African Journal of Biotechnology Vol. 4 (4), pp. 292-302, April 2005
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2005 Academic Journals

*Review*

# Molecular approaches to detect and study the organisms causing bovine tick borne diseases: babesiosis and anaplasmosis

**Ala Lew* and Wayne Jorgensen**

Department of Primary Industries & Fisheries, c/o Locked Mail Bag No. 4 Moorooka 4105 QLD, Australia.

Accepted 24 February, 2004

**This review will summarise the molecular approaches used to detect and analyse the genomes of *Babesia bovis*, *B. bigemina* and *Anaplasma marginale* which cause bovine babesiosis and anaplasmosis. These tick borne diseases are widely distributed in Africa, Asia, Australia, and Central and South America and for example, have been estimated to have an economic impact of US$15.9, $6.9, $6.2, $2.8, $22 million per annum in Australia, Kenya, Zimbabwe, Tanzania, and South Africa, respectively (McLeod and Kristjanson, 1999). The development and uptake of molecular tools to study these pathogens are reviewed to highlight potential directions for future research.**

**Key words:** Review, Babesia, Anaplasma, molecular detection, phylogeny, gene analysis.

## INTRODUCTION

### Babesiosis

The two most economically important species of *Babesia* to the cattle industries of many tropical and sub-tropical countries of the world are *Babesia bigemina* Smith & Kilbourne, 1893 and *B. bovis* Babes, 1888 (Callow, 1984a; Callow, 1984b; Hove et al., 1998). Disease is characterised by fever, weakness, ataxia, haemoglobinuria, anaemia and presence of intraerythrocytic parasites (Wright et al., 1989). Both species belong to the phylum Apicomplexa with *B. bovis* causing more severe disease than *B. bigemina* (de Vos et al., 2000). A major tick vector in Australia and Africa for both species is *Boophilus microplus*, while *B. bigemina* is also transmitted by *B. decloratus* and *Rhipicephalus* spp. in Africa (Friedhoff, 1988).

### Anaplasmosis

Anaplasmosis is an arthropod-borne disease of cattle and other ruminants caused by the intraerythrocytic rickettsiae of the genus *Anaplasma*, Family Anaplasmataceae (Dumler et al., 2001). Based on location within the erythrocyte there are 2 species of *Anaplasma* that infect cattle, *A. marginale* and *A. centrale*. *A. centrale* has only naturally been isolated in South Africa and, due to it's milder pathogenesis has been used as a vaccine to protect against clinical anaplasmosis caused by *A. marginale* in Argentina, Australia, Israel, Malawi, South Africa, Uruguay and Zimbabwe (reviewed by Bock and De Vos, 2001). Clinically, acute anaplasmosis caused by *A. marginale* manifests as a progressive anaemia and jaundice associated with the presence of intraerythrocytic inclusion bodies. Anaplasmosis is more widespread than babesiosis due to a larger number of tick vectors for *A. marginale* in tropical, sub-tropical and temperate regions of the world (Potgieter and Stoltsz, 1994). Only one tick vector for *A. marginale* exists in Australia, *B. microplus* (Callow, 1984a).

## DIAGNOSIS

### Antibody/antigen detection

A characteristic feature of babesiosis and anaplasmosis, is that animals which recover from a primary acute attack become carriers of the respective haemoparasite. Micro-

*Corresponding author. Tel: +617 3362 9502. Fax: +617 3362 9429. E-mail: Ala.Lew@dpi.qld.gov.au.

scopic examination of Giemsa stained blood smears are traditionally used to confirm acute disease states and for the most part, serological methods have been used to detect antibodies in animals which have recovered from infections. Advances in serological diagnosis of babesiosis and anaplasmosis have been reviewed previously (de Vos et al., 2000; McElwain, 2000).

**Molecular techniques**

Polymerase chain reaction (PCR) has been increasingly applied to detect these pathogens in both blood and tick vectors instead of microscopy (Table 1). Although a number of publications report the use of PCR, most publications are based on 6 original methods for all pathogens (Suarez et al., 1991; Fahrimal et al., 1992; Figueroa et al., 1992a; Figueroa et al., 1993; Azambuja et al., 1994; Torioni de Echaide et al., 1998). Many reports summarised in Table 1 compare PCR detection with serology to demonstrate assay specificity. However, the most suitable detection method depends upon whether antigen or antibody detection is relevant for the particular investigation, detection of parasites, or current infection, prevalence studies or evidence of exposure to parasites.

Although PCR is more sensitive than light microscopy (Bose et al., 1995), many of the methods described in Table 1 utilise complicated post-PCR detection methods to further enhance the sensitivity and confirm the specificity of the PCR technique such as PCR-ELISA and PCR-probe hybridisation. A quantifiable PCR technique referred to as real time PCR (also known as 5' *Taq* nuclease assays, fluorogenic probe assays, or TaqMan® assays), are increasingly applied for the detection and identification of animal pathogens and do not require post PCR electrophoresis or processing steps (Belak and Thoren, 2001). Real time assays exploit the 5' nuclease activity of *Taq* DNA polymerase cleaving a dual labelled fluorescent probe which has annealed to a specific sequence between two primers (Livak et al., 1995). To date, the applications of real time PCR for the detection of tick borne disease pathogens have been described for *Theileria sergenti* and *A. phagocytophilum* (Pusterla et al., 1999; Belak and Thoren, 2001; Jeong et al., 2003; Courtney et al., 2004). Real time PCR has engendered wider acceptance of PCR due to its improved rapidity, sensitivity, reproducibility and the reduced risk of carry-over contamination (Mackay, 2004). Currently, real time PCR assays for the *Anaplasma* and *Babesia* species discussed here have only been used for the study of gene expression and not for diagnostic applications (Lohr et al., 2002; Suarez et al., 2003). It may not be feasible for certain laboratories to use PCR-ELISA, PCR-probe hybridisation or real time PCR assays as the application of each of these methods requires specific and expensive reagents and equipment. Microscopy remains the most economic and sustainable method of parasite detection for all laboratories.

Other molecular approaches such as DNA probes (based on the MSP1b gene) have been developed for *in situ* hybridization detection of *A. marginale* in both blood smears and ticks (Ge et al., 1995; Ge et al., 1997; Kocan et al., 1998).

**Methods to detect multiple haemoparasite infections**

Multiplex PCR was initially developed to detect *B. bovis*, *B. bigemina* and *A. marginale* in a single sample (Figueroa et al., 1993). However, the efficiency of numerous primer sets in a single PCR reaction limits the efficiency and consequent sensitivity of each specific assay. The following approaches have been developed to rectify this deficiency by targeting conserved sequences for PCR, followed by either restriction enzyme digest analysis or specific probe hybridisation to identify species. A PCR-RFLP method based on the β-tubulin gene is able to differentiate 7 *Babesia* species and 2 *Theileria* species of cattle and horses (Caccio et al., 2000). Reverse line blot (RLB) hybridisation relies on the initial amplification of 18S and/or 16S rDNA conserved regions followed by hybridisation of the PCR product to a membrane with covalently linked species-specific oligonucleotides. Gubbels et al. (1999) demonstrated that the RLB hybridisation method can be utilised in the epidemiological monitoring of tick borne disease. Subsequent studies demonstrate that RLB is best applied for the detection of a broad range of pathogens in a sample; for example 6 *Theileria* spp. and 3 *Babesia* spp. (Sparagano et al., 2000); 5 *Anaplasma* spp. and 3 *Ehrlichia* spp. (Bekker et al., 2002); 21 species of detect *Ehrlichia*, *Anaplasma*, *Babesia* and *Theileria* (Georges et al., 2001). These approaches have detected these pathogens in tick and animal populations such as the presence of *B. bovis* in Mediterranean countries (Sparagano et al., 2000; Almeria et al., 2001b). RLB hybridisation appears to exhibit sensitivities equitable to that obtained with individual PCRs for some species such as *Theileria parva* (Oura et al., 2004). The potential also exists to expand the application of multiplex real time assays for the detection of multiple tick-borne disease pathogens in one sample (Courtney et al., 2004). Additionally, oligonucleotide micro-array technologies traditionally used for gene expression studies are now also being adapted for diagnostic applications (Lin et al., 2004) and may be used in the future to study and detect tick-borne diseases.

