Trends in Parasitology

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REVIEWS

Review

Babesiosis Vaccines: Lessons Learned, Challenges Ahead, and Future Glimpses

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The incidence and prevalence of babesiosis in animals and humans is increasing, yet prevention, control, or treatment measures remain limited and ineffective. Despite a growing body of new knowledge of the biology, pathogenicity, and virulence of *Babesia* parasites, there is still no well-defined, adequately effective and easily deployable vaccine. While numerous published studies suggest that the development of such anti-*Babesia* vaccines should be feasible, many others identify significant challenges that need to be overcome in order to succeed. Here, we review historic and recent attempts in babesiosis vaccine discovery to avoid past pitfalls, learn new lessons, and provide a roadmap to guide the development of next-generation babesiosis vaccines.

Babesiosis: An Emerging Tick-Transmitted Disease with Significant Global Impact

Babesiosis, caused by unicellular, apicomplexan haemoparasites of the genus *Babesia*, is one of the most widespread tick-transmitted infections in the world with substantial impact on both animal and human health [1,2]. *Babesia* parasites are transmitted by hard ticks of the family Ixodidae and have evolved to infect a wide range of vertebrate hosts [3]. In humans, babesiosis, caused by *Babesia microti*, *B. divergens,* and to a lesser extent, *B. duncani* and *B. venatorum*, has significant and increasing medical importance, with severe, sometimes fatal disease in elderly, marginalised, or immunocompromised individuals [2,4]. The United States Centers for Disease Control and Prevention (CDC) recognised the increasing emergence of human babesiosis in 2011 and added the disease to the list of nationally notifiable conditions [5]. In animals, bovine babesiosis, predominately caused by *B. bovis*, *B. bigemina,* and *B. divergens*, has severe economic impact on livestock industries in tropical, subtropical (*B. bovis* and *B. bigemina*), and temperate (*B. divergens*) regions of the world. More than 500 million cattle are at risk of infection globally, thus posing a sizeable threat to animal health and human livelihood in regions where *Babesia* parasites and their competent tick vectors are coendemic [1,6]. Canine babesiosis, caused by *B. canis* or *B. gibsoni*, is also of substantial veterinary importance, and results in high morbidity and mortality in dogs in Europe, Asia, and the USA [7,8]. Further, changes in climate and extreme weather events, socioeconomic and sociodemographic factors pose a serious and increasing impediment to the control of babesiosis worldwide [3]. Generally, babesiosis manifests clinically as fever, anaemia, haemoglobinuria, and splenomegaly, but can progress to much more severe complications, including acute circulatory shock, cerebral babesiosis, and multiorgan failure, all of which are frequently fatal, even if treated [5].

Current control of bovine or canine babesiosis relies on specific and sensitive diagnosis of parasites in the blood of infected animals with subsequent use of nonsustainable control measures, such as chemotherapeutics (predominantly imidocarb dipropionate or diminazene aceturate), live vaccines with inherent limitations, or control of ticks using acaricides. In reality, a well-defined, effective, safe, sustainable, and easily deployable **subunit vaccine** (see Glossary) is an urgent necessity if we are to effectively control these human, veterinary, and agriculturally important *Babesia* parasites. In this

Highlights

Live vaccines against *Babesia bovis* and *Babesia bigemina* have multiple drawbacks, so effective and easily deployable new subunit vaccines are urgently needed to control these parasites.

Three decades of extensive research into nonlive anti-*Babesia* vaccine development has produced some sweet successes, but also some harsh failures.

Successful vaccination of dogs against *Babesia canis* infection, and gerbils against *Babesia divergens* infection, using recombinant subunit vaccines, strongly suggest that a subunit vaccine against other species of *Babesia* parasites is feasible.

Effective use of next-generation tools will uncover the complex biology of *Babesia* parasites.

Promising, and cutting-edge ways to realise a next-generation babesiosis vaccine are proposed.

Sustainable control of babesiosis will require multiple, innovative, and integrated new research strategies.

A combination of blood-stage vaccines, tick-stage vaccines, and anti-tick vaccines would be a robust strategy to control or eliminate babesiosis.

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review, we consolidate decades of research on *Babesia* vaccines in an attempt to learn new and valuable lessons and identify new strategies to enable the development of an effective and easily deployable vaccine against babesiosis in the not too distant future.

Immunity to *Babesia* Infections: Insights for Future Vaccine Development

Our limited understanding of host immunity to *Babesia* infections is largely based on studies in cattle, due to the lack of a suitable smaller-animal model for bovine babesiosis. Immune responses to *Babesia* parasites involve both innate and adaptive immunity. Infections in asplenic humans or animals tend to be much more severe and fatal than in those with an intact spleen, suggesting a major role of the spleen in parasite clearance and control of the infection [3,9]. Additionally, younger animals are more resistant than older animals to *Babesia* infection, and the immunological basis for this age-related resistance seems to be largely innate, and related to differences in T helper 1 (Th1)-type cytokine responses and the release of **nitric oxide** (**NO**) [3,9]. **γδ T cells** also appear to be involved; however, their precise role in age-related disease resistance currently remains unknown. γδ T cells are essential for clearance of blood-stage *Babesia* parasites in rodent models [10] and they represent a much higher proportion of the mononuclear cell population in young calves when compared to adult animals (70% and 30%, respectively) [11]. Furthermore, antigens derived from the human malaria parasite, *Plasmodium falciparum,* have been shown to stimulate peripheral blood-derived γδ T cells, resulting in proinflammatory cytokine release and protection against *P. falciparum* [12]. Overall, a better understanding of both innate and adaptive immune responses that underpin immunity in young animals, and parasite control and persistence in older animals, respectively, will greatly inform and enhance the design of more effective, future anti-*Babesia* vaccines.

When compared to mammals, the immune system of arthropods is relatively less complex and lacks the ability to develop adaptive immune responses. Furthermore, *Babesia* parasites have coevolved with ticks, their arthropod definitive hosts, by developing the ability to undergo sexual reproduction and biological amplification, apparently without major interference of the tick's immune system. However, investigations of tick immunity, or *Babesia* proteins involved in the modulation of tick immunity, have been scarce, and comparative studies with other invertebrates have provided insight into the tick immune system [13]. Collectively, one may speculate that *Babesia* proteins that modulate the tick immune system in order to establish infection could also be ideal targets for transmission-blocking vaccines. Unfortunately, such antigens remain to be identified and characterised. A better understanding of innate immune responses in young, naïve animals infected with *B. bovis,* adaptive immune responses in adult animals with persistent levels of parasitaemia, and tick immune responses to *Babesia* parasites, is critical to develop strategies to induce a protective immune response in vaccinated individuals, and more research is warranted to close this important knowledge gap.

