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Invited Review

Bovine babesiosis in the 21st century: Advances in biology and functional genomics

Sejal Gohil, Susann Herrmann, Svenja Günther, Brian M. Cooke *

Department of Microbiology, Monash University, Victoria 3800, Australia

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ABSTRACT

Bovine babesiosis caused by the protozoan parasite, *Babesia bovis*, remains a significant cause of avoidable economic losses to the livestock industry in many countries throughout the world. The molecular mechanisms underlying the pathophysiology of severe disease in susceptible cattle are not well understood and the tools available to study the biology of the parasite, including technologies for genetic manipulation, have only recently been developed. Recent availability of multiple parasite genomes and bioinformatic tools, in combination with the development of new biological reagents, will facilitate our better understanding of the parasite. This will ultimately assist in the identification of novel targets for the development of new therapeutics and vaccines. Here we describe some recent advances in *Babesia* research and highlight some important challenges for the future.

1. Bovine babesiosis: an overview

Babesiosis is caused by intraerythrocytic apicomplexan parasites of the genus *Babesia*, which are transmitted by ticks of the Ixodidae family to a variety of vertebrate hosts including domestic, wild and farm animals. With worldwide distribution of the ixodid tick, babesiosis is the second most common blood-borne disease of free living animals (Homer et al., 2000; Hunfeld et al., 2008) and, importantly, is gaining increasing interest as an emerging zoonosis of humans (Homer et al., 2000; Kjemtrup and Conrad, 2000; Zintl et al., 2003; Hunfeld et al., 2008; Leiby, 2011). The cattle industry is particularly affected by *Babesia* parasites as over half of the world's 1.2 billion cattle are at risk of infection and disease (Bock et al., 2004). Over the next decade, in Australia alone, it is estimated that this disease will cost the agricultural sector up to AUD 300 million (Sackett et al., 2006) as a direct consequence of the infection resulting in death, increased abortion rate and sterility, reduced milk and meat production and/or the cost of prevention and treatments (Bock et al., 2004). This estimate, however, is also likely to be an under representation of the local or global economic burden, particularly since information relating to the cost of losses due to decreased productivity or cattle 'tick worry' is often not up to date or readily available.

The most common *Babesia* species infecting cattle and those that are most widely represented in the current literature include *Babesia bovis*, *Babesia bigemina* and *Babesia divergens*. The global distribution of these species is dependent on the ixodid ticks that are responsible for transmission of these parasites between cattle. Both *B. bovis* and *B. bigemina* are most commonly transmitted between cattle by the tick vectors *Rhipicephalus* (*Boophilus*) *microplus* and *Rhipicephalus annulatus* (and *Rhipicephalus decoloratus* for *B. bigemina* alone) and are primarily found in tropical and subtropical regions of the world including Australia, Africa, Asia and the Americas. In contrast, *B. divergens* is transmitted almost exclusively by *Ixodes ricinus* and is localised to northern Europe (Zintl et al., 2003; Bock et al., 2004; Chauvin et al., 2009).

Babesia parasites enter susceptible cattle hosts with the saliva of infected feeding ticks. Unlike the closely related apicomplexan parasites, *Plasmodium* spp. and *Theileria* spp., the lifecycle of *Babesia* parasites within the vertebrate host is relatively simple, with the parasite directly invading the host red blood cells (RBCs) without the need for an exoerythrocytic stage. Once inside the RBC, the parasite divides asexually by binary fission to produce daughter parasites (merozoites) that are released into the circulation upon rupture of the host RBC to continue the cycle of replication. This rapid, perpetual cycle of RBC invasion and destruction accounts for the clinical signs associated with bovine babesiosis, with the clinical severity of disease directly associated with the *Babesia* species responsible for the infection. The pathogenesis of *B. bigemina* and *B. divergens* infection is quite similar, with both parasites principally inducing intravascular haemolysis and associated anaemia, jaundice and haemoglobinuria. Whilst similar symptoms occur later in the course of disease associated with *B. bovis* infections, there is an earlier, more complicated and severe reaction associated with the induction of cytokines and other pharmacologically active

* Corresponding author. Tel.: +61 3 9902 9146; fax: +61 3 9902 9222.
E-mail addresses: editor@ijp.org.au, brian.cooke@monash.edu (B.M. Cooke).

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agents (Bock et al., 2004). *Babesia bovis* also has the ability to dramatically alter the structure and function of the infected RBC (for reviews see Cooke et al., 2005; Gohil et al., 2010). This is accompanied by the accumulation of parasitised RBCs (pRBCs) in the microvasculature in a number of organs including the brain and lungs, and the subsequent development of often fatal clinical complications such as cerebral babesiosis, respiratory distress and multi-organ failure.

Due to the propensity to produce clinically severe and sometimes fatal disease with significant worldwide economic impact, this review will focus largely on *B. bovis*. Here we summarise the current standing of *B. bovis* research, highlighting recent progress in understanding the intricate biology of the parasite associated with advances in the development of biological tools and their use in a post-genomic era; and the challenges in developing new intervention strategies such as effective subunit recombinant vaccines.

