Original article

Immuno-bioinformatic approach for designing of multi-epitope merozoite surface antigen of *Babesia bigemina* and evaluation of its immunogenicity in inoculated calves

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Abstract

Babesiosis is a parasitic disease caused by intraerythrocytic parasites of the genus Babesia, which infect both wild and domestic animals. Merozoite surface antigens (MSAs) have been identified as efficient immunogens in Babesia-infected animals. MSAs play a key role in the invasion process and have been proposed as potential targets for vaccine development. Epitope-based vaccines offer several advantages over whole protein vaccines as the immunogenic proteins are small and can induce both Th1 and Th2 immune responses, which are desirable for protection. However, the MSA, particularly gp45, is polymorphic in *Babesia bigemina*, posing a challenge to vaccine development. The purpose of this study was to develop a recombinant gpME (gp45-multi-epitope) for a vaccine against *Babesia bigemina*. B-cell, T-cell, and HLA epitope predictions were used to synthesize the gpME sequence from the consensus sequence of gp45. The gpME sequence was synthesized and cloned in the pET28α vector through the commercial biotechnology company to get pET28-gpME. The plasmid cloned with the gpME sequence comprising 1068 bp was expressed in a bacterial expression system. A band of 39 kDa of rec-gpME was obtained via SDS-PAGE and Western blotting. Rec-gpME @200ng was injected in calves 3 times at 2 weeks interval. The humoral response was evaluated through the indirect ELISA method. The ELISA with rec-gp45 protein showed a significant value of optical density. The recombinant protein containing multiple epitopes from the MSA gp45 may represent a promising candidate for a vaccine against *Babesia bigemina*.

Keywords: *Babesia bigemina*, multi-epitopes, merozoite surface antigen, gp45, vaccination

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Introduction

Babesiosis is contemplated and the most important challenge to livestock animals (Singh et al. 2009). Round about 80% of the total world population of cattle is present mainly in the area of sub-tropical and tropical, where ticks-borne diseases and ticks are the source of considerable profit losses in bovines (de Castro 1997, Lew-Tabor et al. 2016). It is noteworthy that the reduced production of both dairy and beef cattle in various regions such as Pakistan (Jabbar et al. 2015, Siddique et al. 2020) can significantly impact the livelihoods of impoverished farming communities. Bovine Babesiosis (BB) is caused by *B. bovis*, *B. bigemina,* and *B. divergens*, which are serious challenges to the farm economy and animal health (Uilenberg 2006). It causes severe hemoglobinuria, anemia, and ultimate death (Wagner 2002). BB is transmitted through the saliva of a one-host tick vector (*Rhipicephalus* spp.) into the blood stream of the host (Karim et al. 2017). It is also spread through contaminated syringes, needles, surgical instruments, and blood transfusions (Esmaeilnejad et al. 2015). *B. bovis* and *B. bigemina* transmitted via *Rhipicephalus microplus* are prevalent in the livestock population causing significant economic impacts on the livestock industry in Pakistan (Durrani et al. 2008, Rehman et al. 2019).

Diminazene aceturate and imizole (imidocarb dipropionate) are used against babesial parasites in bovines but their irrational usage might lead to cause drug resistance against these parasites (Mosqueda et al. 2012, Silva et al. 2016, Klafke et al. 2017, Maiorano et al. 2018). This highlights the need for new intervention of various compounds that are effective to kill Babesia and have low toxicity (Yoshida et al. 2017, Tuvshintulga et al. 2019). Production of immunity against babesiosis can be attained by vaccination of bovine owing to its high clinical and economic impact globally (Ciloglu et al. 2018). Vaccines against BB are recently introduced in South Africa, different countries in South America, Israel, and Australia (Jorgensen et al. 1989, Echaide et al. 1993, Mangold et al. 1996, Callow et al. 1997, Bock et al. 2001, Shkap et al. 2005) consisting of bovine blood containing weakened Babesia. But there is a possibility of reverting from attenuated organisms to pathogenic parasites. It is a commonly known fact that the genetic makeup of *B. bigemina* sequences varies widely across different regions of the world (Shkap et al. 2005). Therefore, in the current circumstances, the development of a recombinant protein-based vaccine against *B. bigemina* is imperative.

