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Development of apical membrane antigen-1 based ELISA kit for the diagnosis of *Babesia* infection in cattle

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Abstract

The current finding reports the development of immunocapture-enzyme linked immunosorbent assay (iELISA) with recombinant apical membrane antigen-1 (rAMA-1) to identify an infection of *Babesia* in naturally infected cattle. The 48.00 kDa protein-encoding *rAMA-1* gene was cloned into the pET-28a (+) expression vector and expressed in *Escherichia coli.* The resulting congregate protein was refined under native settings. Towards the evaluation of the diagnostic potential of AMA-1 as a sero-diagnostic reagent, a panel of sera samples from *Babesia* infected cattle and uninfected sera, as well as *Babesia* positive samples with other species, including *B. bigemina*, *B. divergens*, *B. major*, *B. occultans*, were utilized. Additionally, the efficacy of rAMA-1-based serological assays was compared with commercially available kits using 200 samples taken from cattle suspected of babesiosis. The results demonstrated that the iELISA using rAMA-1 exhibited a diagnostic sensitivity of 88.89%, when compared to the commercially available ELISA kit as a reference test. The specificity of this assay was 76.66%. These findings suggest that the iELISA employing rAMA-1 can be utilized on large-scale epidemiological surveys and clinical detection of *Babesia* infection in cattle.

Introduction

Bovine babesiosis stands as a significant challenge to the livestock industry in Pakistan and globally.[1] *Babesia* species are protozoan parasites transmitted by ticks, having the potential to infect livestock, domestic animals, wildlife, and even humans.[2] Various *Babesia* species, including *B. bovis* and *B. bigemina*, with the potential to induce disease are prevalent in both temperate and tropical regions worldwide.[3] Approximately 1.30 billion cattle are at possibility of contracting this contagion. Bovine babesiosis leads to the significant economic losses in the meat and milk industries, emphasizing the critical importance of these protozoa in the domain of veterinary medicine.[4,5] Several kinds of *Babesia*, including *B. microti* and *B. divergens*, have come to light as pathogenic species linked to the development of zoonotic diseases.[6] The prevalence of *Babesia* may be influenced by factors, such as geographical conditions, climate, tick distribution, host availability, and human behavior.[7] Additionally, a certain population may be at the high risk of infection due to the occupational or recreational activities involving exposure to ticks.[8,9] In the United States, *Babesia* is considered a foreign animal disease and has been eradicated from the country since the 1940s. However, it remains a significant concern for livestock producers around the globe, predominantly in Latin America, Africa, and Asia.[10,11] Several studies have revealed a widespread presence in various sections of the Punjab province, Pakistan. Livestock comprises 63.68% in agriculture sector and 14.37% counts of gross domestic product of Pakistan (economic survey 2022-2023). Cattle contribute in 43.00% of Pakistan's total livestock population, encompassing exotic and selectively bred cattle along with 15 indigenous strains.[12] Despite their genetic makeup, most dairy animals in Pakistan produce comparatively less milk due to the lack of disease control. Pakistan's atmosphere is favorable for tick growth and expansion.

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The ticks are major vectors for diversity of pathogens causing an array of disorders in cattle.[13] Out of many highly conserved parasitic proteins characterized, apical membrane antigen-1 (AMA-1) exists in all piroplasm species, exceedingly preserved amongst genera of Apicomplexa phylum. The AMA- 1 is massed in micronemes and just prior to be in the host cell by the parasite, transported to the surface of parasite.[14,15] There are several methods for the detection of babesiosis, including microscopic examination, polymerase chain reaction (PCR),[16] serological testing, immunofluorescence assay, and nucleic acid amplification tests.[17,18] In current study, the *Babesia* enzyme-linked immunosorbent assay (ELISA) kit was developed, and specificity and sensitivity were conferred with commercially accessible ELISA kit.

The sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for analysis. The purpose of developing local immunocapture-enzyme linked immunosorbent assay (iELISA) was the identification of Babesiosis in Pakistan and enhancing efficacy, as well as it's comparison with commercially available diagnostic tools. The benefit of preparing local ELISA kit is its convenient availability and cost effectiveness owing to its trivial amount of protein being utilized in optimization of ELISA.