2. Current advances in understanding the biology of *B. bovis*

Overall, advances in our understanding of the biology of the parasite itself have been slow and arduous, with only a handful of research groups worldwide taking up the challenge to shed light on this haemoprotozoan. Consequently, fundamental biological questions remain unanswered, including the biological characteristics that define sporozoites and gametocytes, the precise duration of the asexual blood stage cycle, and how and when the parasitophorous vacuole, in which the parasite initially resides, disintegrates. Only very recently was the chief obstacle overcome that prevented us from answering these important questions; that is the ability to transiently and stably transform the parasite and genetically manipulate its genome (Suarez et al., 2004, 2006; Suarez and McElwain, 2008, 2009, 2010). With recent advances in comparative genomics, proteomics and transcriptomics, and the ability to culture the parasite in vitro with relative ease (Jackson et al., 2001), there is now great potential to expand our knowledge of the biology of the parasite at a previously unprecedented rate.

2.1. Babesia bovis transfection technologies

Release of the first sequenced and annotated genome of *B. bovis* has, with the application of available transfection technologies, permitted access to the parasite nucleus. Currently, two transfection techniques are available for the genetic manipulation of *Babesia* parasites. These include transient and stable transfection systems, which have been recently reviewed in detail (Suarez and McElwain, 2010). Transfection vectors used in these systems were based on the pBluescript (Stratagene, Santa Clara, CA, USA) plasmid background, and suitable parameters (cuvette size, voltage and capacitance) and transfection methods (electroporation and nucleofection) were tested using both free merozoites and infected RBCs. Overall, transfection efficiency was greater when using high voltage/low capacitance; and transfection using nucleofection resulted in higher parasite viability compared with electroporation, although certain experimental conditions resulted in both techniques demonstrating similar efficiencies (Suarez and McElwain, 2010).

Transient transfection is generally used for short-term expression of reporter genes in order to test promoter efficiency. Recently, this technique was used to characterise the promoter structure in the *ves* multi-gene family of *B. bovis* (Wang et al., 2012) using GFP and luciferase reporter genes. On the other hand, stable transfection relies on the use of selectable markers in order to select for mutant parasite lines long-term. This technology can be applied widely and is used for episomal protein expression as well as for integration of the plasmid into the genome. Currently, only two selectable markers have been reported for transfection of *Babesia* parasites, the identification of which has been based on those in use for the malaria parasite, *Plasmodium falciparum*. The *blasticidin deaminase* gene was the first selectable marker published for use in stable transfection of *Babesia* parasites. This confers resistance on the parasite to blasticidin S hydrochloride and was deemed suitable as the *B. bovis* genome lacks any related blasticidin genes and growth inhibition assays demonstrated the effectiveness of the drug at killing wild-type *Babesia* parasites in vitro (Suarez and McElwain, 2009). More recently, the WR99210/*dhfr* selection system was used to generate the first ever *B. bovis* gene knockout transgenic parasite line by targeted disruption of *B. bovis* thioredoxin peroxidase-1 (TPx-1) (Asada et al., 2012).

The advantage of the development of these *B. bovis* transfection technologies allows any generated mutant parasite lines to not only be studied in vitro, but also in their natural animal host in vivo. The proof of principle behind this concept was recently shown with the successful long-term, stable expression of GFP following inoculation of calves with a GFP-expressing *B. bovis* parasite line (Suarez et al., 2012). While this is an immense step forward for *Babesia* research, the difficulties, cost and level of biological containment associated with large-animal studies restrict such powerful studies to only a very few laboratories worldwide.

2.2. Difficulties with genetic manipulation and functional studies

The tools and reagents available for genetic and functional studies of *B. bovis* are still in their infancy and there is a great deal to be developed and refined, including transfection technologies. Low transfection efficiency has previously been reported for *P. falciparum* (O'Donnell et al., 2002) and is a major obstacle, as exogenous DNA must pass through three membranes to reach the parasite nucleus. This may be overcome by direct transfection of merozoites, as is routinely performed for the rodent malaria parasite, *Plasmodium berghei* (van Dijk et al., 1995), and has recently been shown with at least some degree of success in *B. bovis* (Suarez and McElwain, 2008, 2010). Furthermore, maintenance of the transfected plasmid as an episome within the parasite could be another major difficulty, particularly where integration into the parasite genome is the desired outcome. *Plasmodium falciparum* is known to be a culprit of such behaviour, with stable episomes often forming large concatemers that require multiple drug cycling to eliminate the plasmid (Kadekoppala et al., 2001; O'Donnell et al., 2001). This phenomenon has not yet been described in *B. bovis* parasites, however only a small number of transfected parasite lines have been generated to date. In an attempt to overcome this potential problem in other apicomplexans, negative selectable markers have been used, including the ganciclovir/*thymidine kinase* (Duraisingh et al., 2002) and 5-fluorocytosine/*cytosine deaminase* selection systems (Duraisingh et al., 2002; Maier et al., 2006). Negative selectable markers for *Babesia* parasite transfection have not yet been described. With only *blasticidin deaminase* and dihydrofolate reductase (*dhfr*) utilised to date for positive selection of transfected *Babesia* parasites, the identification of new positive and negative selectable markers will be paramount for the future study of *Babesia* genes, particularly as there is the potential for the parasite to rapidly become resistant to these drugs. Novel selectable markers could be identified with the characterisation of enzymes involved in biosynthetic pathways, which would facilitate the identification of putative drug targets. Furthermore, to overcome the lack of selectable marker genes, site-specific recombinases as shown for *Toxoplasma gondii* with the Cre/Lox-system (Brecht et al., 1999); and the Flp/FRT (van Schaijk et al., 2010; O'Neill et al., 2011) and Cre/Lox-system (O'Neill et al., 2011) used for *P. falciparum* should also be examined for their effectiveness in *B. bovis*.