In the previous studies, splenectomized calves were vaccinated with supplemented antigen fragments of *B. bigemina* which were challenged with *B. bovis* and *B. bigemina*. Protection in *B. bigemina*-immuned animals was also observed against the heterologous challenge of *B. bovis* (Wright et al. 1987). The notable immunogens reported by different scientists directed against *B. bigemina* are RAP-1, gp45, and gp55 proteins (McElwain et al. 1991). Glycosyl-phosphatidylinositol tied up MSAs antigens are considered to illustrate the merozoites' occupation of host erythrocytes. They are promising immunogens due to their critical role in the continuance of Babesia (Wieser et al. 2019). The immunodominant proteins are MSAs in *B. bovis*, gp45 and gp55 proteins in *B. bigemina* from tropical and sub-tropical regions (Carcy et al. 2006). In the previous studies, *B. bigemina* showed amino acid polymorphism in gp45 but it is a promising immunodominant antigen and is considered to be the candidate for a vaccine against *B. bigemina* (McElwain et al. 1991, Fisher et al. 2001). To overcome genetic variability, selective epitopes can serve the purpose of reducing the variation due to polymorphism (Gomara et al. 2007). B-cell-based multi-epitope antigens had been predicted for the diagnostic purpose against parasites like "*Echinococcus*", "*E. multilocularis*" and "*E. granulosus*") (List et al. 2010) and cutaneous and visceral leishmaniasis (Menezes-Souza et al. 2014). Multi-epitopes were also used for vaccination purposes containing T-cell epitopes to induce Th-1 type immunity for intra-cellular parasites like *Theileria annulata* (Kar et al. 2018).

Recently, bioinformatic tools have been employed in making peptide-based vaccines, their adjuvants, and specific antibodies (Khatoon et al. 2018, Kalita et al. 2020a, Kalita et al. 2020b). For the first time, Adu-Bobie et al. (2003) employed an immuno-bioinformatic approach to make a successful vaccine against *Neisseria meningitidis* (Adu-Bobie et al. 2003). Afterwards, different researchers made multi-epitope vaccines via these methods, some of which are presently used via clinical methods (Caro-Gomez et al. 2014, Mehla et al. 2016, Gaafar et al. 2019). As a result, immunological and bioinformatic tools have played a significant role in the identification and development of novel vaccines. In the present study, we utilized these tools to predict B lymphocyte, T lymphocyte, and HTL epitopes of the gp45 protein of *B. bigemina*. A protein sequence consisting of approximately 356 amino acids was cloned into a prokaryotic expression system for expression and inoculation into calves, to evaluate its immunogenicity.

Materials and Methods

Ethics approval

The experiments in this study were approved by the ethical committee of the University of Veterinary and Animal Sciences (UVAS), Lahore (Dr/416, dated: 10-06-2019).

Epitope prediction

To get the consensus sequence of gp45, all of its 25 sequences of gp45 that contain isolates from various regions of Mexico, South Africa, USA, India, and Argentina, from Gene Bank of NCBI were sorted out. The accession number of gp45 were from AGU67936.1 to AGU67947.1, from AEJ89906.1 to AEJ89912 from AVM80891.1 to AVM80893.1,

AAG28757.1 and AAG28759.1. A total of 25 sequences in Fasta format regarding *B. bigemina* were copied and these sequences w e r e lined up by Geneious R8.1.6 (Kearse et al. 2012).