**Differentiation of strains**

It is not possible to identify strains of these species through microscopic examination. However, DNA restriction digestion, restriction fragment length polymorphism (RFLP) and random amplified polymorphic

**Table 1.** Summary of PCR methods used for the detection of *A. marginale*, *B. bovis* and *B. bigemina* and comparison to traditional methods.

| Species | Reference | Method reference and/or gene target | [1]Comparative sensitivity/comments |
|---|---|---|---|
| *Anaplasma marginale* | Stich et al., 1993 | msp1β gene | PCR used to detect *A. marginale* directly in haemolymph in ticks PCR was not compared to other methods. |
| | Figueroa et al., 1993 | Aboytes-Torres 1992 (PhD thesis)/unknown gene | Multiplex PCR method detecting 0.0001% *A. marginale* PPE. |
| | Gale et al., 1996 | PCR – ELISA/ immunodominant antigen (unpublished sequence) | PCR more sensitive than ELISA (Duzgun et al., 1988) detecting 0.00015% PPE. Card test is more sensitive than PCR but PCR is more specific. |
| | Figueroa et al., 1996 | Figueroa et al., 1993/unknown gene | PCR detection comparable with light microscopy. |
| | Cossio-Bayugar et al., 1997 | Figueroa et al., 1993/unkonwn gene | PCR demonstrated higher prevalence than CF test. |
| | Ge et al., 1995 Ge et al., 1997 Kocan et al., 1998 | DNA probe - msp1β | PCR used to prepare probe, probe detects 0.00001% PPE. DNA probe assay more sensitive than CF test and microscopy, probe assay not compared with PCR (used for *in situ* hybridisation). |
| | Torioni de Echaide et al., 1998 | Major surface protein (MSP)5 – nested PCR and specific probe hybridisation | PCR-probe hybridisation method more sensitive than previous PCR assays (Figueroa et al., 1993; Gale et al., 1996). PCR-probe less sensitive than recombinant MSP5 cELISA. (ELISA recommended for epidemiological studies) |
| | Herrero et al., 1998 | Aboytes-Torres 1992 | PCR less sensitive than rMSP5 cELISA (Torioni de Echaide et al., 1998). |
| | Hofmann-Lehmann et al., 2004 | Torioni de Echaide et al., 1998/MSP5 PCR | PCR showed good correlation with microscopy. |
| *Babesia bovis* | Fahrimal et al., 1992 | apocytochrome b gene PCR and probe | PCR/probe assay more sensitive than thick smear microscopy. |
| | Figueroa et al., 1993 | Suarez et al., 1991/60kDa merozoite antigen | Multiplex PCR method detecting 0.00001% *B. bovis* PPE. |
| | Figueroa et al., 1996 | Figueroa et al., 1993 | PCR more sensitive than light microscopy. |
| | Calder et al., 1996 | rRNA gene PCR and probe hybridisation | PCR more sensitive than CF test. |
| | Salem et al., 1999 | Fahrimal et al., 1992 and rDNA PCR tests | Apocytochrome b (extrachromosomal DNA) PCR test more sensitive than rDNA PCR and CF tests. |
| | Almeria et al., 2000a | Fahrimal et al., 1992 | *Babesia* spp. not differentiated using light microscopy. PCR more sensitive than microscopy. |
| | Gayo et al., 2003 | Azambuja et al., 1994/60kDa protein gene | PCR more sensitive than microscopy. |
| | Smeenk et al., 2000 | Fahrimal et al., 1992 | PCR not compared with other method. Nested PCR detecting 0.001% PPE. |
| | Thammasirirak et al. 2003 | Modified Fahrimal et al., (1992) for PCR ELISA | PCR modified into PCR-ELISA format, 1000x more sensitive than thin smears and more sensitive than PCR gel detection. PCR-ELISA low level cross-reaction with *A. marginale.* Antibody screening more sensitive than PCR-ELISA. |
| *Babesia bigemina* | Figueroa et al., 1992b | PCR and probe hybridisation assay using unknown gene target (GenBank S45366) | PCR with probe hybridisation detected 0.0000001% PPE. Test more sensitive than microscopy or CF test. PCR used in combination with probe detection improves sensitivity of PCR 1000x. |
| | Figueroa et al., 1993 | Figueroa et al., 1992b | Multiplex PCR method detected 0.00001% *B. bigemina* PPE. |
| | Figueroa et al., 1996 | Figueroa et al., 1993 | Light microscopy more sensitive than multiplex PCR. |
| | Salem et al., 1999 | apocytochrome b gene and rDNA genes | apocytochrome b (extrachromosomal DNA) PCR test more sensitive than rDNA PCR and CF test. |
| | Almeria et al., 2001a | Figueroa et al., 1992b | *Babesia* spp. not differentiated using light microscopy. PCR more sensitive than microscopy. |
| | Gayo et al., 2003 | Figueroa et al., 1992b | PCR more sensitive than light microscopy. |
| | Smeenk et al., 2000 | Figueroa et al., 1992b | PCR detection not compared with other method 0.000001% PPE. |
| | Hove et al., 1998 | Figueroa et al., 1992b | PCR more sensitive than thin smear microscopy and IFAT. |

[1]Legend of terms: PPE= percent parasitised erythrocytes; IFAT=indirect fluorescent antibody test; PCR=polymerase chain reaction; ELISA=enzyme linked immunosorbent assay; CF=complement fixation.

DNA (RAPD) analysis have been used to demonstrate intra-species variation in *B. bovis*, *B. bigemina* or *A. marginale* (Krueger and Buening, 1988; Eriks et al., 1989; Dalrymple, 1990; Visser et al., 1991; Dalrymple et al., 1992; Rivas et al., 1993; Carson et al., 1994; Lew et al., 1997b; Ngeranwa et al., 1998). Repetitive extragenic palindromic (REP) and enterobacterial repetitive intergenic consensus (ERIC) analysis have been used to demonstrate genetic diversity among *A. marginale* isolates (Ferreira et al., 2001). All of these approaches require pure preparations of isolates or strains which is not always feasible when working with field isolates of these haemoparasites. Dalrymple et al. (1992) suggest that an ideal gene to differentiate strains of parasites is one which is species-specific, single copy, and one which varies in size between strains.

Strains of *A. marginale* have been differentiated using the MSP1a gene (Palmer et al., 2001; Lew et al., 2002a). However, de la Fuente et al. (2003b) have demonstrated that this gene is under positive selection pressure and may not be useful as a marker to characterize geographic isolates. Our studies have indicated that MSP1a is conserved among the Australian populations of *A. marginale* which is possibly due to the limited introduction of this species into Australia and/or presence of only one tick vector (Lew et al., 2002a). Major surface protein 4 (msp4) gene sequences have been used to demonstrate trends in the geographic distribution of *A. marginale* strains (de la Fuente et al., 2001, 2002, 2004).

Two PCR assays based on two immuno-dominant protein genes Bv80 and BvVA1 were developed to differentiate *B. bovis* isolates and strains (Lew et al., 1997b). These methods support the Australian *B. bovis* live vaccine program by both identifying heterogeneous live vaccine strains, and by differentiating vaccine and field strains during babesiosis outbreaks (Lew et al., 1997a; Jorgensen et al., 1998; Bock et al., 2000) A similar approach to differentiate *B. bigemina* strains has been developed (G. Anderson, personal communication).

