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Full Length Article

Identification of *In Vitro* Culture Derived Immunogenic Antigens from Local Isolates of *Babesia bigemina*

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Abstract

Bovine babesiosis is an economically significant tick-borne disease in tropical and subtropical regions of the world. Considering its importance, the current research was as an effort to identify novel antigens of *B. bigemina* to control bovine babesiosis. The present study evaluates the presence of immunogens (exoantigens) produced *in vitro* in local isolates of *B. bigemina*. The blood from naturally infected cattle calves, infested with ticks, showing signs of hemoglobinuria and elevated temperature, were used in this study. It was examined under microscope for intra-erythrocytic bodies, confirmed by PCR and used for *in vitro* propagation of *B. bigemina* parasite using MASP culture technique. The culture supernatants were harvested and pooled every 24 h for three days. These supernatants were lyophilized, stored and rehydrated for SDS-PAGE and immunoblotting. Two proteins of 65 kDa and >180 kDa were identified in immunoblotting with the serum of a cow recovered from natural episode of babesiosis caused by *B. bigemina*. The immunogens of Babesia can be derived from *in vitro* cultivation. Furthermore, the diagnostic and vaccine potential of the identified immunogens should be exploited for developing rapid diagnostic kit and or a vaccine. © 2021 Friends Science Publishers

Keywords: *Babesia bigemina*; Exoantigens; MASP; SDS-PAGE; Western Blot

Introduction

Tick borne diseases (TBD) *i.e.*, blood protozoan diseases, pose a major threat to livestock sector. To meet the increasing demand of meat, milk and other dairy products, majority of high-producing cattle are being imported from other countries. It is, therefore, necessary to check the current status of TBDs of cattle and buffalo in Pakistan to devise their control (Atif *et al.* 2012; Jabbar *et al.* 2015). Babesiosis, among TBDs, is responsible for considerable economic losses in crossbred cattle in tropical and subtropical regions of the world (Passos *et al.* 1998). The principal species causing bovine babesiosis are *Babesia* (*B.) bovis*, *B. bigemina* and *B. divergens* as demonstrated by World Organization for Animal Health (OIE). These parasites belong to phylum Apicomplexa, order Piroplasmida and genus Babesia. It completes its life cycle in vertebrate and an invertebrate host. Ixodidae tick, *Rhipicephalus microplus* is a known-vector of *B. bigemina*, but the main source of spread of disease is transportation of animals from infected areas. High level of parasitemia in cattle is reported due to *B. bigemina* (Young and Morzaria 1986). The most pronounced symptoms of the disease are elevated temperature, anemia, dehydration, hemoglobinuria, and death. The recovered animals remain carriers of the disease and become a source of spread when an uninfected tick takes a blood meal (Alvarez *et al.* 2019). Diagnosis is an important step to prevent the outbreak of babesiosis, but it is difficult to detect by current conventional diagnostic methods due to less parasitemia (Chaudhry *et al.* 2010).

Currently, the disease is being controlled by chemotherapy, ticks control and vaccination. The former two methods leave residues in meat and milk. Further, no chemoprophylaxis schedule is made against the control of babesiosis due to less information on prevalence of the parasites. The effective control for bovine babesiosis is live attenuated vaccine but it has significant drawbacks including danger of disease in immunostressed or immunocompromised animal, maintenance of cold chain and a short storage life (Dominguez *et al.* 2007). The animals may remain in carrier state of disease and become a source of infection to healthy animals *via* tick vectors (Timms *et al.* 1984). Mishandling and lack of satisfactory vaccine are the major challenges for the control of babesiosis (Montenegro-James *et al.* 1992). Nowadays, vaccines are aimed to provide long lasting immunity without health risks, and no withdrawal times.

During infection in vertebrate host, babesia parasite sheds its merozoite surface coat that contains antigens. The parasite also secretes proteins by their organelles, such as

rhoptries and micronemes, to invade host erythrocytes. Cattle can be immunized with these parasitic antigens that are released during its proliferation (Wieser *et al.* 2019). With the advent of Microaerophilous Stationary Phase (MASP) culture technique, the presence of exoantigens is evident in *in vitro* culture supernatants. The immunogenic exoantigens are a potential source of vaccine material (Patarroyo *et al.* 1995). For the prophylactic control of babesiosis, one approach is the use of immunogenic, culture-derived, organism-free exoantigens. The exoantigens can potentially provide better protection against heterologous challenge and apparently there is no risk of the animals moving to carrier state (Timms *et al.* 1984). The parasitic antigens can also be helpful for diagnostic purposes (Ristic and Arellano 1986). Keeping in view the importance of babesiosis, the experiments in present study were designed with the aim to isolate and identify exoantigen/s from parasitic culture media.

