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Review Article

A Comprehensive Review of Tick-Born Blood Protozoan Disease in Cattle: Babesiosis and Vaccination

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Abstract

Babesiosis is a tick-borne blood protozoan cattle disease caused by the genus *Babesia* worldwide and in tropical and subtropical regions. It is mainly caused by *Babesia bovis* and *Babesia bigemina* in cattle. When merozoites enter the erythrocyte, the surface coat of *B. bigemina* produces many merozoite surface antigens. This study is a comprehensive review of the "Tick-Borne Blood Protozoan Cattle Disease: Babesiosis and Vaccination" survey. There is a need to develop effective vaccines to control bovine babesiosis (BB) under field conditions because of the development of drug resistance against it. *B. bigemina* live-attenuated vaccine is available in the form of infected red blood cells (RBCs) in Australia and Israel, which has the danger of disseminating exotic DNA to other countries. A recombinant protein-based vaccine is a good choice to be developed, but very few immunogens have been explored yet to launch vaccination against *B. bigemina*. It is concluded from this study that secretory proteins were characterized, and the 65 kDa protein was found to be immunogenic. Major surface protein (gp45) was found to be antigenic and immunogenic when sera samples of infected and vaccinated calves were screened. The multiepitope-based protein rec-gpME was expressed, and the calves were vaccinated. The vaccinated calves had higher humoral and CTL responses. This rec-gpME could be a potential vaccine candidate against the *B. bigemina* infection. This rec-gpME would be tested on cattle under field conditions in Pakistan in the future.

Keywords: *Babesia bigemina*, rec-gp45, Babesiosis, ELISA, Livestock

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Introduction

The total share of the agriculture sector in Pakistan's gross domestic product (GDP) between 2021 and 2022 was 19.8%, out of which the share of livestock was 53.2%. Subsequently, the total livestock population in Pakistan is about 238.1 million, which includes buffaloes, cattle, camels, sheep, goats, mules, horses, and donkeys. Small-scale–dairy farmers in Pakistan depend on livestock to meet their nutritional needs (1). Among the livestock species, buffaloes and cattle play a vital role in the country from the perspective of dairy farming. The estimated livestock population of buffaloes and cattle in Pakistan is 38.8 million and 46.1 million, respectively. Pakistan is ranked 4th among the highest milk-producing countries in the world (2). In Pakistan, the milk production from buffaloes and cattle from 2021 to 2022 was 35 136 and 20 903 thousand tons, respectively, whereas beef production stood at 2155 thousand tons (3).

Researchers developed vaccines against BB in previous attempts but failed for many reasons. Firstly, the anti-babesial vaccine is not entirely safe for all types of bovines due to the development of clinical manifestations associated with these vaccines (4). Secondly, vaccine contamination with other blood-borne pathogens, and thirdly, lack of heterologous immune response (5,6). All these three factors altogether contribute endlessly to vaccine failure. Developing an effective vaccine against BB to overcome this menace has been a continuous panic and challenge for the last three decades.

Therefore, the current study was designed to characterize the exo-antigens and recombinant major surface antigen (MSA) derived from a local isolate and evaluate the immunogenicity of MSA (gp45). B-cells and T-cells predicted rec-gpME. Potential characterization of rec-gpME, its vaccination trials in calves, and evaluation of immune responses, both cellular and humoral, after the challenge was designed.

Ticks and Tick-Borne Diseases

Economic Impact

In Pakistan, the milk production from buffaloes and cattle from 2021 to 2022 was 35 136 and 20 903 thousand tons, respectively, whereas beef production stood at 2,155 thousand tons (3). Pakistan is an agricultural country,

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and the share of agriculture in the country's GDP was 19.8% during 2021-2022, and the livestock share was 53.2% in agriculture. The total livestock population in Pakistan is about 238.1 million, which includes buffaloes, cattle, camels, sheep, goats, mules, horses, and donkeys. Small-scale dairy farmers in Pakistan depend on livestock to meet their nutritional needs (7). Small and large dairy farmers in the country raise buffaloes and cattle. The estimated livestock population of buffaloes and cattle in Pakistan is 38.8 million and 46.1 million, respectively. Pakistan is ranked 4th among the largest milk-producing countries in the world (8).