Materials and Methods

Collection of samples and identification. Molecular Parasitology Laboratory of the University of Veterinary and Animal Sciences in Lahore, Pakistan, served as the study location. The samples were collected from several farms of Punjab province, Pakistan. *Babesia* **s**uspected blood samples of cattle were collected from the field. Blood was drawn from the caudal or jugular vein; ethylene-diaminetetraacetic acid vacutainer was used to collect some samples. Afterward, the blood collected samples were held in reserve at – 20.00 ˚C for use. In addition to blood, serum was also obtained from the same animals and then stored at – 20.00 ˚C until utilization. From each sample, thin and thick blood smears were prepared. Blood smears were air dried, fixed in absolute alcohol, Giemsa stained and then, examined under a microscope. The University of Veterinary and Animal Sciences Ethical Review Commission in Lahore, Pakistan, accepted this research study (ERC-3355).

DNA extraction. Microscopically positive blood samples were processed to extract DNA using Gene-All DNA extraction kit (GeneAll Biotechnology Co. Ltd., Seoul, Korea) and DNAzol method, following the producer's instructions.[19] The DNA concentration was estimated by the NanoDrop Spectrophotometer (NanoDrop 2000; ThermoScientific, Waltham, USA).[20] A set of primers with specific sequence *ama-F*: GTATCAGCCGCCGACCTCCGTAA GT and *ama-R*: GGCGTCAGACTCCAACGGGGAACCG were used for amplifying the DNA samples.

Polymerase chain reaction and conditions. A set of specific primers for *Babesia* with a product size of 738 bp was selected for amplifying the DNA samples. To confirm the presence of *Babesia*, reaction mixture was prepared. The PCR conditions were followed as described by Sivakumar *et al*.[21] The amplicons generated by PCR were visualized by electrophoresis.[22]

DNA sequencing. The confirmed PCR products were sent to the 1st BASE, Malaysia, for sequencing. The BioEdit (Ibis Therapeutics, Carlsbad, USA) software was used to align and arrange the nucleotide sequences. The sequence was submitted to the NCBI and obtained the accession number of OR640338.

***Babesia AMA-1* production in competent *Escherichia coli*.** The *AMA1* gene sequence was amplified with the primers having BamH1/HindIII enzyme restriction sites (New England Biolabs Ipswich Massachusetts United States). Cloning was performed into the pET-28a (+) vector, and competent cells were prepared chemically.[23] In brief, *E. coli* BL21(DE3) was cultivated in Luria-Bertani broth (Himedia, Mumbai, India), and plasmids were transformed in BL21 following the established protocol.[24]

Restriction analysis and induction of AMA-1 through SDS-PAGE. The transformation restriction analysis was confirmed using BamH1 and HindIII enzymes (New England Biolabs, Ipswich, USA). Concisely, 1.00 µg of plasmid DNA was digested with 3.00 µL of enzymes and an appropriate 10.00 X buffer at room temperature for 1 hr. The expression of recombinant AMA-1 was conceded according to the Kemmer *et al*.[25] The harvested cultures underwent analysis through SDS-PAGE (Bio-Rad Laboratories, Hercules, USA). Subsequently, truncated recombinant proteins were refined using Ni-NTA affinity chromatography kit, following the company's instructions provided by Qiagen (Hilden, Germany).

Serum screening through *Babesia* immunoglobulin G (IgG) ELISA kit. Flat-bottom 96-well polystyrene plate (JET BioFil, Guangzhou, China) was coated with AMA-1 at 0.125 µg mL^{-1} concentration in the coating buffer (50.00 mM NaOH; Pakistan Chemicals, Lahore, Pakistan). This plate was then incubated overnight at 4.00 ˚C, using the washing buffer (0.001 M phosphate buffered saline/0.05% Tween-20 (Chemfine International Co., Wuxi, China) to wash the plate, and procedure was repeated five times, using 300 µL *per* well.[26] Following that, the plate was filled with 4.00% bovine serum albumin (Sigma Aldrich, St. Louis, USA) in 0.01 M phosphate buffered saline (Natureplast, Mondeville, France) at 200 µL *per* well and incubated for 2 hr at 37.00 ˚C. The positive and negative control serum samples were employed in replica wells, while two wells on the plate were kept empty representing blank wells. The plate was then incubated for a specific duration at 37.00 ˚C. Following another round of five washes, anti-bovine secondary antibodies,

