented to vet cold and depressed with graze to chin, initially ate well and appeared to improve, later biting towel aimlessly. Left hanging overnight. When touched, fell and was unable to right, depressed, unable to feed, didn't swallow food put in mouth, became less responsive, purposeless movement, killed.
ABLV-23	97-152661	June 1997	SEQ / Yeronga	Black / M/A	Non-CNS /Animal	< 1	Found moribund in backyard, dog 'sniffing around' but no observed contact. Killed. Rabies-RFFIT1.5 IU/mL.
ABLV-24	97-155574	June 1997	SEQ / Woodend	Black / M/A	CNS / None	NR	Found on ground, apparently behaving abnormally, depressed with head tremor, killed. Rabies-RFFIT< 0.5 IU/mL.
ABLV-25	97-45315	July 1997	North / Kuranda	Spectacled / NR	CNS / Human	1	Bat bit initial rescuer, transferred to carer in cage. Bat very aggressive, vocalising, trying to bite if approached, able to hang, ravenous, next morning still "angry", clearly 'ill" by the afternoon when transported to third person. On arrival hanging but not moving about, wings held loosely, ungroomed, glazed eyes, respiratory difficulty, died within half an hour.
ABLV-26	97-176292	Aug 1997	SEQ / Indooroopilly	Grey / F/A	CNS / None	< 1	Found on ground beside road, paralysis of legs and wings, killed. Pregnant, pup delivered by C-section but died. Pup brain weakly FAT-positive.

Case No.	Laboratory accession number	Month/ Year died	DPI region/ Location found	Species/ Sex/Age	Health / Contact status	Days in care	Summary of history, post-mortem examination and other comments
ABLV-27	97-186955	Oct 1997	SEQ / Beaudesert	Grey / F/A	CNS /None	< 1	Found on ground unable to hang, thin, refusing food, killed. Rabies-RFFIT < 0.5 IU/mL.
ABLV-28	97-192894	Oct 1997	SEQ / Coolamon	Grey / M/A	CNS / None	< 1	Hanging low in tree, depressed, 'walked' on to a stick to be transferred to a shed, thin, didn't fly, no external injuries, killed. PME: bruising to right temporal muscle and periosteum of cranium. Rabies-RFFIT< 0.5 IU/mL.
ABLV-29	97-193673	Oct 1997	SEQ / Strathpine	Black / M/A	CNS / Animal	< 1	Found on ground convulsing, dog with bat, died.
ABLV-30	97-196033	Nov 1997	SEQ / Laidley	Little Red / F/A	CNS / Human	3	Found under tree, ascending paralysis. PME: minimal body fat. Rabies-RFFIT toxic, no result.
ABLV-31	98-102890	Jan 1998	SEQ / Woodend	Little Red / M/A	Non-CNS / None	< 1	Found moribund near colony, killed. PME: contracted patagium suggested electric shock.
ABLV-32	98-104881	Jan 1998	SEQ / Brisbane	Black / M/Juv	CNS / Human	50	Apparently normal 2-3 week old orphan rescued from low in tree, in care for 5 weeks then sudden aggression to cage mate, vocalizing, had spasms and was biting objects. By Day-3 of illness, only able to eat pulped food, appeared to improve with antibiotics and dexamethasone. Day-7, unable to hang, dysphagic, diaorrhea, weight loss, Day-9 died. PME: emaciated. Note: original history provided was only that the bat was suspected of hurting its neck in the cage.
ABLV-33	98-105331	Jan 1998	SEQ / Moorooka	Grey / M/A	CNS / None	< 1	On back in garden "last couple of days", taken to vet, paralysed wings and feet, killed. PME: good NC, food in mouth (dysphagic). Rabies-RFFIT 0.9 IU/mL.
ABLV-34	98-40635	Feb 1998	North / Townsville	Little Red / NR	Non-CNS / None	NR	No visible lesions, semiconscious.
ABLV-35	98-120582	Mar 1998	SEQ / Gumdale	Little Red / F/A	CNS / None	1	Found on back on ground, flaccid paralysis hind limbs, some bleeding from mouth, noisy respiration, unable to spit out pulp when hand fed fruit puree (dysphagic), died next day. PME: pregnant, food in proximal trachea (poss. asphyxiation), urinary bladder distended. Foetal brain FAT-negative.
ABLV-36	98-126853	April 1998	SEQ / Sandgate	Little Red / M/A	CNS / None	1	Found with hind limb paralysis, killed. PME: good NC, NVL.
ABLV-37	98-130425	April 1998	SEQ / Coorparoo	Black / M/Juv	CNS / None	6	Found by public, taken to RSPCA, collected by carer 36 hours later. Initially appeared healthy, progressive weakness, reduced consciousness, unable to swallow, reduced blink reflex, twitching of head and ears, 'clamped' teeth, died overnight 6 days after found. PME: moderate NC, distended urinary bladder.
ABLV-38	98-137634	May 1998	SEQ / Surfers Paradise	Black / M/A	CNS / None	< 1	Found in carpark on ground, quiet with paresis of all limbs, withdrawal reflexes absent in all four limbs, killed. PME: NVL.
ABLV-39	98-140331	May 1998	Central / Rockhampton	Black / F/A	Non-CNS / None	0	Found face-down under tree in backyard. Initially ears twitching, soon died. Not handled until dead. PME: advance autolysis.
ABLV-40	98-140822	May 1998	SEQ / Ipswich	Little Red / M/Juv	CNS / None	< 1	Found in school grounds, and covered by box. Hind limb paralysis, killed. PME: lean NC, well muscled.

Case No.	Laboratory accession number	Month/ Year died	DPI region/ Location found	Species/ Sex/Age	Health / Contact status	Days in care	Summary of history, post-mortem examination and other comments
ABLV-41	98-144576	June 1998	SEQ / Pt Arkwright	YBST / M/A	Non-CNS / Human	6	Found on ground, able to lap a milk/egg mix and flap a bit. Submitted live after carer bitten when bat 'slipped' (not aggressive), killed. PME: encrusted material (food?) on face, multiple Argasid (soft) ticks.
ABLV-42	98-189710	July 1998	North / Townsville	Black / F/A	CNS / None	< 1	Found on ground, very weak, gripping weakly with toes, drank fluids, chewed towel, sticks in mouth, eyes glazed, tremors, laboured respiration, died. Submitted for testing 4 months later. PME: poor NC, pregnant. Foetal brain FAT-negative.
ABLV-43	98-158731	July 1998	SEQ / Woody Point	Black / M/A	CNS / Human	3	Found on ground, no withdrawal right hind, withdrawal and grasping with left hind, excessive yawning, difficulty eating, R_X fluids, vitamins, amino acids. Progressive deterioration, became recumbent, died. PME: dehydrated, poor NC, pale and poor pectoral muscle mass.
ABLV-44	98-165882	Aug 1998	SEQ / Clayfield	Black / F/A	CNS / None	< 1	Found on ground, generalized paresis, unable to hang or right itself, intermittent head tremor, unusually docile, killed. No external injuries, aged, 536 g (light). PME: poor NC, early pregnant.
ABLV-45	98-167351	Aug 1998	SEQ / Helensvale	Grey / F/Juv	Non-CNS / None	< 1	Found on ground, weak, moribund, dehydrated, no external injuries, killed. PME: poor NC, haemorrhage right side neck.
ABLV-46	98-172971	Sept 1998	SEQ / West End	Black / M/A	Non-CNS / Animal	< 1	Bat found in backyard ~ noon on ground with dogs that were approaching it and may have bitten bat, scrambled over fence and into bush during capture ~ 7 pm. Collected by carer, able to hang, and lap fruit juice but weak, deteriorated overnight, killed. PME: very poor NC, no head or bite injuries.
ABLV-47	98-50290	Oct 1998	North / Townsville	Little Red / NR	Non-CNS / None	< 1	Found unwell on grounds of native wildlife sanctuary, killed.
ABLV-48	98-182934	Oct 1998	SEQ / Hervey Bay	Little Red / M/A	Non-CNS / Animal	< 1	Seen in tree, next day in another tree, fell down, lethargic when rescued and died ~1 hr later. PME: poor NC, punctures and soft tissue damage right wing, other puncture wounds and haemorrhage of pectorals (poss cat, small dog, or other bat wounds hence contact status Animal).
ABLV-49	98-183454	Oct 1998	Central / Rockhampton	Little Red / F/A	CNS / Human	< 1	Solitary bat had earlier been observed in Torrelinana tree (in blossom) near house during day (abnormal). Elderly woman with rockmelon went to front door, bat flew from behind her to land on an ornament attached to house, then flew on to woman's head and bit her, then flew back to tree and ate blossom, occasionally vocalising with abnormally harsh voice, and remained in tree while many people arrived (abnormal) including wildlife carers, police, and onlookers. Experienced carer shot bat from tree. Woman had sustained substantial injuries to head, back and hands requiring hospitalization. PME: gun shot injuries.

Case No.	Laboratory accession number	Month/ Year died	DPI region/ Location found	Species/ Sex/Age	Health / Contact status	Days in care	Summary of history, post-mortem examination and other comments
ABLV-50	98-184361	Oct 1998	SEQ / Tennyson	Little Red / F/A	CNS / None	4	Found on ground in garden, marked weakness of one hind limb, unable to hang, fidgety. Day 3, some voluntary movement of both hind limbs but weak, abnormal positioning of wings, Day 4, moribund, CNS exam: conscious, depressed, grinding teeth, poor/ absent corneal, 'sneeze', and gag reflexes, reduced pain reflexes, markedly reduced or absent withdrawal reflexes and voluntary movement of all limbs. Killed. PME: very lean, NVL. Rabies-RFFIT< 0.5 IU/mL.
ABLV-51	98-188455	Nov 1998	SEQ / Maryborough	Black / F/A	CNS / None	< 1	Bat noticed in tree at aged persons complex in morning (abnormal, ~ 3 km from nearest camp), flew at groundsman, who hit it to ground with broom/rake, attempted to fly at man again, hit again, nudged into box. Collected by carer, ravenous, kept coming forward in cage (abnormal), not vocalising, found hanging dead ~ 3 hours later. PME: bruising over right eye and left neck.
ABLV-52	98-189702	Nov 1998	North / Townsville	Little Red / M/A	CNS / Animal	1	Rescued from tree, during which it descended tree towards carer (abnormal), very alert and vocal, vigorous attempts to bite when handled, prone to erections and ejaculated on self during transport. Bruising, swelling, and tooth marks on right arm (self or other bat bites). Wounds R_X with Otoderm and bat placed with other injured bat in aviary ~1830. 2 hours later, heard bats, had cornered and was attacking other bat, biting vigorously. Could not distract bat, had to separate bats manually. Bat then passive, almost torpid, more ejaculate noted, hung in cage corner, occasionally losing grip with one leg and swinging without obvious volition. Within an hour of being found fighting, appeared to be dying. 0530, comatose, 0700 found dead on cage floor. PME: good NC, punctures and cuts to head and bruising of right arm. Note: attacked companion bat held for observation, recovered uneventfully from original injuries, released 56 days later.
ABLV-53	98-189756	Nov 1998	North / Townsville	Black / F/Juv	CNS / None	1	In banana tree all day, agitated and repeatedly ran forward and then away during rescue, hypersalivating. Remained agitated and vocal in small cage, initially no evidence of paralysis. Next morning, lying twitching on cage floor, died. PME: poor NC.
ABLV-54	99-128813	April 1999	SEQ / Bay View	Black / F/Juv	CNS / None	< 1	Found on ground at down at caravan park, lifted with rake to hang off tow-bar. Rescued by carer, very lethargic, unable to hang in cage, paresis of hind limbs and wings, killed. PME: poor NC, dehydrated. Rabies-RFFIT< 0.2 IU/mL.
ABLV-55	99-178735	Aug 1999	Central / Gladstone	Black / F/Juv	CNS / Human	1	Rescued moribund from bamboo, unable to swallow R_X s/c fluids, improved. Responsive but uncoordinated, weak voluntary movement of limbs, very docile, dazed, would bite and hold cloth in mouth, saliva accumulated in throat. Died early next morning. PME: poor NC, dehydrated, large s/c bruise over frontal bone, fractured distal 10th rib, multiple small punctures to back, pectorals and abdomen with s/c haemorrhage and minor muscle damage (small animal bites or s/c fluid administration sites).
ABLV-56	QHSS6067	Oct 1999	Central / Rockhampton	Unidentified *Pteropus* sp. / NR	Non-CNS / Human	NR	Neither QHSS nor referring public health unit could locate records or submitter details.

Case No.	Laboratory accession number	Month/ Year died	DPI region/ Location found	Species/ Sex/Age	Health / Contact status	Days in care	Summary of history, post-mortem examination and other comments
ABLV-57	99-204611	Dec 1999	SEQ / Dutton Park	Black / F/Juv	Non-CNS / None	1	Found on ground in backyard eating peaches with electrical burns to right foot and left wing. Initially alert with weak hind limbs but able to hang, within 4-6 hours depressed and unable to hang. Next morning, depressed and vocalising, killed. PME: poor NC, deep linear burns to 'palm' of right foot, and necrosis of left thumb. Rabies-RFFIT 0.2 IU/mL.
ABLV-58	99-204785	Nov 1999	SEQ / Hervey Bay	Black / F/Juv	CNS / None	< 1	Found ~ 1 m up tree, generalised paresis, would not eat, "fitted", killed next morning. PME: good NC, NVL. Rabies-RFFIT 1.9 IU/mL.
ABLV-59	QHSS 16992373	April 2000	SEQ / Coombabah	Black / F/A	CNS / None	< 1	Found hanging very docile low in bush beneath Cocos palm. Could hang but not using thumbs, hanging with head extended, limp, 'snuffly' respiration, dull eyes, reduced responsiveness to surroundings, poss. unable to swallow, died o'night. PME at QHSS.
ABLV-60	00-132366	April 2000	SEQ / Redland Bay	Black / M/Juv	CNS / Human	3	Seen in Cocos palm tree for 2 days, had fallen out, was put back, later rescued from position part way up tree. When in cage uncharacteristically aggressive, lunging at any movement, bit rescuer through cage wire and towel when carrying cage, 'attacked' water bowel, gabbing, chewing, and pushing it around cage. Next day, still lunging towards passers by, fell in cage and appeared stunned, eyes 'wavering' (nystagmus), recovered and could hang again. Transferred to second carer. Attacked water container again, attempted to bite carer, wings held loosely, thumbs extended (paresis), 'nodding' head, sedated with acepromazine. Day-3, depressed, not eating, when held just lay there. Day-4, hanging but reduced consciousness, flaccid, died ~0800. PME: moderate to good NC, NVL.
ABLV-61	00-177442	June 2000	SEQ / Woodridge	Grey / F/A	Non-CNS / None	< 1	Found on ground, taken to vet, could hang when put in cage, voluntary movement of wings, lethargic, died overnight. Submitted 4 months later.
ABLV-62	00-175973	Sept 2000	SEQ / Labrador	Grey / M/A	CNS / None	< 1	Found thin, weak and dehydrated on ground in garden nursery with corneal ulcer and cataract of right eye, soil in mouth, dehydrated, unable to hang, killed. Rabies-RFFIT< 0.5 IU/mL.
ABLV-63	00-176632	Sept 2000	Central / Gladstone	Black / M/A	CNS / None	1	Woman alerted to bat on ground next door by her dogs, rescued attempting to climb low bush using thumbs, flaccid hind limbs, appeared unafraid and very hungry, swallowed juice and ate fruit, licking objects, appeared alert. During night, found semiconscious, laboured noisy breathing, froth at mouth. Next morning, 'not coherent', respiration. improved, poking tongue in and out. Voluntary movement of wings when handled, killed. PME: poor to moderate NC, NVL.

Case No.	Laboratory accession number	Month/ Year died	DPI region/ Location found	Species/ Sex/Age	Health / Contact status	Days in care	Summary of history, post-mortem examination and other comments
ABLV-64	QHSS 42178795	Oct 2000	Central / Calliope	Black / F/A	CNS / Dual	1	Bat noticed in tree, dead pup found below tree and discarded. Bat flew onto back of lady, husband assisted to remove with shovel, flew back to tree, then on to dog, scratching dog's eye, then on to empty bird cage, also attempted to enter rear door of house. Next day, experienced carer climbed tree to capture, bat climbing towards him in 'pugnacious' manner, bit carer while being carried. That evening, very thirsty, eating, appeared disoriented, biting at things, transferred to larger cage, wandering about aimlessly, very responsive to surroundings, vocalising and attempting to bite at people through wire, appeared distressed, settled when wrapped in towel and returned to smaller cage. Next morning, vocalising, aggressive when approached, still biting objects and cage, appeared confused, killed. No episodes of paresis or dysphagia. PME at QHSS.
ABLV-65	00-179793	Oct 2000	SEQ / Kelvin Grove	Black / M/A	CNS / None	3	Rescued by 2 children who found bat on ground with older children throwing stick and rocks at bat. Nudged bat into school bag and took home, collected by carer. Appeared exhausted, initially unable to hang, hind limb paresis right worse than left. Accepted juice by syringe, later responded to food and ate well, moved to hang. Next day. Partially hanging from side of cage, yawning, accepted juice, later flaccid paresis hind limbs, no grip reflex, Day 3 deteriorating, Died early morning Day 4. PME: poor NC, urinary bladder distended. Rabies-RFFIT 0.91 IU/mL.
ABLV-66	QHSS 42140461	Oct 2000	Central / Rockhampton	Black / M/A	CNS / Dual	< 1	Wild bat behaving abnormally on top of captive bat colony cage, scrambling on cage, would not fly away, behaved aggressively when approached, caught in net and bit person. Transferred to separate large cage, hanging on side of cage, wings out, lunging a people, vocal and baring teeth, jumped towards carer during recapture. No obvious paresis, killed. PME at QHSS. See also ABLV-73.
ABLV-67	00-189691	Oct 2000	SEQ / Logan Village	Grey / M/A	CNS / None	< 1	Found on roof, rescued and transferred to another carer. Hind limb paresis, unable to hang without support, then hanging by thumbs. Later biting cage, other objects and himself. Would open wings and lash out with thumbs if approached. Eventually on bottom of cage, killed. PME: moderate NC, small amount of urine in bladder.
ABLV-68	01-103951	Jan 2001	SEQ / Holland Park	Black / M/A	CNS / None	< 1	Rescued hanging by thumbs and head-butting ~50 cm up base of tree with blood from mouth and hind limb paresis. Vocalising and banging itself on cage, appeared to be attacking towels and cage, unable to hang. Would not settle when left alone with cage covered, could be heard thrashing about violently from other room, chewing cage wires abnormal tongue movements, killed. PME: moderate to good NC, fractured incisor teeth and lacerated gums. Rabies-RFFIT< 0.5 IU/mL.
ABLV-69	01-157670	May 2001	SEQ / Burleigh Heads	Black / M/A	CNS / None	< 1	Found beneath fruiting fig tree at wildlife park. Calm, absent hind limb grip reflexes, unable to hang, no injuries, while drinking fluids from syringe bit its own tongue, rapid respiration, died later that morning. PME: lean NC, NVL.
ABLV-70	01-162141	May 2001	SEQ / Hervey Bay	Black / M/A	CNS / None	< 1	Found at base of fig tree unable to climb, listless. Later biting rungs of cage and own wing, aggressive when approached, not eating, drinking, hind limb paresis, purposeless movement of wings, during night recumbent and biting cage, dead next morning.

Case No.	Laboratory accession number	Month/ Year died	DPI region/ Location found	Species/ Sex/Age	Health / Contact status	Days in care	Summary of history, post-mortem examination and other comments
ABLV-71	01-178364	July 2001	SEQ / Hervey Bay	Black / M/A	CNS / None	1	Found on ground covered with ants, depressed, quiet and placid, hind limb paresis, unable to hang, toileting on self, accepted some fluid next morning, later gasping, died. PME: moderate NC, blood around spinal cord at foramen magnum, congested meninges.
ABLV-72	01-190415	Sept 2001	SEQ / Noosa	Black / M/A	CNS / None	2	Fell from tree, unable to climb, rescued from ground outside Court House. Initially able to invert to urinate, next day unable to hang unaided, taken to vet, altered respiration / auscultation suggested pneumonia R_X antibiotics and finadyne, deteriorated. Day 3 generalised paresis, semi-conscious, killed. PME: lean NC, NVL. Rabies-RFFIT< 0.5 IU/mL.
ABLV-73	QHSS 42145588	Nov 2001	Central / Rockhampton Captive colony	Grey / F/A	CNS / Animal	> 30	Captive colony exposed 30 days earlier to wild ABLV-positive bat on cage (ABLV-66). Dominant female colony bat developed abnormal behaviour, hunched and vocalising in cage, licking vulva, vet exam R_X antibiotics and metacam (NSAID) for suspected urinary tract infection, during transport home, ravenous and aggressive while eating, 'snatching' food, became more aggressive during afternoon, biting cage, hypersalivating, vocalising, had seizures followed by periods of being placid, glassy eyes. Could be heard during the night. Next day very ill, still hanging but semi-comatose, weak voluntary movement, killed. PME at QHSS.
ABLV-74	02-102994	Jan 2002	SEQ / Brisbane CBD	Black / F/A	CNS / None	1	Found on ground in city, paresis of 4 limbs, deteriorated and became unable to hang, killed. PME: poor NC, fractured frontal process of zygomatic arch.

Appendix 6 Case details of 100 CNS flying foxes

Table A6-1 Case details of 100 consecutive submissions of wild Queensland flying foxes with clinical signs suggesting central nervous system disease (August 1997 to July 2002)

Key

Case No.	Positive cases listed in the order in which bats died, not the order in which they were submitted, as some bats were not submitted until 4 or 6 months later.
Diagnosis	Where the diagnosis was ABLV, the ABLV case number is given as per Appendix 5.
A. cantonensis	Sixteen of the 18 cases of neuro-angiostrongylosis referred to here were published in (Barrett *et al.* 2002). The corresponding published case numbers are: CNS-7= Bat 1, CNS-8= Bat 2, CNS-9= Bat 3, CNS-12= Bat 4, CNS-24= Bat 5, CNS-32= Bat 6, CNS-53= Bat 7, CNS-64= Bat 8, CNS-65= Bat 9, CNS-67= Bat 10, CNS-73= Bat 11, CNS-74= Bat 12, CNS-76= Bat 13, CNS-77= Bat 14, CNS-86= Bat 15, CNS-87= Bat 16. CNS- 97 and CNS-99 were not published. Bat 17 of the publication is an archival case from 1992
DPI regions	SEQ = South East Queensland, Central = Central Queensland, North = North Queensland, West = West Queensland, see map, Appendix 1.
Contact status	None = no reported contact with animals or humans, Animal = reported to have had contact with another animal, Human = reported to have bitten or scratched human/s, as defined in Materials and methods, Chapter 2

NR = not recorded, ND = not done, F = Female, M = Male, A = Adult, Juv = Juvenile, PME = Post-mortem examination, NVL = No visible lesions, NC = nutritional condition
H&E = Haematoxylin and eosin stained histological sections of brain +/- other organs as listed
Recovered = Number of worms recovered by maceration of half (not whole) brain, X-ray = results of radiographs of formalin fixed spinal columns removed at post-mortem examination
Pb = Liver lead levels as parts per million dry weight (ppm DW), RNA-Neg = brain negative for ABLV and other lyssavirus RNA by TaqMan® and RT-PCR assay

CNS case no.	Diagnosis	Accession Number	Month died	DPI Region	Species	Sex/Age	Contact status	Comments
CNS-1	ABLV-26	97-176292	8/97	SEQ	Grey	F/A	None	(see Appendix 5)
CNS-2	Spinal fracture	97-177844	9/97	SEQ	Black	F/A	None	Found on ground, vet exam depressed, no deep pain in hind limbs laceration of forehead. PME: pregnant, dislocated thoracic vertebrae fractured frontal and nasal bones with associated haemorrhage. H&E: NVL. X-ray: (by submitting vet) luxation of 9th and 10th thoracic vertebrae.
CNS-3	ABLV-27	97-186955	10/97	SEQ	Grey	F/A	None	(see Appendix 5)
CNS-4	ABLV-28	97-192894	10/97	SEQ	Grey	M/A	None	(see Appendix 5)
CNS-5	ABLV-29	97-193673	10/97	SEQ	Black	M/A	Animal	(see Appendix 5)
CNS-6	ABLV-30	97-196033	11/97	SEQ	Little Red	F/A	Human	(see Appendix 5)
CNS-7	*A. cantonensis*	97-201373	11/97	SEQ	Black	M/A	None	Found on back under mango tree, persistent hind limb paresis, passive disposition, killed 7 days later. PME: NVL. H&E: severe granulomatous meningoencephalitis with a worm section. No worms recovered.
CNS-8	*A. cantonensis*	97-201984	11/97	SEQ	Black	M/A	None	Rescued from wire fence, paresis, poor hind limb reflexes, killed 3 days later. PME: very distended bladder, multiple pale foci in both kidneys. H&E: Severe granulomatous and eosinophilic meningoencephalitis with a worm section. 1 worm recovered.