Losses Due to Ticks and Tick-Borne Diseases

The losses due to ticks and tick-borne diseases (TTBDs) could be categorized as direct losses in production and animal losses, whereas indirect losses include therapeutic and prophylactic costs. If TTBDs are re-established in the cattle industry in the USA, then there would be an annual loss of about US$ 500 million. In Queensland and New South Wales (NSW), US$ 7.8 million per annum was spent on the control of TTBDs. In Sweden, per annum losses reported due to *B. divergens* were US$ 2.5 million (9). The economic losses due to *R. microplus* were estimated at US$ 41 million, according to Brazil's Resource Economic and Australian Bureau of Agriculture in 1994 (10). Based on geographical zones, climatic changes, and natural resources, Pakistan is distributed into different agroecological regions, which affect spatiotemporal patterns of animal diseases. Pakistan falls in the subtropical zone (30° N, 70° E) within South Asia and offers the optimum environmental conditions for developing tick species that transmit various pathogens to the host. About 80% of the world's total cattle population is at risk of TTBDs that cause substantial economic losses due to reduced milk and meat production in tropical and subtropical regions. Globally, the estimated losses due to TTBDs were ~ US$ 22-30 billion annually and an annual shortfall of ~3 billion hides in cattle (11,12). Ticks are well-known vectors of animal and human pathogens. They are the second-largest group of parasites after mosquitoes in the phylum Arthropoda that affect mammals and reptiles.

Babesia

The livestock sector is of substantial socio-economic importance globally because of the production of meat, milk, hide, bones, and hooves. Babes discovered, for the first time, piroplasm in the blood of infected cattle (13). *Babesia* species are widespread worldwide, adversely affecting public health and the livestock sector's economic status (14). Genus Babesia (Apicomplexa; Piroplasmida; Babesiidae) is a protozoal tick-borne parasite that causes a severe disease in livestock and wild animals known as babesiosis. The disease poses a serious challenge to both the farm economy and animal health. It causes severe hemoglobinuria, anemia, icterus, and ultimate death (15). More than 100 species of babesia have been discovered; out of a hundred, only 18 are essential to animals (16). The disease is spread biologically through Ixodid ticks (17). Babesiosis is transmitted through the saliva of a one-host-tick vector (*Rhipicephalus* spp.) into the host's bloodstream. It is also spread through contaminated syringes, needles, surgical instruments, and blood transfusions (18). Babesia species, their vectors, and their distribution are listed in Table 1 (19).

Bovine Babesiosis

The most common species of Babesia infecting cattle are *B. bigemina* and *B. bovis*. Bovine babesiosis (BB) is frequently caused by *B. bigemina,* the most prevalent species, and transmitted by R. microplus, causing substantial economic losses in the dairy sector (20). *B. bigemina* has economic importance in livestock due to the enormous losses caused by this parasite in tropical and subtropical regions. It has been shown that this parasite affects a wide range of cattle breeds that are sensitive to this infection. Its clinical symptoms vary from region to region as the geographic area varies (21). The significant economic impact on livestock sectors was reported in tropical and sub-tropical regions. *B. bigemina* was less virulent in Australia, while in Africa, it was the most pathogenic parasite (22). Cattle with infected erythrocytes with *B. bigemina* develop severe clinical signs like hyperthermia, jaundice, anemia, and hemoglobinuria. The main clinical sign observed in infected calves with babesia infection was anemia, especially hemolytic anemia due to the destruction of red blood cells (RBCs). The main factor involved in the destruction of RBCs is the combat of macrophages in removing the pathogen from the body in babesiosis (23). The ability of infected RBCs to sequester in the capillaries of the lungs, kidneys, and brain results showed in the animal's death (24). Cattle that have recovered from an acute infection become asymptomatic carriers and function as reservoirs for its spread. As a result, the segregation of infected and non-infected animals is inevitable and provides a valuable strategy for disease management and control measures (25).