Materials and Methods

Microscopy

The blood samples were collected from cattle calves naturally infested with ticks and having signs of babesiosis. Thin blood smears were prepared, fixed with absolute ethanol (2 min), stained with Giemsa (1:20 dilution for 30 min). The blood smears were observed under microscope (1000 × magnification) for the presence of intra-erythrocytic protozoans. The parasitaemia was calculated by using following formula:

$$\text{Parasitic erythrocytes (PPE; \%)} = \frac{\text{No. of infected RBCs}}{\text{Total No. of RBCs}} \times 100$$

Polymerase Chain Reaction (PCR)

PCR was performed using SimpliAmp Thermocycler and Taq Polymerase (cat # TAQ005.500), specific primers were used to obtain product size of 321 bp. The PCR mixture was prepared in a final volume of 20 μL. Initial denaturation was given at 95ºC for 5 min, reaction was cycled for 35 times. Each cycle was started with denaturation at 95ºC for 30 sec, annealing at 60ºC for 30 sec, extension step at 72ºC for 30 sec and a final elongation at 72ºC for 10 min was given. After that, these amplified DNA fragments were analyzed on 1.5% agarose gel.

In vitro Culture

To obtain washed infected RBCs, the blood was centrifuged at 1000 × g at 15ºC for 10 min. Supernatant, buffy coat and small portion of upper red blood cells was discarded. The remaining sample was washed with 1X PBS and used in culture of *B. bigemina* and as a source of exoantigens. The parasites *B. bigemina* were cultivated by using MASP culture technique (Levy and Ristic 1980). The atmospheric conditions were 5% CO_2, 2% O_2 and 93% Nitrogen.

Harvesting Exoantigens

Soluble exoantigens were prepared according to method of (Fish *et al.* 2008). The culture supernatant of *B. bigemina* was harvested, according to method of (Nawaz 2018), every 24 h after culture was placed. The supernatant was centrifuged at 6000 × g for 30 min then it was filtered through 0.45 μm filter to remove cell debris and extracellular parasites. This supernatant was lyophilized and stored at 4ºC until use. The lyophilized supernatant was rehydrated with distilled water prior to use in Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

SDS-PAGE

Proteins were separated using methods of (Tsang *et al.* 1983), all SDS-chemicals were obtained from BioRad Laboratories. After casting the apparatus, two discontinuous gels were prepared at room temperature having 5% stacking gel and 12% resolving gel. The gels, when solidified, were placed in Tris-Glycine SDS buffer (Tank buffer). The samples were prepared under reducing conditions using 2X Laemmli buffer (4% SDS, 20% glycerol, 0.004% bromphenol blue, 0.125 M Tris-Cl, pH 6.8, 10% 2-mercaptoethanol) and an equal volume of rehydrated culture supernatant. The samples were heated at boiling temperature of water prior to loading in SDS-PAGE gel. Standard molecular weight markers (Cat # 26616), ranges from 10 to 180 kDa, were used to check the relative mobility of proteins. Initially 80 V, 200 mA for 30 min was applied to linearize the samples on resolving gel, then 120 V, 400 mA for 150 min was applied when samples were passing through resolving gel. The gel was stained with Coomassie Brilliant Blue R-250, and de-stained with de-staining solution (Glacial Acetic Acid, Methanol, and water) until the background was clear (generally over 2 h) and observed for proteins.

Development of Immunoblot

Proteins bands were transferred onto nitrocellulose membrane (NCM) (Trans-Blot Turbo® Transfer System™) for 7 min, 11 volts and 1.3 ampere. The gel and NCM was soaked in Tris-Buffered Saline Tween (TBST). Proceeding transfer, the membrane was washed in TBST buffer. The NCM was than blocked with 5% skimmed milk. The membrane was soaked in serum of babesia recovered animal at 1:100 dilutions in blocking buffer. The membrane was washed again in TBST buffer to ensure washing of spare antibodies. The membrane was then treated with Rabbit anti-bovine IgG conjugated with Alkaline Phosphatase (A0705 Sigma-Aldrich) at 1:3000 dilutions prepared in blocking buffer. The membrane was washed

again to ensure the removal of residual secondary antibody. BCIP (5-bromo-4-chloro-3-indolyl phosphate; Ref no: 34042 Lot no: UH289227) was added over the membrane and placed in dark, frequently checked until the visualization of bands over the membrane. The reaction was stopped by diluting it with several volumes of distilled water. The membrane was dried, photographed and stored for later use.

The schematic flow diagram for the identification of exoantigen has been shown in Fig. 1.