## Molecular Phylogeny

Important for the accurate diagnoses of species is the improved understanding of species phylogeny. Through the advent of PCR and automated sequencing methods, the analysis of gene sequences has led to an explosion of molecular phylogenetic comparisons of species. This has been particularly applicable for studying the fastidious bacterial species of the order Ricketsiales, including the family Anaplasmataceae, by the analysis of 16S rDNA, *groESL* (heat shock protein 60/hsp60 gene) and surface protein genes. Results of these molecular analyses have led to the reassignment of genera *Eperythrozoon* and *Haemobartonella* from the Anaplasmataceae to the family Mycoplasmataceae (Rikihisa et al., 1997). Additionally, a recent comprehensive reorganisation of rickettsial species led to the expansion of the Anaplasmataceae family to include species previously from the genera *Wolbachia*, *Ehrlichia*, *Cowdria* and *Neorickettsia* (Dumler et al., 2001). *A. marginale* remains the type species of the *Anaplasma* genus which now also includes 2 new species, *A. phagocytophilum* and *A. bovis*. 16S rDNA studies of *A. marginale*, *A. centrale* (Theiler, 1910) and *A. ovis* (ovine anaplasmosis) suggest they are members of the same species (<1% sequence heterogeneity). However greater discrimination of *Anaplasma* and related spp. can be demonstrated by analysis of the hsp60 gene (*groESL*) and the RNA polymerase β-subunit protein sequences (Lew et al., 2003; Taillardat-Bisch et al., 2003). These analyses have highlighted that, where possible, sequences of type species and strains from different continents should be compared to confirm molecular phylogenies. For instance, Inokuma et al. (2001a,b) published 2 articles demonstrating the phylogenetic relatedness of *A. centrale* to other Ehrlichiae based on 16S rDNA and citrate synthase gene sequences from an *A. centrale* isolate originating from Japan. Our subsequent analyses based on 16S rDNA demonstrated that the *A. centrale* vaccine strain (Theiler, 1910) is more closely related to *A. marginale* than it is to this '*A. centrale'* isolate from Japan (Lew et al., 2003).

Phylogenetic analysis of the 18S rDNA sequences of *B. bovis* and *B. bigemina* in relation to other Apicomplexa have confirmed monophyly of these 2 related species (Ellis et al., 1992; Allsopp et al., 1993). Alternative approaches using 2 heat shock protein genes *hsp70* and *hsp90* have demonstrated closer similarity of *B. bovis* and *B. microti* than has been demonstrated by rDNA analyses (Ruef et al., 2000). Most molecular phylogenetic studies of piroplasmid species are aiming to define the inter-relationships among species of the Babesiidae and Theileriidae species (Kjemtrup et al., 2000; Zahler et al., 2000; Penzhorn et al., 2001; Criado-Fornelio et al., 2004). The most recent comprehensive review suggests that *Babesia* isolated from ungulates (*B. bigemina*, *B. bovis*, *B. ovis* and *B. caballi*) form a separate clade called 'Ungulibabesids' within a total 5 clades suggested for the piroplasmids (Criado-Fornelio et al., 2003).

## GENE ANALYSIS

Live vaccines consisting of *A. centrale* and attenuated strains of both *Babesia* spp. have been used to control anaplasmosis and babesiosis, respectively, in both Africa and Australia (reviewed by de Vos and Bock, 2000; Bock and de Vos, 2001; Kocan et al., 2003). However, research towards the development of recombinant vaccines have provided the impetus to identify the function of specific genes as well as the mechanisms whereby certain genes manipulate the host immune

system (Palmer et al., 1999; Norimine et al., 2003; Zhang et al., 2003). A summary of genes currently accessible on the GenBank database has been compiled in Table 2. However, discussion of vaccine candidates is beyond the scope of this review.

Most of the sequences listed in Table 2 for *Anaplasma* have resulted from phylogenetic studies described above. The antigenic variation of major surface proteins (MSPs) of tick-borne bacterial pathogens is primarily a mechanism for evasion of the host immune response (Brayton et al., 2001). For this reason, *A. marginale* gene function studies have concentrated on the analysis of the six MSPs identified on *A. marginale* from bovine erythrocytes (reviewed by Kocan et al. 2000). MSP1a, MSP4 and MSP5 are from single genes and are conserved within isolates (Visser et al., 1992; Oberle and Barbet, 1993; Oberle et al., 1993; Viseshakul et al., 2000). However, MSP1b, MSP2 and MSP3 are encoded by polymorphic multigene families (Rurangirwa et al., 2000; Viseshakul et al., 2000; de la Fuente and Kocan, 2001; Kano et al., 2002). *A. marginale* infections persist *in vivo* by the simultaneous clearance and emergence of unique MSP2 and MSP3 variants as demonstrated by cyclic bacteremia and the immune selection for MSP2 and MSP3 proteins (Brayton et al., 2003). MSP1a and MSP1b form the MSP1 complex and have been identified as adhesins involved in the infection of host cells (McGarey et al., 1994; de La Fuente et al., 2001; de la Fuente et al., 2003a). The intensive analysis of sequence variants of these MSPs is reflected in the accession list presented in Table 2 and few other genes have been described for *A. marginale* or *A. centrale*.

Three fold more gene accessions are listed for *B. bovis* than for *B. bigemina* and the list of *B. bovis* accessions have been further sub-categorized to group antigen and ribosomal RNA related sequences separately (Table 2). Similarly to *Anaplasma*, it is evident that studies on *Babesia* have concentrated on major surface proteins or antigens, immuno-dominant antigens and genes which are expressed by polymorphic multigene families such as MSA-1, MSA-2, rhoptry associated proteins (Table 2; Dalrymple et al., 1993; Suarez et al., 1994; Hotzel et al., 1997; McElwain et al., 1998; Fisher et al., 2001; Suarez et al., 2003; Wilkowsky et al., 2003). Other genes sequenced include those used in phylogenetic studies (described above), apicomplexan gene homologues, drug resistance genes and genes implicated in host invasion and/or survival (Silins et al., 1996; Lew et al., 2002b; Bork et al., 2004; Gaffar et al., 2004a; Gaffar et al., 2004b; Gaffar et al., 2004c). As demonstrated by the number of *B. bovis* GenBank accessions, this volume of research activity correlates with the economic importance of this parasite.

## GENOME SEQUENCING

Table 2 contains a limited list of genes when considering the importance of these pathogens to the global cattle industry. Expressed sequence tag (EST) and genome sequencing projects will rectify this deficit of sequence data for both *Babesia* species and *A. marginale*. The sequencing of *A. marginale* strain, St. Maries (USA), is almost complete with currently 1197687 bp of the 1.25 Mb genome completed (GenBank accession number NC_004842 – description only). DNA sequence and predicted proteins are available for BLAST at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi but are not yet downloadable from Entrez at the National Centre for Biotechnology Information (ncbi).

For *B. bovis*, 12565 EST clones have been sequenced (at October 2004) and are available for BLAST at the Sanger Centre web site: http://www.sanger.ac.uk/Projects/B_bovis/. An *B. bovis* genome project has begun with no downloadable data currently available (http://www.vetmed.wsu.edu/research_vmp/babesia-bovis/).

There are no current public announcements for EST or genome analysis of *B. bigemina*.

## FUTURE RESEARCH

Here we have reviewed the molecular approaches which have been developed and applied to study the causative agents of bovine babesiosis and anaplasmosis. Although there are PCR tools to detect and compare species and strains, it appears that the application of novel technologies for *Anaplasma* and *Babesia* detection could still be further developed and improved. Specifically, PCR methods could be improved by the application of real time PCR, and novel tools such as diagnostic micro-arrays could be developed. Molecular based diagnostic applications are most effective when multiple pathogens can be detected in one assay.

The main difficulty in studying gene function in these pathogens has been the limitations in maintaining and manipulating these organisms *in vitro*. More recently, cell culture techniques have advanced and the development of transfection systems for these organisms are also part of on-going research programs (Munderloh et al., 1996; Jackson et al., 2001; Kocan et al., 2003; Suarez et al., 2004). Additionally, detailed genome data will soon be available for at least *A. marginale* and *B. bovis* and methods to study the expression of genes or the analysis of gene knockouts are still required to validate genes as potential drug targets or vaccine candidates.