What Type of Vaccine Is Required to Protect against Babesiosis?

Vaccines to protect against bovine babesiosis could be based on two distinct approaches (Figure 1, Key Figure): (i) vaccines aimed at preventing parasite transmission from tick to host or preventing clinical disease (a blood-stage vaccine), or (ii) vaccines aimed at preventing parasite transmission between hosts and ticks (a tick-stage vaccine). In susceptible adult cattle, *Babesia* infections are characterised by an acute clinical stage, which can become severe and fatal within 10 days after the acquisition of the initial infection. Animals that survive the acute stage of infection remain persistently infected and become long-term carriers of the parasite – a feature that facilitates and perpetuates parasite transmission in endemic areas [1,3]. Despite a set of common clinical syndromes that generally accompany *Babesia* infections, not all *Babesia* parasites cause clinically-identical disease. Significant differences in transmission, virulence, and mechanisms of

Glossary

***Babesia* sensu lato:** *Babesia* species that lack the ability of transovarial transmission.
***Babesia* sensu stricto:** *Babesia* species that display transovarial transmission. *Babesia* species from this lineage are considered as 'true' *Babesia* species.
Gamma-delta (γδ) T cells: cells defined by expression of heterodimeric T cell receptors composed of γ and δ chains. They constitute a small subset of T cells in peripheral blood.
Glycosylation: a common post-translational modification of proteins. Glycosylated proteins contain a carbohydrate moiety, which can alter stability, activity and subcellular localisation of proteins.
Heterologous challenge: animals are vaccinated with parasites or proteins of a particular strain of *Babesia* then challenged with a different strain to assess vaccine efficacy.
Homologous challenge: animals are vaccinated with parasites or proteins of a particular strain of *Babesia* then challenged with the same strain to assess vaccine efficacy.
ISCOMATRIX: the term ISCOMATRIX is derived from ISCOM (immune-stimulating complex). ISCOM is formulated into a matrix by combining saponin, cholesterol, phospholipids, and hydrophobic antigens.
Killed parasites: parasites purified from *Babesia*-infected red blood cells that have been killed/inactivated by a freeze/thaw cycle.
Liposomes: small spherical vesicles prepared from cholesterol and natural non-toxic phospholipids *in vitro*.
Liquid-chromatography mass spectrometry (LC-MS): a robust biophysical detection method used for the analysis and detection of proteins, lipids, metabolites, and other trace components.
Nitric oxide (NO): is synthesised by many cell types involved in immunity and inflammation. It is an important toxic defence molecule against infectious organisms.
Soluble parasite antigens (SPAs): antigens released into the culture medium containing actively growing *Babesia*-infected red blood cells.
Subunit vaccine: a vaccine comprising minimal microbial components necessary to stimulate durable protective immune responses.

pathogenesis exist among different *Babesia* species; this suggests that identification of different therapeutic targets for different species of *Babesia* parasites will be required, as for the development of new and novel vaccines. A pan-protective vaccine containing a single antigen that protects against all species of *Babesia* parasites, while attractive, seems highly unlikely. Vaccines aimed at preventing parasite transmission from tick to host target the sporozoite stage of parasite development and block their invasion into host red blood cells, while a vaccine aimed at preventing clinical disease targets blood-stage parasites to ameliorate the devastating effects of the acute phases of infection (predominantly anaemia and circulatory shock). Such vaccines would allow animals to mount an effective protective immune response prior to parasite-mediated immune modulation of the host to limit parasite numbers and associated pathology. Ideally, a vaccine targeting sporozoites and blood-stage parasites should be able to confer sterile immunity in vaccinated animals to reduce the parasite transmission; however, the realisation of such a vaccine is compounded by the marked difference in the pathogenicity of different *Babesia*

Variable merozoite surface antigens (VMSAs): a large family of antigenically variable proteins expressed on the surface of merozoite-stage *Babesia* parasites.

Virus-like particles: noninfectious, cage-like nanoparticle structures that lack a viral genome but can be spontaneously assembled using viral structural proteins expressed in heterologous expression systems.

