Short Communication

Evidence of bat origin for Menangle virus, a zoonotic paramyxovirus first isolated from diseased pigs

Jennifer A. Barr,[1] Craig Smith,[2] Glenn A. Marsh,[1] Hume Field[2] and Lin-Fa Wang[1]

Correspondence
Lin-Fa Wang
Linfa.wang@csiro.au

[1]CSIRO Australian Animal Health Laboratory, 5 Portarlington Road, Geelong, Victoria 3219, Australia

[2]Department of Agriculture, Fisheries & Forestry, 80 Ann Street, Brisbane, Queensland 4000, Australia

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Menangle virus (MenPV) is a zoonotic paramyxovirus capable of causing disease in pigs and humans. It was first isolated in 1997 from stillborn piglets at a commercial piggery in New South Wales, Australia, where an outbreak of reproductive disease occurred. Neutralizing antibodies to MenPV were detected in various pteropid bat species in Australia and fruit bats were suspected to be the source of the virus responsible for the outbreak in pigs. However, previous attempts to isolate MenPV from various fruit bat species proved fruitless. Here, we report the isolation of MenPV from urine samples of the black flying fox, *Pteropus alecto*, using a combination of improved procedures and newly established bat cell lines. The nucleotide sequence of the bat isolate is 94 % identical to the pig isolate. This finding provides strong evidence supporting the hypothesis that the MenPV outbreak in pigs originated from viruses in bats roosting near the piggery.

Menangle virus (MenPV) is a zoonotic paramyxovirus, tentatively classified in the genus *Rubulavirus*, subfamily *Paramyxovirinae*. MenPV was first identified as the aetiological agent associated with a disease outbreak in pigs at a piggery in 1997 in New South Wales (NSW), Australia (Chant *et al.*, 1998; Philbey *et al.*, 1998). An outbreak of reproductive disease occurred with symptoms including increased fetal death, fetal abnormalities and stillborn piglets. Virus was isolated from the lung, brain and heart of stillborn piglets using BHK21 cells. No disease was observed in postnatal animals, but high-titre neutralizing antibodies were found in adult pigs at two piggeries associated with the disease outbreak (Philbey *et al.*, 1998). The virus was also shown to infect humans; two piggery workers with high-level exposure had serious influenza-type illness and rash during the outbreak, and were later found to have neutralizing antibodies to MenPV (Chant *et al.*, 1998).

Bats were investigated as a source of the MenPV outbreak, as grey-headed flying foxes (*Pteropus poliocephalus*) and little red flying foxes (*Pteropus scapulatus*) were found to be roosting near the piggery involved. The serological survey discovered MenPV-neutralizing antibodies in grey-headed flying foxes, black flying foxes (*Pteropus alecto*) and spectacled flying foxes (*Pteropus conspicillatus*), but not in little red flying foxes in both pre- and post-outbreak serum samples (Philbey *et al.*, 1998). Other species were investigated, including rodents, birds, cattle, sheep, cats and a dog, and all were found to be negative.

A number of recently emerged zoonotic pathogens of bat origin are members of the subfamily *Paramyxovirinae*, including the deadly Hendra virus (HeV) and Nipah virus (NiV), members of the genus *Henipavirus* (Anderson & Wang, 2011; Eaton *et al.*, 2006, 2007; Virtue *et al.*, 2009; Wang & Eaton, 2001). HeV emerged in horses and humans in Queensland, Australia, in 1994 and resulted in the death of 14 horses and one human, and pteropid bats were later found to be the natural reservoir (Halpin *et al.*, 2000; Murray *et al.*, 1995; Young *et al.*, 1996). MenPV emerged only 3 years after the original HeV outbreak, the second novel zoonotic paramyxovirus of bat origin to emerge in Australia at that time (Philbey *et al.*, 1998). MenPV was later found to be most closely related to Tioman virus (TioPV), a paramyxovirus isolated from the urine of pteropid bats on Tioman Island, Malaysia, in 2001, as part of the search for the reservoir host of Nipah virus (Chua *et al.*, 2001). A serological survey of humans on Tioman Island conducted soon after the discovery of TioPV revealed neutralizing antibodies in three out of the 169 serum samples tested (Yaiw *et al.*, 2007). It is unknown whether TioPV causes any disease in humans; however, it has been shown to be able to infect, replicate and be shed in

The GenBank/EMBL/DDBJ accession number for the bat Menangle virus isolate is JX112711.

pigs with no clinical signs other than increased temperature (Yaiw *et al.*, 2008).