Full genome sequence data will enable the comparison of genomes with other related annotated genomes (*Plasmodium* and *Rickettsia* spp.) as well as the development of genome microarrays which will facilitate comprehensive gene expression studies (Boothroyd et al., 2003; Conway and Schoolnik, 2003). Additionally, the completion of the bovine genome (http://www.hgsc.bcm.tmc.edu/projects/bovine) in 2005 and availability of tick genome sequences (eg. *B. microplus* USDA/TIGR) will allow the comparative

**Table 2.** List of *A. centrale*, *A. marginale*, *B. bovis*, and *B. bigemina* GenBank accessions (October 2004).

| Species | Gene(s) | Accession numbers[1] |
|---|---|---|
| *A. centrale* | 16S rDNA | AF283007; AF309869; AF318944; AF414868; AF414869 |
| | citrate synthase | AF304141 |
| | glutathione synthetase D-alanine ligase | M80425 |
| | Heat shock protein 60 | AF414866; AF414867 |
| | Heat shock protein 70 | AY188684 |
| | Major surface protein 2 | AY040556-AY040563; AY132307 |
| | Major surface protein 3 | AY586402 |
| | Major surface protein 4 | AY428090; AY054383 |
| | Major surface protein 5 | AY054384 |
| | unknown | AF352558 |
| *A. marginale* | NC_004842 | whole genome shotgun sequence, 1197687bp St. Maries strain, unfinished |
| | 16S rDNA | M60313; AF309868; AF309867; AF311303; AF414871- AF414873; AF414875-AF414878; AF309866; AJ633048; AY048816 |
| | 23S-5S intergenic spacer | AY048815 |
| | ana29 | AY220298 |
| | ana37 | AY220300 |
| | ana43-like | AY220299 |
| | citrate synthase gene | AF304139; AF304140 |
| | ftsZ gene | AJ010274 |
| | HSP60 | AF165812; AF414859-AF414865 |
| | inorganic pyro-phosphatase gene | AF417515 |
| | Major surface protein 1a | M32868-M32871; AF293062-AF293064; AF345867-AF345871; AF407542-AF407545; AF461133; AF461134; AF352559; AF352560; AF428091-AF428094; AY010242-AY010247; AY127052-AY127064; AY191826; AY245429; AY253141; AY253144; AY283198-AY283200; AY295077; AY355282-AY355284; AY489564; AY602768 |
| | Major surface protein 1b (and msp1b pseudogenes) | M59845; AF110808-AF110810; AF111195; AF111197; AF112480; AF221691- AF221693; AF348137; AF348138 |
| | Major surface protein 2 | U36193; AF107766; AF107767; AF200925- AF200927; AF227261-AF227271; AF290590- AF290599; AF317720- AF317726; AF402257-AF402279; AF540581-AF540593; AF354464-AF354486; AY138955-AY138958; AY241665-AY241668 |
| | msp2 and msp3 pseudogenes | AF305077; AF305503-AF305508 |
| | Major surface protein 3 | U60778- U60780; AF527422- AF527433; AF540565-AF540580; AY127883-AY127898; AY128095- AY128099; AY129828 |
| | Major surface protein 4 | L01987; AF428081-AF428089; AY010246; AY101248-AY010254; AY127065-AY127078; AY191827; AY253142-AY253143; AY283189-AY283192; AY283194-AY283197; AY456001-AY456003; AY714546 |
| | Major surface protein 5 | M93392; AY245428; AY527217; AY714547 |
| | RNA polymerase beta subunit | AF389472 |
| | transcriptional regulator+msp2 | AY132308; AY132310-AY132314 |
| | tRNA-Arg gene | AF081791 |
| *B. bigemina* | 12D3 antigen | A23051 |
| | 18S rDNA | X59604; X59605; X59607; AY648884 |
| | 200kDa antigen | AF142406 |
| | aldo-keto reductase | M93122 |
| | apocytochrome b | AF109354 |
| | Bbg 2.1 antigen | M81569 |
| | Bbg1.1 antigen | M81568 |
| | beta tubulin | AJ289252 |
| | Heat shock protein (small) gene | AF332566; AF332567 |
| | Intergenic spacer region rDNA | AJ538183 |
| | merozoite surface antigen p58 | M60878; M85184-M85187 |
| | merozoite surface protein gp45 | AF298630-AF298632 |
| | plastid | AF040968 |
| | rhoptry associated protein | AF014486; AF014757-AF041768; AF017284-AF017298; AF021246; AF021247; AF021798; AF026272 |
| | rhoptry protein gene | AY146979-AY1146987 |
| | Unknown | AJ538180-AJ538182; S45366 |

**Table 2. Contd.**

| | | |
|---|---|---|
| *B.bovis*[2] | **SURFACE ANTIGENS/PROTEINS:** | |
| | 12D3 antigen | A23049 |
| | 85 kDa merozoite protein gene | M99575 |
| | 225kDA variable antigen (BvVA1) | M80466; M87623; M87624; M80426 |
| | Antigen | M29838 |
| | Apical membrane antigen 1 | AY486101 |
| | ATP-binding protein | U44917-U44919 |
| | Bv80 merozoite protein/ Bb-1/80kDa | A49229; M93125; M93126 |
| | merozoite surface antigen –1 (MSA-1) | AF275908-AF275911 |
| | MSA-2/44kda merozoite surface antigen | M80467; AY052538-AY052542 |
| | merozoite surface protein - pBv42 | M77192 |
| | merozoite surface antigen - 60kDa | M38218 |
| | rhoptry associated protein-1 | L77245; L77326; AF027149; AF030053-AF030062 |
| | rhoptry protein | A16428; L00958-L00961; M91177-M91178 |
| | **RNA GENES AND PROTEINS:** | |
| | BabR locus | K02832-K02834 |
| | phosphoriboprotein P0 | AF498365 |
| | ribosomal protein L12el/Acidic ribosomal protein P2 | M81359; S35440 |
| | 60s ribosomal protein L35; nucleoside monophosphate kinase, mitochondrial protein; ATP binding protein | U34076; U44917; AY170917-AY170919 |
| | small subunit rRNA; 18S rDNA | L19077; L19078; M87566; U06105 |
| | ss rRNA (extrachromosomal) | S57861 |
| | glutamine-dependent carbamoyl-phosphate synthase | U18792 |
| | **OTHER:** | |
| | Actin | AF410769 |
| | apocytochrome b gene | AF053002 |
| | beta tubulin gene | L00978; AJ289247 |
| | Coronin-like protein | AY324186 |
| | DnaJ homolog | AF017149 |
| | HMG-containing protein 1 | JQ1490 |
| | hsp (small) gene | AF331455 |
| | Hsp70 | AF107118 |
| | Hsp90 | AF136649 |
| | iron-dependent superoxide dismutase | U70130; U70131 |
| | L-lactate dehydrogenase | AB112429 |
| | long-chain acyl-CoA synthetase | AF331454; AY534753 |
| | membrane protein; dihydrofolate reductase; thymidilate synthase genes | AY302755; AF333764 |
| | (N)-methyl-aspartate receptor | AF275908; AF275912 |
| | Myosin genes | AF273862- AF273868; AF403045-AF403047 |
| | non-histone protein | M81360 |
| | phosphomannomutase | AF027149; AF028591 |
| | Ras GTPase subclass Rab | AY324134-AY324138 |
| | spherical body protein 4 (SBP4) | AF486506; AF486507 |
| | spherical body protein 3 (SBP3) | AF107117 |
| | thrombospondin-related anonymous protein | AY486102 |
| | unknown | A27286; A27290; A27292; A27294 |
| | Variant erythrocyte surface antigen-1a | AF173158-AF173161; AF195549-AF195570; AY279553-AY279559 |

[1]A dash between accession numbers denotes a series of related accession numbers.
[2]Not including expressed sequence tag – accessed at the Sanger Centre at http://www.sanger.ac.uk/Projects/B_bovis.

analysis of pathogen:host and pathogen:vector interactions. Also applicable to eucaryotic gene expression systems is the potential to utilise gene silencing/RNA interference (RNAi) as a reverse genetic or gene knockdown tool. RNAi silences genes by a natural mechanism using stable dsRNA to trigger messenger RNA (mRNA) destruction. This mechanism has evolved to protect genomes from exogenous (viral) or endogenous (transposon) threats and can also participate in the cellular control of gene expression and development (Cogoni and Macino, 2000). Recently Ullu et al. (2004) reviewed the RNAi mechanisms in protozoan species. Through evidence compiled from both genome data mining for RNAi gene homologues and specific dsRNA gene silencing experiments, they suggested that not all apicomplexan species possess RNAi capability (Ullu et al., 2004). Our preliminary investigations treating *B. bovis* merozoites with *B. bovis* myosin-A specific long dsRNAs and chemically synthesised small interfering (si) RNAs have demonstrated a subsequent decrease in the ability of dsRNA treated merozoites to re-invade red cells in an *in vitro B. bovis* culture system compared with un-treated merozoites (Lew and Jackson, unpublished data). Similar experiments with *P. falciparum* culture systems have shown a down-regulation of genes (McRobert and McConkey, 2002; Malhotra et al., 2002). However, data mining has failed to identify RNAi genes in the *Plasmodium* genome sequence (Ullu et al. 2004). When the *B. bovis* genome sequence is available, this will enable further investigation and study of potential RNAi gene homologues. Additionally, the combination of RNAi and microarray analysis will also prove useful for the understanding of gene regulation networks (Semizarov et al., 2004).