conjugated with alkaline phosphatase (Worthington Biochemical, Lakewood, USA), were added at a dilution of 1 : 5,000 and 100 µL *per* well, followed by 2-hr incubation at 37.00 ˚C. After five more washes, the p-nitrophenyl phosphate substrate (Thermo Scientific) was added at 100 µL per well with a concentration of 1.00 mg mL^{-1} in diethylamin (DEA) substrate buffer (Thermo Fisher, USA). The plate was placed in an incubator at 37.00 ˚C, and the reaction was halted after 15 min by introducing a stop solution of 1.00 M NaOH at a volume of 100 µL *per* well. The optical density was then noted at a specific wavelength using a microplate ELISA reader (ELX-800; BioTek, Winooski, USA).

Microscopic examination. The blood smears were treated with methanol (Merck, Darmstadt, Germany) and fixed for 5 min. Subsequently, they were stained with 5.00% dilution of Giemsa for approximately 30 min. The stained blood smears were then inspected through a light microscope with immersion oil at a magnification of 100 X to detect the incidence of piroplasms.[27]

Statistical analysis. The data was analyzed using SPSS Software (version 20.0; IBM Corp., Armonk, USA) and presented using descriptive statistics including percentages with corresponding 95.00% confidence intervals. The Chi-square test was used to assess the association of various factors with the outcome. A *p* values less than 0.05 was considered statistically significant.

Results

Microscopic examination. The stain revealed a strong concentration of intra-erythrocytic bodies, exhibited a horseshoe-shaped morphology within the red blood cells. Suspected blood samples were examined under a microscope (Fig. 1).

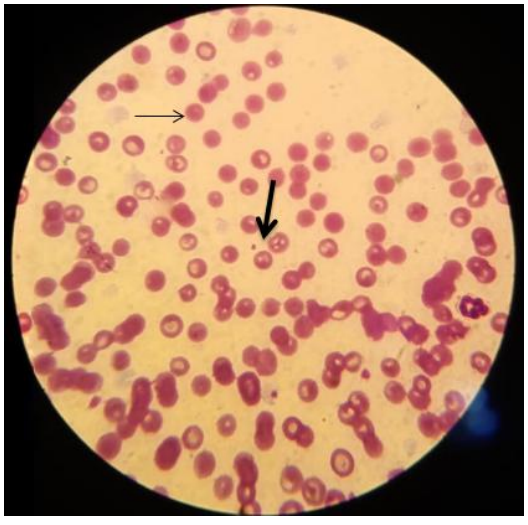

Fig. 1. Giemsa-stained slide revealed characteristics of *Babesia* Infection. Thick arrow illustrates the intra-cellular pathogen and thin arrow shows non-infected erythrocyte.

Polymerase chain reaction identification and confirmation. Out of 200 samples, 30 were identified as positive, *i.e.*, possessing intra-erythrocytic bodies. Subsequently, samples (n = 17) were detected positive with PCR. The *Babesia AMA-1* gene's amplification with PCR was confirmed with 738 bp product using a 100 bp ladder (Fig. 2A).