A significant effort to isolate MenPV from various bat samples was attempted in 2008 (Philbey *et al.*, 2008). Tissues and faeces collected in NSW during 1997–2000 from grey-headed flying foxes, black flying foxes and little red flying foxes were inoculated onto BHK21 cells for virus isolation and also examined by transmission electron microscopy. Paramyxovirus-like particles were observed in faeces samples by transmission electron microscopy; however, no virus was isolated from any of the samples, despite extensive sampling and strong serological evidence of MenPV infection in bats (Philbey *et al.*, 2008).

Lack of virus isolation from bat samples has been a common problem with a number of new emerging zoonotic viruses. A key example is severe acute respiratory syndrome coronavirus (SARS-CoV). Strong serological and molecular evidence of SARS-like CoVs (SL-CoVs) in bats has been discovered. However, attempts to isolate SL-CoVs from various bat species internationally have so far been unsuccessful (Lau *et al.*, 2005; Li *et al.*, 2005). Our group has recently established and characterized primary *P. alecto* cell lines for the purpose of improving virus isolation from bat samples and for in-depth studies of virus–bat interactions (Crameri *et al.*, 2009).

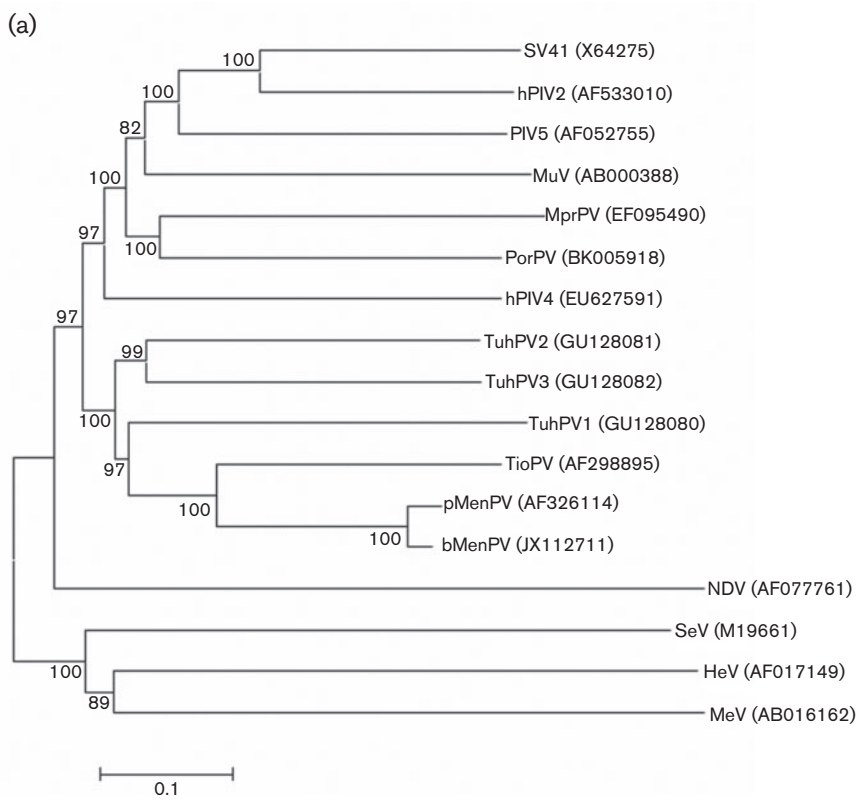
For this study, we focused on virus isolation from bat urine samples as part of the investigation into HeV infection dynamics in different bat populations in Queensland, Australia. Fieldwork was conducted under the Department of Employment, Economic Development and Innovation Animal Ethics Committee permit SA 2008/10/270 and the Queensland Department of Environment and Resource Management Scientific Purposes permit WISP05810609. As described previously, urine samples were collected in September 2009 from a flying fox colony in Cedar Grove, South East Queensland (Field *et al.*, 2011; Marsh *et al.*, 2012). Urine was collected off plastic sheets from underneath a flying fox colony and aliquots were transported to the Australian Animal Health Laboratory (AAHL) in Geelong, Australia, for virus isolation using the primary *P. alecto* cell lines. Although we could not be 100 % sure that bats in this colony are of the same species at different times, they were all black flying foxes (*P. alecto*) at the time of sampling for this study. Due to the high rate of HeV genomic RNA detection by PCR, virus isolation was conducted within the biosafety level 4 facility at AAHL. The samples were thawed at room temperature and centrifuged at 16 000 ***g*** for 1 min to pellet debris. Cleared urine (500 µl) was mixed with 3.5 ml cell culture medium (Dulbecco's modified Eagle's medium nutrient mixture F-12 ham supplemented with 200 U penicillin ml^{-1}, 20 µg streptomycin ml^{-1}, 0.5 µg amphotericin B ml^{-1}, 10 µg ciprofloxacin ml^{-1} and 10 % FCS). The diluted urine was then centrifuged at 1 200 ***g*** for 5 min and 1.6 ml of each supernatant was added to both Vero and *P. alecto* kidney (PaKi) cell (Crameri *et al.*, 2009) monolayers in 25 cm tissue culture flasks. The flasks were rocked for 30 min at 37 °C. An additional 10 ml cell culture medium was added to the flasks and incubated for 7 days at 37 °C. The flasks were observed daily for toxicity, contamination or viral cytopathic effect (CPE).