Multi-epitope protein was designed using MHC-II, Helper T-cells (HTL), MHC-I, epitopes of cytotoxic T-lymphocyte (CTL) and B lymphocytic and a suitable adjuvant by specific linkers as reported (Kalita et al. 2020a, Kalita et al. 2020b). MHC-I, CTL epitopes prediction of gp45 protein has been evaluated using Net CTL 1.2 server. This is a freely available online web server and it anticipates CTL epitopes of gp45 protein on established fundamentals of MHC-I binding attraction, TAP movement effectiveness, and C-terminal cleavage of the proteasome. The threshold value came as 0.75 and it was used to set the deprived and three supertypes (A2, A3, and B7) of alleles that contain 88.3% of worldwide populations taken. Prediction of epitopes (15 amino acids long) of HTL was done using Immune Epitopes & Analysis Resource (IEDB). The IEDB server anticipates epitopes which are based on large-scale datasets of around 17,000. A set of various 26 alleles and these different alleles enclose the more than 99% population. The bepiPred-2.0 server was employed to assess epitope anticipation of B-cell of gp45 protein. This server is based on the random decision forests algorithms, and it consists of 649 antigen-antibodies which were 3D structure datasets that were used for anticipation of antigenic epitopes of B-cell. Linker was added that provides the separation of epitopes. The adjuvant 50S L7/L12 of ribosomes (Uniprot ID: P9WHE3) was built on the N terminal of the fragment and the remaining sequences were joined through a linker of EAAAK. All epitopes of HTL, CTL, and B-cell were conserved in all the 25 sequences of gp45 protein that were retrieved from NCBI and were connected through sequences of GPG-

PG, KK, and AAY consequently to finally construct gpME. Nearly half (50.28 %) of the native gp45 was covered in the construction of gpME.

The following tools were used for the epitope prediction.

1. Uniprot ID: P9WHE3 was employed for the Adjuvant (Yadav et al. 2020).

2. The HTL-epitope prediction was done using IEDB (2.22 prediction method) (Yadav et al. 2020).

3. Bepipred (Linear Epitope Prediction 2.0) was employed for epitopes of B-cell prediction (Rodríguez-Camarillo et al. 2020).

4. NetCTL 1.2 server was employed for the prediction of CTL (Khatoon et al. 2018).

Cloning and expression of gpME

Cloning of gp45 gene was done in pET-28α. Expression and purification of rec-gp45 were done in our laboratory (results are under publication) similar to our previous work on SAG1 and ROP18 of *Toxoplasma gondii* (Nabi et al. 2017, Naeem et al. 2018, Rahman et al. 2021) NEB cutter was used for Restriction analysis of gpME sequence. The gpME sequence was synthesized and cloned along with restriction sites: XhoI site at 5′ and HindIII site at 3′ in pET28a+ vector through Gene Script (https://www.genscript.com/). Competent cells were prepared by the protocol described elsewhere (Rahman et al. 2021). Briefly, *E. coli*; BL21 (DE3) strain chemically competent cells that were in-house prepared and stored at -196°C in cryo-vials were used. The transformation of competent cells was done with pET28-gpME through electroporation. The material from the tube was transferred into 1 mm chilled electroporator cuvettes (Bio-Rad Gene Pulsar X-cell electroporator system). Cuvettes were placed in Gene Pulsar for electroporation. Pulse conditions applied for electroporation were 1.8 kV, 25 µF and 200Ω Super Optimal Broth (SOC) medium was added and mixed immediately after electroporation. Media from cuvettes were transferred to 1.5 ml tubes and were incubated in a shaking incubator at 37°C, for 60 min. Following incubation, 100 µl was poured on LB agar plates containing kanamycin @50 µg/ml. After proper labeling, plates were put upside down in an incubator at 37°C for 24 hrs. Cells of the colonies were incubated in 10 ml LB broth having kanamycin (6 hrs./37°C). The plasmid was extracted using a Gene JET plasmid Miniprep kit (Cat. # K0503) following the given guidelines. The restriction analysis was done to confirm transformation using HindIII and XhoI (Jena Bioscience, Germany). Shortly, restriction enzymes (3 U) and their compatible 10X reaction buffer were employed to assimilate 1 µg of extracted plasmid at 37°C for 1 hr.