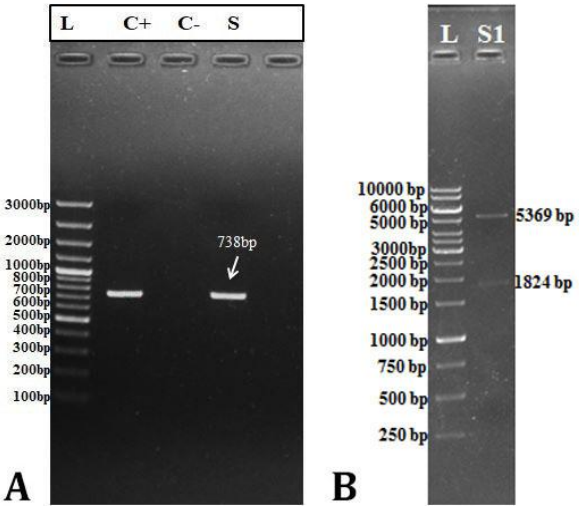

Fig. 2. A) Gel-electrophoresis of 1.20% agarose stained with SYBR Safe revealed a clear 738 bp product with species-specific *Babesia* primers. L: Ladder; C+: Positive control; C-: Negative control; S: Amplified sample at 738. **B)** Agarose gel (0.90%) revealed digestion of plasmid with BamH1 and HindIII. In S1 (digested plasmid), the bands generated by the HindIII and BamH1 restriction enzymes were 1,824 bp and 5,369 bp, respectively. L: 1 kb ladder.

Transformation and restriction analysis of pET-28 a (+)-AMA-1. The BL21 transformed colonies with pET-28a (+) were distinctive as represented in Figure 3A. An undigested plasmid extraction displayed two fragments. The plasmid extraction was performed to confirm the transformation of plasmid in BL21 competent cells. Triple band revealed the plasmid as circular and super coiled, correspondingly displayed in Figure 3B.

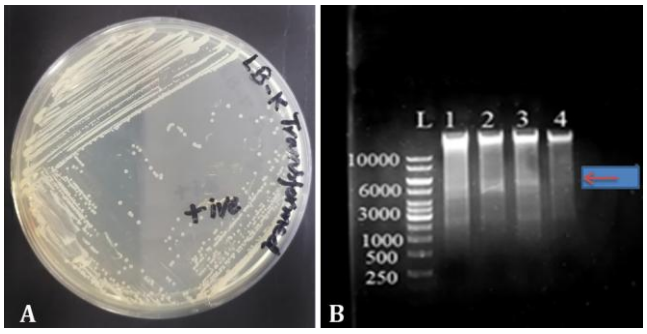

Fig. 3. The growth of transformed white colonies of *apical membrane antigen-1* gene **A)** on Luria-Bertani agar after 24 hr. **B)** The plasmid around 5,000 bp on agarose gel (0.80%) stained with SYBR Safe. L: 1 KB ladder; Lanes 1 - 4: Undigested plasmid.

Plasmid digestion. To confirm the AMA-1 pET28-a (+) transformation in BL21 competent cells, plasmid digestion was performed. The HindIII and BamH1, two restriction enzymes, were utilized to break down plasmids (Fig. 2B).

Induction and expression of AMA-1. The SDS-PAGE was performed to certify the presence of the protein (AMA-1). On SDS gel, proteins got separated depending on their molecular weights. The AMA-1 protein has 48.00 kDa molecular weight. The product of 48.00 kDa was obtained in the induced cultures, whereas nonIsopropyl β-D-1-thiogalatopyranoside (IPTG) induced cultures, like BL21 in Figure 4, did not exhibit any bands. The purification process using Nickel chromatography resulted in a notably rich yield of purification. Purified protein was attained with a distinct product, aligning by 48.00 kDa anticipated molecular weight, at a concentration of 1.20 mg mL^{-1}.

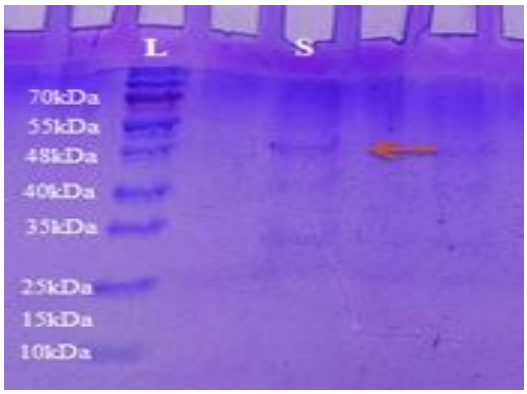

Fig. 4. The sodium dodecyl-sulfate gel (12.00%) stained with coomassie blue illustrates a product of 48.00 kDa. L: Protein marker; S: Sample of protein.