In addition to the isolation of HeV in this batch of urine samples, several other viruses were also isolated. One of them was MenPV. From this particular sample, syncytial CPE was observed in PaKi cell monolayers after 3 days incubation. The tissue culture supernatant was harvested 7 days post-infection and RNA was extracted from the supernatant for PCR analysis using respirovirus–morbillivirus–henipavirus- and paramyxovirus-specific primer sets, as described by Tong *et al.* (2008). PCR products of the expected size were obtained from the paramyxovirus-specific primer set. Sequence analysis indicated that the 582 bp L gene fragment was 96 % identical to the published MenPV sequence, GenBank accession number AF326114 (Bowden & Boyle, 2005; Bowden *et al.*, 2001). The bat virus isolate was designated bMenPV to differentiate it from the pig isolate pMenPV. After purification by three rounds of limiting dilution, a working stock of pMenPV was produced for subsequent analyses.

Next-generation sequencing using the 454 platform was employed to obtain whole genome sequence. Virions from tissue culture supernatant were collected by centrifugation at 30 000 ***g*** for 60 min and resuspended in 140 µl PBS and mixed with 560 µl freshly made virus lysis buffer for RNA extraction using a QIAamp Viral RNA mini kit (Qiagen). Synthesis of cDNA and random amplification was conducted using a modified published procedure (Palacios *et al.*, 2007). Sample preparation for Roche 454 sequencing (454 Life Sciences) was carried out according to their Titanium series manuals, rapid library preparation and emPCR Lib-L SV. Genome assembly and analysis was conducted with Clone Manager 9 (Sci-Ed Software) using the pMenPV genome as a template. The genome size of bMenPV (GenBank accession no. JX112711) was 15 516 nt, exactly the same as that of pMenPV (NC_007620). The overall nucleotide sequence identity of the two genomes was 94 %. The amino acid sequence identities of the deduced proteins between the two viruses were as follows: 99 % for M; 98 % for N, F and L; and 96 % for V, P and HN. In addition, all of the gene-start and -stop signals identified in the pMenPV genome (Bowden & Boyle, 2005; Bowden *et al.*, 2001) were absolutely conserved in the bMenPV genome. As shown in Fig. 1(a), the phylogenetic tree based on whole genome sequences of all known paramyxoviruses in the genus *Rubulavirus* clearly indicates that the two MenPV isolates are most closely related. The same patterns were observed for phylogenetic trees based on amino acid sequences of all major proteins.