CNS case no.	Diagnosis	Accession Number	Month died	DPI Region	Species	Sex/Age	Contact status	Comments
CNS-9	*A. cantonensis* and spinal fracture	97-202152	11/97	SEQ	Black	M/A	None	Found under palm tree. Flaccid paralysis all limbs, unable to right, voluntary movement of head, pupils equal and constricted, no grip or placing reflexes or pain response in feet, urinary and faecal incontinence, killed. PME: distended bladder and colon, retroperitoneal haemorrhage around left adrenal gland. H&E: occasional very small worm sections, virtually no inflammation. 11 3rd stage worm larvae recovered. X-ray: fractured caudal endplate of the 3rd lumbar vertebra. Pb = 0.22 ppm DW.
CNS-10	No diagnosis	97-203983	11/97	North	Little Red	M/Juv	None	Found on ground with suspected tick poisoning and two areas on head that could have been tick sites, Rx tick serum, died 3 days later. PME: no significant abnormalities. H&E: Bacterial colonies in brain and liver suggest parachute septicaemia. X-ray ND. Pb = 0.85 ppm DW. RNA-Neg.
CNS-11	*A. cantonensis*	97-207876	11/97	SEQ	Black	M/A	Human	Rescued from tree on footpath, deteriorated overnight, "frothing", head tilt, one fixed and one dilated pupil, killed. PME: distended urinary bladder. H&E: Occasional very small worm sections, virtually no inflammation. 7 3rdstage worm larvae recovered. Pb = 1.05 ppm DW.
CNS-12	Spinal trauma	97-204696	12/97	SEQ	Black	F/Juv	None	~3 week old pup found on ground under tree with hind limb paralysis, killed. PME: extensive s/c and muscle haemorrhage of abdomen, dorsum, and pelvis, no fracture palpated. H&E: ND. X-ray: ND.
CNS-13	Spinal fracture	98-100690	1/98	SEQ	Grey	F/A	Human	Found with hind limb paralysis, killed. PME: fractured ~T4-T5 thoracic spine. H&E: NVL X-ray: ND.
CNS-14	ABLV-32	98-104881	1/98	SEQ	Black	M/Juv	Human	(see Appendix 5)
CNS-15	ABLV-33	98-105331	1/98	SEQ	Grey	M/A	None	(see Appendix 5)
CNS-16	Hydrocephalus	98-106021	1/98	SEQ	Black	M/Juv	None	~ 15 week old pup found on ground, poorly developed, head tremor, unresponsive killed 8 days later. PME: domed skull with hydrocephalus. RNA-Neg.
CNS-17	Hydrocephalus	98-107032	2/98	SEQ	Black	M/Juv	Human	Rescued at ~10 weeks of age, raised as orphan for 47 days, "strange" behaviour including poor feeder, uncoordinated, not flapping wings, biting own wing and thumbs, wide-based hanging stance, initially quite, then "vocal nashing or fearful screams", bit carer, killed. PME: pronounced hydrocephalus.
CNS-18	Spinal fracture	98-110042	2/98	SEQ	Black	M/A	None	Hanging low in tree by thumbs, "very panicky", hind limb paralysis, killed. PME: fractured thoracic spine, distended bladder. H&E: NVL. X-ray: ND.
CNS-19	No diagnosis	98-120342	3/98	North	Black	M/Juv	None	Pup found hanging low on branch with nail polish on feet claws (ex-orphan). Moribund, somnolent, then series of convulsions / seizures, killed. PME: pale friable liver: H&E: Fatty degeneration of liver. X-ray: ND Pb = 1.62 ppm DW. RNA-Neg.
CNS-20	ABLV-35	98-120582	3/98	SEQ	Little Red	F/A	None	(see Appendix 5)
CNS-21	ABLV-36	98-126853	4/98	SEQ	Little Red	M/A	None	(see Appendix 5)
CNS-22	No diagnosis	98-128821	4/98	SEQ	Black	M/Juv	None	Found on ground being harassed by birds with dilated pupils, killed. PME: fat, NVL. H&E: Pachymeningitis adjacent one optic nerve, bilateral dacryoadenitis. X-ray: ND Pb = 6.56 ppm DW. RNA-Neg.
CNS-23	ABLV-37	98-130425	4/98	SEQ	Black	M/Juv	None	(see Appendix 5)

CNS case no.	Diagnosis	Accession Number	Month died	DPI Region	Species	Sex/Age	Contact status	Comments
CNS-24	*A. cantonensis*	98-136012	5/98	SEQ	Grey	M/A	None	Found on ground surrounded by cats, put up into tree, still there next day, rescued and kept in care for 6 days, no improvement, killed. PME: NVL. H&E: Severe granulomatous and eosinophilic meningitis with large worm sections. 16 worms recovered.
CNS-25	ABLV-38	98-137634	5/98	SEQ	Black	M/A	None	(see Appendix 5)
CNS-26	Hepatopathy	98-138510	5/98	SEQ	Grey	F/Juv	None	Found on ground, weak, no improvement over 7 days, killed. PME: Multiple 0.1-1.0 cm linear to triangular pale green firm areas of discolouration in liver. H&E: brain-NVL, liver-coagulative necrosis indicating severe vascular injury. X-ray: oblique fracture of vertebral body of L5 with dorsal and left lateral displacement. Pb = 1.69 ppm DW. RNA-Neg.
CNS-27	No diagnosis	98-140095	5/98	SEQ	Grey	M/A	None	Hind limb paralysis and respiratory distress, killed 2 days later. PME: no spinal fracture detected. H&E: NVL. X-rays: NVL Pb = 1.6 ppm DW. RNA-Neg.
CNS-28	ABLV-40	98-140822	5/98	SEQ	Little Red	M/Juv	None	(see Appendix 5)
CNS-29	Spinal fracture	98-189861	5/98	North	Black	F/A	None	Found on ground with damaged wings and hind limb paralysis, died few hours later. PME: fractured lumbar spine. H&E: NVL. X-ray: ND.
CNS-30	No diagnosis	98-44761	6/98	North	Black	F/Juv	Animal	Presented to vet moribund, ataxic with swollen head. PME: hyperaemic skin ventral neck, serosanguineous oedema dorsal head and around neck, larynx, face and shoulders. Lethobarb artefact crystal in lungs and heart, liver unremarkable, no thoracic or abdominal fluid. H&E: Low protein oedema of skin of dorsal head/neck with a mild lymphocytic infiltrate. X-ray: ND. Pb = 0.21 ppm DW. RNA-Neg.
CNS-31	Spinal fracture	98-146896	6/98	SEQ	Black	F/A	None	Found trying to cling to base of tree while attacked by Indian Mynas. Hind limb reflexes absent, left wing reflexes depressed, killed. PME: early pregnant, fractured lumbar spine and right zygomatic arch, haemorrhage of right abdominal fat and wall. H&E: NVL. X-ray: fracture of intervertebral disc between 1st and 2nd lumbar vertebra, gross displacement.
CNS-32	*A. cantonensis*	98-150283	6/98	SEQ	Black	M/Juv	None	Hanging low in tree, docile, paresis, initially able to hang. Day 8 “legs much stronger, alert and eating well”, Day 13 eating little, “eyes half-mast, seems weak”. Day 20 no longer able to hang. Day 22, recumbent, cranial nerves normal, poor hind limb reflexes, killed. PME: fat with pectoral muscle wasting, brown meninges. H&E: Very severe granulomatous and eosinophilic meningoencephalitis with multiple worm sections. 20 worms recovered.
CNS-33	Spinal fracture	98-150380	6/98	SEQ	Grey	M/A	None	Found on ground hind limb paralysis with absent reflexes, poor wing reflexes, swollen right wrist, vocal and stressed, killed. PME: fractured frontal bone under skin wound, deep haemorrhage of thoracic spinal muscles, fractured cranial thoracic spine. H&E: NVL. X-ray: corner fracture of 2nd thoracic vertebral body.
CNS-34	Spinal fracture	98-155261	7/98	SEQ	Grey	M/Juv	None	Flaccid paralysis hind limbs, killed. PME: fractured thoracic spine and head of one rib. H&E: NVL. X-ray: collapsed 9th thoracic vertebra.
CNS-35	Spinal fracture	98-158271	7/98	SEQ	Grey	M/A	None	Found ‘tangled’ on fence, hind limb paralysis, absent hind limb reflexes, killed. PME: fractured spine, distended bladder, free blood in chest cavity. H&E: multiple small perivascular haemorrhages. X-ray: ND.
CNS-36	ABLV-42	98-189710	7/98	North	Black	F/A	None	(see Appendix 5)
CNS-37	ABLV-43	98-158731	7/98	SEQ	Black	M/A	Human	(see Appendix 5)

CNS case no.	Diagnosis	Accession Number	Month died	DPI Region	Species	Sex/Age	Contact status	Comments
CNS-38	Chronic skull/brain injury	98-163314	8/98	SEQ	Black	F/NR	Human	Juvenile hanging from guttering with injury to back of head, docile, poor 'doer', moderate left head tilt, used thumbs but didn't flap wings, "nervy" bat in care 4 months then 30 second seizure and bit carer, killed. PME: abnormal skull shape over left temporal and occipital lobes with corresponding cerebral deficits and adhesion of dura to bony proliferation. Bacto: No culture from skull swab.
CNS-39	ABLV-44	98-165882	8/98	SEQ	Black	F/A	None	(see Appendix 5)
CNS-40	Spinal fracture	98-189772	8/98	North	Black	F/A	None	Paralysis hind limbs, bright, feeding well, 2 days later delivered live near–term pup. Dam Rx corticosteroids, no improvement, killed. PME: fractured spine. H&E: NVL. X-ray: ND.
CNS-41	Spinal fracture	98-170411	9/98	SEQ	Black	F/A	None	Paralysis hind limbs, vocal and aggressive. Absent hind limb reflexes, voluntary movement wings, attempting to bite during examination despite acepromazine sedation. PME: pregnant. Fractured spine, fur loss and subcutaneous haemorrhage over temporal muscle. H&E: NVL. X-rays: ND.
CNS-42	Spinal fracture	98-173935	9/98	SEQ	Grey	F/Juv	None	Found on ground, next day had climbed up bush to hang by thumbs, hind limb paralysis, in care 9 days, docile, no improvement, hind limb reflexes absent, visible bump in low thoracic spine, killed. PME: fractured thoracic spine at level of base of heart. H&E: haemorrhages in brain stem. X-ray: ND.
CNS-43	No diagnosis	98-179890	9/98	SEQ	Black	M/A	None	Bat found on ground and put into tree, next day, on ground again, in to care, third day killed. PME: good NC, no spinal fracture detected. H&E: NVL. X-rays: NVL Pb = 0.88 ppm DW. RNA-Neg.
CNS-44	Spinal fracture	98-181970	10/98	SEQ	Little Red	M/A	None	Found in school yard, moribund, hind limb paresis, killed. PME: fractured skull with subdural haemorrhage and "broken neck", deep laceration of pectoral muscle with subcutaneous haemorrhage but no perforation of skin. H&E: haemorrhages in cerebellum and subarachnoid space. X-rays: ND.
CNS-45	Spinal fracture	98-181985	10/98	Central	Little Red	M/A	None	Hind limb paralysis and blood in mouth, killed. PME: fractured thoracic spine, haemorrhage and atelectasis left lung, malenic gut contents (swallowed blood). H&E: NVL. X-ray: fractured mid-body 3rd thoracic vertebra with ventral and right lateral displacement.
CNS-46	ABLV-49	98-183454	10/98	Central	Little Red	F/A	Human	(see Appendix 5)
CNS-47	Exhaustion	98-183841	10/98	SEQ	Black	M/A	Human	Bat rescued from swimming pool in morning, bit man, therefore killed. Wide-based stance, depressed, panting respiration, 'sleepy', very good cranial nerve and wing reflexes, poorer hind limb reflexes, generally depressed but no deficits. Closed eyes as if needing to sleep immediately after strong stimulation and response. PME: lean NC, dehydrated. H&E: NVL. X-ray: NVL. Pb = 3.09 ppm DW. RNA-Neg.
CNS-48	ABLV-50	98-184361	10/98	SEQ	Little Red	F/A	None	(see Appendix 5)
CNS-49	Spinal fracture	98-186762	11/98	SEQ	Grey	F/A	None	Found on ground, unable to hang, hind limb weakness, initially vocal, thought to be agitated/frightened so sedated with acepromazine, then 'screaming' beating wings, thrashing in cage, biting at own feet, chewing cage bars. Poor or absent hind limb reflexes except deep pain response, poor wing responses, killed. PME: Fractured thoracic spine between ribs 8 and 9, ~10 mL free blood in thorax, urine in bladder, colon full, pale friable liver. H&E: NVL. X-ray: ND.
CNS-50	ABLV-51	98-188455	11/98	SEQ	Black	F/A	None	(see Appendix 5)
CNS-51	ABLV-52	98-189702	11/98	North	Little Red	M/A	None	(see Appendix 5)

CNS case no.	Diagnosis	Accession Number	Month died	DPI Region	Species	Sex/Age	Contact status	Comments
CNS-52	ABLV-53	98-189756	11/98	North	Black	F/Juv	None	(see Appendix 5)
CNS-53	*A. cantonensis*	98-199503	12/98	SEQ	Grey	F/Juv	Animal	Owner alerted to weak and uncoordinated bat by dogs. Owner killed bat by drowning! PME: very poor NC. H&E: severe eosinophilic and granulomatous meningoencephalitis with one worm section. No worms recovered.
CNS-54	No diagnosis	99-117491	3/99	Central	Black	M/Juv	None	Bat acting strangely, chewing himself, aggressive, erratic behaviour. PME: NVL H&E: NVL. X-ray: NVL. Pb = 0.1 ppm DW. RNA-Neg.
CNS-55	No diagnosis	99-126934	3/99	SEQ	Black	F/A	None	Found on lawn, hind limb paralysis, vocal, killed. PME: full bladder, no fracture apparent. H&E: NVL. X-ray: ND Pb: ND. RNA-Neg.
CNS-56	ABLV-54	99-128813	4/99	SEQ	Black	F/Juv	None	(see Appendix 5)
CNS-57	Bacterial encephalitis (*P. multocida*)	99-136960	4/99	SEQ	Black	F/Juv	Human	Found with laceration on nose, had seizure, appeared "in pain", hanging with all limbs, uncoordinated, maggots under wing. PME: poor NC, subcutaneous pocket of blood and pus over cranium and neck. Mass of thinly encapsulated caseous pus extending from the caudal pharynx ventral to epaxial muscles of neck and laterally along trachea, eroding through ventral aspect of atlanto-axis joint capsule and extending proximally and distally within the spinal canal and caudal cranial cavity. Also 2 irregular areas of fibrosis with pus on the dorsal thorax near scapula margins and puncture wounds through skin of head, neck, and back. Bacto: *P. multicida* from brain, cervical lymph node and subcutis.
CNS-58	Spinal fracture	99-141686	5/99	SEQ	Black	M/A	None	Found 3-4 metres in tree hanging by thumbs, hind limb paralysis, wings strong. PME: fractured left zygomatic arch with haemorrhage in adjacent temporal muscle, fractured lumbar spine. H&E: NVL. X-ray: fracture at intervertebral space of 2nd and 3rd lumbar, gross displacement.
CNS-59	Chronic injury and mydriasis	99-147385	5/99	SEQ	Black	M/Juv	None	Pup found on ground with wounds on head and right shoulder. Appeared normal as infant, once hanging was uncoordinated, head held to right, learned to fly. During preparation for release 6 months later seen to 'shake', held wings 'loosely', head tilt persisted. Thought to be improving, found dead 1 month later with wound to back of head. PME: large open necrotic wound with maggots on back extending from right scapulae with fibrosis, scapula fixed and displaced medially and caudally, fibrosis back of neck and greatly reduced range of head movement, hepatomegaly, low grade chronic peritonitis. H&E: NVL. X-rays: ND. Bacto: ND Pb = 1.94 ppm DW. RNA-Neg.
CNS-60	Spinal fracture	99-155102	6/99	SEQ	Black	M/A	None	Heard thump on roof, bat seen flopping on ground attempting to climb tree and falling down. Rescued hanging low in tree by thumbs with hind limb paralysis, visible bump in back, hind limb reflexes absent. PME: fractured right zygomatic arch and spine at thoraco-lumbar junction with associated s/c haemorrhage. Also subdural haematoma. H&E: some small haemorrhages. X-ray: ND.
CNS-61	Head trauma	99-174173	7/99	SEQ	Black	M/A	None	Found moribund, responded to fluids and Amoxil. 2 days later developed neurological signs of weakness and contorted body, killed. PME: subdural haemorrhage over occipital lobes. H&E: NVL.
CNS-62	ABLV-55	99-178735	8/99	Central	Black	F/Juv	Human	(see Appendix 5)
CNS-63	No diagnosis	99-192936	10/99	SEQ	Grey	F/Juv	Human	Dam and pup found on ground, pup in unusual position on dam. Dam killed humanely (reason unknown) during next 5 days later pup had reduced appetite, diarrhoea, distress calls, lethargy, and seizures, killed. PME: petechiation both lungs. H&E: NVL Bacto: lung-no aerobic growth. X-ray: ND. Pb: ND. RNA-Neg.

CNS case no.	Diagnosis	Accession Number	Month died	DPI Region	Species	Sex/Age	Contact status	Comments
CNS-64	*A. cantonensis*	99-192944	10/99	SEQ	Black	F/Juv	None	Found in tree, crashed when attempted to fly, dehydrated, generalized weakness, died same day. PME: poor NC, NVL. H&E: eosinophilic and granulomatous meningoencephalitis with multiple worm sections. 1 worm fragment recovered.
CNS-65	*A. cantonensis*	99-192952	10/99	SEQ	Grey	F/A	None	Seen in tree, next day on ground, ascending hind limb paresis. Day 3 delivered stillborn pup. Persistent paresis, then day-16 head tilt, head tremor, nystagmus, pallor, killed. PME: fat, NVL. H&E: severe granulomatous meningitis and multiple worm sections. 14 worms recovered.
CNS-66	Spinal fracture	99-193425-1	10/99	SEQ	Grey	F/A	Human	Found on ground with hind limb paralysis and dystocia, vet caesarean (pup died). Demeanour improved after a few days, paralysis persisted, killed. PME: gross displacement of fractured thoracic vertebrae at level of base of heart some post-op ascites. H&E: NVL. X-ray: fractured vertebral body and caudal endplate of 7th thoracic vertebra, severe ventral displacement.
CNS-67	*A. cantonensis*	99-193425-2	10/99	SEQ	Grey	M/A	None	Found on ground, hind limb paresis, depressed, intermittent shivering, no improvement over 8 days, killed. PME: NVL. H&E: moderate nonsuppurative meningitis with sections of worms. 8 worms recovered.
CNS-68	Spinal fracture	99-201976	11/99	SEQ	Black	M/A	None	Bat found in backyard with hind limb paralysis, aggressive, not calmed by acepromazine sedation. Vocalising and attempting to bite during CNS exam, hind limb reflexes absent or very depressed. PME: No spinal fracture seen. H&E: NVL. X-ray: subluxation of 2nd and 3rd lumbar vertebra. RNA-Neg.
CNS-69	ABLV-58	99-204785	12/99	SEQ	Black	F/Juv	None	(see Appendix 5)
CNS-70	Spinal fracture	00-125390	3/00	SEQ	Black	M/A	None	Found caught in trees, paralysis, unable to fly, killed 2 days later. PME: good NC, fractured mid-thoracic spine, distended bladder. H&E: ND. X-ray: fractured vertebral body of 9th thoracic vertebra, ventral displacement.
CNS-71	ABLV-60	00-132366	4/00	SEQ	Black	M/Juv	Human	(see Appendix 5)
CNS-72	No diagnosis	00-133981	5/00	SEQ	Grey	F/Juv	None	Found under palm tree eating fruit, hanging weakly with wings held loosely, could invert to toilet. During next 4 days deteriorated, lying in bottom of cage, killed. PME: NVL H&E: Vacuolar change in obex. X-rays: NVL Pb = 4.65 ppm DW. RNA-Neg.
CNS-73	*A. cantonensis*	00-137464	5/00	SEQ	Black	F/Juv	None	Captive born, released. 2-3 weeks later found depressed in tree near cage containing dam, easily captured, movements ‘stiff’ and slow. Deteriorated over 24 hrs, nystagmus, died. PME: good NC, congested meninges and petechial cerebral haemorrhages. H&E: very severe granulomatous and eosinophilic meningoencephalitis with large worm sections. 20 worms recovered.
CNS-74	*A. cantonensis*	00-138193	5/00	SEQ	Black	F/Juv	None	Found on ground, depressed, wide based stance, drooped wings, ate well, day-3 recumbent, killed. PME: Good NC, petechial haemorrhages in cerebral cortices. H&E: very severe granulomatous and eosinophilic meningoencephalitis with large worms sections. 31 worms recovered.
CNS-75	No diagnosis	00-141945	6/00	North	Spectacled	M/A	None	Found in garden, thin dehydrated, hungry but difficulty chewing and unable to spit out pulp, tongue sticking our one side, difficulty hanging with wide-based stance, wings hanging to side, eyes dull and unfocused. Next day seizures, head nodding, died. PME: marked autolysis, NVL. H&E: NVL. X-rays: NVL. Bacto: brain-mixed aerobic culture, no significant isolates. Pb = 0.28 ppm DW. RNA-Neg.
CNS-76	*A. cantonensis*	00-145676	6/00	SEQ	Black	M/Juv	None	Found on ground depressed, deteriorated over 7 days, profoundly depressed, generalized paresis, nystagmus, killed. PME: petechiae on cerebrum, cloudy ventral meninges. H&E: Very severe granulomatous and eosinophilic meningoencephalitis with large nematodes. 43 worms recovered.