Protein quantification. The protein quantification was performed according to the Thermo Scientific Pierce™ Bradford Protein Assay Kit method (Fig. 5).

***Babesia* IgG ELISA kit for screening of sera.** Out of a total of 84 samples examined using K, 57.10% tested positive, while 27.30% tested negative (Table 1 and Fig. 6). The analytic presentation of K was evaluated based on the ability to detect positive and negative sera. The sensitivity was 88.89% and specifically was 76.66%. The value of Chi-square was 36.67, which was significant statistically ($p < 0.05$), as indicated in Table 2.

Table 1. Protein concentration (µg mL^{-1}).

Samples	Samples (Optical density)	Protein concentration (µg mL^{-1})
S1	0.584	405
S2	0.561	329
S3	0.551	295
S4	0.548	285
S5	0.658	652

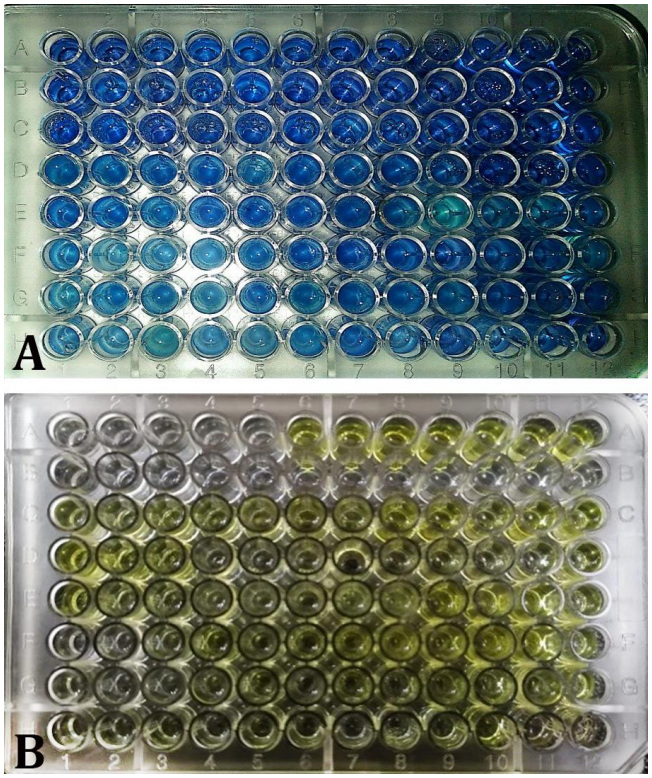

Fig. 5. A) Protein quantification plate subsequent to the addition of the Bradford reagent reaction solution. The blue color signifies the reaction between the Bradford reagent and protein, while the dark color represents a robust reaction between the Bradford solution and protein (apical membrane antigen-1). **B)** The enzyme-linked immunosorbent assay (ELISA) plate after addition of p-nitrophenyl phosphate substrate (1.00 mg mL^{-1}). Yellow color illustrates substrate and protein reaction. Protein coated rAMA-1 = 0.45 µg mL, serum dilution = 1:50, and serum samples = 84. Control negative samples = 5 (A1 - A5) and control positive samples = 7 (A6 - A12). The ELISA optical density reading at 15 min and cut-off value 2.50 times more than control negative.

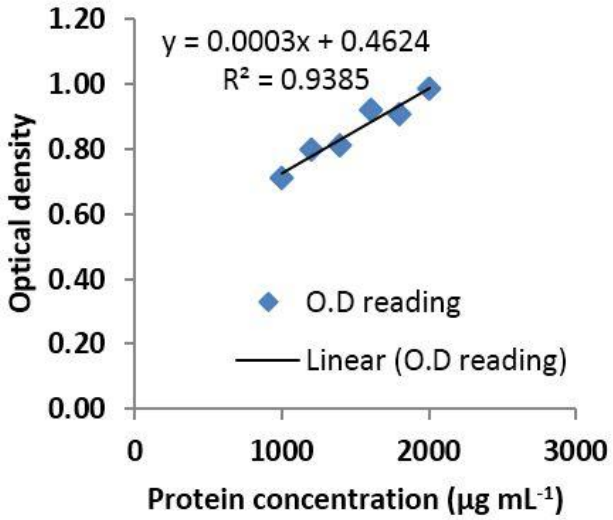

Fig. 6. Analytical calibration for protein concentration.