Transformed colonies were inoculated in 1 ml (LB containing kanamycin), and incubated (37°C/24 hrs.) until OD600 come to 2. Next, the culture was added to 100 ml broth of LB kanamycin, and incubated (8 hrs./37°C) when OD600 come to 0.5. Subsequently, 1 mM IPTG (Isopropyl ß-D-1thiogalactopyranoside) (Invitrogen™, Waltham, Massachusetts, USA, Cat #15529019) was added to the broth, incubated (7 hrs./37°C) to induce the expression of a protein. For protein purification, induced cells were lysed as per the optimized procedure (Teimouri et al. 2019). Shortly, pelleted cells were resuspended in bacterial lysis buffer (8 M urea, 500 mM NaCl and 20 mM NaH_2PO_4) for a few minutes at pH 8.0. The ultrasound (15-20 kHz) generated a sonic wave, which was utilized to lyse cells (Shehadul Islam et al. 2017). After the addition of lysis buffer to bacteria, the lysed suspension was sonicated 15 times for 30 pulses with 1 min intervals. The lysate-containing tube was placed in a container with ice and water during sonication. The quantification of protein was done by Bicinchoninic Acid kit (Cat. # 786-628) following the manufacturer's guidelines. Furthermore, the induced cells were lysed using 5x SDS buffer (100 mM DTT, 10% SDS, 50% glycerol, 0.313 M Tris–HCl of pH 6.8 and 0.05% bromophenol blue). The un-induced and induced cells were verified through 12% SDS-PAGE for purification of rec-gpME. The chromatographic purification of rec-gpME protein was carried out using HisPurTM affinity of nickel-nitrilotriacetic acid (Ni-NTA) columns (Thermo Fisher Scientific, Zhongzheng, Taipei City, Taiwan, Cat # 88228).

SDS PAGE and Western blotting

The purified rec-gpME protein was verified through 12% SDS-PAGE with 5% stacking gelelectrophoresis and Western blotting. For immune-blotting analysis, 30 µl of purified rec-gpME protein was added in the wells of 12% SDS-PAGE gel. After SDS-PAGE electrophoresis, one gel was stained via Coomassie brilliant blue, and the other gel was transferred onto nitrocellulose membrane (NCM), 3% skim milk solutions were used to block non-specific binding sites of NCM and washed (Phosphate--buffered saline (PBS)/Tween 20).The membrane was incubated with sera of vaccinated calf (25°C/ 1 hr and washed (PBS/Tween20). The bounded antibodies were detected by Rabbit Anti-bovine IgG (Sigma-Aldrich, Cat# A0705)-AP, diluted 1:2000 in 5% non-fat milk. Alkaline phosphatase activity was recognized through BCIP/NBT liquid substrate system (Sigma-Aldrich, USA). Protein ladder (Thermo Fisher Cat# 26619) was used a standard. Expressed rec-gpME was analyzed with SDS- PAGE and Western blotting

as described earlier (Rahman et al. 2021). The sera of vaccinated cattle calves were used as primary antibody (Umber et al. 2020) and Rabbit Anti-Bovine IgG (Sigma-Aldrich Cat # A0705) was employed as Secondary Antibody conjugated with AP.

Inoculation trials with rec-gpME

In this research study, a total of six calves (6-8 months) of Holstein Friesian breed were used forimmunization purposes; the calves were reared for 10 days in UVAS research pens, given "ad-lib" water and food. The calves were divided into 2 groups: Group B (immunization) and Group A (control) and were tested negative for *B. bigemina* through thick blood smear andPCR screening. Each experimental group contained three calves. The calves of group B were immunized with 200 µg of rec-gpME using 2 ml of oil-based adjuvant Montanide TM injected subcutaneously three times with 2 weeks intervals. The animals in control group A were injected with an equal volume of PBS only. The baseline humoral antibody response was measured in Group A (3 calves), which served as a control group having healthy animals.