CNS case no.	Diagnosis	Accession Number	Month died	DPI Region	Species	Sex/Age	Contact status	Comments
CNS-77	*A. cantonensis*	00-147973	6/00	SEQ	Black	F/A	None	Seen on ground under palm tree, rescued next day, depressed, unable to hang, hind limb paresis, killed. PME: good NC, NVL. H&E: two sections of small worms, no inflammation. No worms recovered.
CNS-78	No diagnosis	00-148284	6/00	North	Spectacled	F/Juv	None	Unable to climb, hung low in tree and low in cage. Bit hard on to towel and “shook it like a dog”. PME: (incomplete examination) moderate NC, ants over body. H&E: NVL. X-rays: NVL. Pb = 0.82 ppm DW. RNA-Neg.
CNS-79	Spinal fracture	00-169326	8/00	SEQ	Grey	F/A	None	Blood on nose, breathing heavily, hind limb paralysis, wings held weakly, depressed, tremors, hind limb reflexes absent, died. PME: pregnant, good NC, fractured mid-thoracic spine, free blood in thorax and abdomen, fractured ribs on right. Large subdural haemorrhage over caudoventral and caudo-dorsal aspects of both cerebral hemispheres and cerebellum (contrecoup), dirt impacted nostrils. H&E: subarachnoid haemorrhage. X-ray: two spinal fractures: at intervertebral disc of 7th and 8th thoracic vertebra with right displacement and at intervertebral disc of 3rd and 4th lumbar vertebra, also right displacement.
CNS-80	Bacterial encephalitis & neck trauma	00-171936	9/00	SEQ	Grey	F/A	Animal	Found on ground with wing paralysis, died overnight. PME: puncture wounds and extensive subcutaneous haemorrhage to neck suggestive of dog or other animal attack. H&E: moderate meningoencephalitis and subarachnoid haemorrhage with infiltrates of predominantly lymphocytes and macrophages and some polymorphonuclear cells and prominent bacterial colonies. Bacto: ND.
CNS-81	Head trauma	00-171944	9/00	SEQ	Grey	M/Juv	None	Found on ground lethargic, unable to hang, killed. PME: fat, fractured left zygomatic arch, haemorrhage of left temporal muscles and large subdural haemorrhages of right and left cerebral hemispheres. No spinal fractures detected. H&E: haemorrhage in brain stem, frontal cortex and subarachnoid space. X-ray: NVL.
CNS-82	ABLV-62	00-175973	9/00	SEQ	Grey	M/A	None	(see Appendix 5)
CNS-83	ABLV-63	00-176632	9/00	Central	Black	M/A	None	(see Appendix 5)
CNS-84	ABLV-65	00-179793	10/00	SEQ	Black	M/A	None	(see Appendix 5)
CNS-85	No diagnosis	00-180726	10/00	SEQ	Grey	F/A	Animal	Flew into (hit) deck of house, rescued. Initially weak down one side, over next 14 days, eating but no improvement, rarely moving within cage, killed. PME: fat, no evidence of head trauma, limbs appeared normal, chronic thumb and foot injuries on left. H&E: NVL X-ray: ND. Pb: ND. RNA-Neg.
CNS-86	ABLV-67	00-189691	10/00	SEQ	Grey	M/A	None	(see Appendix 5)
CNS-87	*A. cantonensis*	00-186762	11/00	SEQ	Grey	F/A	None	Found low in bush, would not fly, docile. Hung with wide-based stance, wings held loosely. By day-9 inappetant, depressed, killed. PME: good NC, cloudy ventral meninges, 1 worm recovered from surface of caudal cerebellum/brain stem.
CNS-88	*A. cantonensis*	00-189346	11/00	SEQ	Black	M/A	None	Found low on fence, rescued 3 days later, moribund, recumbent, no voluntary limb movement, killed. PME: moderate NC, NVL. H&E: mild non-suppurative meningoencephalitis with sections of worms. Bacto: mixed aerobic culture, no significant isolates. 3 worms recovered.
CNS-89	No diagnosis	00-195246	12/00	SEQ	Black	F/Juv	None	Pup found on driveway, good growth but slow feeder, not flapping, placid, not active or social,” no sparkle”, carer suspect brain damage, killed 43 after rescue. PME: head shape brain grossly unremarkable. H&E: NVL Pb: ND. RNA-Neg.
CNS-90	ABLV-68	01-103951	1/01	SEQ	Black	M/A	None	(see Appendix 5)

CNS case no.	Diagnosis	Accession Number	Month died	DPI Region	Species	Sex/Age	Contact status	Comments
CNS-91	Fractured skull	01-108921	2/01	SEQ	Little Red	M/A	None	Found in parking lot salivating and fitting, killed. PME: multiple skull fractures with cerebral haemorrhages and large s/c haemorrhage over temporal muscles extending down neck. H&E: extensive haemorrhage and disruption of cerebellum and parietal cortex with subarachnoid haemorrhage.
CNS-92	Fractured skull	01-119571	3/01	SEQ	Black	M/A	None	Left on vet doorstop in box. Recumbent, "semi-paralysed" no external injuries detected, killed. PME: good NC with fractured skull associated s/c haemorrhage and fractured right elbow. H&E: haemorrhage and disruption of frontal and parietal cortex with malacia associated with mild lymphocytic meningitis.
CNS-93	Head trauma	01-134141	3/01	SEQ	Black	F/Juv	None	Found on driveway, lethargic and not wanting to move, killed. PME: large subdural haemorrhage over left cerebral cortex, ecchymosis of right cerebral cortex and s/c haemorrhage extending from neck, over right temporal muscle to right eye. H&E: focal haemorrhages in frontal cortex. X-ray: ND.
CNS-94	No diagnosis	01-154853	5/01	SEQ	Black	M/Juv	Human	Found crawling along lawn, no visible injuries, very lethargic, hung in cage, died 12 hours later. PME: poor NC, bruising to left shoulder, bladder partly distended, 1 cm crusty alopecia over left pectoral muscle, moderately congested meninges. H&E: NVL, Kidney-Megaschizonts (merocysts) of *Hepatocystis pteropi* in endothelial cells. Focal dermatophytosis 'ringworm'. X-ray: ND. Pb = 0.86 ppm DW. RNA-Neg.
CNS-95	ABLV-69	01-157670	5/01	SEQ	Black	M/A	None	(see Appendix 5)
CNS-96	Head trauma	01-158916	5/01	SEQ	Black	M/A	None	Seen hanging in tree for 2 days then on ground 1 day. Able to hang, alert but "not able to move limbs well" (paresis?), deteriorated over 6 days, killed. PME: congestion of meninges and bilateral patches of petechial haemorrhages and 'bruising' over parietal lobes. H&E: focus of multiple haemorrhages in parietal cerebrum with hyperplastic endothelium and gitter cells.
CNS-97	*A. cantonensis*	01-161772	5/01	SEQ	Black	M/Juv	None	Found on ground, unable to hang, poor grip reflexes, urinary incontinence Rx Antirobe. Day-3 able to grip but weak. Day-6 climbs slowly about cage with urinary incontinence Day-10, voluntary movement legs and wings not dribbling urine (improved), remains weak. Day-17 "flicker in eyes" (nystagmus), slight head tilt. Day-20 flicker left eye only, right eye no palpebral reflex. Day-23 Rx corticosteroids. Day-25 some improvement after steroids, hanging, weak, no right palpebral reflex and corneal ulcer, eating very slowly horizontal nystagmus , Day-27 has deteriorated, very depressed, not hanging, poor or absent grip reflex, killed. PME: good NC, poor pectoral muscle mass (wastage), cloudy ventral meninges with patchy discolouration of the cerebral cortexes and ~10 petechial haemorrhages. Very distended bladder. 3 worms recovered from cut surface near 3rd ventricle. H&E: severe non-suppurative meningoencephalitis with sections of worms.
CNS-98	ABLV-70	01-162141	5/01	SEQ	Black	M/A	None	(see Appendix 5)
CNS-99	*A. cantonensis*	01-169415	7/01	SEQ	Black	F/Juv	None	Found on trellis in garden, weak, depressed, paresis, wings held loosely, aggressive, distressed, and fearful, deteriorated over 24 hours, killed. PME: moderate NC, full bladder and colon. H&E: 2 sections of small worms, minimal inflammation.
CNS-100	ABLV-71	01-178364	7/01	SEQ	Black	M/A	None	(see Appendix 5)

Appendix 7 Case controls for neuro-angiostrongylosis

Nineteen normal, wild flying foxes were caught in urban Brisbane within 2 weeks of one or more sick flying foxes with neuro-angiostrongylosis being found, as follows;

Table A7-1 Temporal and geographical matching of case control samples for neuro-angiostrongylosis

Month/s wild normal flying foxes caught in urban Brisbane	Corresponding cases of neuro-angiostrongylosis
November, December 1997	CNS-7, 8 and 9 found sick at Cleveland, Brisbane and Nambour respectively
May 1998	CNS-24 found sick at Ashgrove
June 1998	CNS-32 found sick at Bundamba

Table A7-2 Species, age, and sex matching of case control samples for neuro-angiostrongylosis

Class of flying fox	Number with angiostrongylosis n=18	Number clinically normal n=19
Black adult female	3	8
Black adult male	5	1
Black juvenile female	3	2
Black juvenile male	3	0
Grey adult female	1	1
Grey adult male	2	2
Grey juvenile female	1	1
Grey juvenile male	0	4

Fewer Black flying foxes had been caught during months in which cases of neuro-angiostrongylosis were occurring, resulting in an inability to match each class exactly. All available Black flying foxes were examined. Substitutions were made in an effort to preserve species, then age, then sex matches

Table A7-3 Details of 19 wild-caught, presumably normal flying foxes examined histologically for evidence of subclinical neuro-angiostrongylosis (All negative)

Accession number	Species	Age	Sex	Month, Year and Site of Capture
97-195560	Black	Adult	Female	Nov 97 Norman Creek, East Brisbane
97-195575	Black	Adult	Female	Nov 97 Norman Creek, East Brisbane
97-195583	Black	Adult	Female	Nov 97 Norman Creek, East Brisbane
97-195591	Black	Adult	Female	Nov 97 Norman Creek, East Brisbane
97-203012	Black	Adult	Female	Nov 97 Norman Creek, East Brisbane
97-203020	Black	Adult	Female	Nov 97 Norman Creek, East Brisbane
97-203056	Black	Adult	Female	Dec 97 Norman Creek, East Brisbane
98-143194-19	Black	Adult	Female	June 98 Norman Creek, East Brisbane
98-143194-21	Black	Adult	Male	June 98 Norman Creek, East Brisbane
98-143194-17	Black	Juvenile	Female	June 98 Norman Creek, East Brisbane
98-143194-18	Black	Juvenile	Female	June 98 Norman Creek, East Brisbane
98-141060-9	Grey	Adult	Female	May 98 Indooroopilly Is.
98-141060-3	Grey	Adult	Male	May 98 Indooroopilly Is.
98-143186-13	Grey	Adult	Male	June 98 Norman Creek, East Brisbane
98-141060-12	Grey	Juvenile	Female	May 98 Indooroopilly Is.
98-141060-1	Grey	Juvenile	Male	May 98 Indooroopilly Is.
98-141060-4	Grey	Juvenile	Male	May 98 Indooroopilly Is.
98-141060-6	Grey	Juvenile	Male	May 98 Indooroopilly Is.
98-141060-8	Grey	Juvenile	Male	May 98 Indooroopilly Is.

Appendix 8 Liver lead determination in normal flying foxes

Determination of average percent dry matter of normal, formalin-fixed flying fox livers

Bat	Weights	% Dry matter
Bat 99-165731-53	Wet liver weight = 1.1312 Dry liver weight = 0.2613	% Dry matter = 23.1
Bat 99-165731-54	Wet liver weight = 1.7084 Dry liver weight = 0.3885	% Dry matter = 22.7
Bat 99-165731-58	Wet liver weight = 1.3767 Dry liver weight = 0.3344	% Dry matter = 24.3
Bat 99-165731-51	Wet liver weight = 1.2852 Dry liver weight = 0.2752	% Dry matter = 21.4

Average % Dry matter = 22.8

Relationship between fresh wet weights, formalin-fixed wet weights, and dry weights

Accurate liver Pb results were determined as *ppm dry weight*, using desiccated formalin fixed liver.

Approximations of the equivalent formalin-fixed wet weight lead concentration (ppm wet weight) were calculated using the average percent dry matter (see above) as follows.

$$\text{ppm wet weight} = \text{ppm dry weight} \times \frac{22.8}{100}$$

The approximate ppm wet weight results are generally comparable to published fresh wet weight values for flying foxes and other species. However, as the relationship between the fresh wet liver weights and formalin-fixed wet liver weights is unknown, it is preferable to compare and analyse these results as dry weight concentrations (ppm dry weight) where possible.

Table A8-1 Liver lead levels in 50 clinically normal flying foxes captured at two sites in urban Brisbane during November 1997 to July 1999
Note: table continues over page

DPI accession number	Species	Capture site	Sex	Age	Year	Month	Pb ppm dry weight[1]
98-143193/17	Black	Norman creek	F	Juv	98	6	5.35
98-143193/18	Black	Norman creek	F	Juv	98	6	5.65
98-143193/20	Black	Norman creek	F	Adult	98	6	8.05
98-143193/19	Black	Norman creek	F	Adult	98	6	16.05
98-143193/21	Black	Norman creek	M	Adult	98	6	17.36 (21.1)
99-165746/56	Black	Norman creek	F	Adult	99	7	5.63
99-165746/54	Black	Norman creek	F	Juv	99	7	5.99
99-164766/47	Black	Norman creek	F	Adult	99	7	6.9 (6.15)
99-165746/53	Black	Norman creek	M	Adult	99	7	7.02
99-165746/57	Black	Norman creek	F	Adult	99	7	7.16
99-165746/58	Black	Norman creek	F	Adult	99	7	10.04
99-165746/55	Black	Norman creek	F	Adult	99	7	10.47
99-165746/52	Black	Norman creek	F	Adult	99	7	10.67
98-162624/28	Black	Norman creek	M	Adult	98	8	3.06
98-162624/22	Black	Norman creek	M	Adult	98	8	10.46
98-162624/29	Black	Norman creek	M	Adult	98	8	10.57
98-162624/25	Black	Norman creek	M	Adult	98	8	10.74
98-162624/27	Black	Norman creek	M	Adult	98	8	11.67
98-162624/24	Black	Norman creek	M	Adult	98	8	12.56
98-162624/23	Black	Norman creek	M	Adult	98	8	13.06
98-162624/31	Black	Norman creek	M	Adult	98	8	16.68
98-162624/26	Black	Norman creek	M	Adult	98	8	18.29
98-162624/32	Black	Norman creek	F	Adult	98	8	19.77 (16.0)
97-203004	Black	Indooroopilly Is.	F	Adult	97	11	3.52
97-195591	Black	Norman creek	F	Adult	97	11	13.22
98-143186/13	Grey	Norman creek	M	Adult	98	6	0.27
98-143186/14	Grey	Norman creek	M	Adult	98	6	0.8
98-141186/16	Grey	Norman creek	M	Juv	98	6	0.81
98-143186/15	Grey	Norman creek	M	Juv	98	6	1.76
99-157162/43	Grey	Norman creek	F	Adult	99	6	6.95 (6.07)
99-165731/50	Grey	Norman creek	F	Adult	99	7	1.5
99-165731/51	Grey	Norman creek	F	Adult	99	7	4.04
99-164774/49	Grey	Norman creek	F	Juv	99	7	4.34
98-162744/34	Grey	Norman creek	F	Adult	98	8	5.92
97-195575	Grey	Norman creek	F	Adult	97	11	13.24
98-141060/2	Grey	Indooroopilly Is.	M	Adult	98	5	0.10 (0.08)

DPI accession number	Species	Capture site	Sex	Age	Year	Month	Pb ppm dry weight[1]
98-141060/6	Grey	Indooroopilly Is.	M	Juv	98	5	0.13
98-141060/4	Grey	Indooroopilly Is.	M	Juv	98	5	0.26
98-141060/12	Grey	Indooroopilly Is.	F	Juv	98	5	0.26
98-141060/10	Grey	Indooroopilly Is.	F	Juv	98	5	0.44
98-141060/9	Grey	Indooroopilly Is.	F	Adult	98	5	0.45
98-141060/5	Grey	Indooroopilly Is.	M	Adult	98	5	0.47
98-141060/3	Grey	Indooroopilly Is.	M	Adult	98	5	0.83
98-141060/1	Grey	Indooroopilly Is.	M	Juv	98	5	0.88
98-141060/11	Grey	Indooroopilly Is.	F	Adult	98	5	0.96
98-141060/7	Grey	Indooroopilly Is.	M	Juv	98	5	1.28
98-141060/8	Grey	Indooroopilly Is.	M	Juv	98	5	1.35
98-180974/38	Grey	Indooroopilly Is.	F	Juv	98	10	0.74
98-196145/40	Grey	Indooroopilly Is.	M	Adult	98	12	1.68
98-196145/39	Grey	Indooroopilly Is.	M	Adult	98	12	2.41

[1] Values in brackets (n=5) are for duplicate samples submitted to the DNR Natural Resources Sciences Laboratories. All other values are results of testing at the DPI Trace Metal Laboratory. The results from the two laboratories were considered consistent. The duplicate results were not used in any further analysis.

Statistical analysis of the relationships between flying fox characteristics and Pb levels

Known characteristics that could have been associated with Pb levels included species, capture site and date, age, and sex. However there is a very close association between some of these terms in the data collected. In particular relationships between species, capture site, and capture date show three main groups.

- **Greys-IIs-May** Grey-headed flying foxes caught at Indooroopilly Island during May 1998 (n=12)
- **Greys-NC-JJA** Grey-headed flying foxes caught at Norman Creek Island during June, July or August 1998 or 1999 (n=9)
- **Blacks-NC-JJA** Black flying foxes caught at Norman Creek during June, July or August 1998 or 1999 (n=23)

The remaining six flying foxes were caught in October, November or December 1997 or 1998 and include: 1 Black from Norman Creek (Pb = 13.22 ppm DW), 1 Black from Indooroopilly Is. (Pb = 3.52 ppm DW), 1 Grey from Norman Creek (Pb = 13.34 ppm DW), and 3 Greys from Indooroopilly Is (Pb = 0.74, 1.68, and 2.41 ppm DW).

There are clear differences in the levels of liver Pb between the three main groups (see Figure A8-1 and Figure A8-2). The differences were tested by analysis of variance with the log (x + 1) transformation before analysis to stabilize variance. All analysis was done by Tony Swain (ARI) using the statistical package GenStat 2000.

Figure A8-1 Liver Pb levels of 50 wild flying foxes from urban Brisbane

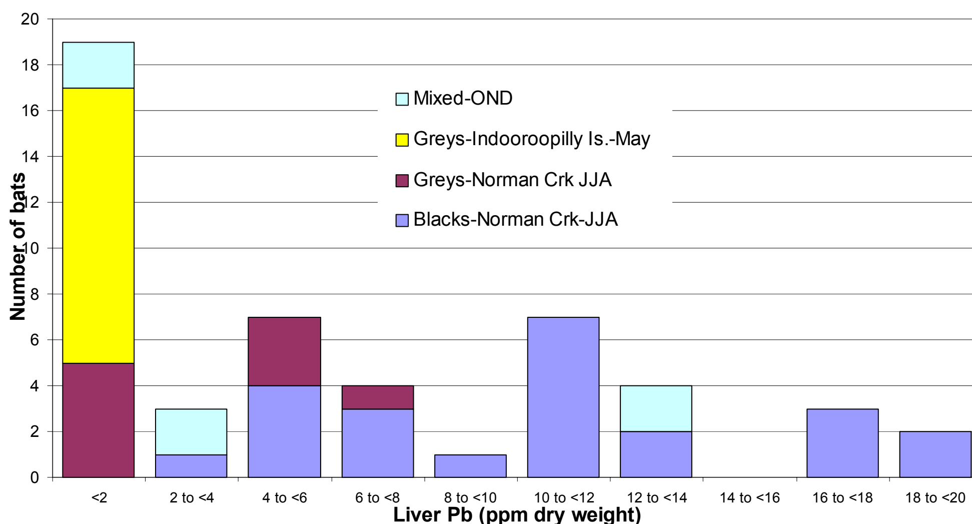
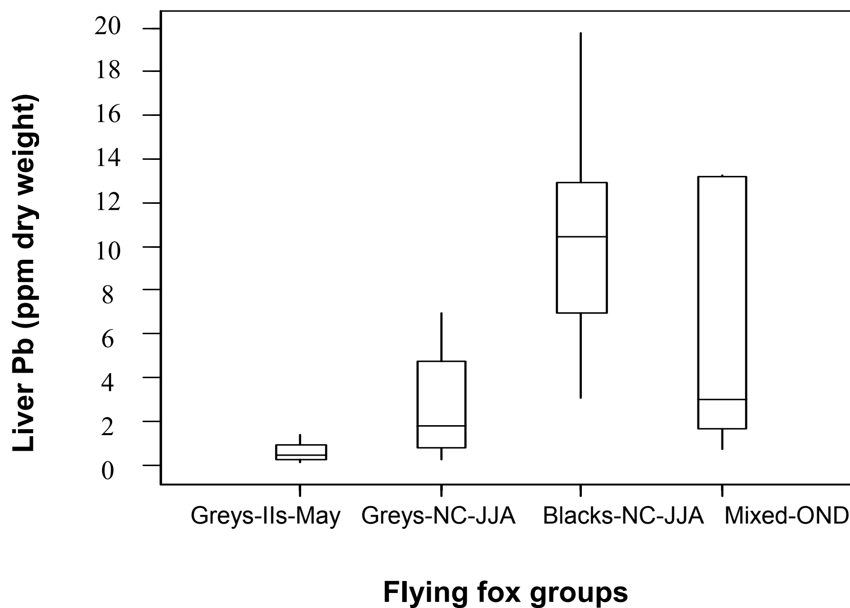


Figure A8-2 Box plot summary: liver Pb in 50 wild urban Brisbane flying foxes



The predicted liver Pb levels for each group from the regression model are:

- **Blacks-NC-JJA** Predicted Pb = 9.68 ppm dry weight
- **Greys-NC-JJA** Predicted Pb = 2.27 ppm dry weight
- **Greys-Ils-May** Predicted Pb = 0.57 ppm dry weight

In terms of statistically different liver Pb levels ($p = 0.01$)

Blacks-NC-JJA > Greys-NC-JJA > Greys-Ils-May

The effects of year, age and sex when added to a general linear model containing the three main groups were not statistically significant ($p = 0.63$, 0.19 and 0.43 respectively)

In addition, the low levels and tight distribution of values for the Grey-headed flying foxes caught at Indooroopilly Is. in May 1998 suggests that this group has had minimal exposure to sources of lead. All values for this group are less than 1.5 ppm dry weight and there is a gap in the values at this point, further suggesting that this is an indicator level.