Table 2. Evaluation of K and C for anti-*Babesia* immunoglobulin G detection in 84 sera samples.

Results	Apical membrane antigen-1 (%)
Sensitivity	88.89%
Specificity	76.66%
Positive predictive value	87.27%
Negative predictive value	79.31%
Disease prevalence	64.28%
Odd ratio	26.29
Chi-square	36.67
***p*-value**	0.000

Sera analysis through *Babesia* IgG ELISA kit. An ELISA was conducted using cattle serum for screening of babesiosis as represented in Figure 7. A total of 84 samples were tested by C and K. Notably, 48 samples were identified as entirely positive by K and C, while 23 samples were entirely negative by K and C.

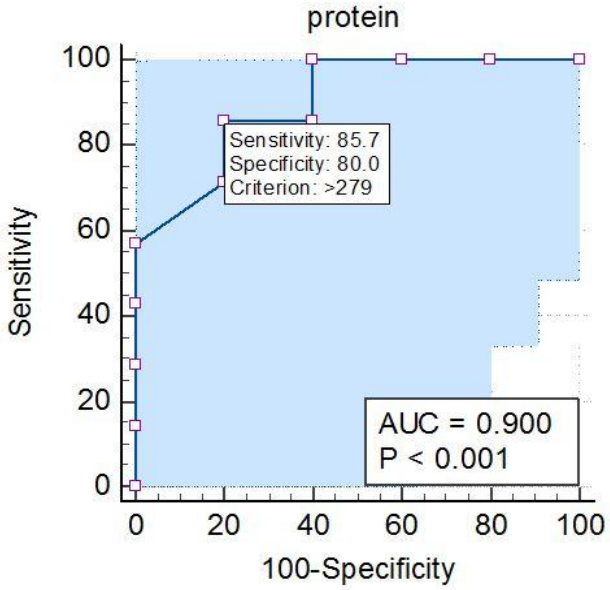

Fig. 7. The curve evaluating the effectiveness of K in distinguishing positive and negative individuals, recognized by K and C to determine the cut-off value. AUC: Area under curve.

Discussion

Babesia is a warily imperative pathogen renowned to cause bovine babesiosis globally with substantial economic impact, resulting in enormous losses in the dairy industry.[28] *Babesia* is pervasive in numerous regions, posing a noticeable threat to the livestock industry.[29] This malady has a need for prompt diagnosis and veterinary intervention to curtail financial harm. Different techniques and procedures have been defined and executed regularly to reveal specific antibodies in cattle exposed to *Babesia* spp.[30] The iELISA is one of the best practices executed for the analysis of hemoparasites, owing to its great sensitivity and specificity. The current research affirms 88.89% sensitivity and diagnosis specificity is 76.66% with the *Babesia* AMA-1 by analysis of receiver operating characteristic curve. The technique assists with linking two diagnostic techniques, as the receiver operating characteristic curve is a substitute to select the accuracy.