A fully annotated *Anaplasma* genome will be available sooner than for *Babesia* and this data will be beneficial towards the development of improved control methods (Kocan et al., 2003). Most of the vaccine candidate research to date has concentrated on exploiting immuno-dominant antigens/proteins, however it has been suggested that hidden antigens may be more efficacious potential vaccine candidates (Newton and Meeusen, 2003; Nielsen et al., 2003). Alternatively, genomic host and pathogen analyses may elucidate possible novel host immune defence mechanisms which could be used to develop protective treatments (Wilkowsky et al., 2003; Brown et al., 2004; Norimine et al., 2004). Indeed, RNAi may be exploited to protect the bovine host from pathogen invasion by manipulating the host immune system as proposed for human disease therapeutics (Wall and Shi, 2003; Caplen, 2004; Lee and Rossi, 2004).

**REFERENCES**


Allsopp MTE, Cavalier-Smith T, De Waal DT, Allsopp BA (1993). Phylogeny and evolution of the piroplasms. Parasitology 108: 147-152.

Almeria S, Castella J, Ferrer D, Ortuno A, Estrada-Pena A, Gutierrez JF (2001a). Bovine piroplasms in Minorca (Balearic Islands, Spain): a comparison of PCR-based and light microscopy detection. Vet. Parasitol. 99: 249-259.

Almeria S, Castella J, Ferrer D, Sparagano O, Estrada-Pena A (2001b). First report of *Babesia bovis* in Spain. Vet. Rec. 149:716-717.Azambuja CJ, Gayo V, Solari MA (1994). Biotechnology applied to the detection of infectious agents in cattle. Diagnosis of *Babesia bovis* by PCR. Rev. Bras. Parasitol. Vet. 3: 1-4.

Bekker CP, de Vos S, Taoufik A, Sparagano OA, Jongejan F (2002). Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. Vet. Microbiol. 89: 223-238.

Belak S, Thoren P (2001). Molecular diagnosis of animal diseases: some experiences over the past decade. Expert Rev. Mol. Diagn 1: 434-443.

Bock RE, De Vos AJ (2001). Immunity following use of the Australian tick fever vaccine: a review of the evidence. Aust. Vet. J. 79:832-839.

Bock RE, Lew AE, Minchin CM, Jeston PJ, Jorgensen WK (2000). Application of PCR assays to determine the genotype of *Babesia bovis* parasites isolated from cattle with clinical babesiosis soon after vaccination against tick fever. Aust. Vet. J. 78: 179-181.

Boothroyd JC, Blader I, Cleary M, Singh U (2003). DNA microarrays in parasitology: strengths and limitations. Trends Parasitol. 19:470-476.

Bork S, Okamura M, Boonchit S, Hirata H, Yokoyama N, Igarashi I (2004). Identification of *Babesia bovis* L-lactate dehydrogenase as a potential chemotherapeutical target against bovine babesiosis. Mol. Biochem. Parasitol. 136: 165-172.

Bose R, Jorgensen WK, Dalgliesh RJ, Friedhoff KT, deVos AJ (1995). Current state and future trends in the diagnosis of babesiosis. Vet. Parasitol. 57: 61-74.

Brayton KA, Knowles DP, McGuire TC, Palmer GH (2001). Efficient use of a small genome to generate antigenic diversity in tick borne ehrlichial pathogens. Proc. Nat. Acad. Sci. USA. 98: 4130-4135.

Brayton KA, Meeus PF, Barbet AF, Palmer GH (2003). Simultaneous variation of the immunodominant outer membrane proteins, MSP2 and MSP3, during *Anaplasma marginale* persistence *in vivo*. Infect. Immun. 71: 6627-6632.

Brown WC, Palmer GH, Brayton KA, Meeus PF, Barbet AF, Kegerreis KA, McGuire TC (2004). CD4+ T lymphocytes from *Anaplasma marginale* major surface protein 2 (MSP2) vaccinees recognize naturally processed epitopes conserved in MSP3. Infect. Immun. 72: 3688-3692.

Caccio S, Camma C, Onuma M, Severini C (2000). The β-tubulin gene of *Babesia* and *Theileria* parasites is an informative marker for species discrimination. Int. J. Parasitol. 30: 1181-1185.

Calder JAM, Reddy GR, Chieves L, Courtney CH, Littell R, Livengood JR, Norval RAI, Smith C, Dame JB (1996). Monitoring *Babesia bovis* infections in cattle by using PCR-based tests. J. Clin. Microbiol. 34: 2748-2755.

Callow LL (1984a). Arthropod-borne rickettsias of the blood. In: Protozoal and rickettsial diseases. Animal Health in Australia, Vol. Australian Bureau of Animal Health, AGPS 5, Canberra, pp. 174-201.

Callow LL (1984b). Piroplasms. In: Animal Health in Australia, Protozoal and rickettsial diseases, Vol. 5. Australian Bureau of Animal Health, AGPS, Canberra, pp. 121-160.

Caplen NJ (2004). Gene therapy progress and prospects. Downregulating gene expression: the impact of RNA interference. Gene Ther. 11: 1241-1248.

Carson CA, Brandt HM, Jensen JB, Bailey CW, Allen GK (1994). Use of random amplified polymorphic DNA analysis to compare *Babesia bovis* and *B. bigemina* isolates. Parasitol. Res. 80:312-315.

Cogoni C, Macino G (2000). Post-transcriptional gene silencing across kingdoms. Curr. Opin. Genet. Dev. 10: 638-643.

Conway T, Schoolnik GK (2003). Microarray expression profiling: capturing a genome-wide portrait of the transcriptome. Mol. Microbiol. 47: 879-889.

Cossio-Bayugar R, Rodriguez SD, Garcia-Ortiz MA, Garcia-Tapia D, Aboytes-Torres R (1997). Bovine anaplasmosis prevalence in northern Veracruz state, Mexico. Prev. Vet. Med. 32: 165-170.

Courtney JW, Kostelnik LM, Zeidner NS, Massung RF (2004). Multiplex real-time PCR for detection of *Anaplasma phagocytophilum* and *Borrelia burgdorferi*. J. Clin. Microbiol. 42: 3164-3168.

Criado-Fornelio A, Gonzalez-del-Rio MA, Buling-Sarana A, Barba-Carretero JC (2004). The "expanding universe" of piroplasms. Vet. Parasitol. 119: 337-345.

Criado-Fornelio A, Martinez-Marcos A, Buling-Sarana A, Barba-Carretero JC (2003). Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe. Part II. Phylogenetic analysis and evolutionary history. Vet. Parasitol. 114: 173-194.

Dalrymple BP (1990). Cloning and characterization of the rRNA genes and flanking regions from *Babesia bovis*: use of the genes as strain discriminating probes. Mol. Biochem. Parasitol. 43:117-124.

Dalrymple BP, Jorgensen WK, DeVos AJ, Wright IG (1992). Analysis of the composition of samples of *Babesia bovis* and the influence of different environmental conditions on genetically distinct subpopulations. Int. J. Parasitol. 22: 731-737.