Conclusion

Why there should be statistically different liver Pb levels among two species of flying fox (*P. alecto* and *P. poliocephalus*) camped at two apparently similar camp sites only 7.2 km apart in urban Brisbane, is not known. Further investigation of this unexpected result was beyond the scope of this project.

Appendix 9 Heminested RT-PCR for lyssavirus RNA

A hemi-nested reverse transcriptase (RT) PCR assay, as described in Heaton *et al.* (1997), is used at QHSS for the detection of known and potentially unknown variants of ABLV. The assay uses a combination of primers capable of amplifying the RNA of all seven lyssavirus genotypes, including both the pteropid and Yellow-bellied sheathtail-variants of ABLV (Smith *et al.* 2002). Primers were designed to amplify regions with high homology on the nucleocapsid (N) protein genes (see Table A9-1 and Table A9-2). This combination of primers is presumed to be likely to amplify the RNA of any further, as yet unrecognised variants of ABLV, such as may be identified among other species of Australian microchiroptera.

Table A9-1 Oligonucleotide primers for primary amplification of lyssavirus RNA in hemi-nested RT-PCR at QHSS

Primer name[1]	Direction	Position on genome[2]	Nucleotide sequence 5'-3', + genomic sense
JW12	Forward	55-73	ATG TAA CAC C(C/T)C TAC AAT TG
JW6 (DPL)	Reverse	660-641	CAA TTC GCA CAC ATT TTG TG
JW6 (E1E2)	Reverse	660-641	CAG TTG GCA CAC ATC TTG TG
JW6 (M)	Reverse	660-641	CAG TTA GCG CAC ATC TTA TG

Table A9-2 Oligonucleotide primers for second-round amplification of lyssavirus RNA in hemi-nested RT-PCR at QHSS

Primer name[1]	Direction	Position on genome[2]	Nucleotide sequence 5'-3', + genomic sense
JW12	Forward	55-73	ATG TAA CAC C(C/T)C TAC AAT TG
JW10 (DLE2)	Reverse	636-617	GTC ATC AAA GTG TG(A/G) TGC TC
JW10 (ME1)	Reverse	636-617	GTC ATC AAT GTG TG(A/G) TGT TC
JW10 (P)	Reverse	636-617	GTC ATT AGA GTA TGG TGT TC

[1] Letters in parentheses refer to the genotype on which the primer designs were based. D-Duvenhage virus, P-Pasteur virus (GenBank PV-X03673), L-Lagos bat virus, E1-European bat lyssavirus 1, E2-European bat lyssavirus 2, M-Mokola virus (GenBank S59448).

[2] Numbering corresponds to nucleotide positions within the Pasteur virus + sense genome, (GenBank X03673)

Adapted from Heaton *et al.* (1997).

The hemi-nested RT-PCR assay was demonstrated to be more sensitive than the FAT for the detection of rabies virus in brain samples that had undergone extensive deterioration (to the point of liquefaction and incubated at 37°C for 360 hrs) (Heaton *et al.* 1997). Because of this higher sensitivity in sub-optimal samples, and the potential to amplify sequences from the RNA of unrecognised variants of ABLV, the hemi-nested RT-PCR is indicated whenever poor sample quality or the possibility of a new ABLV variant is suspected. The hemi-nested RT-PCR is currently performed at QHSS on all brain samples from *non*-Yellow-bellied sheathtail microchiroptera, due to the possibility of other ABLV-variants being present in these bat species.

Appendix 10 Nucleotide sequences

Figure A10-1 Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences
Multiple sequence alignment of the + sense 5' nucleotide sequences of 28 new pteropid-variant ABLV isolates (green)**, 3 new YBST-variant ABLV isolates** (orange), **other available ABLV sequences** (ABLHumanPt and ABLBallina (lime), **and AHHLYBST** (gold) **and the Nobivac rabies vaccine strain (Pasteur virus RIVM,** blue)**.**

Key

Base position 1 corresponds to position 1 of the Pasteur virus + sense genome (GenBank X03673).

Primer binding sites are indicated in pale font, over and underlined, and labelled with the primer name and an arrow indicating the direction of primer extension.

The start and stop of the N protein mRNA sequence are indicated by vertical arrows (positions 59 and 1486).

The start and stop codons of the N protein coding sequence (positions 71-73 and 1,421-1,423), N-P intergenic sequence (CT^{1489}), and start codon for the P protein (positions 1519-1521) are boxed and labelled.

Bases that differ from the predominant base at each position and are like that of one or more ybst-ABLV sequence are shown in yellow (e.g. A).

Bases that differ from the predominant base at each position and are like that of the Nobivac rabies sequence are shown in blue (e.g. A).

Bases that differ from the predominant base at each position and are unlike either the predominant base of the ybst-ABLV or the Nobivac sequences are highlighted in green (e.g. T).

Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 1 of 16)**

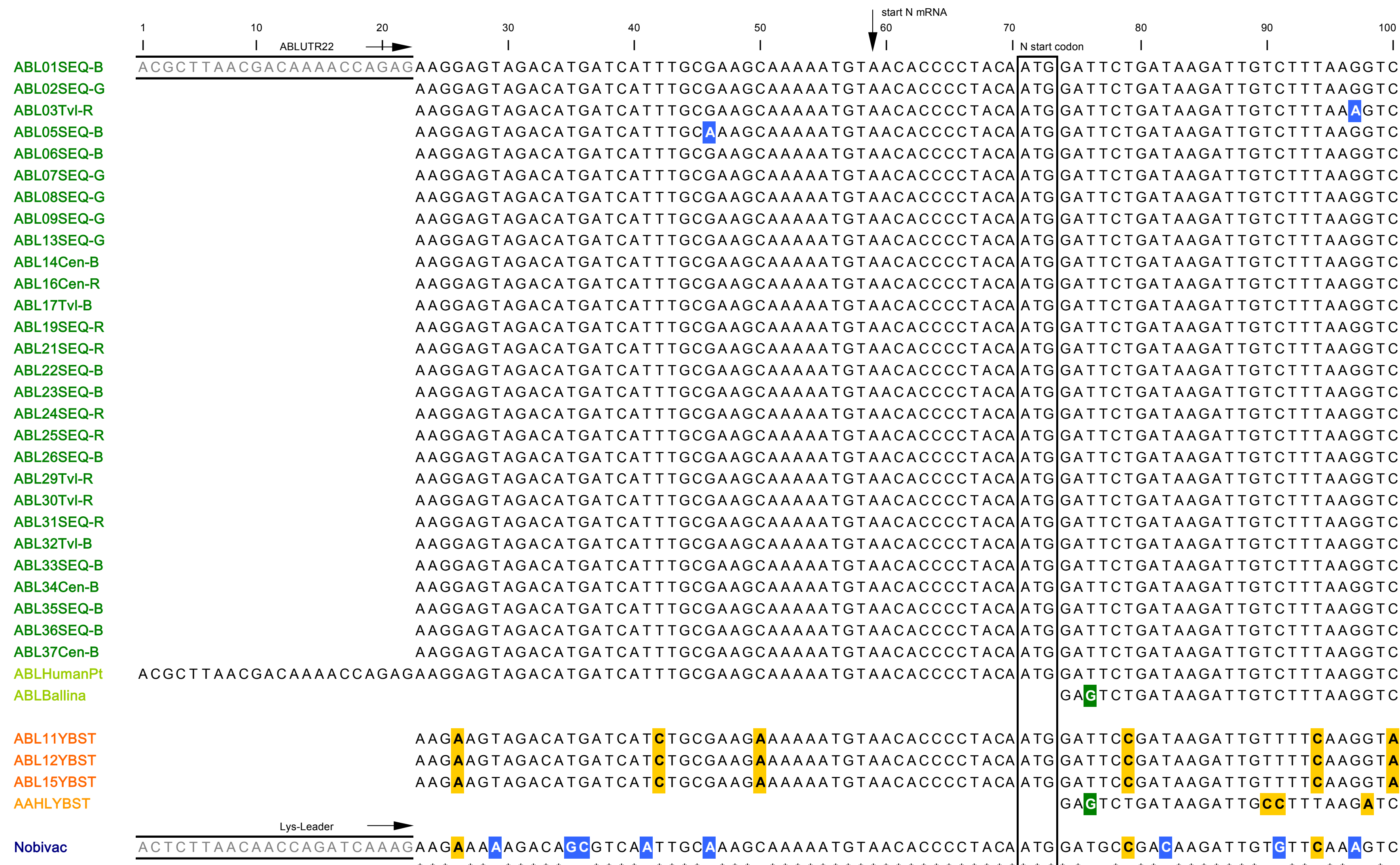


Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 2 of 16)**

```
            101      110       120       130       140       150       160       170       180       190       200
ABL01SEQ-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL02SEQ-G  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL03TvI-R  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL05SEQ-B  AACAATCAGTTGGTGTCTGTCAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL06SEQ-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL07SEQ-G  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL08SEQ-G  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL09SEQ-G  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL13SEQ-G  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCTGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL14Cen-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL16Cen-R  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL17TvI-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL19SEQ-R  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL21SEQ-R  AACAATCAGTTGGTTTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL22SEQ-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGCATAACTCTTG
ABL23SEQ-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAGTATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL24SEQ-R  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL25SEQ-R  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL26SEQ-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL29TvI-R  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAGTATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL30TvI-R  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL31SEQ-R  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL32TvI-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL33SEQ-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL34Cen-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL35SEQ-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL36SEQ-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABL37Cen-B  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATTAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABLHumanPt  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATCAAAGACCAAAAGAAGCCTAGTATAACTCTTG
ABLBallina  AACAATCAGTTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAATATGAGTACAAATATCCCGCTATACAAGACCATACGAAGCCTAGTATAACTCTTG

ABL11YBST   AACAATCAATTGGTGTCTGTTAAGCCTGAGGTGATAGTAGATCAGTATGAATATAAGTACCCTGCAATCAAAGATCAGAGGAAGCCTAGCATTACTCTTG
ABL12YBST   AACAATCAATTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAGTATGAATATAAGTACCCTGCAATCAAAGATCAGAGGAAGCCTAGCATTACTCTTG
ABL15YBST   AACAATCAATTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAGTATGAATATAAGTACCCTGCAATCAGAGATCAGAGGAAGCCTAGCATTACTCTTG
AAHLYBST    AACAATCAATTGGTGTCTGTTAAGCCGGAGGTGATAGTAGATCAGTATGAATATAAGTACCCTGCAATCAAAGATCAGAGGAAGCCTAGCATTACTCTTG

Nobivac     AATAATCAGGTGGTCTCTTTGAAGCCTGAGATTATCGTGGATCAATATGAGTACAAGTACCCTGCCATCAAGGATTTGAAAAAGCCTTGTATCACCCTAG
            ** *****  **** *** * ***** *** * ** ** ***** ***** ** ** ** ** ** **    **    *  ****** * ** ** ** *
```

Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 3 of 16)**

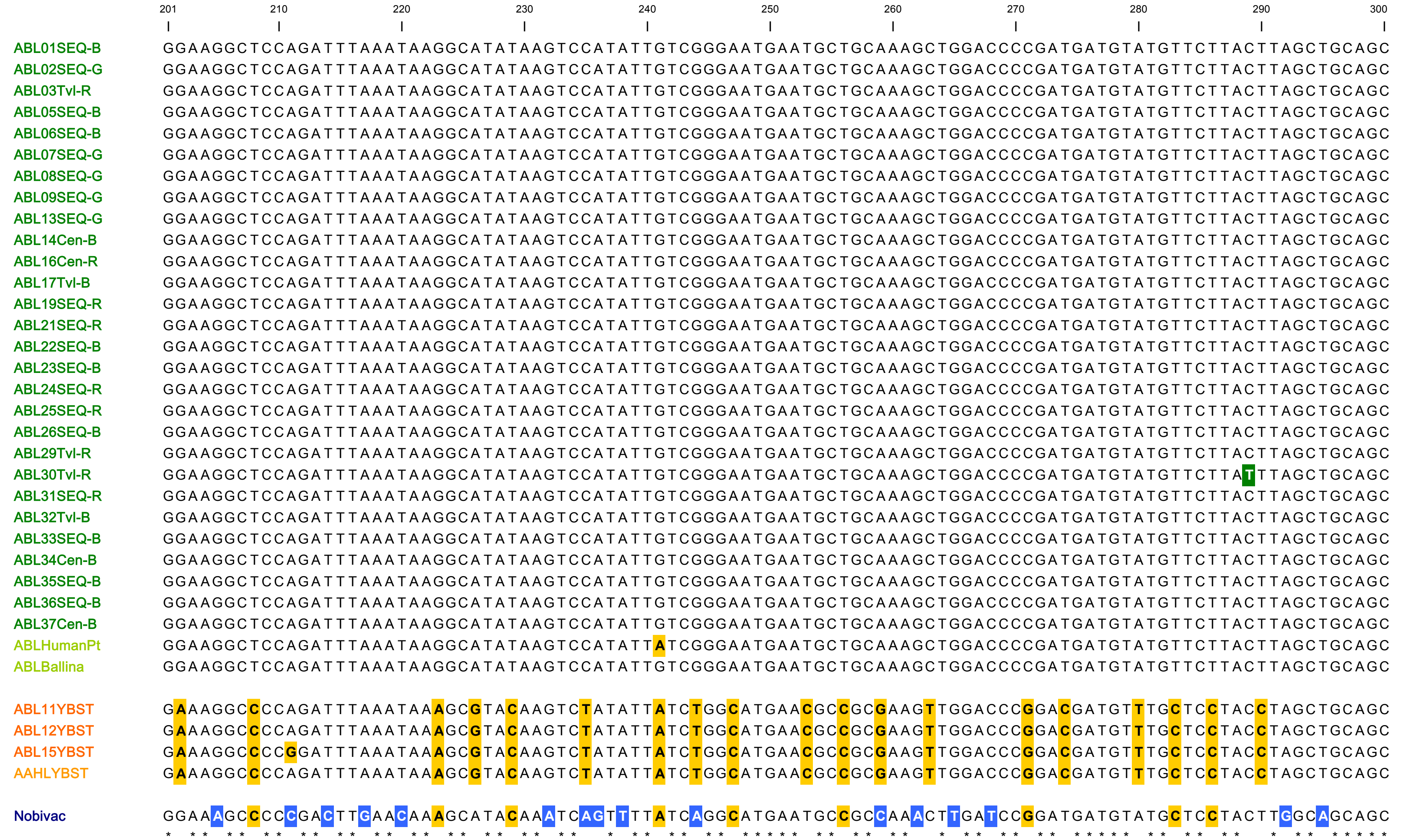


Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 4 of 16)**

```
             301      310       320       330       340       350       360       370       380       390       400
ABL01SEQ-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATCACACCAGCCACCCTTGTTGAC
ABL02SEQ-G   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL03TvI-R   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL05SEQ-B   TATGGAGTTATTTGAGGGGGTTTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL06SEQ-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL07SEQ-G   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL08SEQ-G   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL09SEQ-G   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL13SEQ-G   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL14Cen-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL16Cen-R   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL17TvI-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL19SEQ-R   TATGGAGTTATTTGAGGGGGTCTGCCCTGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL21SEQ-R   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL22SEQ-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL23SEQ-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL24SEQ-R   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL25SEQ-R   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL26SEQ-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL29TvI-R   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL30TvI-R   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL31SEQ-R   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL32TvI-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL33SEQ-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL34Cen-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL35SEQ-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL36SEQ-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABL37Cen-B   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABLHumanPt   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTTTGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC
ABLBallina   TATGGAGTTATTTGAGGGGGTCTGCCCAGAAGATTGGACGAGTTATGGGATTATGATTGCCAGAAAAGGAGATAAAATTACACCAGCCACCCTTGTTGAC

ABL11YBST    TATGGAGTTATTTGAGGGGATCTGCCCAGAGGACTGGACAAGTTACGGGATTTTAATTGCCAGAAAAGGAGACAAAATCACACCGGCTACTTTAGTTGAC
ABL12YBST    TATGGAGTTATTTGAGGGGATCTGCCCAGAGGACTGGACAAGTTACGGGATTTTAATTGCCAGAAAAGGAGACAAAATCACACCGGCTACTTTAGTTGAC
ABL15YBST    TATGGAGTTATTTGAGGGGATCTGCCCAGAGGACTGGACAAGTTACGGGATTTTGATTGCCAGAAAAGGAGACAAAATCACACCGGCTACTTTAGTTGAC
AAHLYBST     TATGGAGTTATTTGAGGGGATCTGCCCAGAGGACTGGACAAGTTACGGGATTTTGATTGCCAGAAAAGGAGACAAAATCACACCGGCTACTTTAGTTGAC

Nobivac      AATGCAGTTCTTTGAGGGGACATGTCCGGAAGACTGGACCAGCTATGGAATCCTGATTGCACGAAAAGGAGATAGGATCACCCCAAACTCTCTAGTGGAG
              *** **** *********   ** ** ** ** ***** ** ** ** **  * *****  ********** *  ** ** **     *  * ** **
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Figure A10-1 (continued) Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 5 of 16)

```
             401      410       420       430       440       450       460       470       480       490       500
ABL01SEQ-B   ATAAAGAGAACAGATATTGAGGGTAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC  --> N5'Seq
ABL02SEQ-G   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL03Tvl-R   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACATGCTTCTTTAGTAGGTCTTC  <-- N5'Seq
ABL05SEQ-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACATGCTTCTTTAGTAGGTCTTC
ABL06SEQ-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL07SEQ-G   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL08SEQ-G   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL09SEQ-G   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL13SEQ-G   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACATGCTTCTTTAGTAGGTCTTC
ABL14Cen-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL16Cen-R   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL17Tvl-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL19SEQ-R   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL21SEQ-R   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL22SEQ-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACATGCTTCTTTAGTAGGTCTTC
ABL23SEQ-B   ATAAAGAGAACAGACATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL24SEQ-R   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL25SEQ-R   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL26SEQ-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL29Tvl-R   ATAAAGAGAACAGACATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL30Tvl-R   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACATGCTTCTTTAGTAGGTCTTC
ABL31SEQ-R   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL32Tvl-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL33SEQ-B   ATTAAGAGAACAGATATTGAGGGCAACTGGGCATTGACAGGAGGACAGGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL34Cen-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL35SEQ-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTTACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL36SEQ-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABL37Cen-B   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC
ABLHumanPt   ATAAAGAGAACAGATATTGAGGGCAATTGGGCATTGACGGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACATGCTTCTCTAGTAGGTCTTC
ABLBallina   ATAAAGAGAACAGATATTGAGGGCAACTGGGCATTGACTGGAGGACAAGACTTGACCAGGGATCCCACAGTAGCAGAACACGCTTCTTTAGTAGGTCTTC

ABL11YBST    ATAAGGAGGACAGATATTGAGGGTAGCTGGGCTCTAGCAGGGGGGCAGGACTTTACCAGAGACCCTACAATCGCAGAGCATGCATCTCTGGTGGGTCTTC
ABL12YBST    ATAAGGAGGACAGATATTGAGGGTAGCTGGGCTCTAGCAGGGGGGCAGGACTTTACCAGAGACCCTACAATCGCAGAGCATGCATCTCTGGTGGGTCTTC
ABL15YBST    ATAAGGAGGACAGATATTGAGGGCAGCTGGGCTCTAGCAGGGGGGCAGGACTTTACCAGAGACCCTACAATCGCAGAGCATGCATCTCTGGTGGGTCTTC
AAHLYBST     ATAAGGAGAACAGATATTCAGGGCAGCTGGGCTCTGGCAGGGGGGCAGGACTTTACCAGAGACCCTACAATCGCAGAGCATGCATCTCTGGTGGGTCTTC
                                                                                                  <-- NobiVSeq1R
Nobivac      ATAAAGCGTACTGATGTAGAAGGGAATTGGGCTCTGACAGGAGGCATGGAATTGACAAGGGACCCCACTGTCTCTGAACATGCATCTTTAGTCGGTCTTC
             ** * * * ** **  *  * ** *  *****  *  * * **    **  ** ** ** ** ** ** **  *  * ** **  ** *** * ** *******
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Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 6 of 16)**

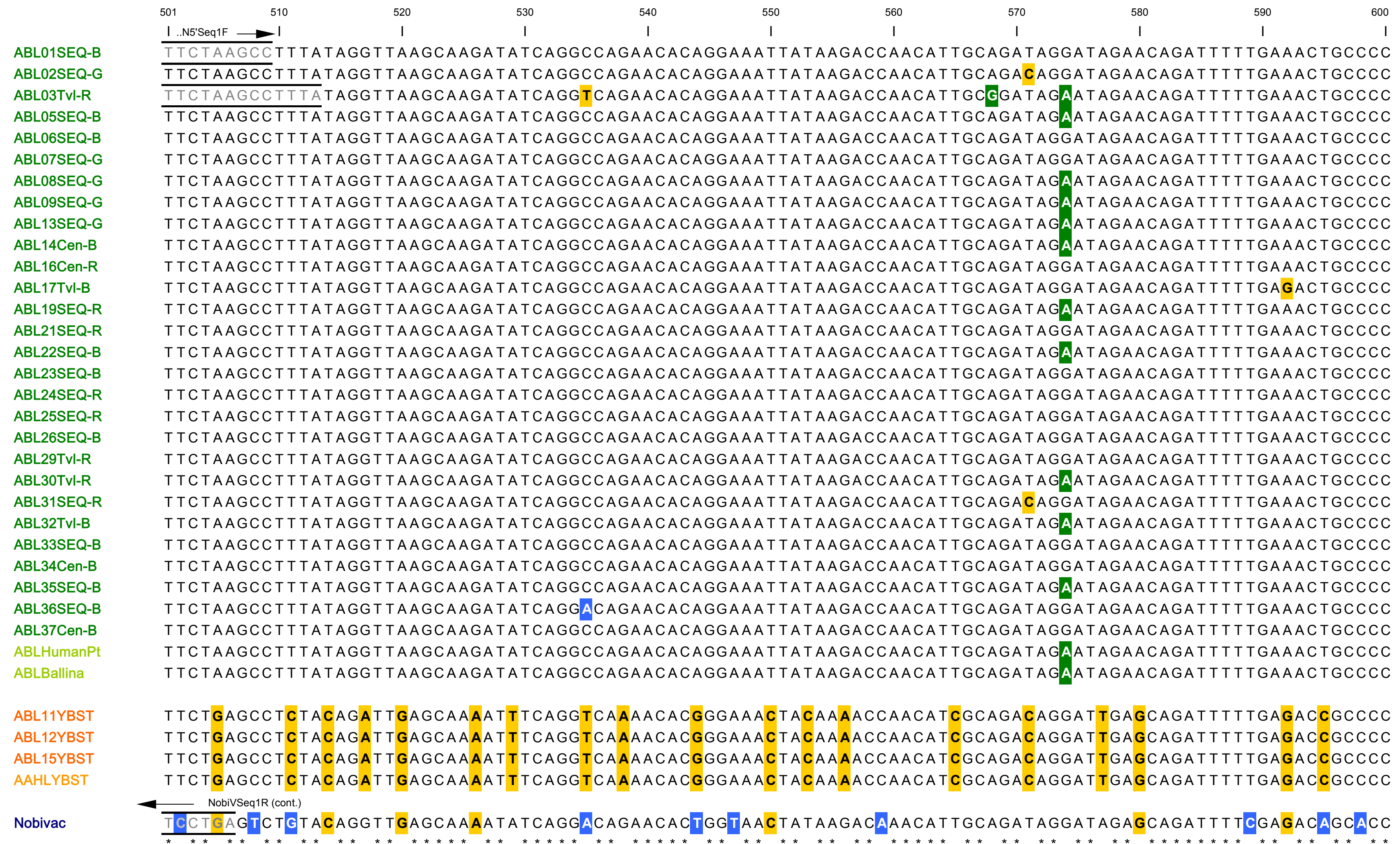

Figure A10-1 (continued) Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 7 of 16)