Virus neutralization assays were conducted in duplicate to determine the cross-reactivity of bMenPV, pMenPV and the closely related TioPV. Sera used in this study were from pigs experimentally infected with pMenPV or TioPV (Yaiw *et al.*, 2008) and rabbits immunized with inactivated

(a)



(b)

Amino acid position		2	8	250	347	355	358	392	484	542
F (554 aa)	bMenPV	I	I	M	T	K	D	A	S	L
	pMenPV	M	M	I	M	N	E	S	N	S
HN (595 aa)		13	14	32	34	48	54	72	113	187
	bMenPV	D	G	Q	P	L	I	S	V	K
	pMenPV	E	E	H	S	S	V	G	I	Q
		273	282	293	300	364	367	368	440	474
	bMenPV	I	V	A	L	A	T	S	Y	L
	pMenPV	V	I	S	I	D	S	N	F	R
		480	493	504	517	564	566			
	bMenPV	W	A	P	S	T	P			
	pMenPV	R	V	S	A	I	S			

Fig. 1. (a) Phylogenetic tree based on the full genome sequences of selected paramyxoviruses (maximum likelihood, 1000 bootstrap). Virus names and their abbreviations are as follows: Hendra virus (HeV); Human parainfluenza virus 2 (hPIV2); Human parainfluenza virus 4b (hPIV4); Menangle virus (porcine isolate - pMenPV); Menangle virus (bat isolate - bMenPV); Measles virus (MeV); Mapeura virus (MprPV); Mumps virus (MuV); Newcastle disease virus (NDV); Parainfluenza virus 5 (PIV5); Porcine rubulavirus (PorPV); Sendai virus (SeV); Simian virus 41 (SV41); Tioman virus (TioPV); Tuhoko virus 1 (TuhPV1); Tuhoko virus 2 (TuhPV2); Tuhoko virus 3 (TuhPV3). Bar, 0.1 amino acid substitutions per site. (b) Amino acid residue differences between the F and HN proteins of bMenPV and pMenPV, respectively. Non-conserved differences are highlighted by shading.

pMenPV or TioPV (L.-F. Wang, unpublished). Serum-neutralizing titres against each virus are shown in Table 1. The sera raised to pMenPV were able to neutralize bMenPV, albeit with slightly lower titres than the homologous virus, but unable to neutralize TioPV. Conversely, the sera raised to TioPV did not neutralize bMenPV or pMenPV. The two to fourfold difference in neutralizing antibody titres between the two viruses can be explained by the amino acid sequence difference observed (Fig. 1b). There are a total of nine and 24 amino acid residue differences for the F and HN proteins, respectively. Although F may play a role in virus neutralization, the HN protein is the main target of neutralization

Table 1. Neutralizing antibody titres to bMenPV, pMenPV and TioV

Serum	bMenPV	pMenPV	TioPV
MenPV pig 1	1 : 80	1 : 320	Neg
MenPV pig 5	1 : 80	1 : 320	Neg
MenPV rabbit 2	1 : 80	1 : 160	Neg
MenPV rabbit 4	1 : 160	1 : 320	Neg
TioPV rabbit (Myer)	Neg	Neg	1 : 320
TioPV rabbit (Mark)	Neg	Neg	1 : 320
TioPV pig P298	Neg	Neg	1 : 160
TioPV pig P299	Neg	Neg	1 : 320

for rubulaviruses. In this respect, it is worth noting that not only do the HN proteins of the two viruses have more amino acid residue differences than the F proteins, but also that these differences are more widely spread along the molecule and there are also more non-conserved changes.