Dalrymple BP, Peters JM, Goodger BV, Bushell GR, Waltisbuhl DJ, Wright IG (1993). Cloning and characterisation of cDNA clones encoding two *Babesia bovis* proteins with homologous amino- and carboxy-terminal domains. Mol. Biochem. Parasitol. 59: 181-190.

de La Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM (2001). Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. Int. J. Parasitol. 31: 135-153.

de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM (2003a). Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. Vet. Microbiol. 91: 265-283.

de la Fuente J, Kocan KM (2001). Expression of *Anaplasma marginale* major surface protein 2 variants in persistently infected ticks. Infect. Immun. 69: 5151-5156.

de La Fuente J, Passos LM, Van Den Bussche RA, Ribeiro MF, Facury-Filho EJ, Kocan KM (2004). Genetic diversity and molecular phylogeny of *Anaplasma marginale* isolates from Minas Gerais, Brazil. Vet. Parasitol. 121: 307-316.

de la Fuente J, Van Den Bussche RA, Garcia-Garcia JC, Rodriguez SD, Garcia MA, Guglielmone AA, Mangold AJ, Friche Passos LM, Barbosa Ribeiro MF, Blouin EF, Kocan KM (2002). Phylogeography of New World isolates of *Anaplasma marginale* based on major surface protein sequences. Vet. Microbiol. 88: 275-285.

de la Fuente J, Van Den Bussche RA, Kocan KM (2001). Molecular phylogeny and biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae: Ehrlichieae). Vet. Parasitol. 97: 65-76.

de la Fuente J, Van Den Bussche RA, Prado TM, Kocan KM (2003b). *Anaplasma marginale* msp1alpha genotypes evolved under positive selection pressure but are not markers for geographic isolates. J. Clin. Microbiol. 41: 1609-1616.

de Vos AJ, Bock RE (2000). Vaccination against bovine babesiosis. Ann. N Y Acad. Sci. 916: 540-545.

de Vos AJ, Molloy JB Jorgensen WK (2000). Bovine babesiosis. In: Office International des Epizooties. (Ed) Manual of recommended diagnostic techniques and requirements for biological products, Vol. IV, pp. 412-422.

Dumler J, Barbet A, Bekker C, Dasch G, Palmer G, Ray S, Rikihisa Y, Rurangirwa F (2001). Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. 51: 2145-2165.

Duzgun A, Schuntner CA, Wright IG, Leatch G, Waltisbuhl DJ (1988). A sensitive ELISA technique for the diagnosis of *Anaplasma marginale* infections. Vet. Parasitol. 29: 1-7.

Ellis J, Hefford C, Baverstock PR, Dalrymple BP, Johnson AM (1992). Ribosomal DNA sequence comparison of *Babesia* and *Theileria*. Mol. Biochem. Parasitol. 54: 87-96.

Eriks IS, Palmer GH, McGuire TC, Barbet AF (1989). Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. J. Clin. Microbiol. 27: 279-284.

Fahrimal Y, Goff WL, Jasmer DP (1992). Detection of *Babesia bovis* carrier cattle by using polymerase chain reaction amplification of parasite DNA. J. Clin. Microbiol. 30: 1373-1379.

Ferreira AM, Suzart S, Vidotto O, Knowles DP, Vidotto MC (2001). Use of repetitive DNA elements to define genetic relationships among *Anaplasma marginale* isolates. FEMS Microbiol. Lett. 197:139-143.

Figueroa JV, Alvarez JA, Canto GJ, Ramos JA, Mosqueda JJ, Buening GM (1996). Comparative sensitivity of two tests for the diagnosis of multiple hemoparasite infection of cattle. Ann. N Y Acad. Sci. 791: 117-127.

Figueroa JV, Chieves LP, Byers PE, Frefichs WM, Buening GM (1992a). Evaluation of DNA-based probe for the detection of cattle experimentally infected with *Babesia bigemina*. In: E.P.J. Gibbs (Ed) Tropical Veterinary Medicine: Current issues and perspectives, Vol. 653. Annals of New York Academy of Sciences, New York.

Figueroa JV, Chieves LP, Johnson GS, Buening GM (1992b). Detection of *Babesia bigemina*-infected carriers by polymerase chain reaction amplification. J. Clin. Microbiol. 30:2576-2582.

Figueroa JV, Chieves LP, Johnson GS, Buening GM (1993). Multiplex polymerase chain reaction based assay for the detection of *Babesia bigemina*, *Babesia bovis* and *Anaplasma marginale* DNA in bovine blood. Vet. Parasitol. 50: 69-81.

Fisher TG, McElwain TF, Palmer GH (2001). Molecular basis for variable expression of merozoite surface antigen gp45 among American isolates of *Babesia bigemina*. Infect. Immun. 69: 3782-3790.

Friedhoff KT (1988). Transmission of *Babesia*. In: M. Ristic (Ed) Babesiosis of Domestic Animals and Man. CRC Press, Florida, pp. 23-52.

Gaffar FR, Wilschut K, Franssen FF, de Vries E (2004a). An amino acid substitution in the *Babesia bovis* dihydrofolate reductase-thymidylate synthase gene is correlated to cross-resistance against pyrimethamine and WR99210. Mol. Biochem. Parasitol. 133:209-219.

Gaffar FR, Yatsuda AP, Franssen FF, de Vries E (2004b). A *Babesia bovis* merozoite protein with a domain architecture highly similar to the thrombospondin-related anonymous protein (TRAP) present in *Plasmodium* sporozoites. Mol. Biochem. Parasitol. 136:25-34.

Gaffar FR, Yatsuda AP, Franssen FF, de Vries E (2004c). Erythrocyte invasion by *Babesia bovis* merozoites is inhibited by polyclonal antisera directed against peptides derived from a homologue of *Plasmodium falciparum* apical membrane antigen 1. Infect. Immun. 72:2947-2955.

Gale KR, Dimmock CM, Gartside M, Leatch G (1996). *Anaplasma marginale*: detection of carrier cattle by PCR-ELISA. Int. J. Parasitol. 26:1103-1109.

Gayo V, Romito M, Nel LH, Solari MA, Viljoen GJ (2003). PCR-based detection of the transovarial transmission of Uruguayan *Babesia bovis* and *Babesia bigemina* vaccine strains. Onderstepoort J. Vet. Res. 70: 197-204.

Ge N-L, Kocan KM, Ewing SA, Blouin EF, Edwards WL, Murphy GL, Dawson LJ (1997). Use of a nonradioactive DNA probe for detection of *Anaplasma marginale* infection in field cattle: comparison with complement fixation serology and microscopic examination. J. Vet. Diagn. Invest. 9: 39-43.

Ge N-L, Kocan KM, Murphy GL, Blouin EF (1995). Detection of *Anaplasma marginale* DNA in bovine erythrocytes by slot-blot and *in situ* hybridization with a PCR-mediated digoxigenin-labeled DNA probe. J. Vet. Diagn. Invest. 7: 465-472.

Georges K, Loria GR, Riili S, Greco A, Caracappa S, Jongejan F, Sparagano O (2001). Detection of haemoparasites in cattle by reverse line blot hybridisation with a note on the distribution of ticks in Sicily. Vet. Parasitol. 99: 273-286.

Gubbels JM, de Vos AP, van der Weide M, Viseras J, Schouls LM, de Vries E, Jongejan F (1999). Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. J. Clin. Microbiol. 37: 1782-1789.

Herrero MV, Perez E, Goff WL, Torioni de Echaide S, Knowles DP, McElwain TF, Alvarez V, Alvarez A, Buening GM (1998). Prospective study for the detection of *Anaplasma marginale* Theiler, 1911 (Rickettsiales:Anaplasmataceae) in Costa Rica. Ann. NY Acad. Sci. 849: 226-233.

Hofmann-Lehmann R, Meli ML, Dreher UM, Gonczi E, Deplazes P,

Braun U, Engels M, Schupbach J, Jorger K, Thoma R, Griot C, Stark KD, Willi B, Schmidt J, Kocan KM, Lutz H (2004). Concurrent infections with vector-borne pathogens associated with fatal hemolytic anemia in a cattle herd in Switzerland. J. Clin. Microbiol. 42:3775-3780.

Hotzel I, Suarez CE, McElwain TF, Palmer GH (1997). Genetic variation in the dimorphic regions of RAP-1 genes and rap-1 loci of *Babesia bigemina*. Mol. Biochem. Parasitol. 90:479-489.