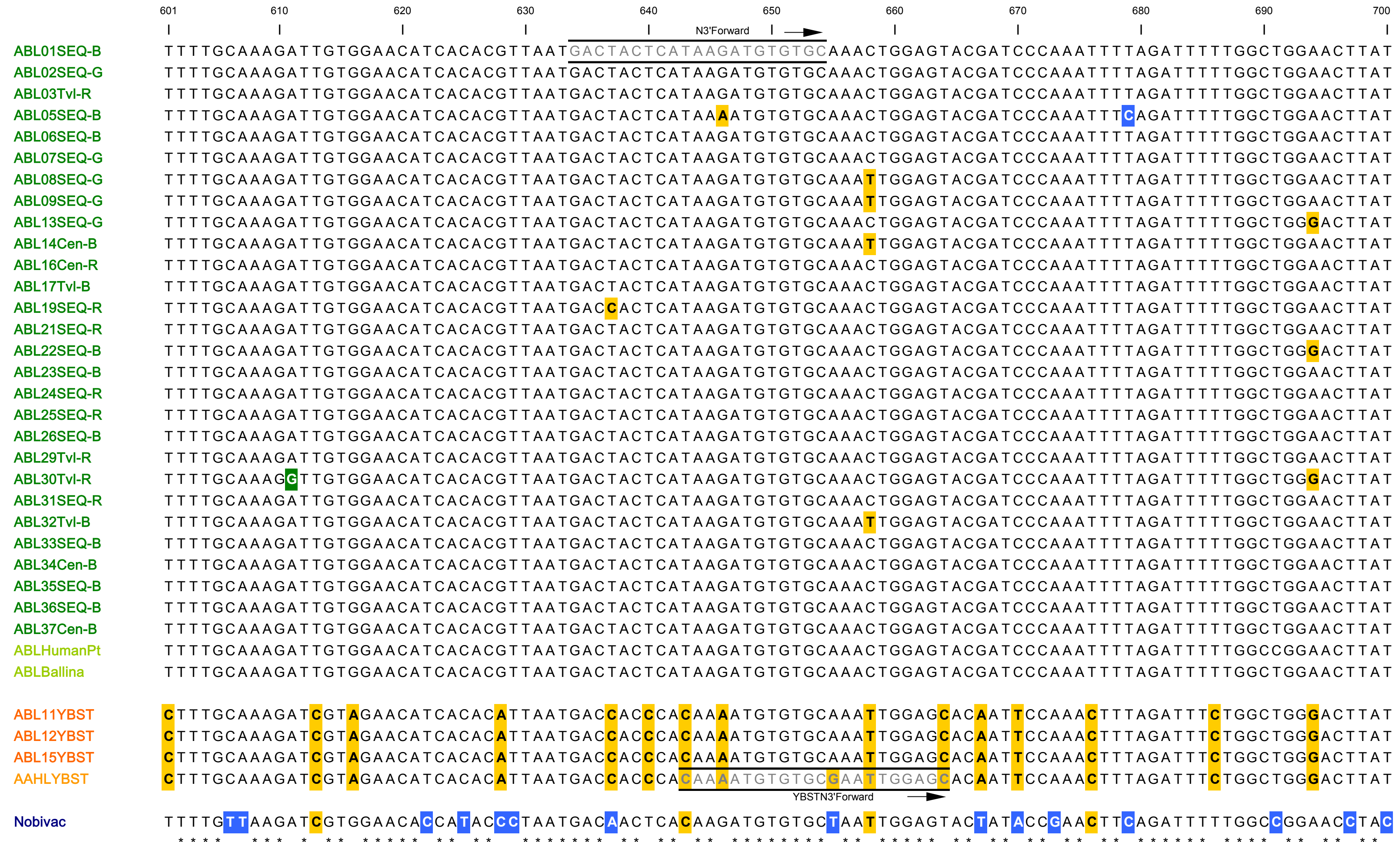

Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 8 of 16)**

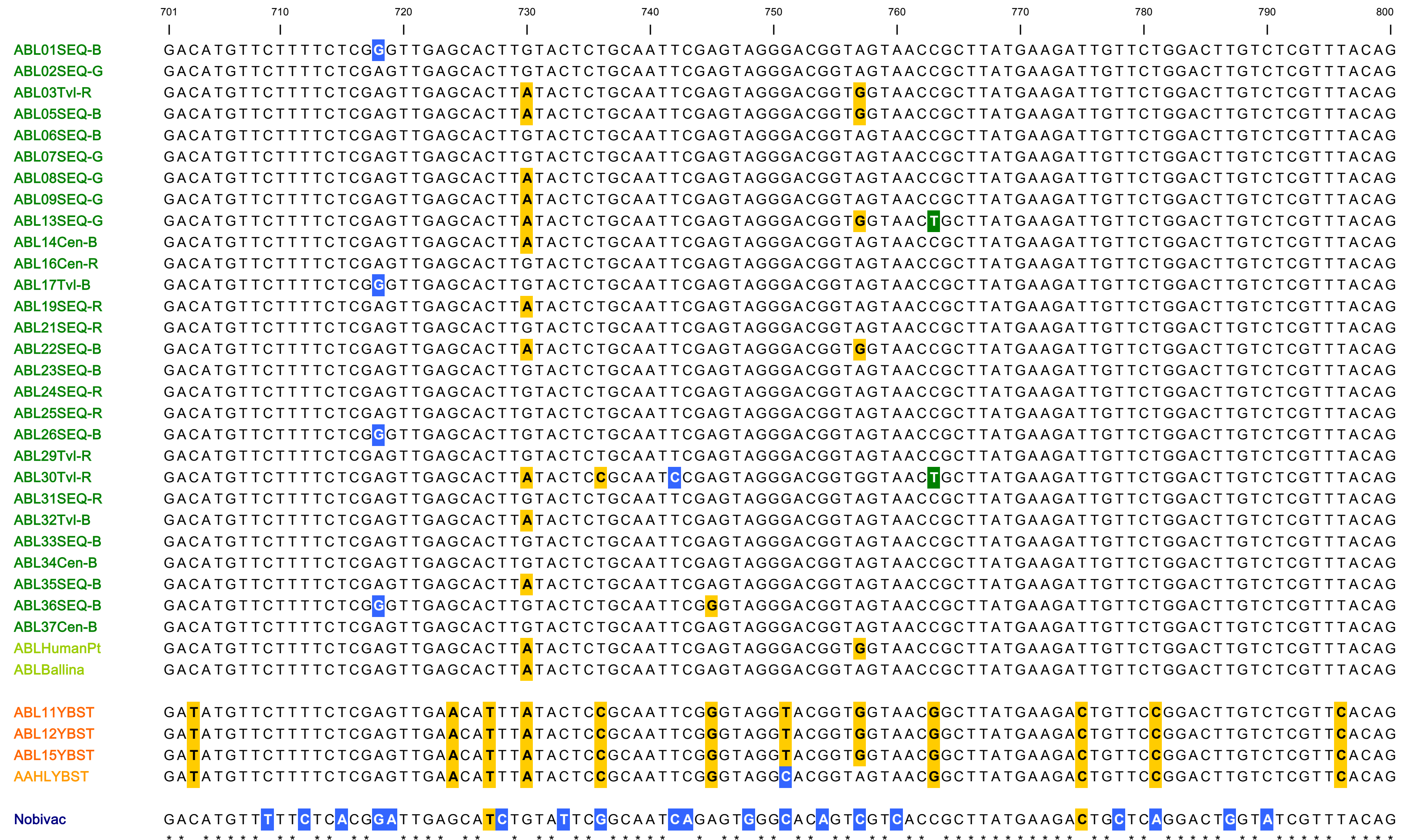

Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 9 of 16)**

```
              801      810       820       830       840       850       860       870       880       890      900
              |        |         |         |         |         |         |         |         |         |        |
ABL01SEQ-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL02SEQ-G    GGTTTATCAAACAAATCAACCTCACTGCTAGGGAAGCAATACTGTATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL03Tvl-R    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL05SEQ-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL06SEQ-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL07SEQ-G    GGTTTATCAAACAGATCAACCTCACCGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL08SEQ-G    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL09SEQ-G    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL13SEQ-G    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAGGAAATTAGAAGAATGTTTGAACCCGGACA
ABL14Cen-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL16Cen-R    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL17Tvl-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL19SEQ-R    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL21SEQ-R    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTCGAACCCGGACA
ABL22SEQ-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL23SEQ-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCGATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL24SEQ-R    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL25SEQ-R    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL26SEQ-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL29Tvl-R    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCGATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL30Tvl-R    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL31SEQ-R    GGTTTATCAAACAAATCAACCTCACTGCTAGGGAAGCAATACTGTATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL32Tvl-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL33SEQ-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL34Cen-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL35SEQ-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL36SEQ-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABL37Cen-B    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABLHumanPt    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA
ABLBallina    GGTTTATCAAACAGATCAACCTCACTGCTAGGGAAGCAATACTATATTTCTTCCATAAGAACTTCGAAGAAGAAATTAGAAGAATGTTTGAACCCGGACA

ABL11YBST     GATTCATCAAGCAAATAAACCTCACTGCTAGGGAAGCGATACTTTACTTCTTCCATAAGAACTTTGAGGAGGAAATCAGGAGAATGTTTGAGCCCGGACA
ABL12YBST     GATTCATCAAGCAAATAAACCTCACTGCTAGGGAAGCGATACTTTACTTCTTCCATAAGAACTTTGAGGAGGAAATCAGGAGAATGTTTGAGCCCGGACA
ABL15YBST     GATTCATCAAGCAAATAAACCTCACTGCTAGGGAAGCGATACTTTACTTCTTCCATAAGAACTTTGAGGAGGAAATCAGGAGAATGTTTGAGCCCGGACA
AAHLYBST      GATTCATCAAGCAAATAAACCTCACTGCTAGGGAAGCGATACTTTACTTCTTCCATAAGAACTTTGAGGAGGAAATCAGGAGAATGTTTGAGCCCGGACA

Nobivac       GGTTCATAAAGCAGATCAATCTCACCGCAAGGGAAGCAATACTATATTTCTTCCACAAGAACTTTGAGGAAGAGATAAGAAGAATGTTCGAGCCAGGGCA
              * ** ** ** ** ** ** ***** ** ******** ***** ** ******** ******** ** ** ** ** ** ******** ** ** ** **
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Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 10 of 16)**

Figure A10-1 (continued) Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 11 of 16)

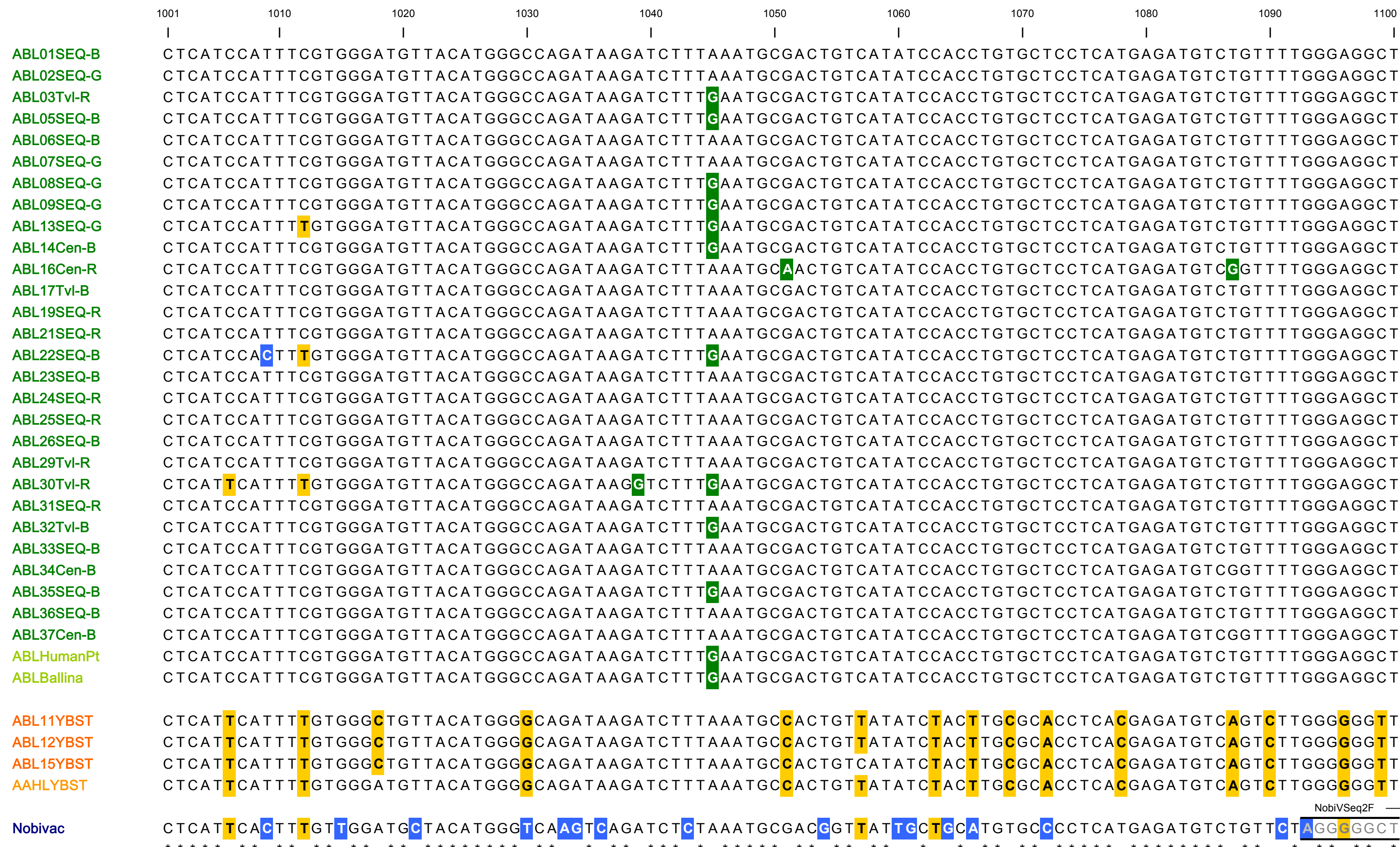

Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 12 of 16)**

```
            1101      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
                                                          <-- N3'Seq2R                         N3'Seq2F -->
ABL01SEQ-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL02SEQ-G  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL03TvI-R  ATCTTGGGGAAGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTACAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL05SEQ-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL06SEQ-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAGCTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL07SEQ-G  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL08SEQ-G  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL09SEQ-G  ATCTTGGGGAGGAGTTTTTCGGGAAAGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL13SEQ-G  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGGTTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL14Cen-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL16Cen-R  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL17TvI-B  ATCTTGGGGAAGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL19SEQ-R  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL21SEQ-R  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAGAC
ABL22SEQ-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL23SEQ-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL24SEQ-R  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL25SEQ-R  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL26SEQ-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL29TvI-R  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL30TvI-R  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL31SEQ-R  ATCTTGGGGAGGAGTTTTTTGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL32TvI-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL33SEQ-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTCGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL34Cen-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL35SEQ-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL36SEQ-B  ATCTTGGGGAAGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABL37Cen-B  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAGAAC
ABLHumanPt  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAAATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC
ABLBallina  ATCTTGGGGAGGAGTTTTTCGGGAAGGGGACGTTTGAACGGAGATTCTTCAGAGATGAGAAAGAACTGCAAGATTATGAGGCAGCAGAGTCTATGAAAAC

ABL11YBST   ACCTAGGGGAAGAGTTCTTCGGGAAAGGGACATTTGAGAGAAGATTCTTCAGAGACGAGAAAGAACTTCAGGATTATGAAGCAGCAGAGGCCATGAAGAT
ABL12YBST   ACCTAGGGGAAGAGTTCTTCGGGAAAGGGACATTTGAGAGAAGATTCTTCAGAGACGAGAAAGAACTTCAGGATTATGAAGCAGCAGAGGCCATGAAGAT
ABL15YBST   ACCTAGGGGAAGAGTTCTTCGGGAAAGGGACATTTGAGAGAAGATTCTTCAGAGACGAGAAAGAACTTCAGGATTATGAAGCAGCAGAGGCCATGAAGAT
AAHLYBST    ACCTAGGGGAAGAGTTCTTCGGGAAAGGGACATTCGAGAGAAGATTCTTCAGAGACGAGAAAGAACTTCAGGATTATGAAGCAGCAGAGGCCATGAAGAT
              NobiVSeq2F (cont.) -->
Nobivac     ATTTGGGAGAGGAATTCTTCGGAAAAGGGACATTTGAAAGAAGGTTCTTCAGAGACGAGAAAGAACTTCAAGAATATGAGGCGGCTGAACTAACAAAGTC
            *  * ** ** ** ** ** ** ** ***** ** **  * ** ********* * ******** ** ** ** ***** ** ** **    *  *    
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Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 13 of 16)**