To investigate whether there was any gross difference in the infection of cells derived from known susceptible host species between the two MenPV isolates, we compared the infectivity of the viruses in five different cell lines: Vero, PaKi, HeLa, PK15 (pig kidney) and LoVo (human colorectal adenocarcinoma). The other four cell lines were chosen based on the following: Vero, the most commonly used cells for the propagation of other paramyxoviruses; PK15, from pigs, which is the only known susceptible host other than bats and humans; HeLa, a commonly used human cell line; LoVo, a human cell line deficient in furin protease production, which is required for the processing of the F protein for some, but not all, paramyxoviruses. Cell monolayers were prepared in eight-well chamber slides by seeding at a concentration of 30 000 cells per well in 300 µl cell medium. After incubation overnight at 37 °C, the cell monolayers were infected with an m.o.i. 0.01 of either bMenPV or pMenPV and fixed with 100 % ice-cold methanol at 24 or 48 h post-infection. The chamber slides were stained with rabbit sera against pMenV, following the previously published methods (Chua *et al.*, 2011; Tu *et al.*, 2004). As shown in Fig. 2, the two virus isolates had almost identical staining patterns in five different cells. Infection in Vero and PaKi cells was best, followed by that in the human cell lines HeLa and LoVo. It was surprising to find that the viruses grew very poorly in PK15 cells, considering that MenPV was able to infect and cause disease in pigs. It is not clear, at present, why this was the case. However, it is commonly known that virus susceptibility of a host species and its derived cell lines may not match all the time. For example, our group discovered previously that HeLa USU cells were not susceptible to either HeV or NiV infection, due to the lack of expression of the major entry receptor molecule (Bonaparte *et al.*, 2005). It is also interesting to note that although the virus was able to spread to neighbouring cells at the 48 h point in HeLa and LoVo cells, there was no CPE detected at this time point. In fact, no CPE was observed even at 10 days post-infection.

In conclusion, virological, molecular and serological data presented in this paper conclusively demonstrate that bMenPV and pMenPV are two strains of the same virus

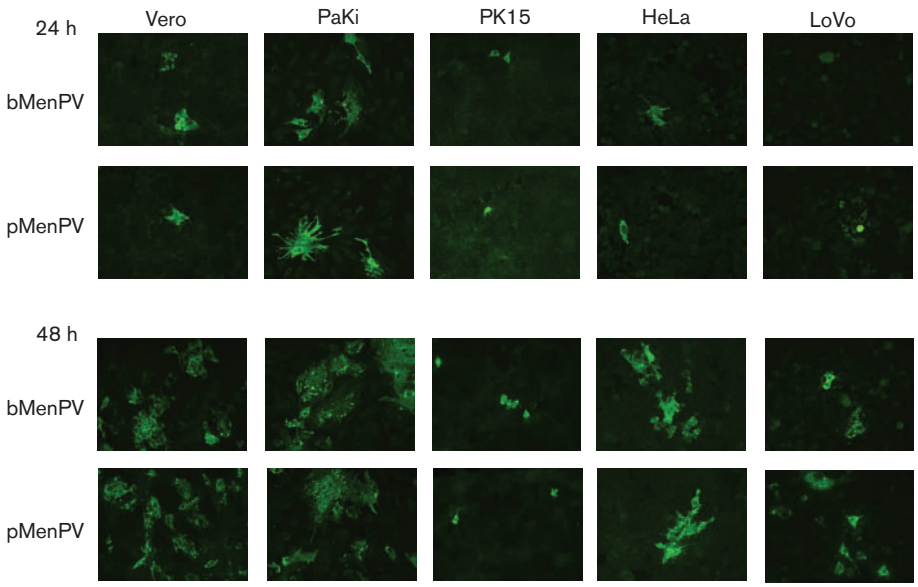


Fig. 2. Immunofluorescent staining of five cell lines infected with bMenPV and pMenPV. Infected cells in chamber slides were fixed with 100 % ice-cold methanol at 24 or 48 h post-infection, followed by staining with rabbit anti-pMenPV antibody and anti-rabbit Alexa Fluor 488 conjugate (Invitrogen). Mock infection was conducted for each of the five cell lines and all of them gave completely negative staining (not shown).