Diagnostic Development on Bovine Babesiosis

Microscopy Method

Conventionally, the microscopic method is still adopted, and a less expensive method is used to diagnose Babesia infection. The microscopic examination of infected blood through Giemsa staining is a standard method used to diagnose babesia, but its specificity and sensitivity are limited because it gives false negative results (26). Using an oil immersion lens, the thin blood film slide stained with Giemsa stain is observed at 1000X magnification

Table 1. Babesia, Hosts, Vectors, and Their Distribution

Species	Vectors	Countries	Hosts
B. bigemina	*R. microplus, R. everts, B. decoloratus*	Asia, Africa, southern Europe, Australia, and America	Cattle
B. bovis	*B. geigyi, R. microplus, B. annulatus*	Asia, Africa Australia, and Europe	Cattle, Buffalo
B. ovis	*Rhipicephalus bursa*	Africa, Asia	Goat & Sheep
B. divergens	*Ixodes persulcatus, Ixodes ricinus*	Ireland, United Kingdom, Spain, Northwest Europe	Cattle
B. major	*Hemaphysalis punctate*	Africa, Asia, and Europe	Cattle
B. ovata	*H. longicornis*	Asia	Cattle
B. trautmanni	*Boophilus* spp.	Africa, Former USSR	Pig
B. motasi	*Hemaphysalis punctate, Rhipicephalus bursa*	Asia, Africa, and Europe	Sheep & Goat
B. gibsoni	*Rhipicephalus sanguineus, Hemaphysalis* spp.	America, Europe, Asia, Africa	Dog
B. canis	*Hyalomma spp., Dermacentor* spp., *Rhipicephalus sanguineus, Hemaphysalis* spp.	Australia, North America, South Europe, Africa, and Asia.	Dog
B. caballi	*H. truncatum, H. marginatus, R. evertsi evertsi*	Asia, America, Africa, and Europe	Horse and Donkey

power. The sensitivity of this approach is relatively high (27). In most cases, *B. bigemina* is usually present in venous blood samples. However, due to the very small quantity of infected erythrocytes, numerous thick and thin blood smears from a single suspected animal must be microscopically inspected. A thin smear is better for visualizing the organisms in *B. bigemina* infections. Parasites emerge as attached, small pyriform pairs with sizes ranging from 1.5 to 1.9 µm and ring stages with diameters ranging from 1.5 to 1.8 µm. As a result, light microscopy can easily present parasites (28). *B. bigemina* can be found in blood circulation as merozoite, ranging from 2.3 µm to 5 µm in length (29).

Conventional Polymerase Chain Reaction

DNA-based detection has been performed due to its reliable sensitivity and specificity than the microscopic method (30). Polymerase Chain Reaction (PCR) makes millions of copies *in vitro* from a single DNA fragment. The purpose is to detect DNA specific to *B. bigemina* infecting the host blood. The PCR test has the following advantages: it is faster, more sensitive, and precise. The assay's specificity is confirmed by sequencing the target amplified amplicons using specific primers. The drawback of this method is the need for a technical expert to do that experiment (31).

Real-Time Polymerase Chain Reaction

In this method, fluorogenic probes release fluorescent signals during DNA amplification to analyze the parasite genome. The visibility without gel electrophoresis, quick findings, closed automated amplification, little chance of cross-contamination, and quantifiable results are the most significant advantages of this method over the conventional PCR procedure. A signal proportionate to the amount of the amplified product in real-time must be produced to detect and quantify the amount of the target DNA. This assay has many forms and makes use of fluorescence technology for detection. Compared to conventional PCR, RT-PCR uses less template material for the whole test. The equipment is expensive, and the test requires more technical ability and competence to overcome other significant drawbacks of traditional PCR (32). Additionally, the RT-PCR technique is more sensitive than the microscopic examination of Giemsa-stained blood smears and has higher specificity, repeatability, and sensitivity. These qualities encourage its application in detecting and measuring chronic infections in animals. A quantitative PCR (qPCR) created as a TaqMan test was crucial in experimental and field surveys as a duplex format for diagnosing *B. bovis and B. bigemina*. A fluorescence resonance energy transfer (FRET) probe was also used to identify B. divergens and distinguish *B. bovis, B. bigemina*, and other species. Compared to traditional PCR tests, the RT-PCR technique has identified Babesia infection in blood samples at 1000-fold lower quantities (33). Modern qPCR technology has improved sensitivity, specificity, and variability to provide great validity and reliability in detecting chronologically infected cattle (34).