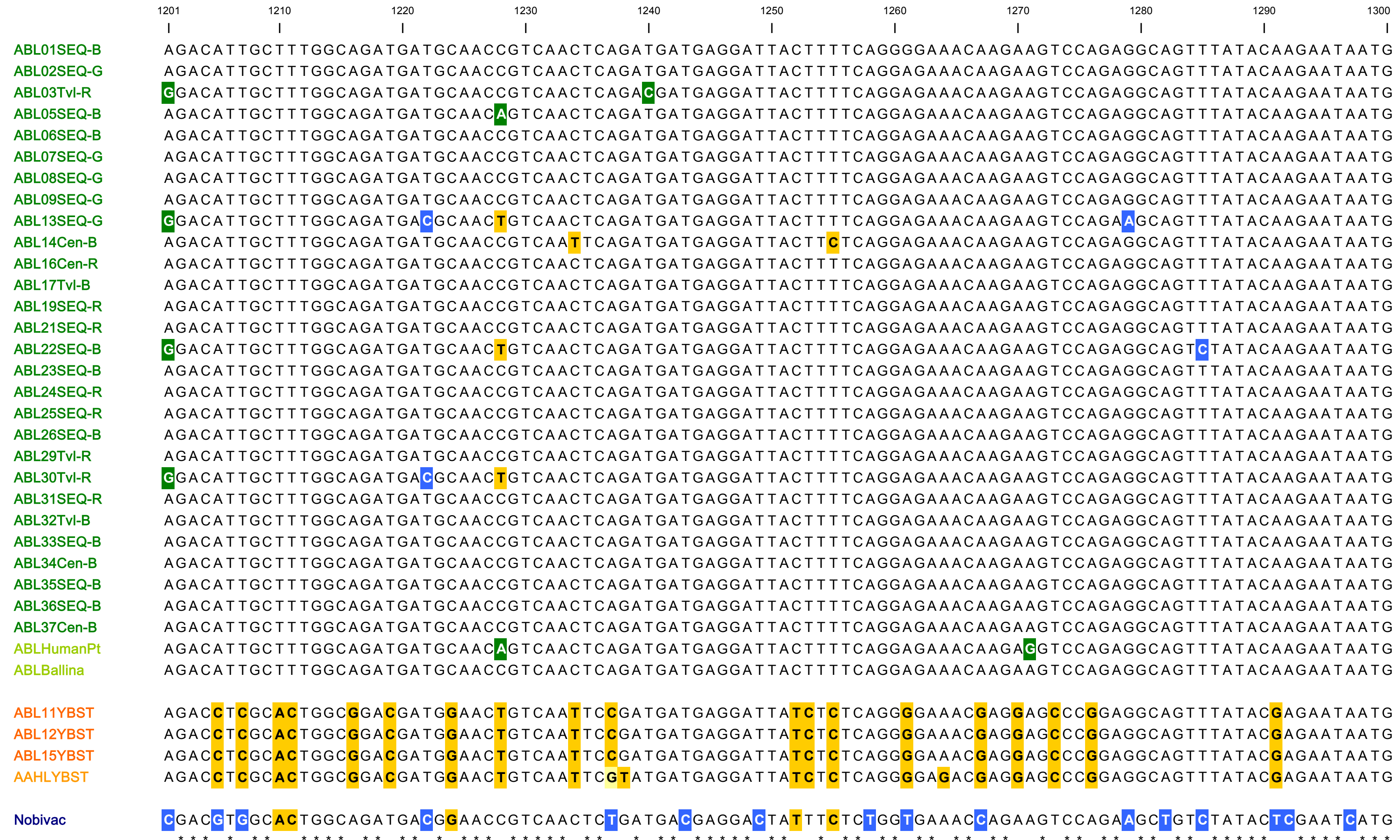

Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 14 of 16)**

```
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ABL01SEQ-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL02SEQ-G   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCAAACTCATTTGCGGAGTTTCTCAACA
ABL03TvI-R   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL05SEQ-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL06SEQ-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL07SEQ-G   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL08SEQ-G   ATGAACGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL09SEQ-G   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL13SEQ-G   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL14Cen-B   ATGAACGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL16Cen-R   ATGAATGGTGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL17TvI-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCAGAGTTTCTCAACA
ABL19SEQ-R   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL21SEQ-R   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL22SEQ-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL23SEQ-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCAAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAATA
ABL24SEQ-R   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL25SEQ-R   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL26SEQ-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL29TvI-R   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAATA
ABL30TvI-R   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL31SEQ-R   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL32TvI-B   ATGAACGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL33SEQ-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL34Cen-B   ATGAATGGTGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL35SEQ-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL36SEQ-B   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABL37Cen-B   ATGAATGGTGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABLHumanPt   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA
ABLBallina   ATGAATGGGGGTCGACTTAAAAGGTCACACATTAGGAGATACATCTCAGTCAGCTCCAACCATCAGTCCCGACCGAACTCATTTGCGGAGTTTCTCAACA

ABL11YBST    ATGAATGGAGGCCGACTCAAGAAGTCACACATCAGGAGGTACATCTCAGTGAGTTCTAACCATCAGTCTCGACCGAACTCGTTTGCTGAATTCCTAAACA
ABL12YBST    ATGAATGGAGGCCGACTCAAGAAGTCACACATCAGGAGGTACATCTCAGTGAGTTCTAACCATCAGTCTCGACCGAACTCGTTTGCTGAATTCCTAAACA
ABL15YBST    ATGAATGGAGGCCGACTCAAGAAGTCACACATCAGGAGGTACATCTCAGTGAGTTCTAACCATCAGTCTCGACCGAACTCGTTTGCTGAATTCCTAAACA
AAHLYBST     ATGAATGGAGGCCGACTCAAGAAGTCACACATCAGGAGGTACATCTCAGTGAGTTCTAACCATCAGTCTCGGCCGAACTCGTTTGCTGAATTCCTAAACA

Nobivac      ATGAATGGAGGTCGACTGAAGAGATCTCATATACGGAGATATGTCTCAGTCAGTTCCAATCATCAAGCCCGTCCAAACTCATTCGCCGAATTTTTAAACA
             ***** ** ** ***** ** ** ** ** **  **** **  ******* ** ** ** *****  * ** ** ***** ** *  ** **  * ** *
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Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 15 of 16)**

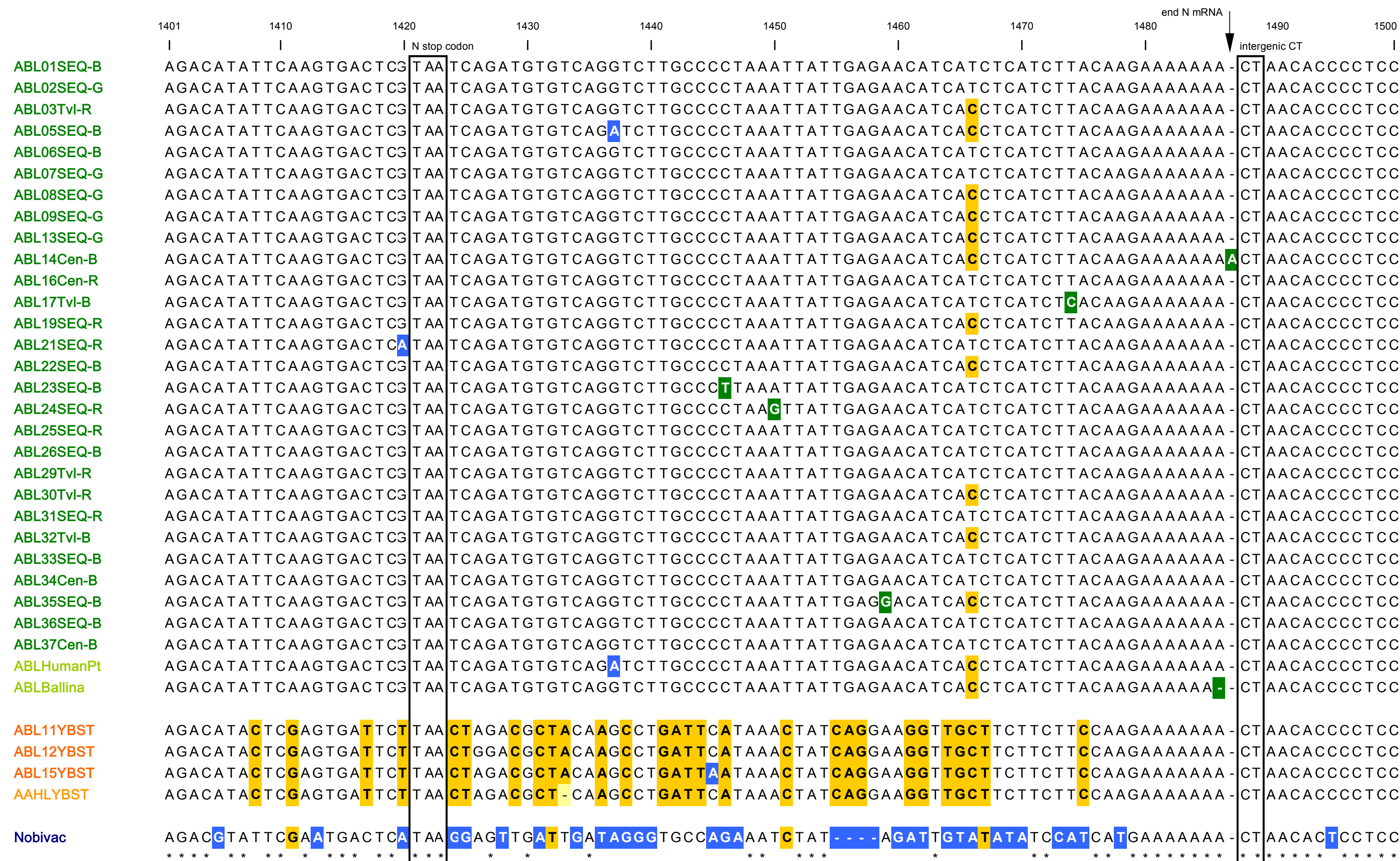
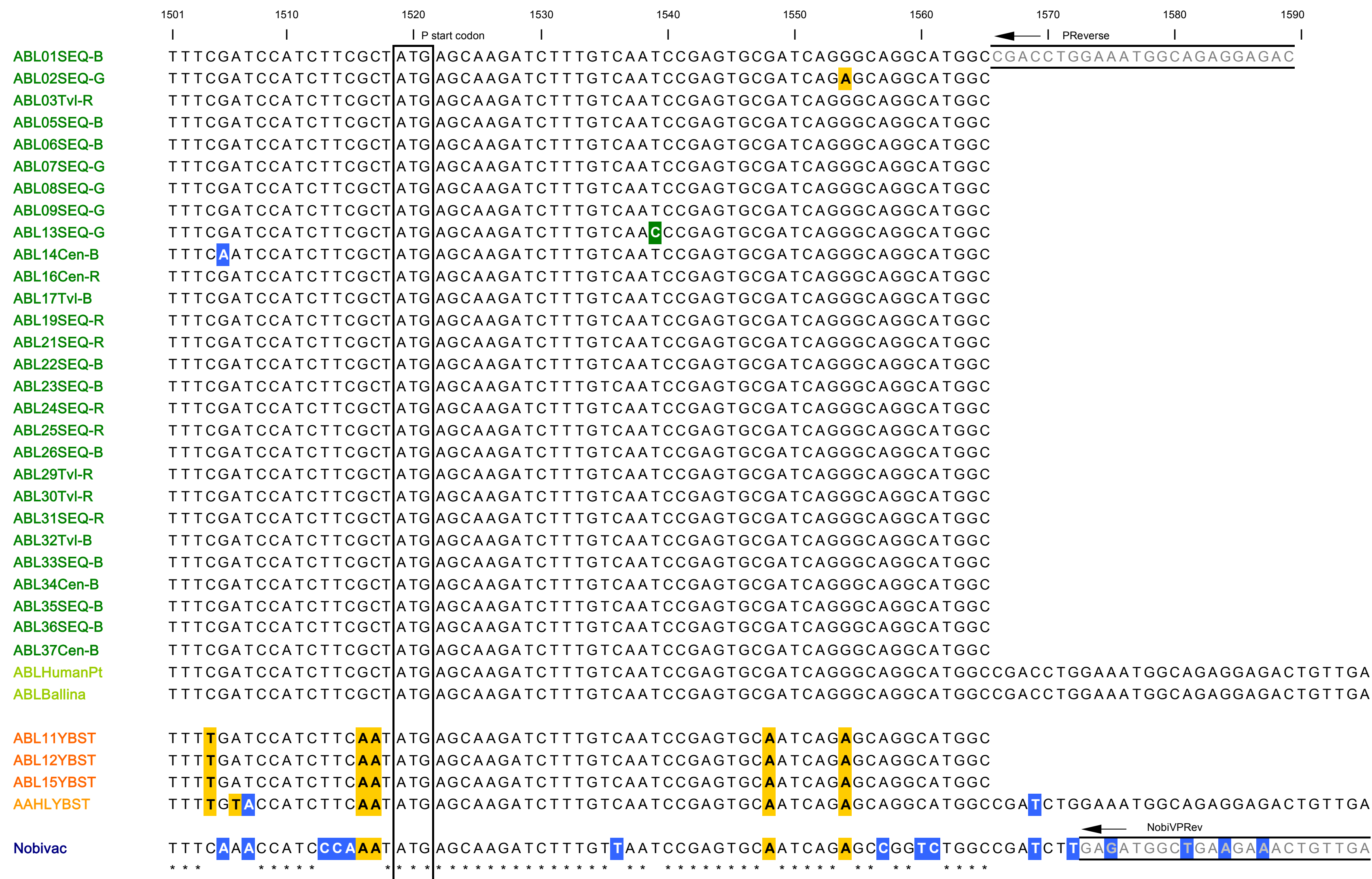


Figure A10-1 (continued) **Nucleotide alignment of ABLV (n=34) and Nobivac (n=1) sequences (page 16 of 16)**



Appendix 11 Response to Nobivac rabies vaccine

Individual rabies-RFFIT titres for 36 flying foxes vaccinated with the Nobivac rabies vaccine and one control flying fox (no Nobivac) are shown in Table A11-1. Rabies-RFFITs were kindly performed by Ross Lunt and Kim Newberry at the AAHL (see Appendix 2).

Key to Table A11-1

Bat no. Unique last three digits of bat microchip number

Species **Black** = Black flying fox (*Pteropus alecto*) (n=13)
Grey = Grey-headed flying fox (*Pteropus poliocephalus*) (n=24)

Sex **M** = male (n=19)
F lact = female that gave birth to and lactated for a pup (n=11)
F abort = female that aborted a pre-term foetus (n=4)
F np = female that was not pregnant (n=3)

Age class **Juv** = juvenile, pre-pubescent flying fox 1-3 years of age (n=10)
A = adult flying fox > 3 years of age (n=27)

Capt. weight Weight of flying fox on the day of capture on PV-day –52 to –60

Method **Nobivac** = flying fox vaccinated with KLH *in* Nobivac vaccine on PV-day 0 ± PV-day 28 (Phase 1), and subsequently vaccinated with High-dose KLH + Alhydrogel on PV-day 85 (Phase 2)
Alhydrogel = flying fox vaccinated with Nobivac vaccine and a separate injection of KLH + Alhydrogel on PV-day 0 ± PV-day 28 (Phase 1), and subsequently vaccinated with High-dose KLH + Alhydrogel on PV-day 85 (Phase 2)
No Nobivac Alhydrogel = single control flying fox vaccinated with KLH + Alhydrogel only on PV-day 0 (no separate Nobivac vaccination) during Phase 1, and subsequently vaccinated with high dose KLH + Alhydrogel on PV-day 85 (Phase 2)

Boost day 28 Flying fox that did (**Yes**) or did not (**No**) receive a booster vaccination on PV-day 28.

The data for individuals shown in Table A11-1 are illustrated in Figure A11-1 to Figure A11-6. The graphs have been truncated at a rabies-RFFIT titre of 25 IU/mL for clarity, values for titres in excess of 25 IU/mL can be read from Table A11-1.

Table A11-1 Response to vaccination with Nobivac (rabies) vaccine in Black and Grey-headed flying foxes (n=37)

Bat details									rabies-RFFIT titres (IU/mL)									
									PV-day									
Microchip number	Bat No	Group	Species	Sex	Age	Capt. weight	Method	Boost day-28	0	7	14	21	28	42	56	85	127	171
5034144778	778	1	Grey	Male	Juv	405	Nobivac	No	0	0.1	1.6	3.1	2.8	2.6	5.7	5	7.3	7.2
41066E1B66	B66	1	Grey	Male	Juv	373	Nobivac	No	0	0.1	0.9	0.7	0.9	1	2.6	0.7	1	0.8
40366C4256	256	1	Grey	Male	A	673	Nobivac	No	0	0.1	1	3.3	3.5	2.3	2.2	2.5	3	1
400F2E2332	332	1	Grey	F lact	A	666	Nobivac	No	0	0.2	1.2	2.3	0.8	0	0.1	0.6	0.2	0.7
4105485215	215	1	Grey	F lact	A	710	Nobivac	No	0	0.1	1.1	2.9	3.5	0.8	1.3	1.2	1.8	2.2
40374E6362	362	1	Grey	F lact	A	630	Nobivac	No	0	0.3	0.6	1.7	0.9	0	0	0.4	0.9	0.9
4105523A75	A75	2	Black	Male	A	846	Alhydrogel	No	0	0.3	1.5	3.1	6.2	5.2	3.6	1.2	0.4	0.8
0001BB1B30	B30	2	Black	Male	A	774	Alhydrogel	No	0	1.4	3.1	17.1	10.5	10.3	4.6	1.5	2.6	1.9
4036384B32	B32	2	Black	Male	A	800	Alhydrogel	No	0	0.8	1	4.4	14.3	11	10.3	3.1	1.3	1.2
50340F2CIB	CIB	2	Black	Male	A	884	Alhydrogel	No	0	0.7	0.6	2.1	1.5	0.5	0.2	0.5	1.6	0.9
50341B5E4B	E4B	2	Black	F np	A	552	Alhydrogel	No	0	3.6	7.2	7.6	4.6	5.2	5.5	7.3	11.8	10.2
41055B595B	95B	2	Black	F lact	A	855	Alhydrogel	No	0	0.1	1.1	2.5	1.5	1.1	1.9	5	2	DIED
50066A1361	361	2	Grey	Male	Juv	410	Alhydrogel	No	0	0.6	1	3.5	4.6	1	0.8	0.5	0.4	0.8
41054F2179	179	2	Grey	F np	Juv	401	Alhydrogel	No	0	0.3	2.6	8.5	8.7	5.2	5.9	3.1	7.8	4.5
4035220813	813	2	Grey	Male	A	626	Alhydrogel	No	0	0.5	0.1	1.4	3.1	2.6	0.9	0.5	1.8	1.1
410556384D	84D	2	Grey	F lact	A	634	Alhydrogel	No	0	0.3	2	3.3	4.1	2.3	2.9	3.1	5	10
41066D3A35	A35	2	Grey	F lact	A	604	Alhydrogel	No	0	0.8	1.8	2	1	0.2	0.8	0.7	0.9	1.1
0001BBA11A	11A	2	Grey	F abort	A	692	Alhydrogel	No	0	0.1	0.8	0.8	1	1	1.1	0.6	1	10

Table A11-1 (continued): rabies-RFFIT titres (IU/mL) page 2 of 2

Microchip number	Bat No	Group	Species	Sex	Age	Capt. weight	Method	Boost day-28	PV-day									
									0	7	14	21	28	42	56	85	127	171
50325D1655	655	3	Grey	Male	Juv	427	Nobivac	Yes	0	0.7	0.5	3.6	4.6	16.3	21.5	10	13.3	8.4
41050E1F19	F19	3	Grey	Male	Juv	385	Nobivac	Yes	0	0.2	2	3.3	4.1	11.3	11.8	2.9	8.9	5.1
41055B2119	119	3	Grey	F np	Juv	440	Nobivac	Yes	0	0.1	7.2	29	9.7	53.6	12.5	11.3	9.8	11.7
400F3F6551D	51D	3	Grey	F lact	A	651	Nobivac	Yes	0	0.1	1	1	0.9	4.7	0.7	0.6	0.3	0.7
4105391A24	A24	3	Grey	F lact	A	760	Nobivac	Yes	0	0.1	0.9	1.7	2.1	5	0.7	0.5	0	0.2
5006683B43	B43	3	Grey	F abort	A	460	Nobivac	Yes	0	1.5	4.9	7.5	1.4	10	6.1	2.9	4.8	1.5
41054D0424	424	4	Black	Male	A	845	Alhydrogel	Yes	0	0.1	0.5	1.2	2.1	13.4	11.8	3.3	6.5	3.6
41064A610B	10B	4	Black	Male	A	848	Alhydrogel	Yes	0	1.4	1.4	2.9	4.3	18.5	11.1	2.9	2.5	0.9
0001DC383F	83F	4	Black	Male	A	975	Alhydrogel	Yes	0	0.1	0.6	0.7	1.1	19.2	12.5	9.1	19.1	10.2
4017293F43	F43	4	Black	Male	A	851	Alhydrogel	Yes	0	0.8	0.2	0.5	0.8	17.5	3	1.1	1	0.2
4036732565	565	4	Black	F lact	A	655	Alhydrogel	Yes	0.2	1.8	2.5	3.3	1	20.1	7.3	2.9	1	1
405C0E6D58	D58	4	Black	F lact	A	777	Alhydrogel	Yes	0	0.3	1.2	3.5	5	14.6	11.8	31.5	28.1	9
400F1D4174	174	4	Grey	Male	Juv	356	Alhydrogel	Yes	0.2	1.3	4.6	22.4	4.1	63	13.4	5.4	7.2	4.8
5034150402	402	4	Grey	Male	Juv	402	Alhydrogel	Yes	0	1.1	4	6.3	1.5	15.2	11.8	2.9	7.8	2.2
4010276D03	D03	4	Grey	Male	Juv	464	Alhydrogel	Yes	0	1.5	2	2.9	4.1	7.6	5.9	1.4	1	1.2
4056740707	707	4	Grey	F lact	A	611	Alhydrogel	Yes	0	0.1	0.7	2.9	1.1	10	3.7	1.8	0.5	0.9
40366D2E64	E64	4	Grey	F abort	A	642	Alhydrogel	Yes	0	0.1	1.3	2.5	1.8	6.6	2.8	0.7	0.9	0.4
50341F2B7A	B7A	4	Grey	F abort	A	696	Alhydrogel	Yes	0	0.3	0.7	0.5	0.8	5	1.2	0.9	1.6	0.2
50335F1775	775	Control	Black	Male	A	834	No Nobivac Alhydrogel	No	0	0	0	0	0	0	0	0	0	0

Figure A11-1 Response to Nobivac rabies vaccine: Group 1 Grey headed flying foxes (n=6)
Vaccinations: PV-day 0 KLH *in* Nobivac
PV-day 85 High dose KLH + Alhydrogel (no Nobivac)

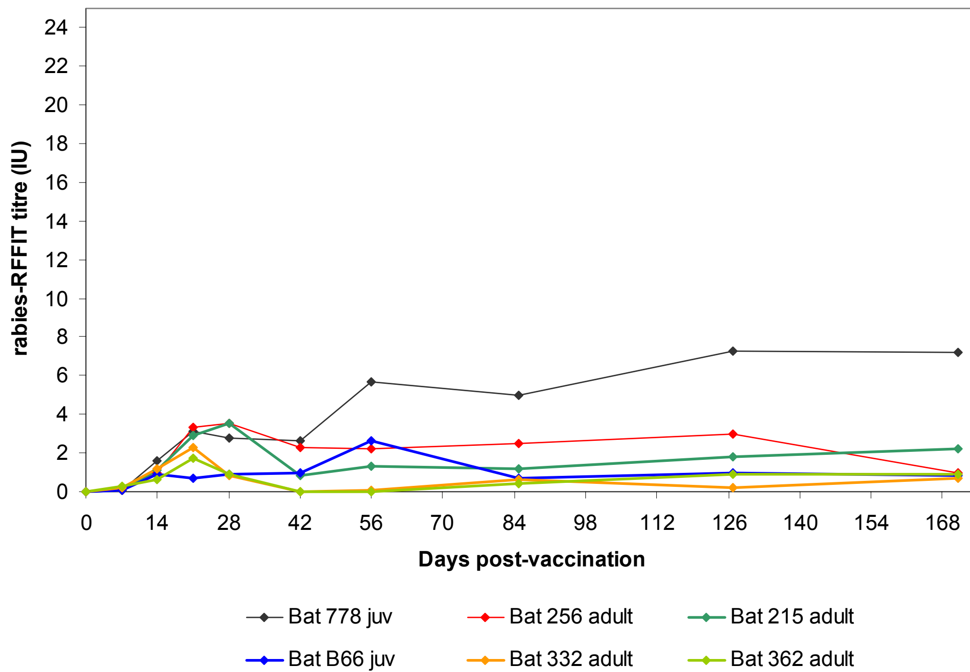


Figure A11-2 Response to Nobivac rabies vaccine: Group 3 Grey headed flying foxes (n=6)
Vaccinations: PV-day 0 KLH *in* Nobivac