Key Figure

Vaccine-Targetable Processes in the Life Cycle of *Babesia* Parasites

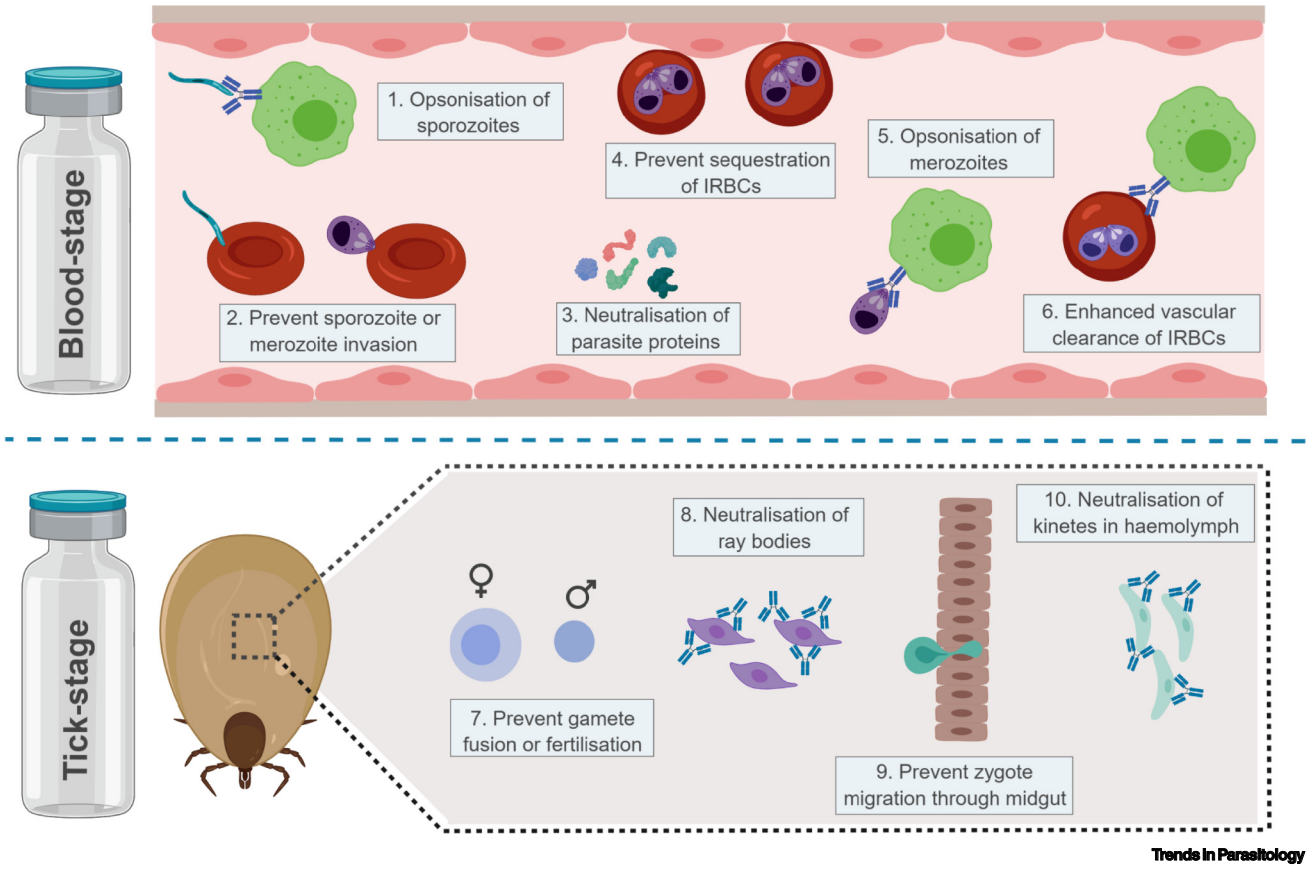

Figure 1. Recombinant subunit vaccines against *Babesia* parasites could potentially target various points in the multistage parasite life cycle. Parasite proteins expressed on the surface of sporozoites, merozoites, or the surface of parasite-infected red blood cells (IRBCs) are considered as key targets for blood-stage vaccine development to prevent parasite transmission and clinical disease [3,65]. Proteins involved in sexual-stage development of the parasite are considered as key targets for future tick-stage antiparasite vaccine development to prevent parasite transmission between hosts [3].

parasites. Thus, failure of blood-stage vaccines to provide sterile immunity against *Babesia* parasites in vaccinated animals would likely lead to persistent infection, albeit at low parasite density, and potentially result in persistent parasite transmission in endemic areas. Transmission-blocking vaccines, comprising tick-stage parasite antigens, that are able to block development of sexual-stage parasites in the tick could be combined with a blood-stage vaccine to prevent parasite transmission from ticks to bovine hosts in endemic areas; however, no such transmission-blocking vaccines are currently available, although this is an area of current intensive research. Historically, live vaccines containing virulence-attenuated parasites have been used to prevent the development of clinical disease associated with the presence of blood-stage parasites; however, widespread use of live vaccines is hampered by several drawbacks (Box 1). From the 1980s, babesiosis vaccine research was focussed on the development of safe and efficacious nonlive vaccines (blood-stage vaccines) to overcome the limitations of live vaccines by using the following key approaches: (i) vaccines containing **killed parasites** or native proteins; (ii) vaccines containing **soluble parasite antigens (SPAs**); and (iii) vaccines containing recombinant parasite proteins [9,14,15]. Despite a relatively large volume of work in this area, there are currently no commercially viable and sustainable vaccines available for the control of babesiosis. Overall, the magnitude of efforts dedicated to developing an effective babesiosis vaccine has faced both promising successes and harsh failures.

The Rise and Fall of Nonliving Vaccines

From the late 1960s, the focus of babesiosis vaccine research and development has swiftly moved toward nonlive vaccines using killed parasites or SPAs (sometimes referred to as

Box 1. Looking Back at the Good Old Days – Live Vaccine Research

Observations made in the USA during the late 19th century showed that animals that recovered from natural *Babesia* infections usually developed a long-lasting immunity, and inoculation of blood from recovered animals into recipient susceptible cattle produced less severe disease [63]. These findings led to the development of the first vaccine formulations containing blood from animals that had recovered from an acute infection and were widely used in many countries to vaccinate cattle against babesiosis [64,65]. Australia remains at the forefront of babesiosis vaccine development with the landmark discovery of virulence-attenuation of *Babesia* parasites following multiple passages of the parasite in splenectomised animals that laid the groundwork for modern live-vaccine development [64]. Following extensive contributions from researchers in Australia, a standardised protocol to produce a live vaccine against bovine babesiosis was realised and still remains in use in many countries. The live vaccine can only be applied to young (less than 12 months old) calves, and it effectively reduces the mortality associated with babesiosis in cattle. However, these vaccines have serious limitations, including the variable degree of protection afforded, potential tick transmissibility, reversion to virulence, viability, and logistical difficulties associated with vaccine production and deployment [63]. Despite these drawbacks, valuable lessons were learned from decades of live vaccine use in Australia to inform the future babesiosis vaccine development efforts. Even with these limitations, live-attenuated vaccines are still the cornerstone of babesiosis control in several countries and can certainly be improved while effective subunit vaccines remain under development.