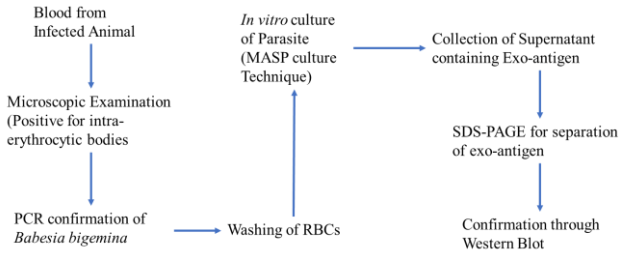

Fig. 1: A schematic flow diagram for identification of Exoantigen/s

Results

Microscopic Evaluation

The presence of intra erythrocytic bodies was seen and 3% parasitemia was observed in the blood of infected animals (Fig. 2).

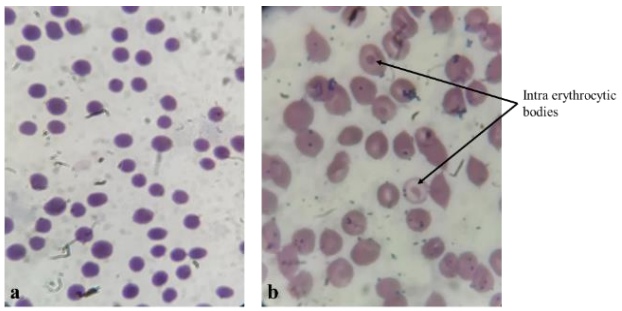

Fig. 2: a. Microscopic image of blood smear of a healthy animal while **b**. Microscopic image of blood smear of *Babesia* positive calf showing intra-erythrocytic bodies

PCR

The amplicon size was observed at 321 bp (Fig. 3) on 1.5% agarose gel, the control positive was developed in Molecular Laboratory, Department of Parasitology, UVAS, Lahore, by (Umber *et al.* 2020).

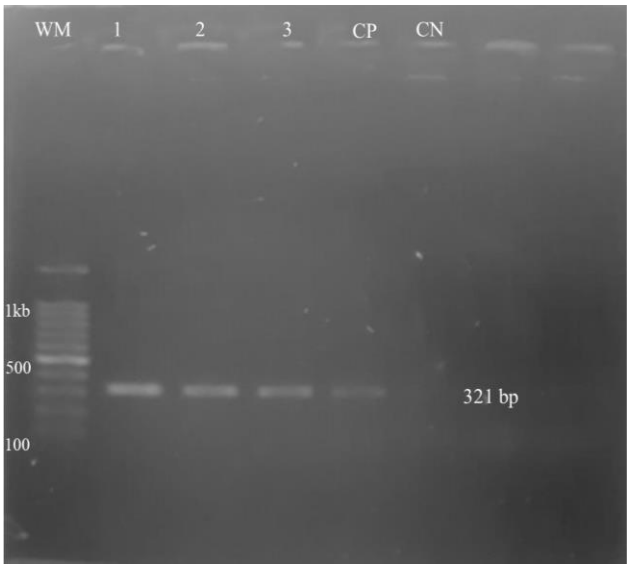

Fig. 3: PCR results on 1.5% agarose gel. WM indicates weight marker, 1, 2 and 3 are PCR products. CP and CN are control positive and negative, respectively

MASP Culture

Increased parasitemia level leads to low oxygen concentration that resulted in darkening of RBCs culture. The bright red color indicates no or less proliferation of parasites in culture media, while a color change indicates proliferation of babesia parasites in blood cells.

SDS-PAGE Analysis

The SDS-PAGE analysis revealed many dense proteins in all wells throughout the gel. Several proteins were seen overlapping each other (Fig. 4). In sample 1, more prominent bands are present at 27, 32, 37, 45 and 60 kDa. In sample 2, these bands are present at 32, 36, 40, 68 and >180 kDa, while in sample 3, the prominent protein bands can be seen at 40, 68 and >180 kDa.

Development of Immunoblot

The blot was developed using serum of recovered animal as a source of primary antibody, and a commercial secondary antibody conjugated with Alkaline Phosphatase. BCIP, as a substrate show a colour change at 65 kDa and > 180 kDa in supernatants.

Discussion

Being more economic, thin and thick Giemsa-stained smears are prime choice for detection of blood protozoans, but these have many drawbacks including false +ve/-ve results. Currently, highly sensitive and specific molecular tools are being used for accurate diagnosis of Babesia at species level (Alvarez *et al.* 2019), such as PCR based assays which are used for rapid detection in live and dead animals (Singh *et al.* 2007).

