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Review

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

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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-

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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 L12eI/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).

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