Here are some lessons learned from live vaccine research:

- Combination of multiple vaccine strains was vital to achieve protection against heterologous challenge with virulent strains in the field.
- Cross protection against different species of *Babesia* parasites has been observed previously. Cattle previously immunised against *B. bigemina* showed relative resistance to subsequent challenge with *B. bovis*. Similarly, *B. microti*-preimmune mice showed resistance to subsequent challenge with *B. rodhaini.*
- Extended duration of immunity after parasite clearance in adult cattle. Animals that were completely recovered from previous *B. bovis* infection showed protection against reinfection with *B. bovis*, and these animals lacked *B. bovis*-specific antibodies prior to reinfection, demonstrating the importance of T cell immunity in protection against *B. bovis*.
- Poorly understood and controversial role of nitric oxide (NO) in parasite clearance.
- $CD4^+$ T cell activation and interferon (IFN)-γ production are vital for the development of protective immune responses against *B. bovis* infection.
- Younger animals are more resistant to challenge with virulent strains of *Babesia* parasites, and there is a crucial role for the spleen in this age-related protection.

exoantigens). Pioneering studies in Australia showed that killed parasite extract, used as vaccines against *B. bovis*, could generate effective protection levels similar to live vaccines [16,17] (Table 1). Subsequently, significant progress was achieved in the identification of protective antigens in killed parasite fractions, and three protective antigens (12D3, 11C5, and 21B4) were identified following extensive testing in cattle trials (see below). At a similar time, SPAs released into the peripheral blood during *Babesia* infection were shown to protect dogs and rats against *B. canis* and *B. rodhaini*, respectively, providing the rationale for the development of SPA-based vaccines [18]. Furthermore, the development of *in vitro* systems to culture *Babesia* parasites (*B. bovis* (1980), *B. canis* (1982), *B. bigemina* (1985), and *B. divergens* (1991)) were breakthrough discoveries [19] that allowed the production of adequate amounts of parasite material to be tested in experimental SPA vaccines (i.e., soluble antigens released into the culture medium) (Table 1). SPA vaccines prepared from *in vitro*-cultured *B. bovis* parasites demonstrated the proof of concept to protect cattle against tick challenge [20]. SPA vaccines were subsequently tested against other species of *Babesia* parasites, including *B. bigemina*, *B. canis*, *B. divergens*, and *B. orientalis* with promising outcomes [21–24]. The level of protection against *B. bovis* afforded by SPA vaccines was geographically variable, ranging from no protection in animals in the USA [25] to high levels of protection in South America [21] (significantly different levels of parasitaemia, packed cell volume reduction, and fever in vaccinated animals compared to control animals). Furthermore, even though trials in South America showed excellent protection against **homologous challenge** with *B. bovis*, the level of protection against **heterologous challenge** with *B. bovis* was lower and more variable, raising concerns about the development of an effective universal vaccine in endemic regions. The variable protection against heterologous challenge is a well established phenomenon described in both nonlive and live vaccines against babesiosis in animals, in addition to malaria in humans [26–28]. Similarly, SPAs derived from *B. divergens* and *B. orientalis* were shown to protect gerbils and buffalo, respectively, from homologous and heterologous parasite strains [23,24], demonstrating the feasibility of developing SPA-based vaccines against *Babesia* in livestock.

Initial efforts to develop a vaccine against canine babesiosis used SPA derived from the plasma of infected dogs and showed significant protection against infection in vaccinated naïve dogs. Vaccinated dogs showed lower levels of peripheral parasitaemia and less reduction in haematocrit when compared to nonvaccinated animals, and animals recovered from a challenge infection once an antibody response against SPA was detected in serum [18]. These promising results led to the development of the first commercial vaccine based on SPA derived from the plasma of a single *B. canis* strain isolated in France [29]. With the advent of an *in vitro* culture system for *B. canis*, production of this SPA vaccine was scaled up, and, as a result, was a great commercial success in the dog vaccine industry for many years. The *B. canis* SPA vaccine demonstrated excellent protection against homologous challenge, but limited protection against heterologous challenge was achieved [14,30]. Antigenic variation in merozoite- and infected red blood cell (IRBC)-surface proteins is the likely cause of SPA vaccine failure against heterologous parasites. Furthermore, the lack of protective immunity against heterologous *B. canis* parasites was overcome by combining SPA from *B. canis* and *B. rossi*, and vaccination with a mixture of SPA from *B. canis* and *B. rossi* protected dogs against heterologous *B. canis* challenge [31]. Despite the success of this SPA-based vaccine to protect dogs against babesiosis, the vaccine was recently withdrawn from the market by its manufacturer, for commercial reasons.

Progress towards Development of Subunit Anti-*Babesia* Vaccines

Following successful vaccination of animals against *Babesia* using killed parasites or crude parasite extract, the development of subunit vaccines started by using native parasite antigens isolated using monoclonal antibodies (generated with pure parasite fractions) or by size

Table 1. Experimental Anti-*Babesia* Vaccines

Vaccine antigen	Host	Adjuvant and number of vaccinations	Challenge infection	Protection	Refs
Parasite extracts					
B. bovis killed parasites	Cattle	FCA; 3	2 weeks (homologous)[a]	+++	[16,17,87]
B. bovis crude insoluble extract	Cattle	FCA; 3	2 weeks (heterologous)[a]	+++	[87]
B. bovis crude soluble extract	Cattle	FCA; 3	2 weeks (heterologous)[a]	++	
B. bovis crude soluble fraction	Cattle	FCA; 3	2 weeks (heterologous)[a]	+	[88]
B. bovis soluble fraction (fractionised)	Cattle	FCA; 3	1 month (homologous)[a]	+	[89]
SPA vaccines					
B. bovis SPA	Cattle	Saponin; 2	3 months[b]	++	[20]
B. bovis SPA	Cattle	Saponin; 2	8 weeks (heterologous)[a]	+	[25]
B. bovis SPA	Cattle	Quil-A; 2	2 weeks and 23 weeks (heterologous)[a]	+	[90]
B. bovis SPA	Cattle	Quil-A; 2	3 months (heterologous)[a]	+	[91]
		Quil-A; 2	1 month (heterologous)[a]	+	
B. bovis SPA	Cattle	Quil-A; 2	3 months (homologues and heterologous)[a]	+++	[92]
B. bigemina SPA	Cattle	Quil-A; 2		+++	
B. bovis SPA *B. bigemina* SPA	Cattle	Quil-A; 2	Field trial	++	[93]
B. divergens SPA	Cattle	Quil-A; 3	3 weeks (homologous)[a]	+++	[23]
B. bovis SPA	Cattle	Saponin; 2	4 weeks (heterologous)[a]	+++	[94]
B. canis SPA	Dogs	Saponin ; 2	3 weeks (homologous)[a]	+++	[95]
B. canis SPA	Dogs	Quil-A ; 2	3 weeks (heterologous)[a]	+++	[31]
B. orientalis SPA	Buffaloes	FCA; 2	4 weeks (homologous)[a]	+++	[24]
B. bovis SPA	Cattle	Saponin; 2	6 weeks (heterologous)[a]	+++	[27]
Native subunit vaccines					
B. bovis purified native antigen (2C3, 15B1, 18A5)	Cattle	FCA; 2	2 weeks (homologous)[a]	+++ (15B1) – (2C3 and 18A5)	[66]
B. bovis native antigen (29 kDa)	Cattle	FCA; 2	4 weeks (homologous)[a]	++	[96]
B. bovis native 70 kDa or RAP1	Cattle	FCA; 4	16 weeks (homologous)[a]	++	[74]
B. bovis purified native antigens	Cattle	FCA; 3	8 weeks (homologous)[a]	±	[97]
B. bigemina native RAP1	Cattle	FCA; 5	1 week (homologous)[a]	+++	[75]
B. bovis native antigen 11C5	Cattle	FCA; 2	4 weeks (homologous)[a]	++	[98]
Recombinant subunit vaccines					
B. bovis r5-10 peptide	Cattle	Quil-A and FCA; 3	Five months[b]	–	[99]
B. bovis rMSA-1	Cattle	Saponin; 4	3 weeks (homologous)[a]	–	[100]
B. bovis r11C5	Cattle	Saponin; 3	4 weeks[b]	+	[101]
B. bovis rRAP-1	Cattle	RIBI; 4	5 months (homologous)[a]	–	[49]
B. bovis r11C5 and r12D3	Cattle	Montanide ISA 50V, Quil A and DEAE dextran; 2	2 weeks (heterologous)[a]	++	[68]
B. bovis rRAP-1	Cattle	FCA and FIA; 3	6 weeks (heterologous)[a]	–	[27]
B. divergens rBd37	Gerbils	Saponin; 2	3 weeks (heterologous)[a]	+++	[33]
B. bovis rMSA-1, rMSA-2c and r12D3	Cattle	Montanide 75; 2	2 weeks (heterologous)[a]	+	[70]