Indirect immunological Assays

If there are extremely few parasites in the bloodstream of cattle raised in babesiosis-endemic regions, well below the threshold of direct detection methods, indirect serological techniques, including the complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA), and indirect fluorescent antibodies test (IFAT), are frequently employed. The primary drawback of these tests is that even with high antibody titers, the diseases may not always indicate parasitic infection. Also, false-negative samples can be identified even in the presence of circulating parasites (35). Serological techniques have the drawback of presenting with a cross-reaction between antibodies to *B. bovis* and *B. bigemina* and are not always reliable in detecting persistently infected animals. Overall, as antibodies typically remain for varying lengths of time, even in *B. bovis*, *B. bigemina*, or *B. divergens* in cleared

animals, it is impossible to discriminate between prior exposure and current infections using serological tests. Although, in enzootic stable regions, BB lacks clinical signs throughout the year, many cattle are babesia infected. When it occurs, cattle have high antibody titers that can passively be passed to calves through the colostrum. As a result, it is falsely seropositive for Babesia infection, which seems to be seropositive (35).

Indirect Fluorescent Antibodies Test

IFAT is a widely used test that combines the stringent specificity associated with immunological approaches with the high sensitivity of fluorescence microscopy. The antibodies obtained from the cultures of B. bigemina, B. divergens, and B. bovis are used to detect the parasite. Despite its widespread usage, cross-reactivity is a key limitation for species-specific diagnosis, although IFAT is a more sensitive and specific method (20).

Enzyme-Linked Immunosorbent Assay

The crude antigens of merozoites from the infected RBCs were used to execute the ELISA. The antibodies against *B. bigemina, B. bovis*, and *B. divergens* were screened using several ELISA techniques. The capacity of serum antibodies to prevent monoclonal antibody (MAb) binding was used as the basis for a competitive enzyme-linked immunosorbent assay (cELISA). To identify positive cattle, the suppression of MAb binding to a particular epitope on the purified recombinant *B. bovis* RAP-1 C terminal construct by serum antibodies was evaluated (36). The method was modified to prevent cross-reactivity with bovine sera positive for B. bigemina. The *B. bigemina* epitope found in the RAP-1 C terminus was also used to set up the experiment. The test's strong positive and negative predictive values, 100% and 95.9%, respectively, were seen in a region with a prevalence of 75% (37). Recently, a chimeric multi-antigen included in a fragment was synthesized by combining B-cell and T-cell epitopes of three *B. bovis* antigens, e.g., heat shock protein, MSA-2c, and RAP-1. This was used in an indirect ELISA, and 95.9% and 94.3% are the reported sensitivity and specificity rates, respectively. However, the sera of *B. bigemina*-infected calves were tested, and cross-reactivity was observed. ELISA has generally replaced IFAT because of the simplicity and objectivity of the interpretation of the serological test and the ability for automatization to handle a greater number of samples in a day (38).

Prevention and Control of Bovine Babesiosis

Prevention

Both diagnostic methods, conventional microscopic examination, and nucleic acid identification, are mainly used to diagnose BB. A few drugs, like diminazene aceturate and imidocarb, were being used for chemotherapy against babesiosis. Recently, many drugs have been developed and evaluated to control the disease (39). The rate at which parasites develop resistance against the drugs is challenging for developing and industrialized countries until and unless it is the main accessible manipulation to control the disease (40).

Control of Bovine Babesiosis

Although many vaccines are present against different pathogens, a very small number of anti-parasitic vaccines are available. Protective immunity can be induced by pre-munition against BB, developed by *B. bigemina* and *B. bovis* (41). On the other hand, live vaccines have limited adoption due to technical and biological drawbacks (42). The various commercial vaccines for BB are available on the market, as shown in Table 2.

Exo-antigens From the Supernatant of *In Vitro* Culture

Soluble antigens separated from the parasite's culture medium in vitro could provide protective immunity against the parasite. The immunological assay of soluble proteins obtained from the culture medium of *B. bovis* revealed that MASP (micro-aerophilous stationary phase) contained three antigens. These antigens were analyzed through various assays to characterize their physicochemical and antigenic properties (50).