PV-day 28 KLH *in* Nobivac
PV-day 85 High dose KLH + Alhydrogel (no Nobivac)

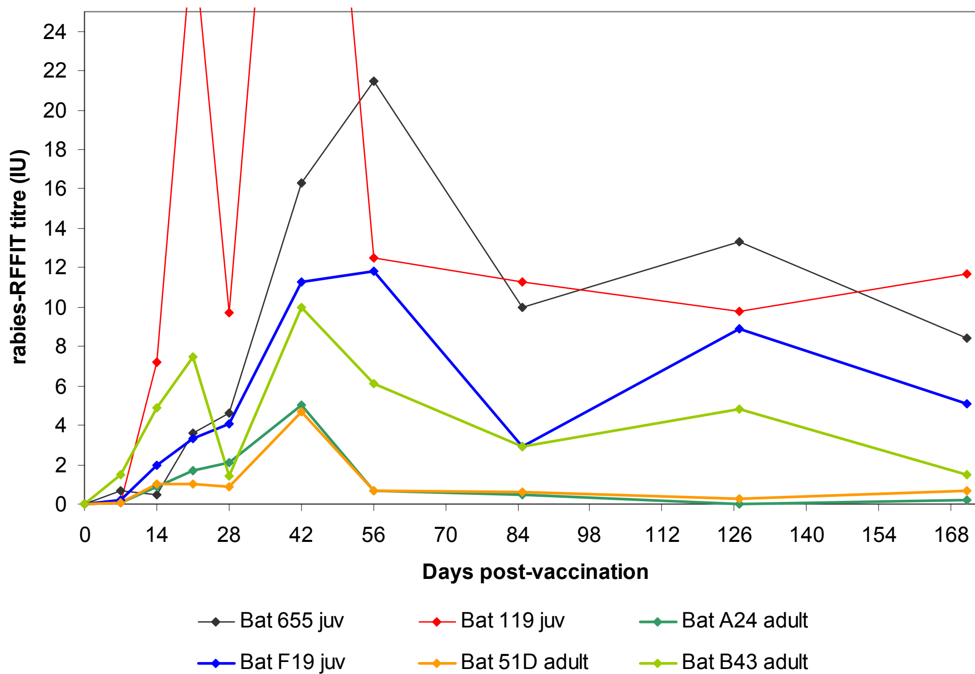


Figure A11-3 Response to Nobivac rabies vaccine: Group 2 Grey-headed flying foxes (n=6)
Vaccinations: PV-day 0 Nobivac and KLH + Alhydrogel
PV-day 85 High dose KLH + Alhydrogel (no Nobivac)

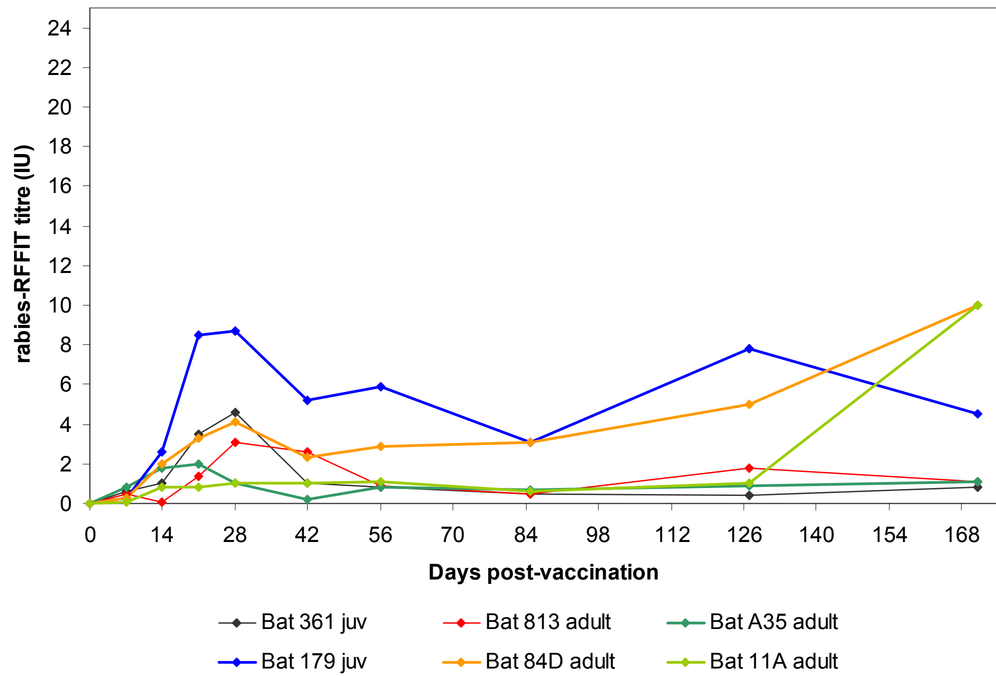


Figure A11-4 Response to Nobivac rabies vaccine: Group 4 Grey headed flying foxes (n=6)
Vaccinations: PV-day 0 Nobivac and KLH + Alhydrogel
PV-day 28 Nobivac and KLH + Alhydrogel
PV-day 85 High dose KLH + Alhydrogel (no Nobivac)

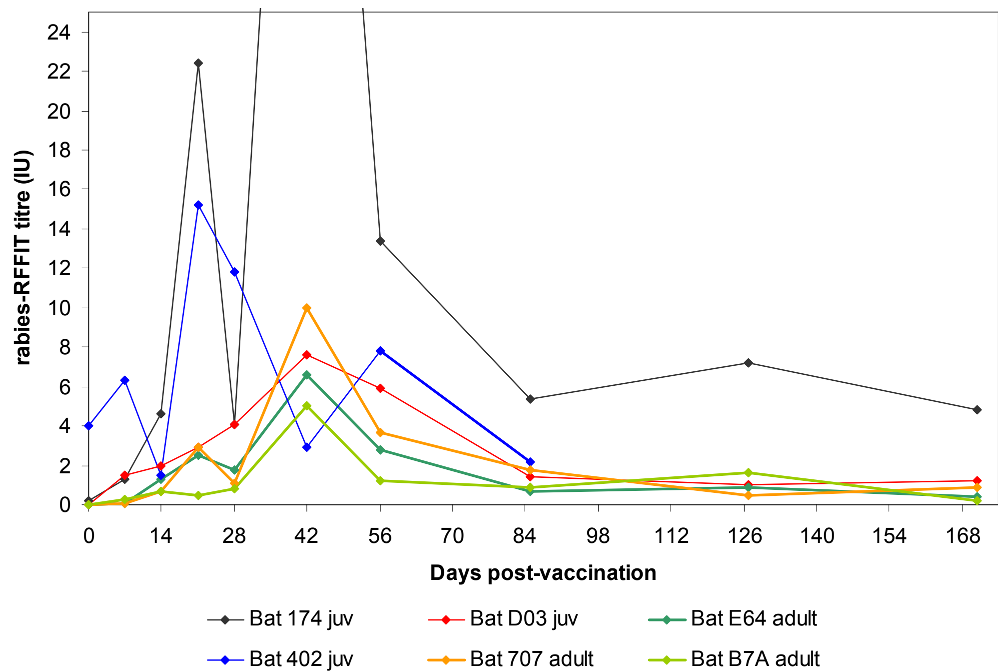


Figure A11-5 Response to Nobivac rabies vaccine: Group 2 Black flying foxes (n=6)
Vaccinations: PV-day 0 Nobivac and KLH + Alhydrogel
PV-day 85 High dose KLH + Alhydrogel (no Nobivac)

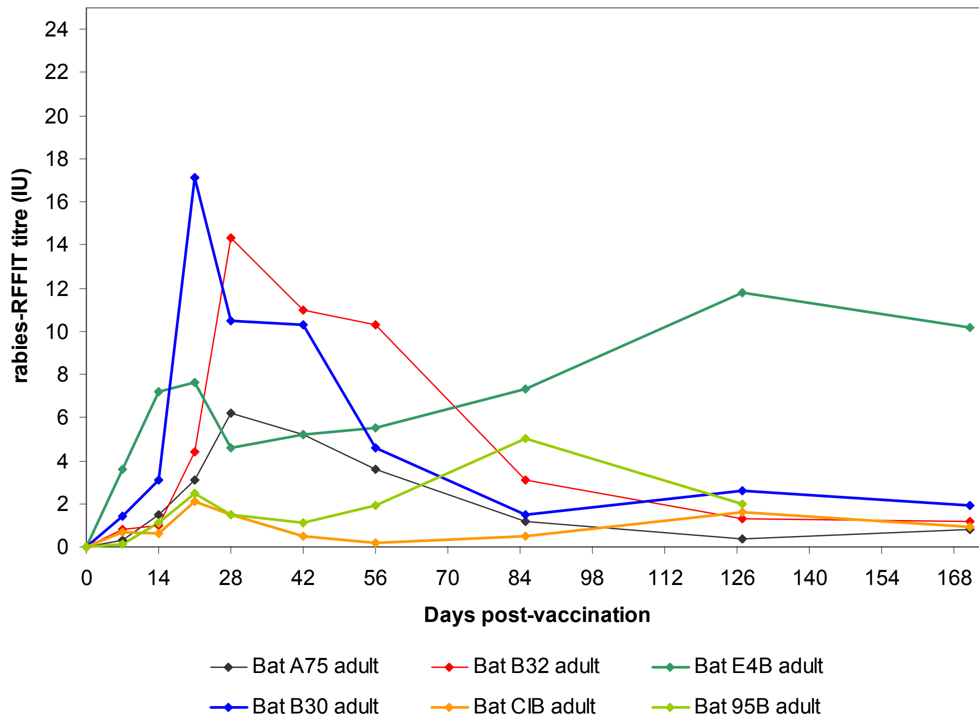
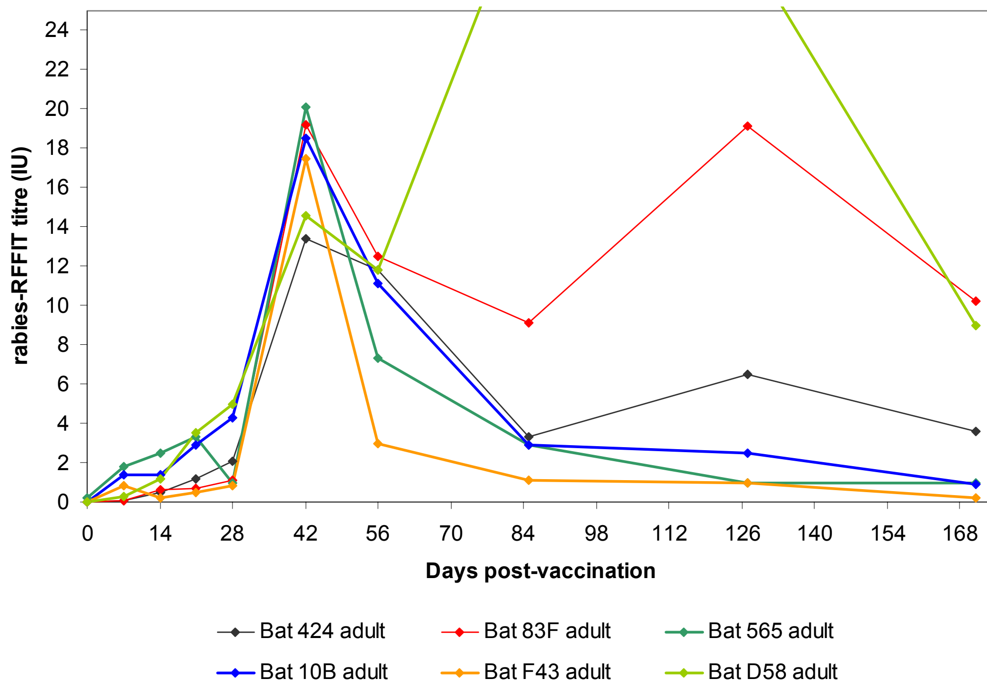


Figure A11-6 Response to Nobivac rabies vaccine: Group 4 Black flying foxes (n=6)
Vaccinations: PV-day 0 Nobivac and KLH + Alhydrogel
PV-day 28 Nobivac and KLH + Alhydrogel
PV-day 85 High dose KLH + Alhydrogel (no Nobivac)



Appendix 12 Response to vaccination with KLH

Individual KLH ELISA titres for 37 flying foxes vaccinated with KLH either combined with the Nobivac rabies vaccine or as a separate injection with the adjuvant Alhydrogel are shown in Table A12-1. Titres were determined relative to a positive control standard (percent absorbance) in a KLH ELISA developed by the candidate (see Chapter 8).

Key to Table A12-1

Bat no.	Unique last three digits of bat microchip number
Species	**Black** = Black flying fox (*Pteropus alecto*) (n=13)
	Grey = Grey-headed flying fox (*Pteropus poliocephalus*) (n=24)
Sex	**Male** = male (n=19)
	F lact = female that gave birth to and lactated for a pup (n=11)
	F abort = female that aborted a pre-term foetus (n=4)
	F np = female that was not pregnant (n=3)
Age class	**Juv** = juvenile, pre-pubescent flying fox 1-3 years of age (n=10)
	A = adult flying fox > 3 years of age (n=27)
Capt. weight	Weight of flying fox on the day of capture on PV-day –52 to –60
Method	**Nobivac** = flying fox vaccinated with KLH *in* Nobivac vaccine on PV-day 0 ± PV-day 28 (Phase 1), and subsequently vaccinated with High-dose KLH + Alhydrogel on PV-day 85 (Phase 2)
	Alhydrogel = flying fox vaccinated with Nobivac vaccine and a separate injection of KLH + Alhydrogel on PV-day 0 ± PV-day 28 (Phase 1), and subsequently vaccinated with High-dose KLH + Alhydrogel on PV-day 85 (Phase 2)
	No Nobivac Alhydrogel = single control flying fox vaccinated with KLH + Alhydrogel only on PV-day 0 (no separate Nobivac vaccination) during Phase 1, and subsequently vaccinated with high dose KLH + Alhydrogel on PV-day 85 (Phase 2)
Boost day 28	Flying fox that did (**Yes**) or did not (**No**) receive a booster vaccination on PV-day 28.

Actual KLH responses for individuals in each group and species are illustrated in Figure A12-1 to Figure A12-6.

Table A12-1 Response to vaccination with KLH immunogen in Black and Grey-headed flying foxes (n=37)

Bat details									Phase 1 KLH-ELISA titres (percent absorbance)								Phase 2 titres		
Microchip number	Bat No.	Group	Species	Sex	Age class	Capt. weight	Method	Boost Day 28	PV-day 0	7	14	21	28	42	56	85	99	127	171
5034144778	778	1	Grey	Male	Juv	405	Nobivac	No	1.5	33.7	60.5	66.0	52.8	37.8	30.3	19.3	94.9	87.6	83.7
41066E1B66	B66	1	Grey	Male	Juv	373	Nobivac	No	0.6	1.8	10.6	14.6	11.4	10.3	8.7	6.9	101.5	85.6	38.7
40366C4256	256	1	Grey	Male	A	673	Nobivac	No	1.1	16.4	41.4	42.8	32.4	20.0	16.4	21.3	97.7	90.6	53.7
400F2E2332	332	1	Grey	F lact	A	666	Nobivac	No	2.1	15.9	53.5	53.8	38.6	23.3	20.7	9.5	89.3	59.8	30.2
4105485215	215	1	Grey	F lact	A	710	Nobivac	No	5.2	45.4	46.3	45.1	27.2	19.2	15.1	7.1	100.9	86.9	47.1
40374E6362	362	1	Grey	F lact	A	630	Nobivac	No	0.6	18.6	53.3	47.6	26.5	9.3	5.2	6.0	96.6	79.4	53.4
4105523A75	A75	2	Black	Male	A	846	Alhydrogel	No	0.4	5.2	67.6	34.4	84.4	56.7	39.5	21.0	100.3	86.7	62.3
0001BB1B30	B30	2	Black	Male	A	774	Alhydrogel	No	0.5	12.7	70.9	74.4	77.4	65.9	57.2	53.0	101.1	94.0	79.6
4036384B32	B32	2	Black	Male	A	800	Alhydrogel	No	5.3	47.7	94.8	82.6	82.1	79.5	67.4	59.2	104.3	95.0	72.1
50340F2CIB	CIB	2	Black	Male	A	884	Alhydrogel	No	0.5	59.6	61.6	48.8	35.8	25.2	19.1	14.4	101.1	75.5	53.2
50341B5E4B	E4B	2	Black	F np	A	552	Alhydrogel	No	0.5	38.1	73.8	75.8	67.2	54.4	45.5	31.2	111.3	96.3	72.3
41055B595B	95B	2	Black	F lact	A	855	Alhydrogel	No	3.9	23.5	78.9	74.0	67.7	31.1	18.7	16.1	101.2	83.5	70.9
50066A1361	361	2	Grey	Male	Juv	410	Alhydrogel	No	3.4	34.5	79.7	91.0	80.1	50.4	35.1	22.8	102.6	94.5	69.9
41054F2179	179	2	Grey	F np	Juv	401	Alhydrogel	No	0.6	43.2	78.3	92.9	92.2	83.3	64.7	46.9	99.6	97.1	100.5
4035220813	813	2	Grey	Male	A	626	Alhydrogel	No	2.6	4.2	50.3	84.6	78.5	39.0	23.0	7.7	103.8	85.5	35.6
410556384D	84D	2	Grey	F lact	A	634	Alhydrogel	No	10.3	36.8	92.3	96.0	90.9	42.8	64.5	12.8	97.0	75.9	35.5
41066D3A35	A35	2	Grey	F lact	A	604	Alhydrogel	No	0.9	9.2	70.4	75.8	53.2	28.3	24.6	11.2	100.1	79.8	33.7
0001BBA11A	11A	2	Grey	F abort	A	692	Alhydrogel	No	3.3	23.7	79.8	80.8	77.3	43.5	36.9	28.7	97.2	94.1	62.5

Table A12-1 (continued): KLH-ELISA titres (PA) page 2 of 2

Microchip umber	Bat No.	Group	Species	Sex	Age class	Capt. weight	Method	Boost Day 28	PV-day										
									0	7	14	21	28	42	56	85	99	127	171
50325D1655	655	3	Grey	Male	Juv	427	Nobivac	Yes	2.4	48.2	60.0	66.1	46.7	78.0	59.7	31.3	92.5	80.1	54.0
41050E1F19	F19	3	Grey	Male	Juv	385	Nobivac	Yes	0.0	1.1	9.4	21.0	17.9	75.9	49.7	20.8	108.2	81.2	59.6
41055B2119	119	3	Grey	F np	Juv	440	Nobivac	Yes	0.8	6.4	29.7	68.0	22.1	85.1	57.6	39.9	94.8	94.7	79.6
400F3F6551D	51D	3	Grey	F lact	A	651	Nobivac	Yes	4.5	25.5	58.9	82.9	44.3	51.8	37.8	19.4	82.7	63.3	26.9
4105391A24	A24	3	Grey	F lact	A	760	Nobivac	Yes	15.4	37.3	56.6	54.3	33.4	34.4	22.0	16.6	45.1	44.3	26.2
5006683B43	B43	3	Grey	F abort	A	460	Nobivac	Yes	2.0	48.1	76.8	74.1	55.3	69.6	47.7	25.6	97.3	77.9	45.5
41054D0424	424	4	Black	Male	A	845	Alhydrogel	Yes	0.9	14.7	56.4	78.2	58.7	73.5	64.3	50.5	86.7	77.7	56.6
41064A610B	10B	4	Black	Male	A	848	Alhydrogel	Yes	0.3	9.7	54.4	59.0	47.4	83.8	57.3	37.2	100.2	55.1	29.2
0001DC383F	83F	4	Black	Male	A	975	Alhydrogel	Yes	0.2	22.8	58.3	70.6	50.1	75.7	36.2	13.4	106.8	85.4	50.8
4017293F43	F43	4	Black	Male	A	851	Alhydrogel	Yes	0.0	2.9	35.9	32.7	32.7	68.7	27.7	12.3	102.6	60.6	16.6
4036732565	565	4	Black	F lact	A	655	Alhydrogel	Yes	0.5	5.3	53.8	55.6	55.8	91.4	59.7	28.6	92.0	69.9	45.4
405C0E6D58	D58	4	Black	F lact	A	777	Alhydrogel	Yes	2.3	37.3	85.4	76.6	61.9	79.8	54.5	37.5	107.1	101.1	98.5
400F1D4174	174	4	Grey	Male	Juv	356	Alhydrogel	Yes	1.0	8.9	90.6	81.3	37.0	104.3	96.3	73.4	106.1	105.9	95.4
5034150402	402	4	Grey	Male	Juv	402	Alhydrogel	Yes	1.9	7.3	69.0	102.0	88.1	103.8	84.8	45.8	100.9	94.7	103.1
4010276D03	D03	4	Grey	Male	Juv	464	Alhydrogel	Yes	3.7	18.4	89.9	88.8	91.2	97.0	85.6	72.4	111.2	104.2	96.8
4056740707	707	4	Grey	F lact	A	611	Alhydrogel	Yes	1.2	19.6	75.7	73.4	55.1	94.8	63.0	33.9	97.2	78.4	41.8
40366D2E64	E64	4	Grey	F abort	A	642	Alhydrogel	Yes	2.4	35.3	88.4	80.3	18.3	77.3	50.6	19.1	111.6	86.7	32.6
50341F2B7A	B7A	4	Grey	F abort	A	696	Alhydrogel	Yes	1.2	27.1	74.3	86.3	74.8	79.4	67.8	37.3	102.1	92.7	59.2
50335F1775	775	Control	Black	Male	A	834	Alhydrogel No Nobivac	No	4.1	18.5	79.8	91.0	90.7	64.2	48.7	30.0	99.8	95.6	86.1

Extrapolated duration of positive KLH-titre

The length of time that KLH-ELISA titres in individual flying foxes could have been expected to remain positive (titre >20 PA) following either Phase 1 vaccinations alone or following both Phase 1 and the PV-day 85 high-dose KLH + Alhydrogel booster (Phase 2) was estimated by mathematical extrapolation of the known titres.

The estimated duration for each flying fox is shown in Table A12-2.

Table A12-2 Extrapolated duration of positive KLH titres following Phase 1 and Phase 2 vaccinations

Bat No	Group	Species	Sex	Age	Method	Boost Day 28	Extrapolated duration of positive titre >20 PA (days)	
							Phase 1	Phase 2
778	1	Grey	Male	Juv	Nobivac	No	76	592
B66	1	Grey	Male	Juv	Nobivac	No	21	194
256	1	Grey	Male	A	Nobivac	No	52	228
332	1	Grey	F lact	A	Nobivac	No	58	182
215	1	Grey	F lact	A	Nobivac	No	43	209
362	1	Grey	F lact	A	Nobivac	No	32	227
A75	2	Black	Male	A	Alhydrogel	No	83	251
B30	2	Black	Male	A	Alhydrogel	No	232	370
B32	2	Black	Male	A	Alhydrogel	No	282	288
CIB	2	Black	Male	A	Alhydrogel	No	56	219
E4B	2	Black	F n p	A	Alhydrogel	No	114	268
95B	2	Black	F lact	A	Alhydrogel	No	59	292
361	2	Grey	Male	Juv	Alhydrogel	No	81	281
179	2	Grey	F np	Juv	Alhydrogel	No	168	1000+
813	2	Grey	Male	A	Alhydrogel	No	62	189
84D	2	Grey	F lact	A	Alhydrogel	No	90	190
A35	2	Grey	F lact	A	Alhydrogel	No	56	187
11A	2	Grey	F abort	A	Alhydrogel	No	88	261
655	3	Grey	Male	Juv	Nobivac	Yes	95	235
F19	3	Grey	Male	Juv	Nobivac	Yes	84	228
119	3	Grey	F np	Juv	Nobivac	Yes	103	445
51D	3	Grey	F lact	A	Nobivac	Yes	83	181
A24	3	Grey	F lact	A	Nobivac	Yes	72	199
B43	3	Grey	F abort	A	Nobivac	Yes	89	207

Table A12-2 (continued): Extrapolated KLH positive titre durations

Bat No	Group	Species	Sex	Age	Method	Boost Day 28	Extrapolated duration of positive titre >20 PA (days)	
							Phase 1	Phase 2
424	4	Black	Male	A	Alhydrogel	Yes	142	259