species. This study provides strong evidence supporting the original hypothesis that the outbreak of MenPV infection in pigs and humans in 1997 was probably a result of a spillover from bats roosting near the piggery. It is worth noting that the predominant species of bats near the Menangle piggery are the grey-headed flying fox (*P. poliocephalus*) and the little red flying fox (*P. scapulatus*). Future studies are required to determine whether the genetic difference observed between pMenPV and bMenPV is a result of host species difference or geographical separation of hosts.

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References

Anderson, D. E. & Wang, L.-F. (2011). New and emerging paramyxoviruses. In *The Biology of Paramyxoviruses*, pp. 435–458. Edited by S. K. Samal. Norfolk: Caister Academic Press.

Bonaparte, M. I., Dimitrov, A. S., Bossart, K. N., Crameri, G., Mungall, B. A., Bishop, K. A., Choudhry, V., Dimitrov, D. S., Wang, L. F. & other authors (2005). Ephrin-B2 ligand is a functional receptor for Hendra virus and Nipah virus. *Proc Natl Acad Sci U S A* **102**, 10652–10657.

Bowden, T. R. & Boyle, D. B. (2005). Completion of the full-length genome sequence of Menangle virus: characterisation of the polymerase gene and genomic 5′ trailer region. *Arch Virol* **150**, 2125–2137.

Bowden, T. R., Westenberg, M., Wang, L. F., Eaton, B. T. & Boyle, D. B. (2001). Molecular characterization of Menangle virus, a novel paramyxovirus which infects pigs, fruit bats, and humans. *Virology* **283**, 358–373.

Chant, K., Chan, R., Smith, M., Dwyer, D. E., Kirkland, P. & The NSW Expert Group (1998). Probable human infection with a newly described virus in the family *Paramyxoviridae*. *Emerg Infect Dis* **4**, 273–275.

Chua, K. B., Wang, L. F., Lam, S. K., Crameri, G., Yu, M., Wise, T., Boyle, D., Hyatt, A. D. & Eaton, B. T. (2001). Tioman virus, a novel paramyxovirus isolated from fruit bats in Malaysia. *Virology* **283**, 215–229.

Chua, K. B., Voon, K., Yu, M., Ali, W. N., Kasri, A. R. & Wang, L. F. (2011). Saffold virus infection in children, Malaysia, 2009. *Emerg Infect Dis* **17**, 1562–1564.

Crameri, G., Todd, S., Grimley, S., McEachern, J. A., Marsh, G. A., Smith, C., Tachedjian, M., De Jong, C., Virtue, E. R. & other authors (2009). Establishment, immortalisation and characterisation of pteropid bat cell lines. *PLoS ONE* **4**, e8266.

Eaton, B. T., Broder, C. C., Middleton, D. & Wang, L. F. (2006). Hendra and Nipah viruses: different and dangerous. *Nat Rev Microbiol* **4**, 23–35.

Eaton, B. T., Mackenzie, J. S. & Wang, L.-F. (2007). Henipaviruses. In *Fields Virology*, pp. 1587–1600. Edited by D. M. Knipe, D. E. Griffin, R. A. Lamb, S. E. Straus, P. M. Howley, M. A. Martin & B. Roizman. Philadelphia: Lippincott Williams & Wilkins.

Field, H., de Jong, C., Melville, D., Smith, C., Smith, I., Broos, A., Kung, Y. H., McLaughlin, A. & Zeddeman, A. (2011). Hendra virus infection dynamics in Australian fruit bats. *PLoS ONE* **6**, e28678.

Halpin, K., Young, P. L., Field, H. E. & Mackenzie, J. S. (2000). Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *J Gen Virol* **81**, 1927–1932.