(continued on next page)

Table 1. (continued)

Vaccine antigen	Host	Adjuvant and number of vaccinations	Challenge infection	Protection	Refs
B. canis rCBA	Dogs	Saponin; 3	2 weeks (homologous)[a]	+++	[34]
Recombinant subunit vaccines					
Profilin from *B. microti, B. bovis and B. bigemina*	Mice	FCA; 4	2 weeks (heterologous *B. microti*)[c]	++	[102]
B. microti MSA	Mice	FCA and FIA; 3	2 weeks (homologous)[c]	++	[103]

Abbreviations: CBA, canine babesiosis antigen; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; MSA, merozoite surface antigen; r, recombinant; RAP, rhoptry-associated protein; SPA, soluble parasite antigen.
[a]Direct intravenous challenge (needle) with *Babesia* parasites.
[b]Natural challenge via infected ticks.
[c]Intraperitoneal challenge.

fractionation. A handful of native antigens isolated from parasite fractions showed protective responses against *Babesia* infections in animals (Table 1) and provided a promising lead for recombinant subunit vaccine development. In the late 1980s, subunit vaccine development against babesiosis gained momentum with the advent of recombinant DNA technology. Several studies have evaluated a variety of different proteins of *B. bovis* as subunit vaccines to protect cattle; however, variable levels of protection against parasite challenge impose a significant and ongoing challenge to the development of effective subunit vaccines against *B. bovis* (Box 2 and Table 1).

Box 2. Subunit Vaccines against *B. bovis*

Several immunodominant antigens have been identified from killed parasite extracts using monoclonal antibodies, including the 70 kDa immunodiffusion (ID) antigen, the 200 kDa antigen, and an antigen of more than 1500 kDa in molecular weight (the 3C1 antigen) [66]. All of these immunodominant proteins showed a lack of protection against *B. bovis* in cattle trials despite inducing high antibody titres in vaccinated animals. In addition, the protective effect of 12D3 was abolished in combination with 3C1, confirming the suppressive properties of immunodominant antigens. Early studies with recombinant forms of two nonimmunodominant antigens, 11C5 and 12D3, either alone or in combination, produced promising results; however, the challenge infection used less-virulent strains than the circulating field strains [67]. Further studies with these antigens produced variable results and were reported in published reviews as 'unpublished data'. Furthermore, re-evaluation of 11C5 and 12D3, alone or in combination, showed less protection compared to the earlier studies against a virulent *B. bovis* strain [68], confirming the partial protective nature of these antigens.

Antibodies against members of **variable merozoite surface antigens** (**VMSAs**) of *B. bovis* were able to neutralise merozoite invasion into red blood cells and stimulate T cell lines isolated from *B. bovis*-infected cattle [69]. Therefore, VMSAs were considered to be potential targets for new subunit vaccines. Two immunodominant antigens from the VMSA family, MSA-1 and MSA-2c, either alone or in combination with 12D3, failed to prevent the development of clinical symptoms after *B. bovis* challenge [70]. These studies demonstrated that *in vitro* neutralisation tests are poor predictors of protective immune responses. Furthermore, in the case of live vaccines, significant sequence polymorphisms have been reported for MSA-1 and MSA-2 in vaccine breakthrough isolates compared to vaccine strains, suggesting a partial role for members of the VMSA family in evading the vaccine-induced protective responses [71].

The *B. bovis* rhoptry-associated protein, RAP-1, also termed 21B4, was a component of the protective 11C5 and 12D3 vaccines (described above [67]), and it was also identified in independent studies as a vaccine candidate antigen, and named Bv60 [72]. Rhoptry-associated proteins (RAPs) with potential involvement in red blood cell invasion were shown to protect against *B. bigemina* and *B. bovis* in native form [73–75]; however, recombinant RAP-1 failed to protect against *B. bovis* challenge, despite stimulating IFN-γ producing $CD4^+$ T cells and IgG [49]. *B. bovis* RAP-1 protein possessed the features of appropriate vaccine antigens, including major histocompatibility complex (MHC) class II recognition, conserved B cell and T cell epitopes, and high immunoreactivity. Intriguingly, these are also common features of immunodominant antigens, which raises the question as to whether potentially subdominant antigens might be critical for inducing protection against *Babesia*. The recently discovered RAP-1-related antigen, RRA, with marked characteristics of a subdominant antigen, may represent an alternative reasonable target for a subunit vaccine [76]. Collectively, these data demonstrate the difficulties faced in the identification of protective antigens in *B. bovis* and the need for native-like proteins to elicit protection in vaccinated animals.