Furthermore, the area under the curve, acquired in recent investigation, is reflected moral (1.000), ensuring thus the capacity to categorize, as well as avoid incorrect classification, considering positive and negative samples.[31] Serodiagnosis of bovine babesiosis is a crucial policy for monitoring and preventing the spread of the disease. Currently, in Pakistan, there are no commercially available ELISA kits specifically designed for diagnosing bovine babesiosis caused by *Babesia* spp. The preparation of native parasite antigen reveals challenges; *i.e.*, being laborious and tedious, and requiring the care of merozoites in contributor bovine red blood cells. Microscopy and PCR were initially used as screening and reference tests for the diagnosis of babesiosis. According to the recent research findings, 15.00% of the tested cattle were found positive for *B. Bovis*, using both microscopy and PCR techniques. Contrary to earlier research,[32] these methods detect *B. bovis*, but revealed a lesser occurrence of babesiosis in cattle matched to the recent findings. In comparison with other animal species, the present study demonstrates a considerably advanced prevalence of *B. bovis* in cattle, suggesting the notable presence of babesiosis within this group. Despite the limitations of these tests, serological methods, specifically ELISA, appear to be the best feasible, as well as cost-effective option for conducting diagnostic purpose.[32] In current investigation, a cattle *Babesia* IgG ELISA kit was prepared with *AMA-1* gene using similar procedure as earlier one, which displayed better sensitivity and specificity in comparison with conventional methods. The ELISA kit surpassed microscopy and PCR in its ability to detect both active and past infections, scalability, and measurement of the immune response. It is usual for sequence conservation among the antigens chosen for diagnostic purposes to result in a loss of species-specificity, and recombinant proteins from other protozoa have been utilized to diagnose distinct species within the same genus. Prior investigation for diagnosis of bovine trypanosomiasis using ELISA revealed different levels of cross-reactivity. Another iELISA based on a recombinant antigen against *Theileria* spp. exhibited cross-reactivity amongst *T. uilenbergi* and *T. Luwenshuni* in small ruminants. Additionally, in a current description of an indirect ELISA for the bovine *Theileria* based on three immune-dominant proteins, the researchers described a resilient cross-reactivity in cattle infected with *T. annulata*, as well as *T. sinensis*. Generally, these results unveil one of the main tasks for the assortment of immune-dominant antigens for diagnosis.[33] As compared to earlier studies, the utilization

of AMA-1 was remarkable, owing to its unwavering reactivity with naturally infected cattle sera. Immuno-dominant antigens with comparable molecular weights have been formerly documented in numerous *Babesia* species, including *B. bigemina*, *B. divergens*, and *B. microti*.[34] Consequently, the application of ELISA with AMA-1 antigen having an admirable species specificity rate and an adequate sensitivity permits the recognition of antibodies to *B. bovis* in naturally infected cattle.

The SDS-PAGE revealed that MAb H9P2C2 effectively identified a protein band of approximately 30.00 kDa in *B. bovis* infected erythrocytes.[35] Monoclonal antibodies were employed to create a competitive-inhibition ELISA by directing a *B. bigemina* merozoite antigen of 58.00 kDa. This antigen exhibited strong reactivity with immune sera in SDS.[36,37] The constructed 48.00-kDa antigen was used to develop a sero-diagnostic test. Concentration of AMA-1 coating was set at 0.125 µg mL^{-1}, besides the optimum dilution of cattle sera was determined as 1:100. The sera and antibodies were incubated for 2 hr at 37.00 ˚C. After adding the substrate, it was found that the optimal reading time for optical density was 15 min. These standardized settings revealed favorable diagnostic test results for the cattle *Babesia* IgG ELISA kit. Cattle *Babesia* IgG ELISA kit was employed to evaluate the 84 bovine sera. The results exhibited that 57.10% of cattle tested positive for *Babesia* using IgG ELISA kit. Interestingly, the maximum rate of sero-positivity for *Babesia*-specific IgG antibodies was perceived in older ones. In this context, the current ELISA assay holds significant potential as a valuable means for examining epidemiological condition at a provincial level. This, in turn, facilitates prompt decision-making processes, like vaccination, relocation, or acaricides application. Based on present findings, the recombinant AMA-1 Bbo-ELISA proves highly suitable for conducting epidemiological surveys, as demonstrated in current investigation conducted in distinct areas of Punjab province, Pakistan, where former infection tolls were not documented. Conducting such surveys would provide a comprehensive understanding of the disease prevalence and enable the assessment of its economic impact in areas where *Babesia* spp. infection is present.

In conclusion, AMA-1 was successfully transformed into BL21 competent cells. The AMA-1 was expressed and characterized through SDS-PAGE in the host *E. coli*. Additionally, AMA-1 was coated on ELISA wells and evaluated using cattle serum samples. It can be suggested that this AMA-1 variant can be utilized for diagnostic studies in cattle, proving helpful in the diagnosis of babesiosis in food animals of Pakistan.

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Conflict of interest

The contributors claim no conflicting interest.

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