Lau, S. K., Woo, P. C., Li, K. S., Huang, Y., Tsoi, H. W., Wong, B. H., Wong, S. S., Leung, S. Y., Chan, K. H. & Yuen, K. Y. (2005). Severe acute respiratory syndrome coronavirus-like virus in Chinese horseshoe bats. *Proc Natl Acad Sci U S A* **102**, 14040–14045.

Li, W., Shi, Z., Yu, M., Ren, W., Smith, C., Epstein, J. H., Wang, H., Crameri, G., Hu, Z. & other authors (2005). Bats are natural reservoirs of SARS-like coronaviruses. *Science* **310**, 676–679.

Marsh, G. A., de Jong, C., Barr, J. A., Tachedjian, M., Smith, C., Middleton, D., Yu, M., Todd, S., Foord, A. J. & other authors (2012). Cedar virus: a novel henipavirus isolated from Australian bats. *PLoS Pathog* **8**: e1002836. doi:10.1371/journal.ppat.1002836

Murray, K., Selleck, P., Hooper, P., Hyatt, A., Gould, A., Gleeson, L., Westbury, H., Hiley, L., Selvey, L. & other authors (1995). A morbillivirus that caused fatal disease in horses and humans. *Science* **268**, 94–97.

Palacios, G., Quan, P. L., Jabado, O. J., Conlan, S., Hirschberg, D. L., Liu, Y., Zhai, J., Renwick, N., Hui, J. & other authors (2007). Panmicrobial oligonucleotide array for diagnosis of infectious diseases. *Emerg Infect Dis* **13**, 73–81.

Philbey, A. W., Kirkland, P. D., Ross, A. D., Davis, R. J., Gleeson, A. B., Love, R. J., Daniels, P. W., Gould, A. R. & Hyatt, A. D. (1998). An apparently new virus (family *Paramyxoviridae*) infectious for pigs, humans, and fruit bats. *Emerg Infect Dis* **4**, 269–271.

Philbey, A. W., Kirkland, P. D., Ross, A. D., Field, H. E., Srivastava, M., Davis, R. J. & Love, R. J. (2008). Infection with Menangle virus in flying foxes (*Pteropus* spp.) in Australia. *Aust Vet J* **86**, 449–454.

Tong, S., Chern, S. W., Li, Y., Pallansch, M. A. & Anderson, L. J. (2008). Sensitive and broadly reactive reverse transcription-PCR assays to detect novel paramyxoviruses. *J Clin Microbiol* **46**, 2652–2658.

Tu, C., Crameri, G., Kong, X., Chen, J., Sun, Y., Yu, M., Xiang, H., Xia, X., Liu, S. & other authors (2004). Antibodies to SARS coronavirus in civets. *Emerg Infect Dis* **10**, 2244–2248.

Virtue, E. R., Marsh, G. A. & Wang, L. F. (2009). Paramyxoviruses infecting humans: the old, the new and the unknown. *Future Microbiol* **4**, 537–554.

Wang, L.-F. & Eaton, B. T. (2001). Emerging paramyxoviruses. *Infect. Dis. Rev.* **3**, 52–69.

Yaiw, K. C., Crameri, G., Wang, L., Chong, H. T., Chua, K. B., Tan, C. T., Goh, K. J., Shamala, D. & Wong, K. T. (2007). Serological evidence of possible human infection with Tioman virus, a newly described paramyxovirus of bat origin. *J Infect Dis* **196**, 884–886.

Yaiw, K. C., Bingham, J., Crameri, G., Mungall, B., Hyatt, A., Yu, M., Eaton, B., Shamala, D., Wang, L. F. & Thong Wong, K. (2008). Tioman virus, a paramyxovirus of bat origin, causes mild disease in pigs and has a predilection for lymphoid tissues. *J Virol* **82**, 565–568.

Young, P. L., Halpin, K., Selleck, P. W., Field, H., Gravel, J. L., Kelly, M. A. & Mackenzie, J. S. (1996). Serologic evidence for the presence in *Pteropus* bats of a paramyxovirus related to equine morbillivirus. *Emerg Infect Dis* **2**, 239–240.