Promising progress was achieved in developing subunit vaccines against *B. divergens* or *B. canis* with the identification of a single, albeit different, protective antigen for each of these two different *Babesia* species [32–34]. Bd37 is a GPI-anchored glycoprotein that was identified in SPA fractions from *B. divergens*, elicited promising protection against homologous and heterologous parasite challenge in gerbils that was related to the presence of hydrophobic residues in the vaccine antigen [32,33]. Canine *Babesia* antigen (CBA), was identified in SPA fractions from *B. canis*. Vaccination of dogs with recombinant CBA protected them against challenge with homologous *B. canis* parasites [34]; however, its efficacy against challenge with heterologous parasites has never been reported (Table 1).

Over the past three decades, efforts toward the development of subunit vaccines against babesiosis have revealed mixed outcomes, with protective antigens identified against *B. canis* and *B. divergens* but not for *B. bovis.* Failure to identify protective antigens against *B. bovis*, albeit with only a handful of antigens tested, could be attributed to the enhanced pathogenicity of *B. bovis* when compared to other *Babesia* species. However, the protective efficacy of SPA vaccine candidates against challenge with homologous *B. bovis* strains [19], and the identification of protective antigens in *B. canis* and *B. divergens* [33,34], strongly suggest that a subunit vaccine against *B. bovis* and other *Babesia* parasites (*B. bigemina* and *B. orientalis*) is feasible. Protection induced by vaccination against *Babesia* parasites appears to be both antibody-mediated and cell-mediated, as demonstrated by studies on *B. bovis* in cattle and *B. canis* in dogs. A closer examination of subunit vaccine studies in *B. bovis* suggests some potential experimental limitations: (i) recombinant *B. bovis* antigens tested as subunit vaccines have been expressed in *Escherichia coli*, which may be associated with a lack of conformational structure in the vaccine antigens that might have interfered with antigen processing and presentation; and (ii) a very limited number of antigens have been tested in vaccine trials to date. *B. bovis* antigens that have been tested as subunit vaccines so far contain 1 to 11 predicted disulphide bonds, and their production in *E. coli*, which lacks the ability to perform certain post-translational modifications that might be important to reproduce the native conformation of the protein, could have affected an appropriate stimulation of optimal, vaccine-mediated immune responses (Box 3).

Furthermore, during the last few decades, subunit vaccine development efforts have increasingly focussed on the use of appropriate adjuvants. Adjuvants aid in improving the immunogenicity of antigens by either controlling the rate of release, induction of local inflammation, recruitment of antigen-presenting cells (APCs) for antigen uptake, or cytokine secretion [35]. Saponin, an

Box 3. Considerations for Vaccine Antigen Design

Vaccine antigens produced in *E. coli* expression systems lack eukaryotic post-translational modifications, including **glycosylation** and the ability to form disulphide bonds (cytoplasmic protein expression in *E. coli*) that can affect protein conformation and certain pathogen-associated molecular patterns (PAMPs) [77]. This can result in a lack of PAMP-mediated activation of innate immune responses and effective downstream adaptive responses. In addition, the presence of disulphide bonds is an important determinant for vaccine antigen presentation to MHC class II molecules and effective T cell responses [78,79]. The importance of post-translational modifications in vaccine antigen design, and in particular, glycosylation, has been increasingly recognised in recent times, and vaccine antigens from apicomplexan parasites in a native conformation with post-translational modifications could generate effective vaccine-mediated immune responses [80,81]. Furthermore, the recognition of pathogen glycans is known to be mediated by the pathogen recognition receptors (PRRs) on antigen-presenting cells (APCs), such as Toll-like receptors and c-type lectin receptors, and this process is crucial to initiate the innate immune response and subsequent adaptive immunity. The activation of PRRs on APCs by sugar-associated PAMPs can lead to the activation of nuclear transcription factors, such as NF-κB and interferon regulatory factors, to initiate intracellular pathways towards the production of inflammatory cytokines [82]. Production of recombinant proteins in mammalian expression systems (e.g., human embryonic kidney 293 and Chinese hamster ovary cell lines) will provide near-identical native conformation in addition to glycosylation of recombinant proteins and achieve effective stimulation of the host immune system similar to native parasite proteins.

Box 4. A Potent Adjuvant Activity of ISCOM-Based Adjuvants

Saponins are steroids or triterpenoid glycosides widely distributed in plants, marine animals, and certain bacteria [83]. Saponin, vaccine antigens, phospholipids and cholesterol have been incorporated into cage-like nanoparticle structures or ISCOMs (40–50 nm in diameter) and delivered as a vaccine [84,85]. Several promising vaccine antigens from influenza virus, *Mycobacterium tuberculosis*, dengue virus, or *Trypanosoma cruzi* have been incorporated into ISCOMs to provide effective protection in subunit vaccines [85], and these observations suggest that ISCOM nanoparticles could also be efficient adjuvants in subunit vaccines against babesiosis. The mechanism of ISCOM nanoparticle adjuvant (i.e., **ISCOMATRIX**) was extensively studied in mice and humans, providing comprehensive data on the adjuvant action of ISCOMATRIX [85,86]. The key features of ISCOMATRIX include induction of Th1 and Th2-type immune responses, cross presentation of antigen to the MHC class I pathway, higher induction of IFN-γ, tumour necrosis factor (TNF)-α and NO, natural killer (NK) cell activation, and efficient presentation of antigen to resident and lymphoid dendritic cells [84–86]. Induction of cell-mediated responses with elevated Th1-type cytokine responses (vital for resistance against *Babesia* infection in animals), along with induction of higher antibody responses, suggests that ISCOMATRIX or ISCOM-based nanoparticles would be a most suitable adjuvant for subunit vaccines against babesiosis.

adjuvant commonly used in experimental anti-*Babesia* vaccines, appears to aid the induction of strong immune responses and is usually associated with protection in vaccinated animals; this warrants the inclusion of saponin as a gold standard adjuvant in future babesiosis vaccine studies. In addition, effective delivery of saponin in immune stimulating complexes (ISCOMs) appears to improve its adjuvant potency (Box 4), suggesting that a similar approach in subunit vaccines against babesiosis could improve protection levels in vaccinated animals.