10B	4	Black	Male	A	Alhydrogel	Yes	99	176
83F	4	Black	Male	A	Alhydrogel	Yes	77	211
F43	4	Black	Male	A	Alhydrogel	Yes	75	166
565	4	Black	F lact	A	Alhydrogel	Yes	89	209
D58	4	Black	F lact	A	Alhydrogel	Yes	101	853
174	4	Grey	Male	Juv	Alhydrogel	Yes	159	659
402	4	Grey	Male	Juv	Alhydrogel	Yes	104	1000+
D03	4	Grey	Male	Juv	Alhydrogel	Yes	178	559
707	4	Grey	F lact	A	Alhydrogel	Yes	93	200
E64	4	Grey	F abort	A	Alhydrogel	Yes	83	184
B7A	4	Grey	F abort	A	Alhydrogel	Yes	103	238
775	Control	Black	Male	A	No Nobivac Alhydrogel	No		

The data for individuals shown in Table A12-1 and Table A12-2 are illustrated in Figure A12-1 to Figure A12-6. Actual titres in Table A12-1 are represented by solid lines. The calculated extrapolations to the day at which the titre would no longer be positive (KLH positive threshold = 20 PA, Table A12-2) are shown in broken lines. The graphs have been truncated to 400 days for clarity. Values for the extrapolated duration of positive KLH titres in excess of 400 days can be read from Table A12-2.

Figure A12-1 Response to KLH: Group 1 Grey-headed flying foxes (n=6)
Actual (solid lines) **and extrapolated** (broken lines) **KLH titres**
Vaccinations: PV-day 0 KLH *in* Nobivac
PV-day 85 High-dose KLH + Alhydrogel

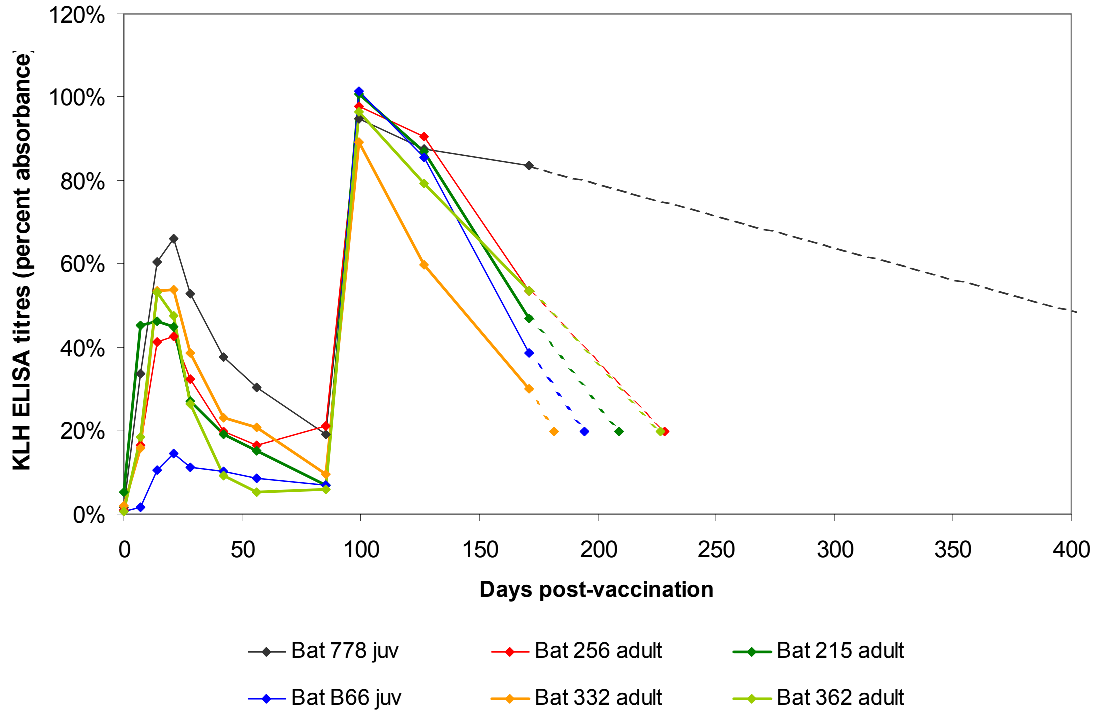


Figure A12-2 Response to KLH: Group 3 Grey-headed flying foxes (n=6)
Actual (solid lines) **and extrapolated** (broken lines) **KLH titres**
Vaccinations: PV-day 0 KLH *in* Nobivac
PV-day 28 KLH *in* Nobivac
PV-day 85 High-dose KLH + Alhydrogel

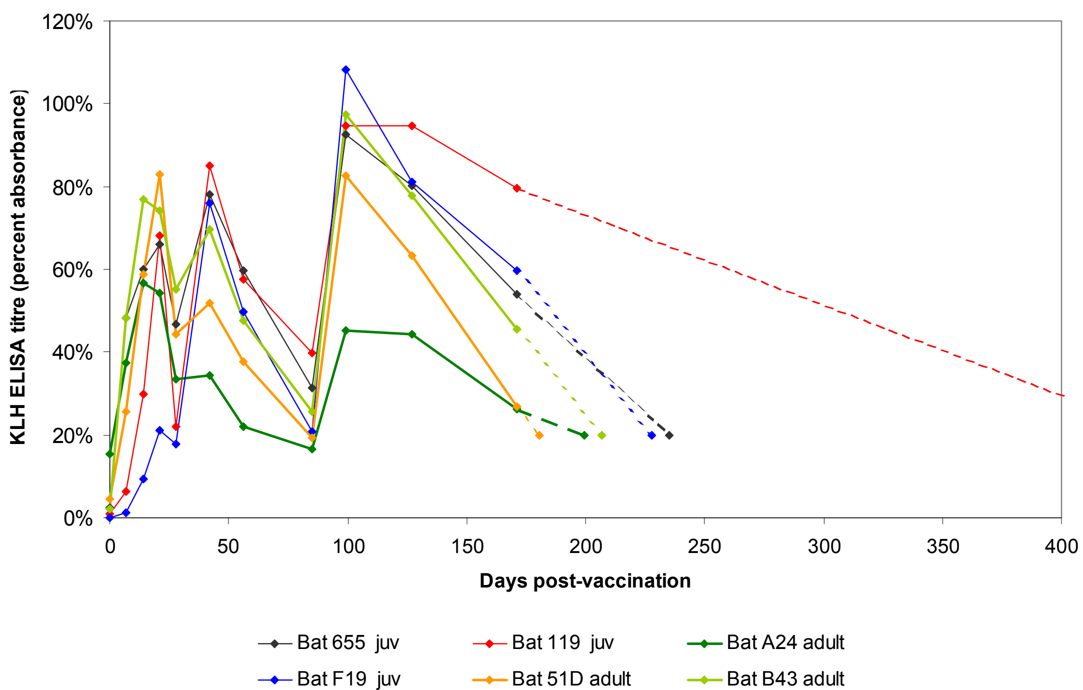


Figure A12-3 Response to KLH: Group 2 Grey-headed flying foxes (n=6)
Actual (solid lines) **and extrapolated** (broken lines) **KLH titres**
Vaccinations: PV-day 0 Nobivac and KLH + Alhydrogel
PV-day 85 High-dose KLH + Alhydrogel

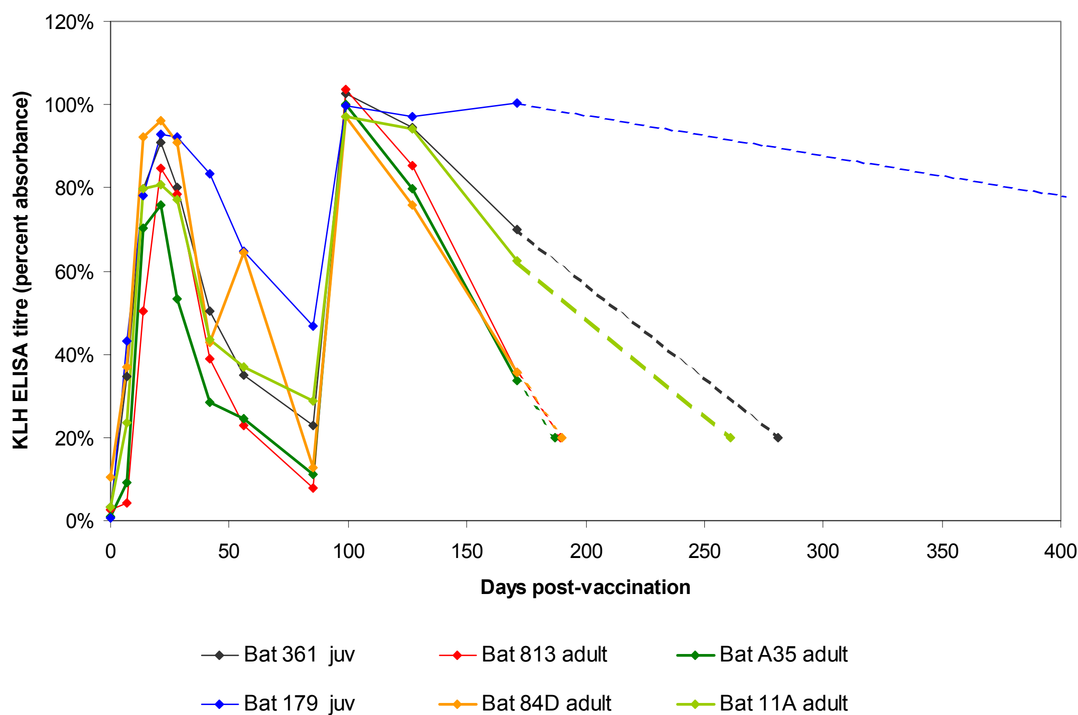


Figure A12-4 Response to KLH: Group 4 Grey-headed flying foxes (n=6)
Actual (solid lines) **and extrapolated** (broken lines) **KLH titres**
Vaccinations: PV-day 0 Nobivac and KLH + Alhydrogel
PV-day 28 Nobivac and KLH + Alhydrogel
PV-day 85 High-dose KLH + Alhydrogel

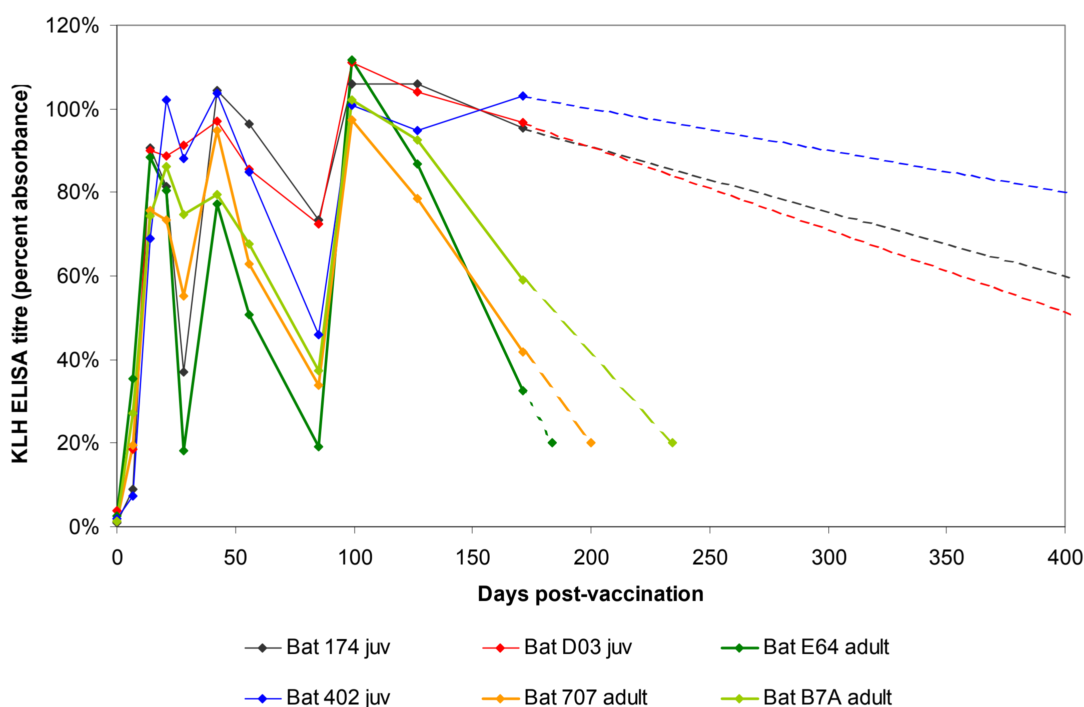


Figure A12-5 Response to KLH: Group 2 Black flying foxes (n=6)
Actual (solid lines) **and extrapolated** (broken lines) **KLH titres**
Vaccinations: PV-day 0 Nobivac and KLH + Alhydrogel
PV-day 85 High-dose KLH + Alhydrogel

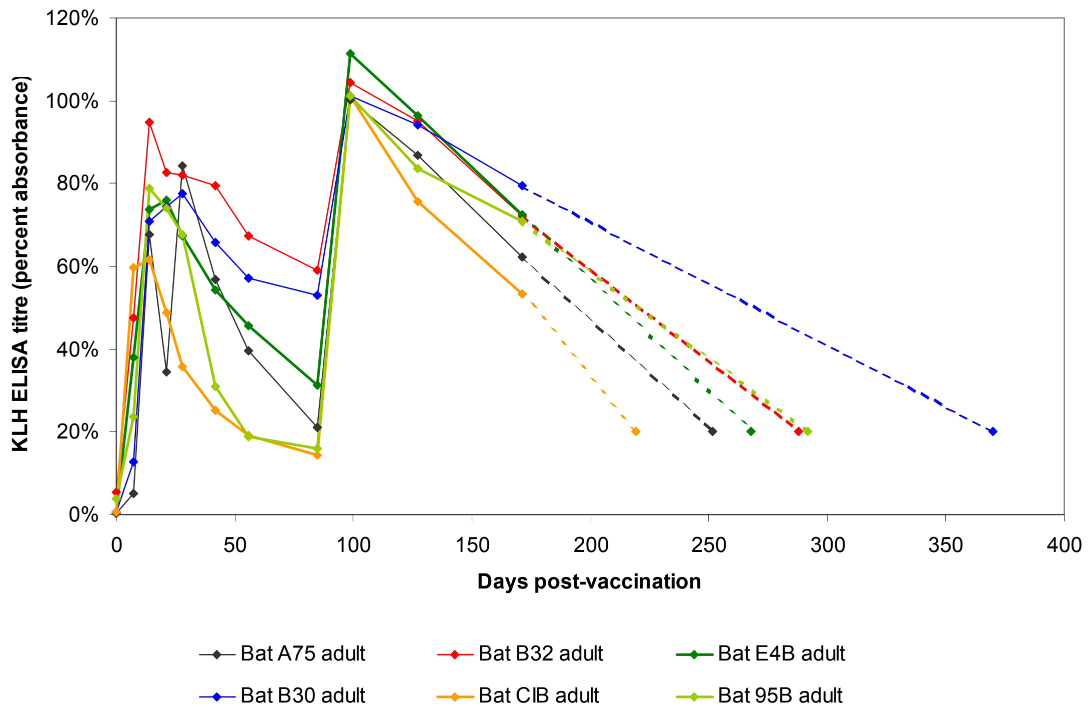
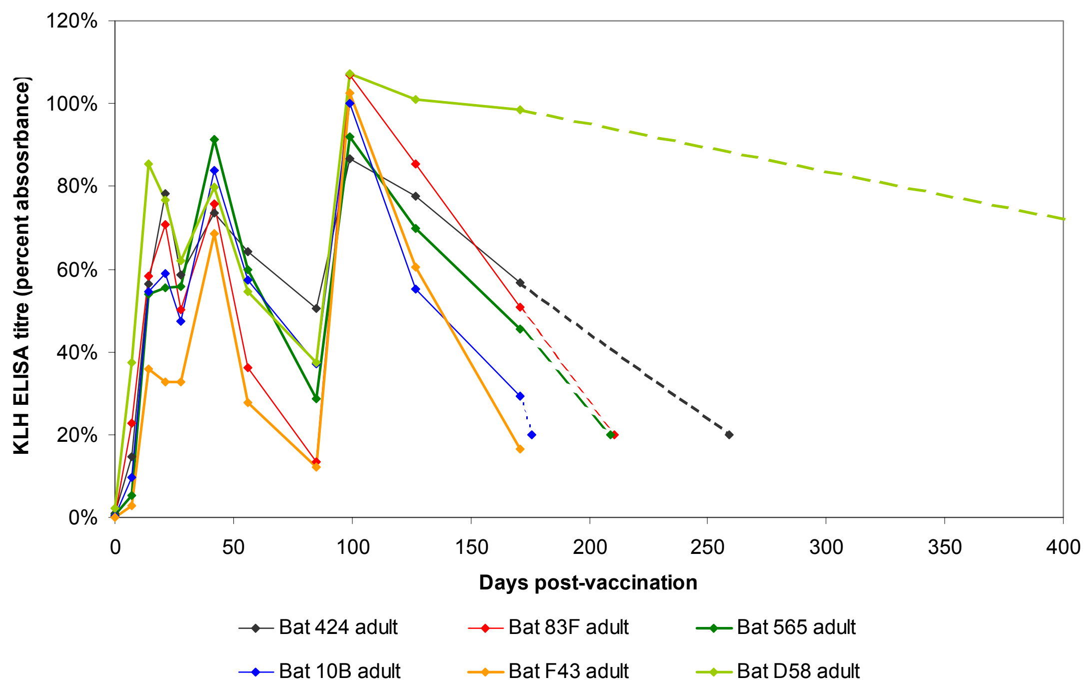


Figure A12-6 Response to KLH: Group 4 Black flying foxes (n=6)
Actual (solid lines) **and extrapolated** (broken lines) **KLH titres**
Vaccinations: PV-day 0 Nobivac and KLH + Alhydrogel
PV-day 28 Nobivac and KLH + Alhydrogel
PV-day 85 High-dose KLH + Alhydrogel



Appendix 13 Response in pups to vaccination of dam during (Phase 1) and after pregnancy (Phase 2)

The rabies-RFFIT and KLH ELISA titres for 11 pups born to flying foxes born between PV-day 48 and 83 of the vaccination trial, the corresponding titres of their dams and other relevant details are shown in Table A13-1.

All pups were born towards the end of Phase 1, i.e. a minimum of 28 days (Bat 51D) and a maximum of 82 days (Bat 95B) after the dams last Phase 1 vaccination on PV-day 28, and 2 to 37 days prior to the high-dose KLH booster on PV-day 85.

Table A13-1 Response in pups to vaccination of adult female flying foxes during (Phase 1) and after (Phase 2) pregnancy

--- not done
* In addition to the Phase 1 vaccination protocols described in this table, all adult bats (not pups) were vaccinated with a high-dose KLH + Alhydrogel booster on PV-day 85.
† Where the exact date of birth for individual pups was not known (see Appendix 14 Determination of pup age), the possible range of dates for the birth is shown, followed by a nominal birth-PV-day in parentheses.
Red font Nominally positive *pup* rabies-RFFIT titres (> 0.5 IU/mL) and positive *pup* KLH ELISA titres (>20 PA) are shown in red font.

Details of dam			Details of pup				Rabies-RFFIT titres (IU/mL)				KLH ELISA titres (PA)			
							PV-days				PV-days			
Bat No.	Species	Vaccination*	Pup No.	Date of birth†	Sex		85	99	127	171	85	99	127	172
332	Grey	Group 1 KLH *in* Nobivac PV-day 0	745	17 Sept PV-day 48	F	Pup	---	0	0	0	---	5.6	2.4	0.4
						Dam	0.6	---	0.2	0.7	9.5	89.3	59.8	30.2
215	Grey	Group 1 KLH *in* Nobivac PV-day 0	512	19 or 20 Sept PV-day 50 or 51 (PV-day 50)	Male	Pup	0.3	0	0	0	7.8	3.6	3.3	7.9
						Dam	1.2	---	1.8	2.2	7.1	100.9	86.9	47.1
362	Grey	Group 1 KLH *in* Nobivac PV-day 0	610	30 Sept - 22 Oct PV-days 61-83 (PV-day 62)	Male	Pup	---	---	0	---	---	---	1.3	---
						Dam	0.4	---	0.9	0.9	6.0	96.6	79.4	53.4
95B	Black	Group 2 Nobivac, KLH +Alhydrogel PV-day 0	760	17 or 20 Oct PV-days 78-82 (PV-day 78)	Male	Pup	---	---	**1.2**	**3.0**	---	---	6.4	0.9
						Dam	5.0	---	2.0	---	16.1	101.2	83.5	70.9
A35	Grey	Group 2 Nobivac, KLH +Alhydrogel PV-day 0	F2C	19 or 20 Sept PV-day 50 or 51 (PV-day 50)	F	Pup	0	---	0	0	18.4	---	3.3	0.7
						Dam	0.7	---	0.9	1.1	11.2	100.1	79.8	33.7

Table A13-1 (continued): Pup and dam rabies-RFFIT and KLH-ELISA titres

Bat No.	Species	Vaccination*	Pup No.	Date of birth†	Sex		Rabies-RFFIT titres (IU/mL) 85	99	127	172	KLH ELISA Titres (PA) 85	99	127	172
84D	Grey	Group 2 Nobivac, KLH +Alhydrogel PV-day 0	251	19 or 20 Sept PV-day 50 or 51 (PV-day 50)	Male	Pup	---	**0.5**	0	0	---	16.9	5.5	0.5
						Dam	**3.1**	**---**	**5.0**	**10.0**	**12.8**	**97.0**	**75.9**	**35.5**
51D	Grey	Group 3 KLH *in* Nobivac PV-day 0 + 28	056	25 Sept PV-day 56	Male	Pup	---	---	0	0	---	---	5.2	1.6
						Dam	**0.6**	**---**	**0.3**	**0.7**	**19.4**	**82.7**	**63.3**	**26.9**
A24	Grey	Group 3 KLH *in* Nobivac PV-day 0 + 28	423	30 Sept - 22 Oct PV-days 61-83 (PV-day 62)	F	Pup	---	**1.4**	---	0	---	11.9	---	10
						Dam	**0.5**	**---**	**0**	**0.2**	**16.6**	**45.1**	**44.3**	**26.2**
565	Black	Group 4 Nobivac, KLH +Alhydrogel PV-day 0 + 28	227	17 or 20 Oct PV-days 78-82 (PV-day 78)	F	Pup	---	---	**9.5**	**1.6**	---	---	**44.6**	11.9
						Dam	**2.9**	**---**	**1.0**	**1.0**	**28.6**	**92.0**	**69.9**	**45.4**
D58	Black	Group 4 Nobivac, KLH +Alhydrogel PV-day 0 + 28	926	17 or 20 Oct PV-days 78-82 (PV-day 78)	Male	Pup	---	---	**22.8**	**2.2**	---	---	**42.8**	15.8
						Dam	**31.5**	**---**	**28.1**	**9.0**	**37.5**	**107.1**	**101.1**	**98.5**
707	Grey	Group 4 Nobivac, KLH +Alhydrogel PV-day 0 + 28	411	30 Sept - 22 Oct PV-days 61-83 (PV-day 62)	F	Pup	---	---	0	0	---	---	7.8	1.2
						Dam	**1.8**	**---**	**0.5**	**0.9**	**33.9**	**97.2**	**78.4**	**41.8**

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Appendix 14 Determination of pup age

Disturbance of the colony was kept to a minimum in order to reduce the incidence of pup losses/ abortion. Consequently, new mothers were not identified until captured for sampling on PV-days 56 and 85. The arrival of pups was detected by daily observation of the colony for increased pup numbers. While the correct date of birth for every pup is not known, known or nominal birth dates were determined by cross-referencing the increasing pup numbers and species with captured mother/pup pairs on PV-days 56 and 85 as shown in Table A14-1.

Table A14-1 Pup detection and determination of known or nominal birth dates

Date	Species	Comments
17 Sept	1 Grey	First pup observed, possibly 1-3 days old. Dam identified as Bat No 332 on 18 Sept
19 Sept	2 Greys	Second and third Grey pups observed
20 Sept	1 Grey	Fourth Grey pup observed with wet umbilical cord
25 Sept	1 Grey	Fifth Grey with a new born pup with a long umbilical cord
25 Sept		**Capture and sampling PV-day 56**
		5 Grey mother/pup pairs captured and identified Bat 332 from 17 Sept Bat 51D with a newborn pup = 25 Sept Bats 215, A35, and 84D from 19 and 20 Sept
		As it is unclear which dam gave birth on each day, the nominal birth date for Bats 215, A35 and 84D is 19 Sept
30 Sept	1 Grey	Sixth Grey pup observed with wet umbilical cord
2 Oct	1 Grey	Seventh Grey pup observed with wet fur.
17 Oct	2 Blacks	First and second Black pups observed with wet umbilical cords
20 Oct	1 Black	Third Black pup observed, no wet cord seen, poss. 0-2 days old
22 Oct	1 Grey	Eight Grey pups counted, no wet cord seen*
24 Oct		**Capture and sampling PV-day 85**
		3 new Grey mother/pup pairs captured and identified as Bats A24, 707 and 362 that gave birth between 30 Sept, 2 Oct and ~ 22 Oct. As it is unclear which dam gave birth on each day, the nominal birth date for Bats A24, 707 and 362 is 1 Oct, being ± 1 day from the correct birth date for two pups, and up to 21 days early for the third
		3 Black mother/pup pairs captured and identified as Bats 565, 95B, and D58 from 17 or 20 Sept. As it is unclear which dam gave birth on each day, the nominal birth date for Bats 565, 95B, and D58 is 17 Oct, being correct for two pups and up to 3 days early for the third.

* By this time, the presence of 11 pups made counting difficult. As none of the pups on 22 Oct appeared to have a wet or long umbilical cord, indicating a recent birth, the 'eighth' pup may have been born any time between 2 Oct and 22 Oct, but probably much closer to 22 Oct.

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