Screening of sera through ELISA using rec-gp45 as antigen

Serum samples were collected pre- and post-immunization from group A and group B to evaluate the antibody titer. ELISA was done to recognize Immunoglobulin G (IgG) antibodies confirming the procedure described elsewhere (Rashid et al. 2011). Shortly, 0.125 µg/ml of rec-gp45 was coated on the wells of ELISA plate (JET BioFil, Hong Kong, China, Code # TCP011096) with 50 mM bicarbonate buffer and incubated (4°C) overnight. ELISA plates were washed (3 times). Saturation of the plate was done with 4 % BSA/PBS followed by incubation (37°C/2 hrs.). PBS was added into positive and negative sera for serial dilutions with a two-fold factor. Diluted sera were dispensed into each well. The plate was incubated again (37°C/2 hrs.). The second washing was done (3 times). Primary antibodies of serum were detected by incubating (37°C/2 hrs.) with Rabbit Anti-Bovine IgG-AP (1:10,000). Followed by three washing, phosphatase activity was detected through pNPP. (Cat. 41480004-1, Bioworld® USA) @1 mg/ml in 1M Diethanolamine (Cat. # 40400060-3, Bioworld®). OD values were taken with an ELISA reader (Model ELX 800, BioTeck, USA) at 405 nm.

Statistical analysis

The antibody titers were analyzed with the Mann-Whitney test. Statistical differences were signifi-

B-cell epitopes	score
IWSASTHPFFDKFYKD	0.92
AVSGATTHGGDARGVN	0.89
SRMEIHAPDAVKLPNL	0.88
AVGARTLREEMHIVSK	0.87
HGGDARGVNPTPGVTS	0.87
TSRTSDPFGMAVAEQP	0.87
LSTLDESNSRMEIHAP	0.84
TDPVIVPGTSRTSDPF	0.84
FSLLNLFDEYEANLSP	0.84
TSESTSLRSGAAEASP	0.83
PVLPEGVPQREKVADI	0.81
DAVQVNGERAVSGATT	0.81
EKWIFHDSLVVELRVP	0.81
QPIVGMYGENKPVLPE	0.8
GVVVREDAEGDAVQVN	0.8

NetCTL-1.2 predictions using MHC supertype A2, A3 and B7	Epitope	Score
gp45		
A2 super type	MLATFSIAA	1.1938
	MMLATFSIA	0.9787
	ALCCLIAQV	1.1017
	TLREEMHIV	0.8014
	KLLDVQPIV	1.4663
	GMYGENKPV	0.9937
	KVADINAKL	1.0733
	KLATLKALI	0.9283
	ALIDGEKWI	0.8047
	WIFHDSLVV	0.9079
	SMALKLETL	0.9484
	FLSTEATDV	1.1469
	TLDESNSRM	0.8155
	YAFEPFAML	0.9001
	AMLLIVMSA	1.1101
A3 super type	IVGMYGENK	0.7986
	INAKLATLK	0.8676
	GVNRYVFFK	1.5082
	YVFFKGLLK	1.5722
	GLLKSMALK	1.6586
	MALKLETLY	0.9105
	KAIELNLAK	0.9461
	FLKVFYNSK	0.9019
	ASTHPFFDK	1.2733
	HPFFDKFYK	0.9116
	TTSESTSLR	1.0587
	GAAEASPKK	0.8164
B7 super type	LATFSIAAL	1.1637
	QVRASTDPV	0.9584
	QPAVGARTL	1.4357
	GARTLREEM	0.7850
	KVADINAKL	0.8287
	SPLFSLLNL	1.3037
	SPRDIWSAS	1.1043
	TPGVTSSTT	0.7763
	SPKKASYAF	1.6369
	KASYAFEPF	0.9374
	IVMSAAFAF	0.8705

Prediction method: IEDB recommended 2.22 | Low adjusted_rank = good binder

Allele	#	Start	End	Length	Peptide	Percentile Rank
HLA-DRB1*01:01	1	344	358	15	EPFAMLLIVMSAAFA	0.38
HLA-DRB1*01:01	1	345	359	15	PFAMLLIVMSAAFAF	0.56
HLA-DRB1*01:01	1	130	144	15	VFFKGLLKSMALKLE	1.30
HLA-DRB1*01:01	1	129	143	15	YVFFKGLLKSMALKL	1.30
HLA-DRB1*01:01	1	343	357	15	FEPFAMLLIVMSAAF	1.80
HLA-DRB1*01:01	1	127	141	15	NRYVFFKGLLKSMAL	2.00
HLA-DRB1*01:01	1	128	142	15	RYVFFKGLLKSMALK	2.20
HLA-DRB1*01:01	1	131	145	15	FFKGLLKSMALKLET	2.30
HLA-DRB1*01:01	1	132	146	15	FKGLLKSMALKLETL	2.50
HLA-DRB1*01:01	1	126	140	15	VNRYVFFKGLLKSMA	2.60