Considering that *Babesia* parasites are transmitted by ticks, it is logical to propose that strategies designed to control tick vectors would also be likely to decrease parasite load in the field and consequently decrease infection in susceptible animals. A commercially available recombinant anti-tick vaccine based on the immune-protective tick mid-gut antigen Bm86 from *Rhipicephalus microplus* has been used to attempt to control ticks; however, the variable level protection achieved has prompted the search for alternative tick-expressed antigens for future vaccine development [36], and several new tick antigens are currently being explored as potential new vaccine targets, targeting functions such as blood coagulation and digestion (reprolysins), tick feeding, and tick biology (Aquaporins) [36–38]. Anti-tick vaccines are outside the scope of this current review but these have been reviewed recently elsewhere [36,39]. We suggest that targeting antigens in ticks, together with parasite-expressed antigens, in a single, next-generation, multivalent subunit vaccine could offer a novel and very effective approach to control *Babesia* infection, disease, and parasite transmission.

The Road to Novel, Next-Generation Babesiosis Vaccines

Novel Vaccine Antigen Discovery

Currently, only a limited number of antigens have been tested as subunit vaccines against *Babesia* parasites (*B. bovis*, *B. bigemina*, *B. divergens*, *B. canis*, and *B. microti*), representing only a small fraction of predicted protein-coding genes (~3400–3700) in the genomes of these parasites [40–42]. In the current postgenomic era, annotated genomes of *Babesia* parasites provide a large-scale, still relatively untapped, dataset for novel candidate antigen selection; however, prioritisation of candidates for evaluation in animal trials is still a significant limitation. Reverse vaccinology approaches (i.e., mining parasite genomes for antigens that are predicted to be secreted or surface expressed) have provided a comprehensive list of potential new vaccine candidates; however, this list remains large, warranting additional criteria to prioritise rational vaccine candidate antigen selection [42,43]. Proteomic approaches including enzymatic processing of IRBCs or whole parasites or secreted parasite antigens, followed by **liquid-chromatography mass spectrometry (LC-MS)**, provide a promising option to identify or quantify the global protein profile of *Babesia* parasites, providing a cutting-edge option to validate *in silico* predictions. However, proteomic studies in *Babesia* are limited [44–47], and uncovering the protein profile

of blood-stage parasites (e.g., merozoites or sporozoites), proteins expressed on the surface of IRBCs, or SPAs released into the culture medium, will significantly improve the data set for future vaccine candidate selection.

Vaccine Antigen Discovery Exploiting Antibody and T Cell Responses

Identification of parasite proteins using 'omics' approaches is likely to provide many potential new vaccine antigens; however, the key question of prioritising the candidate vaccine antigens to test in animal trials remains a complex issue. The selection of candidate vaccine antigens from a large list of potential antigens can be aided by carefully using humoral responses and cell-mediated responses from naturally protected animals to identify and select antigens that are associated with protective immune responses in animals. It is important to note that this approach has been tried against *B. bovis* (specifically focussing on merozoite surface proteins) and failed to identify protective vaccine antigens [9]. However, adopting the same approach, and focussing on antigens expressed on the surface of IRBCs and SPAs, in addition to merozoites, could potentially identify promising, new potentially protective antigens. Antibodies from animals that showed protection against infection with *Babesia* parasites (potentially from different genetic backgrounds) could be used to screen parasite proteins in order to select conserved and potentially protective antigens. Antibody-secreting cells, isolated from the spleen of younger animals infected with *Babesia*, can be used to identify the targets of local antibody responses in the spleen during an acute infection, and this robust method has been applied to identify vaccine antigens against parasites of other livestock [48]. Further, more advantage could be taken of cell-free protein systems to synthesise a large set of parasite proteins and use them in protein arrays to screen for antibodies in protected animals. Parasite proteins produced in a cell-free system could also be used in *ex vivo* T cell assays to uncover antigens with a strong potential to induce a T cell response using immune cells from protected animals [9,49]. *Ex vivo* assays include T cell proliferation against *ex vivo* antigens followed by measurement of antigen-induced cytokines (IFN-γ, TNF-α, etc.), and NO responses [50]. Based on data obtained with live vaccines and SPA vaccines, it is unlikely that neither T cell immunity nor the antibody-mediated immunity function as an independent mechanism to offer protection against babesiosis, suggesting that antigens targeted by both arms of the immune response will be ideal vaccine targets. Once a list of potential candidate antigens has been refined, using protein microarray and T cell assays, next-generation transfection technologies could be used to validate these candidate antigens [3], and those offering promising protective immune responses could be further tested as potential new subunit vaccines.

Exploiting Exosomes for the Discovery of Novel Anti-*Babesia* Vaccine Targets

Exosomes are small extracellular vesicles (~40 nm in diameter) which serve as carriers for proteins, nucleic acids, and lipids in host–parasite interactions and parasite–parasite interactions [51,52]. Secretion of exosomes has been reported for a number of parasites, and exosomes are present in soluble parasite fractions, but remain to be explored in *Babesia* [53]. Host cell-derived exosomes have been increasingly studied in *Plasmodium* spp., with important roles identified in nonclassical protein secretion, pathogenesis, immune modulation, and parasite–host interactions. Further, *Plasmodium yoelii*-infected, reticulocyte-derived exosomes have been shown to protect against *P. yoelii* infection in mice [54,55], providing a strong precedence to study *Babesia*-IRBC-derived exosomes as a source of novel vaccine targets. Currently, there are no reports in the literature of IRBC-derived exosomes in *Babesia* parasites; however, further studies in this area are clearly warranted.