Hove T, Sithole N, Munodzana D, Masaka S (1998). Isolation and characterization of a *Babesia* species from *Rhipicephalus evertsi evertsi* ticks picked off a sable antelope (*Hippotragus niger*) which died of acute babesiosis. Onderstepoort. J. Vet. Res. 65:75-80.

Inokuma H, Brouqui P, Drancourt M, Raoult D. (2001a) Citrate synthase gene Sequence: a new tool for phylogenetic analysis and identification of *Ehrlichia*. J. Clin. Microbiol. 39:3031-3039.

Inokuma H, Yutaka T, Kamio T, Raoult D, Brouqui P (2001b). Analysis of the 16S rRNA gene sequence of *Anaplasma centrale* and its phylogenetic relatedness to other Ehrlichiae. Clin. Diagn. Lab. Immunol. 8:241-244.

Jackson LA, Waldron SJ, Weier HM, Nicoll CL, Cooke BM (2001). *Babesia bovis*: culture of laboratory-adapted parasite lines and clinical isolates in a chemically defined medium. Exp. Parasitol. 99:168-174.

Jeong W, Kweon CH, Kang SW, Paik SG (2003). Diagnosis and quantification of *Theileria sergenti* using TaqMan PCR. Vet. Parasitol. 111:287-295.

Jorgensen WK, Jeston PJ, Bowles PM, Croft J, Lew AE, Molloy JB, Dalgliesh RJ (1998). Relationships between vaccine and virulent strains of *Babesia bovis* during co-infection in calves. Aust. Vet. J. 76:57-58.

Kano FS, Vidotto O, Pacheco RC, Vidotto MC (2002). Antigenic characterization of *Anaplasma marginale* isolates from different regions of Brazil. Vet. Microbiol. 87:131-138.

Kjemtrup AM, Thomford J, Robinson T, Conrad PA (2000). Phylogenetic relationships of human and wildlife piroplasm isolates in the western United States inferred from the 18S nuclear small subunit RNA gene. Parasitology 120:487-493.

Kocan KM, de la Fuente J, Guglielmone AA, Melendez RD (2003). Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. Clin. Microbiol. Rev. 16:698-712.

Kocan KM, Ge N-L, Blouin EF, Murphy GL (1998). Development of a non-radioactive DNA probe and in situ hybridization for detection of *Anaplasma marginale* in ticks and cattle. Ann. NY Acad. Sci. 849:137-145.

Krueger C, Buening GM (1988). Isolation and restriction endonuclease cleavage of *Anaplasma marginale* DNA in situ in agarose. J. Clin. Microbiol. 26:906-910.

Lee NS, Rossi JJ (2004). Control of HIV-1 replication by RNA interference. Virus Res. 102:53-58.

Lew AE, Bock RE, Croft JM, Minchin CM, Kingston TG, Dalgliesh RJ (1997a). Genotypic diversity in field isolates of *Babesia bovis* from cattle with babesiosis after vaccination. Aust. Vet. J. 75:575-578.

Lew AE, Bock RE, Minchin CM, Masaka S (2002a). A msp1alpha polymerase chain reaction assay for specific detection and differentiation of *Anaplasma marginale* isolates. Vet. Microbiol. 86:325-335.

Lew AE, Dalrymple BP, Jeston PJ, Bock RE (1997b). PCR methods for the discrimination of *Babesia bovis* isolates. Vet. Parasitol. 71:223-237.

Lew AE, Dluzewski AR, Johnson AM, Pinder JC (2002b). Myosins of *Babesia bovis*: Molecular characterisation, erythrocyte invasion, and phylogeny. Cell Motil Cytoskeleton 52:202-220.

Lew AE, Gale KR, Minchin CM, Shkap V, Theo de Waal D (2003). Phylogenetic analysis of the erythrocytic *Anaplasma* species based on 16S rDNA and GroEL (HSP60) sequences of *A. marginale*, *A. centrale*, and *A. ovis* and the specific detection of *A. centrale* vaccine strain. Vet. Microbiol. 92:145-160.

Lin B, Vora GJ, Thach D, Walter E, Metzgar D, Tibbetts C, Stenger DA (2004). Use of oligonucleotide microarrays for rapid detection and serotyping of acute respiratory disease-associated adenoviruses. J. Clin. Microbiol. 42:3232-3239.

Livak KJ, Flood SJ, Marmaro J, Giusti W, Deetz K (1995). Oligonucleotides with fluorescent dyes at opposite ends provide a quenched system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Applic. 4:357-362.

Lohr CV, Rurangirwa FR, McElwain TF, Stiller D, Palmer GH (2002). Specific expression of *Anaplasma marginale* major surface protein 2 salivary gland variants occurs in the midgut and is an early event during tick transmission. Infect .Immun. 70:114-120.

Mackay IM. (2004) Real-time PCR in the microbiology laboratory. Clin. Microbiol. Infect. 10:190-212.

McElwain RF (2000). Bovine anaplasmosis. In: Office International des Epizooties (Ed) Manual of recommended diagnostic techniques and requirements for biological products, Vol. IV, pp. 399-411.

McElwain TF, Hines SA, Palmer GH (1998). Persistence of antibodies against epitopes encoded by a single gene copy of the *Babesia bovis* merozoite surface antigen 1 (MSA-1). J. Parasitol. 84:449-452.

McGarey DJ, Barbet AF, Palmer GH, McGuire TC, Allred DR (1994). Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. Infect. Immun. 62:4594-4601.

McLeod R, Kristjanson P (1999). Final report of joint esys/ILRI/ACIAR TickCost project- Economic impact of ticks and tick-borne disease to livestock in Africa, Asia and Australia. International Livestock Research Institute, Nairobi, Kenya.

McRobert L, McConkey GA. (2002) RNA interference (RNAi) inhibits growth of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 119:273-278.

Malhotra P, Dasaradhi PV, Kumar A, Mohmmed A, Agrawal N, Bhatnagar RK, Chauhan VS. (2002) Double-stranded RNA-mediated gene silencing of cysteine proteases (falcipain-1 and -2) of *Plasmodium falciparum*. Mol. Microbiol. 45:1245-1254.

Munderloh UG, Blouin EF, Kocan KM, Ge NL, Edwards WL, Kurtti TJ (1996). Establishment of the tick (Acari:Ixodidae)-borne cattle pathogen *Anaplasma marginale* (Rickettsiales:Anaplasmataceae) in tick cell culture. J. Med. Entomol. 33:656-664.

Newton SE, Meeusen EN (2003). Progress and new technologies for developing vaccines against gastrointestinal nematode parasites of sheep. Parasite. Immunol. 25:283-296.

Ngeranwa JJN, Venter EH, Penzhorn BL, Soi RK, Mwanzia J, Nyongesa (1998). Characterization of *Anaplasma* isolates from eland (*Taurotragus oryx*) Pathogenicity in cattle and sheep and DNA profiles analysis. Vet. Parasitol. 74:109-122.

Nielsen M, Lundegaard C, Worning P, Lauemoller SL, Lamberth K, Buus S, Brunak S, Lund O (2003). Reliable prediction of T-cell epitopes using neural networks with novel sequence representations. Protein Sci. 12:1007-1017.

Norimine J, Mosqueda J, Palmer GH, Lewin HA, Brown WC (2004). Conservation of *Babesia bovis* small heat shock protein (Hsp20) among strains and definition of T helper cell epitopes recognized by cattle with diverse major histocompatibility complex class II haplotypes. Infect. Immun. 72:1096-1106.

Norimine J, Mosqueda J, Suarez C, Palmer GH, McElwain TF, Mbassa G, Brown WC (2003). Stimulation of T-helper cell gamma interferon and immunoglobulin G responses specific for *Babesia bovis* rhoptry-associated protein 1 (RAP-1) or a RAP-1 protein lacking the carboxy-terminal repeat region is insufficient to provide protective immunity against virulent *B. bovis* challenge. Infect. Immun. 71:5021-5032.

Oberle SM, Barbet AF (1993). Derivation of the complete msp4 gene sequence of *Anaplasma marginale* without cloning. Gene 136:291-294.

Oberle SM, Palmer GH, Barbet AF (1993). Expression and immune recognition of the conserved MSP4 outer membrane protein of *Anaplasma marginale*. Infect. Immun. 61:5245-5251.