After obtaining washed RBCs in an *in vitro* culture of Babesia species maintained through the MASP culture technique, the culture's supernatants were stored after lyophilization at 4 °C after maintaining the culture for 24 hours. These supernatants contained soluble antigens (51). Pure RBCs can be obtained by centrifugation, removing plasma and the buffy coat layer. Washing with PBS is recommended to obtain more purity (52).

RBCs obtained from the calf infected with *B. bigemina* were washed at 3000 × g with normal saline. The cells were lysed with cold distilled water. The lysate of the supernatant was fractionated by Sephadex columns at pH = 8.6. The elution was checked at 280 nm and 413 nm. The Sephadex contained all the antigens that were active in haemagglutination tests (53). *B. bigemina*-infected RBC suspension was separated. The suspension was disrupted with an oscillator rather than centrifugation. The sediment was extracted and used as a source of crude antigens (54). Antigens separated from the culture of *B. bovis*-infected RBCs on the membrane of RBCs and directly on the parasite membrane. These antigens are potent immunogens in the control of BB antigens (55).

Crude Parasitic Antigens

Crude antigens are primarily used in immunological assays to detect parasites. As the babesial parasite lives exclusively in RBCs, specific antigens are purified to enhance immune assays' specificity (56). *B. canis* recombinant proteins were separated by electrophoresis on SDS-PAGE. These proteins were transferred to

Table 2. Commercially Available Vaccines Against Bovine Babesiosis

Country	Vaccine Name	Composition	Storage	Reference
Uruguay	HEMOVAC C HEMOVACUNA/DILAVE	*B. bigemina* *B. bovis* *Anaplasma centrale*	Ultra-frozen Refrigerated	(43)
South Africa	FROZEN African Redwater Vaccine for cattle FROZAN Asiatic Redwater Vaccine for cattle	*B. bigemina* *B. bovis*	Ultra-frozen	(44)
Mexico	VACUNA CONTRA LA BABIOSIS BOVINA	*B. bigemina* *B. bovis*	Ultra-frozen	(45)
Colombia	ANABASAN	*B. bigemina* *B. bovis*	Ultra-frozen	(46)
Brazil	EMBRAVAC HEMOPAR ERITROVAC N2 ERITROVAC	*B. bigemina* *B. bovis*	Ultra-frozen	(47)
Australia	Combavac 3 in 1 concentrate Trivalent tick fever vaccine	*B. bigemina* *B. bovis*	Ultra-frozen Refrigerated	(48)
Argentina	Vacuna contra la babesiosis y la Biojaja	*B. bigemina* *B. bovis*	Refrigerated Ultra-frozen	(49)

nitrocellulose membrane (NCM), and immunoblots were developed using anti-mouse IgG conjugated with HRP (57).

Immunization With Recombinant Major Surface Antigens of *Babesia bigemina*

AMA1, glycosylphosphatidylinositol-anchored protein (gp45/55), and rhoptry-associated proteins (Rap-1) are vaccine candidates against B. bigemina used to immunize cattle. B. bigeminal's merozoite surface antigens, like gp45, and the merozoite surface antigen of *B. bovis* played the same antigenic role (58).

The gp45 antigen activates an immune response in animals when infected with the *B. bigemina* strain. The clone JG-29 of *B. bigemina* was used to examine the molecular basis for polymorphism. gp45 was cloned and sequenced from *B. bigemina* (*JG*-29 strain), and then the full-length protein recognized by anti-gp45 antibodies was expressed. *Escherichia coli* strains have been used in cloning and the production of proteins. *E. coli* is among the best choices for recombinant protein growth. It has become the most popular platform for protein expression. There are multiple benefits to using *E. coli: its fast,* unparalleled growth kinetics, short replication time (approx. 20 minutes), and ability to attain high cell density cultures (59,60). It has been used in recombinant protein production (61). The swift and easy transformation of exogenous DNA in E. coli makes its use more common. Transformation of the plasmid into *E. coli* can be completed in 5 minutes (62) using competent cells like Top 10, BL-21, DH5α, etc. *E. coli* strains are used to clone and express recombinant proteins in suitable media (63). The gp45 and gp58 are two merozoite surface glycoproteins of *B. bigemina* having 45 kDa and 58 kDa sizes, respectively. These proteins have been expressed using strains of *E. coli*. Both have antigenic variations similar to MSA-1 and 2 of *B. bovis*. Immunization of calves with purified gp45 significantly reduced parasitemia.