Development of Efficacious Subunit Anti-*Babesia* Vaccines

Strategies for identifying novel antigens for the development of new subunit vaccines (targeting sprozoites, or merozoites, or enhanced vascular clearance of IRBCs) would be similar for

***Babesia* sensu stricto** (*B. bovis, B. bigemina*, *B. divergens* ,and *B. canis*) and ***Babesia* sensu lato** species (*B. microti-like* and *B. conradae*) (Figure 1); however, the identity of the target antigens might vary depending on differences in virulence factors between different *Babesia* parasite species [56]. Furthermore, *Babesia* parasites show extensive antigenic diversity, and notable differences in the pathogenicity among different species, making it difficult to envisage the development of a vaccine that would be universally effective against all species of *Babesia* parasites. Reflecting on previous efforts at subunit vaccine development against *Babesia* parasites of livestock (particularly *B. bovis*), it may not be surprising that subunit vaccines comprising single antigens have failed to protect against *B. bovis* challenge, particularly heterologous challenge. Ideally, an effective subunit vaccine for babesiosis would be based on a combination of conserved antigens that induce protective responses against *Babesia* parasites in proof-of-concept vaccine trials. New antigens, or new antigen combinations, with the potential to elicit synergetic effects as subunit vaccines against *Babesia* would be advantageous to achieve an effective subunit vaccine against babesiosis. Novel antigens, identified in proof-of-concept vaccine trials, shown promising efficacy as subunit vaccines against *Babesia*, either alone or in combination, can be potentiated to elicit stronger and durable protection with the use of state-of-the-art modern delivery systems, such as **liposomes** or **virus-like particles**. It will also be important not to exclude antigens eliciting low/moderate immune responses, such as subdominant antigens, in future pilot studies, since combination of these antigens in a cocktail vaccine could improve overall protective efficacy. Vaccine antigens that have been tested as potential subunit vaccines against *Babesia* in previous studies with low/moderate protection could be reproduced using an appropriate expression system, including new vaccine antigens identified in future studies in a cocktail subunit vaccine to achieve effective protection against the parasite. Looking ahead to the future of effective management/control of babesiosis, it would be highly valuable to combine blood-stage, sexual-stage, and tick vaccine antigens in a multicomponent vaccine. This approach would, in turn, progressively reduce *Babesia* transmission in endemic areas and provide herd immunity that ultimately could lead to elimination of babesiosis. Another attractive approach to overcome strain-specific protective immunity might be an attempt to overpower the parasite by targeting not just a few antigens, but a large number of antigens in a genetically modified whole-parasite vaccine.

Novel Whole-Parasite Vaccines

A novel and alternative approach to control apicomplexan parasites, including *Babesia*, is through the development of transmission-blocking vaccines (TBVs) [3,57]. Several lines of research, using genetic manipulation methods, may lead to more globally acceptable live vaccines that are nontransmissible, impaired in their ability to revert to virulence, or to the production of attenuated parasites that could be used as antigen-delivery systems, thus potentially converting live vaccines against *Babesia* into multipurpose vaccines. The recent production of a transgenic *B. bovis* parasite line lacking two genes involved in the development of sexual-stage parasites suggests that this is a feasible goal in the longer term [58]. Extensive knowledge developed in TBV development against *Plasmodium* has informed the selection of possible candidates for new TBVs against *Babesia*, and this strategy is supported by the high degree of conservation in the mechanisms of sexual-stage parasite development among related apicomplexans. Several sexual, and tick-stage antigens are currently in a pipeline to be tested as TBVs to control *B. bovis*, including HAP-2 [59], CCp1-3 [60], and 6-Cys [58]. The concept that an effective TBV could be developed against vector-transmitted apicomplexan parasitic diseases is supported by ongoing efforts to develop such a vaccine against malaria. Several *P. falciparum* antigens have been identified as promising TBV candidates in preclinical studies, including Pfs230, Pfs25, and Pfs48/45 [57]. Evaluation of Pfs25, and Pvs25 (its ortholog in *P. vivax*), as TBVs in Phase 1a clinical trials demonstrated transmission-blocking activity of these antigens, and functionally active vaccine-

induced antibodies blocked the development of mosquito-stage parasites in a dose-dependent manner [61,62].

Concluding Remarks

A century of babesiosis vaccine research has resulted in many exciting discoveries – from live vaccines to state-of-the-art molecular tools to study *Babesia* parasite biology and manipulate their genomes. There is a clear need to accelerate the development of next-generation control measures to improve animal and human health and countermand limitations in current control measures and the growing challenges posed by pathogen evolution and geographical spread. The hope of sustainable control of babesiosis must rest on the development of novel control measures (i.e., subunit vaccines) and a better understanding of the disease and the complex biology of the parasite (see Outstanding Questions). Lessons learnt from previous decades of vaccine development efforts against *Babesia* parasites of animals can be readily applied to human babesiosis vaccine development. Ultimately, a multicomponent vaccine containing antigens from multiple parasite stages (i.e., blood-stage and sexual stages in ticks) would be desirable to control disease severity and potentially achieve lower disease transmission and improved livestock production globally. To be achievable, this goal will require focussed and sustained efforts considering the lack of new protective antigens identified for *Babesia* parasites and rapid antigenic variation in parasites circulating in the field. In terms of an effective and protective *Babesia* vaccine, the future looks bright but will undoubtedly be full of new challenges and disappointments. We are certain, however, that current and future studies of parasite–vector–host interactions, parasite biology, and host immunology will bring the essential new information to allow the rational design of novel, sustainable, and effective control strategies to effectively manage babesiosis.

Outstanding Questions

What are the virulence factors involved in the superior pathogenicity of *B. bovis* compared to other species of *Babesia* parasites?

What is the comprehensive protein profile of SPA, surface antigens of blood-stage parasites, and IRBC-surfaced-expressed proteins?

What are the correlates of protective immunity in *Babesia* infections? How could we best overcome antigenic variation in *Babesia* parasites and design novel and efficient subunit vaccines?

How can we achieve sterile immunity against *Babesia* infections? Is this a realistic, future goal?

What are the glycans present in *Babesia* parasites, and how are they involved in pathogenesis and immune evasion?

Can we exploit glycan-based interactions with ticks for new transmission-blocking vaccines?

What are the immune-modulating factors in ticks, and can these be exploited as targets for transmission-blocking vaccines?

Are exosomes secreted by *Babesia*-*IRBCs*? If so, what proteins are present, and might they represent new and attractive vaccine candidates?

Is there any potential bottleneck in the understanding of *Babesia* parasite biology (e.g., understanding sexual reproduction in the parasites' tick vectors) that can be explored to design new disease-control strategies?

Do we have adequate expression systems to produce more 'native-like' *Babesia* antigens that might improve the efficacy of future anti-*Babesia* vaccines?

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