Oura CA, Bishop RP, Wampande EM, Lubega GW, Tait A (2004). Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda. Int. J. Parasitol. 34:603-613.

Palmer GH, Rurangirwa FR, Kocan KM, Brown WC (1999). Molecular basis for vaccine development against the ehrlichial pathogen *Anaplasma marginale*. Parasitol. Today 15:281-286.

Palmer GH, Rurangirwa FR, McElwain TF (2001). Strain composition of the ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian reservoir for tick transmission. J. Clin. Microbiol. 39:631-635.

Penzhorn BL, Kjemtrup AM, Lopez-Rebollar LM, Conrad PA (2001).

Babesia leo n. sp. from lions in the Kruger National Park, South Africa, and its relation to other small piroplasms. J. Parasitol. 87:681-685.
Potgieter FT, Stoltsz WH (1994). Bovine anaplasmosis. In: R.C. Tustin (Ed) Infectious diseases of Livestock, with special reference to Southern Africa, Vol. 1. Oxford University Press, London, pp. 408-430.
Pusterla N, Huder JB, Leutenegger CM, Braun U, Madigan JE, Lutz H (1999). Quantitative real-time PCR for detection of members of the *Ehrlichia phagocytophila* genogroup in host animals and *Ixodes ricinus* ticks. J. Clin. Microbiol. 37: 1329-1331.
Rikihisa Y, Kawahara M, Wen B, Kociba G, Fuerst P, Kawamori F, Suto C, Shibata S, Futohashi M (1997). Western immunoblot analysis of *Haemobartonella muris* and comparison of 16S rRNA gene sequences of *H. muris*, *H. felis*, and *Eperythrozoon suis*. J. Clin. Microbiol. 35: 823-829.
Rivas D, Corona B, Rojas R (1993). Differentiation of two isolates of *Anaplasma marginale* using the polymerase chain reaction technique with arbitrary primers (PCR-AP). Rev. Salud Anim. 15: 339-343.
Ruef B, Ward TJ, Oxner CR, Conley PG, Brown WC, Rice-Ficht AC (2000). Phylogenetic analysis with newly characterized *Babesia bovis* hsp70 and hsp90 provides strong support for paraphyly within the piroplasms. Mol. Biochem. Parasitol. 109: 67-72.
Rurangirwa FR, Stiller D, Palmer GH (2000). Strain diversity in major surface protein 2 expression during tick transmission of *Anaplasma marginale*. Infect. Immun. 68: 3023-3027.
Salem GH, Liu X, Johnsrude JD, Dame JB, Roman Reddy G (1999). Development and evaluation of an extra chromosomal DNA-based PCR test for diagnosing bovine babesiosis. Mol. Cell Probes 13: 107-113.
Semizarov D, Kroeger P, Fesik S (2004). siRNA-mediated gene silencing: a global genome view. Nucleic Acids Res. 32: 3836-3845.
Silins GU, Blakeley RL, Riddles PW (1996). Characterisation of genes encoding a nucleoside monophosphate kinase and a L35 ribosomal protein from *Babesia bovis*. Mol. Biochem. Parasitol. 76: 231-244.
Smeenk I, Kelly PJ, Wray K, Musuka G, Trees AJ, Jongejan F (2000). *Babesia bovis* and *B. bigemina* DNA detected in cattle and ticks from Zimbabwe by polymerase chain reaction. J. S. Afr. Vet. Assoc. 71: 21-24.
Sparagano O, Loria GR, Gubbels MJ, De Vos AP, Caracappa S, Jongejan F (2000). Integrated molecular diagnosis of *Theileria* and *Babesia* species of cattle in Italy. Ann. N Y Acad. Sci. 916: 533-539.
Stich RW, Bantle JA, Kacan KM, Fekete A (1993). Detection of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in hemolymph of *Dermacentor andersoni* (Acari:Ixodidae) with the polymerase chain reaction. J. Med. Entomol. 30: 780-788.
Suarez CE, Mcelwain TF, Echaide I, De Echaide ST, Palmer GH (1994). Interstrain conservation of *Babesia* RAP-1 surface-exposed B-cell epitopes despite rap-1 genomic polymorphism. Infect. Immun. 62: 3576-3579.
Suarez CE, Palmer GH, Florin-Christensen M, Hines SA, Hotzel I, McElwain TF (2003). Organization, transcription, and expression of rhoptry associated protein genes in the *Babesia bigemina* rap-1 locus. Mol. Biochem. Parasitol. 127: 101-112.
Suarez CE, Palmer GH, Jasmer DP, Hines SA, Perryman LE, McElwain TF (1991). Characterization of the gene encoding a 60-kilodalton *Babesia bovis* merozoite protein with conserved and surface exposed epitopes. Mol. Biochem. Parasitol. 46: 45-52.
Suarez CE, Palmer GH, LeRoith T, Florin-Christensen M, Crabb B, McElwain TF (2004). Intergenic regions in the rhoptry associated protein-1 (rap-1) locus promote exogenous gene expression in *Babesia bovis*. Int. J. Parasitol. 34: 1177-1184.
Taillardat-Bisch AV, Raoult D, Drancourt M (2003). RNA polymerase beta-subunit-based phylogeny of *Ehrlichia* spp., *Anaplasma* spp., *Neorickettsia* spp. and *Wolbachia pipientis*. Int. J. Syst. Evol. Microbiol. 53: 455-458.
Thammasirirak S, Siriteptawee J, Sattayasai N, Indrakamhang P, Araki T (2003). Detection of *Babesia bovis* in cattle by PCR-ELISA. Southeast Asian J. Trop. Med. Public Health 34: 751-757.
Theiler A (1910). *Anaplasma marginale* (gen and spec., nov.). The marginal points in the blood of cattle suffering from a specific disease. Report of the Government on Veterinary Bacteriology in Transvaal, Department of Agriculture 1908-1909.
Torioni de Echaide S, Knowles DP, McGuire TC, Palmer GH, Suarez CE, McElwain TF (1998). Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. J. Clin. Microbiol. 36: 777-782.
Ullu E, Tschudi C, Chakraborty T (2004). RNA interference in protozoan parasites. Cell Microbiol. 6: 509-519.
Viseshakul N, Kamper S, Bowie MV, Barbet AF (2000). Sequence and expression analysis of a surface antigen gene family of the rickettsia *Anaplasma marginale*. Gene 253: 45-53.
Visser ES, Ambrosio RE, DeWaal DT (1991). An *Anaplasma centrale* DNA probe that differentiates between *Anaplasma ovis* and *Anaplasma marginale* DNA. Vet. Microbiol. 28: 313-325.
Visser ES, McGuire TC, Palmer GH, Davis WC, Shkap V, Pipano E, Knowles DP (1992). The *Anaplasma marginale* msp5 gene encodes a 19-kilodalton protein conserved in all recognized *Anaplasma* species. Infect. Immun. 60: 5139-5144.
Wall NR, Shi Y (2003). Small RNA: can RNA interference be exploited for therapy? Lancet 362: 1401-1403.
Wilkowsky SE, Farber M, Echaide I, Torioni de Echaide S, Zamorano PI, Dominguez M, Suarez CE, Florin-Christensen M (2003). *Babesia bovis* merozoite surface protein-2c (MSA-2c) contains highly immunogenic, conserved B-cell epitopes that elicit neutralization-sensitive antibodies in cattle. Mol. Biochem. Parasitol. 127: 133-141.
Wright IG, Goodger BV, Buffington GD, Clark IA, Parrodi F, Waltisbuhl DJ (1989). Immunopathophysiology of babesial infections. Trans R Soc. Trop. Med. Hyg. 83 Suppl:11-13.
Zahler M, Rinder H, Gothe R (2000). Genotypic status of *Babesia microti* within the piroplasms. Parasitol. Res. 86: 642-646.
Zhang Y, Palmer GH, Abbott JR, Howard CJ, Hope JC, Brown WC (2003). CpG ODN 2006 and IL-12 are comparable for priming Th1 lymphocyte and IgG responses in cattle immunized with a rickettsial outer membrane protein in alum. Vaccine 21: 3307-3318.