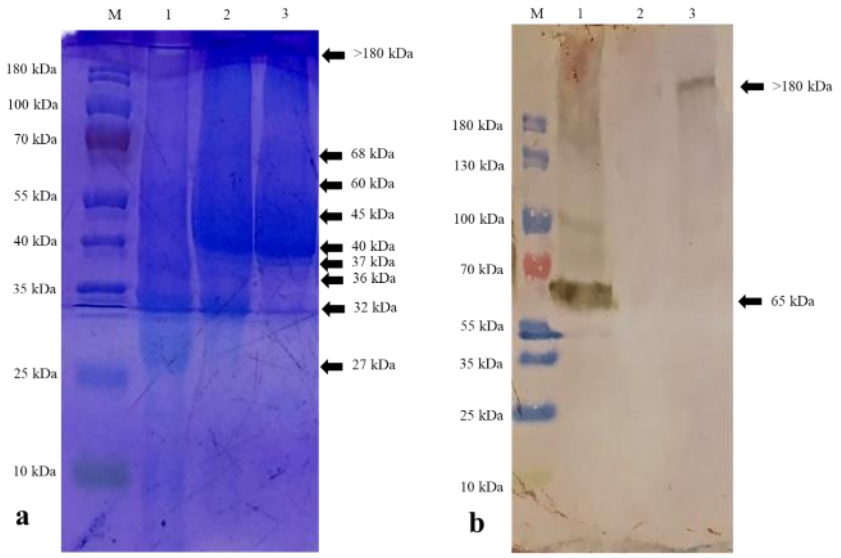

Fig. 4: a. SDS-PAGE analysis of proteins in culture supernatant, 12% SDS-PAGE stained with Coomassie Brilliant blue R-250 **b.** Western Blot analysis of supernatant proteins separated on SDS-PAGE, immunoblot was developed using serum of *B. bigemina* recovered cattle. M is weight marker, whereas 1, 2 and 3 are supernatant cultures

The molecular probes used in this study were developed previously in our laboratory for the detection of *B. bigemina* (Umber *et al.* 2020) and routinely used for the evaluation of parasite in the blood of diseased animals. The molecular methods are more analytical, sensitive and specific but these are not economic, require higher expertise and technical skills.

The soluble antigens are efficient immunogens against bovine babesiosis for the induction of protective immunity (James 1984). Exo-antigens are immunogens naturally released from parasite into blood plasma or supernatant in case of *in vitro* cultures (James 1989). The merozoite surface coat of Babesia has antigens, which are responsible for production of antibodies that react with parasite and cause lysis of merozoites. Exoantigens provide a potent, efficacious, and safe control for immunoprophylaxis against bovine babesiosis (Montenegro-James 1989). The profile of exoantigen revealed two immunogenic bands, at 65 kDa and greater than 180 kDa, identified by anti–*B. bigemina* serum, collected from naturally infected animal with *B. bigemina*. Further investigations are needed for identification of amino acid sequences, protein classification and to understand the role of these immunogenic proteins in parasitic life cycle. Our results indicate that these antigens can potentially induce protective immunity as these were identified with anti–*B. bigemina* serum from an animal recovered after a natural infection.

Antigens produced in *in vitro* culture supernatants have been used in a commercial vaccine against canine babesiosis (Pirodog®) (Moreau *et al.* 1989). An improved vaccine (Nobivac® Piro) with combination of different soluble antigens conferred greater protection against heterologous infection (Schetters *et al.* 2007; Freyburger *et al.* 2011). The immunoprotective efficacy of *in vitro* produced *B. bigemina* exoantigens have been reported (Beniwal *et al.* 1997). The present study also corroborates the presence of novel exoantigens in *in vitro* culture supernatants of *B. bigemina*. Since, the cultures require biological components they have less hazard of contamination with extraneous agents than live attenuated vaccines (Pipano 1997). Until optimal vaccines are developed, the culture derived soluble Babesia immunogens may offer the best combination of potency, efficacy and safety to fulfil the critical need for immunoprophylactic control against bovine babesiosis.

Conclusion

The immunogens of Babesia can be derived from *in vitro* cultivation. The diagnostic and vaccine potential of these identified immunogens should be exploited for development of rapid diagnostic kit and/ or a vaccine.

Acknowledgements

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Author Contributions

AA, HA and IR conceptualized, designed the experiment; AA, MK, SA, AMA and ZR acquisition of Data, analysis, and interpretation, drafting of manuscript; HA and IR supervised, proofread the experiment, and give access to research components.

Conflict of Interest

The authors declare that they have no competing interests.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

The study was approved from the Animal Welfare and Ethic Society of University of Veterinary and Animal Sciences Lahore, Pakistan with No. DR/ 496, Dated May 30, 2019.

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