Fig. 1. 3 B-cell, 6 CTL, and 5 HTL epitopes of gp45 protein. Highlighted epitopes having the highest score of epitopes of CTL and B-cell and lower percentile rank epitopes of HTL were included in the sub-unit vaccine construct.

cant at $p \leq 0.05$. Data were assessed by GraphPad Prism 7 (GraphPadSoftware, La Jolla, CA, USA).

Results

Designing of gp-ME

All sequences of gp45 from the database were retrieved and aligned using Geneious R8.1.6 software to get the conserved sequence. Subsequently, we identified potential B lymphocytic cell epitopes, HTL epitopes, and CTL epitopes that were conserved across all gp45 protein sequences obtained from the NCBI database. To enhance the immunogenicity of the vaccine construct, a protein fragment of 356 amino acids containing a 50-amino acid adjuvant from L7/L12 (P9WHE3) ribosomes was synthesized. All 5 HTL, 3 B-cell, and 6 CTL epitopes (Fig. 1) were connected with suitable linkers. All epitopes of CTL were connect-

ed via AAY linker whereas the KK linker was employed to join the epitopes of B-cell. GPGPG linker was employed to join the epitopes of HTL and subsequently, an adjuvant was connected to epitopes of HTL epitopes through an EAAAK linker, as shown in Fig. 2.

Predicted epitopes were found across the antigen sequence as shown in Fig. 2. The peptides were synthesized and cloned in pET-28α vector.

The epitope of HTL has been connected through EAAAK linker and GPGPG, AAY, and KK linkers has was employed for the epitopes of intra-HTL, B-cell, and intra-CTL. Hence, the final gpME protein constructs were composed of a total of 356 amino acid residues as shown in Fig. 3.

SDS-PAGE and Western Blot Analysis

After the expression and purification, rec-gpME was subjected to 12% polyacrylamide gel electropho-

MAKLSTDELLDAFKEMTLLELSDFVKKFEETFEVTAAAPVAVAAAGAAPAGAAVEAAE
EQSEFDVILEAAGDKKIGVIKVVREIVSGLGLKEAKDLVDGAPKPLLEKVAKEAADEAK
AKLEAAGATVTVKEAAAKEPFAMLLIVMSAAFAGPGPGPFAMLLIVMSAAFAFGPGPG
VFFKGLLKSMALKLEGPGPGYVFFKGLLKSMALKLGPGPGFEPFAMLLIVMSAAFAAY
MLATFSIAAAAYKLLDVQPIVAAYGVNRYVFFKAAYGLLKSMALKAAYSPKKASYAFA
AYQPAVGARTLKKIWSASTHPFFDKFYKDKKAVSGATTHGGDARGVNKKSRMEIHAP
DAVKLPNL