A study reported using the pGEM-T vector for cloning (64) and pET28α (+) as a sub-cloning and expression vector for recombinant proteins. PCR selected positive clones after cloning into pGEM-T and then reconfirmed them using restriction analysis (64,65). Various molecules, including thrombospondin-related anonymous protein (TRAP), rhoptry associated protein-1 (RAP-1), spherical body proteins (SBPs), and AMA-1, are secreted by the apical organelles. These are potential immunogenic vaccine targets. The genetic diversity and distribution of RAP-1 and AMA-1 were examined. Sets of primers were used for each gene's amplification. The selected PCR products underwent cloning, which was carried out into the pGEM-T vector.

In another study, the recombinant p200 protein was characterized and used in an ELISA kit to diagnose the presence of antibodies in infected sera. In the p200 protein, bovine B-cell epitopes were found with a size of 7 kDa band on SDS-PAGE, and it was proven to be a potential candidate for developing an ELISA kit to couple with the antibodies of *B. bigemina*. Other recombinant proteins, like glutathione S-transferase (GST), with a size of 26 kDa, were cloned in the PpGE XII T vector, expressed in *E. coli and* BL-21 cells, and used in ELISA coating. The recombinant proteins against different parasitic diseases have been described in Tables 3, 4, 5, and 6.

Epitope-Based Vaccine

Genetic and antigenic diversity severely hinders the development of an effective vaccine against any specific infection. Therefore, for effective design and assessment, knowledge of the diversity and polymorphism in the population is essential. Such information could also offer priceless insights into how parasites and hosts interact (71-73). New approaches to designing and manufacturing innovative epitope-based vaccinations have been made

Table 3. Babesia Recombinant Vaccines (66)

Protein	Babesia Specie	Vaccine Type
Glutathione S-transferase	*B. canis and B. bovis*	Subunit vaccine
Bd-37	*B. bigemina*	Exo-antigen *in vitro* culture
SBP-1, MSA-1 & MSA-2	*B. bovis*	Subunit vaccine
Ribosomal Phosphoprotein PO	*B. divergens* & *B. bovis*	

Table 4. Theileria Subunit Vaccines (67-69)

Vaccine Candidates	Species	Type
SPAG1	*T. annulata*	Recombinant vaccine
TAMS1		
p67	*T. parva*	

Table 5. Trypanosoma Recombinant Vaccines

Vaccine Candidates	Species	Type	References
Beta-tubulin	*T. evansi*	Recombinant vaccine	(68)
MAP p15	*T. brucei*		(69)

Table 6. Toxoplasma Recombinant Vaccines (70)

Vaccine Candidates	Species	Type
SAG1	*T. gondii*	Recombinant vaccine
MIC1 & MIC 4		
GRA2 and GRA6		
GRA4 and ROP2		
GRA 1 and SAG 1		
GRA7, MIC2, MIC3 & SAG1		

possible by increased bioinformatic tool expertise and advancements in recombinant DNA technology (74-76). The least immunogenic component of any antigenic determinant (epitope) can elicit a particular immune response (77-79).

Conclusions and Recommendations

It is concluded from this study that secretory proteins were characterized, and the 65 kDa protein was found to be immunogenic. Major surface protein (gp45) was found to be antigenic and immunogenic when sera samples of infected and vaccinated calves were screened. Owing to the polymorphic nature of gp45, a multi-epitope fragment containing 388 amino acids (1164 bp) was designed using bioinformatic tools. This multiepitope-based protein (rec-gpME) was expressed, the calves were inoculated, and elevated humoral and CTL responses were observed in vaccinated calves. This rec-gpME could be a potential vaccine candidate against the *B. bigemina* infection. This rec-gpME would be tested on cattle under field conditions in Pakistan in the future.

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Competing Interests

There is no conflict of interest.

Ethical Approval

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