Fig. 2. Multi-epitope base vaccine construct of gpME protein of *Babesia bigemina* adjuvant-EAAAK-HTL epitope-AAY-CTL
epitope-KK-B-cell epitope.
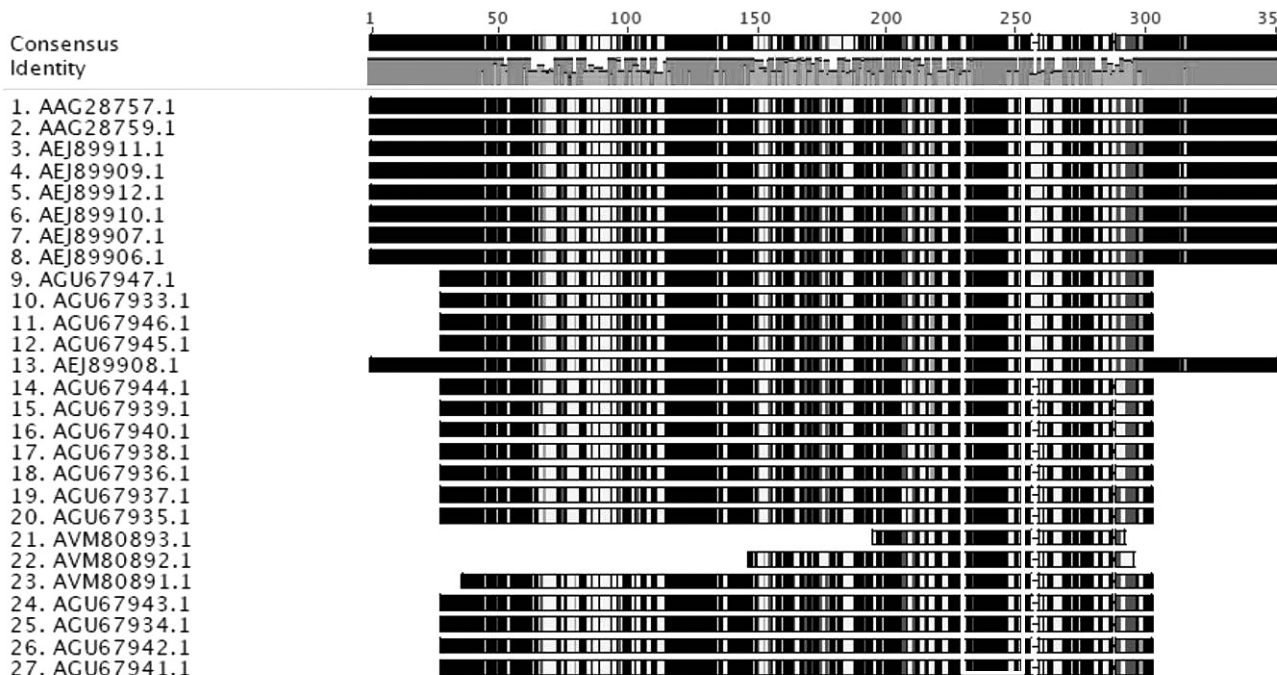
Fig. 3. Selected epitopes of CTL, HTL, and B-cell were found conserved among all sequences of gp45 that were retrieved from NCBI.
Epitopes were selected from the black shaded area in the consensus sequence out of t h e conserved region obtained after
alignment 50.28 % of the native protein (gp45) was covered in gpME.

resis for SDS-PAGE analysis. Figure 4 shows the
protein band of rec-gpME (356 amino acids) with
a molecular weight of about 39 kDa that was visualized
through Coomassie-Blue staining. The expressed
rec-gpME protein reacted with sera of the immunized
calf which showed that our rec- gpME was immuno-
genic and a brown band with 39 kDa was found as
shown in Fig. 4.

Humoral response

The sera of vaccinated and non-vaccinated animals
were tested with rec-gp45 as shown in Fig. 5. The mean
OD of the vaccinated group containing the high-affinity
IgGs showed a significant difference ($p<0.05$) as com-
pared to the control group. A remarkable difference was
also observed before and after immunization in the test
group but no apparent difference was seen in control
group. Vaccinated animals showed specific IgG response
with an OD value of 2.460 ± 0.3151.

Discussion

Gaining accurate knowledge of antigenic epitopes
from multi-domain or large proteins is a challenging
and laborious task due to difficulties associated with
low expression, improper purification, and constraints
related to structural studies of proteins. Moreover,
research related to immunity and vaccine development
is experimentally rigorous and costly. However, in the
last decade or two, various powerful computational
tools have been developed that facilitate the handling
of genomic and proteomic data for useful purposes.
One of the best examples is reverse vaccinology
and structural bioinformatics which is used to lessen the
efforts of screening different immuno-dominant epi-
topes to design effective vaccines. Correspondingly,
immuno-informatic approaches have been used to dis-
close the important immuno-dominants from the data-
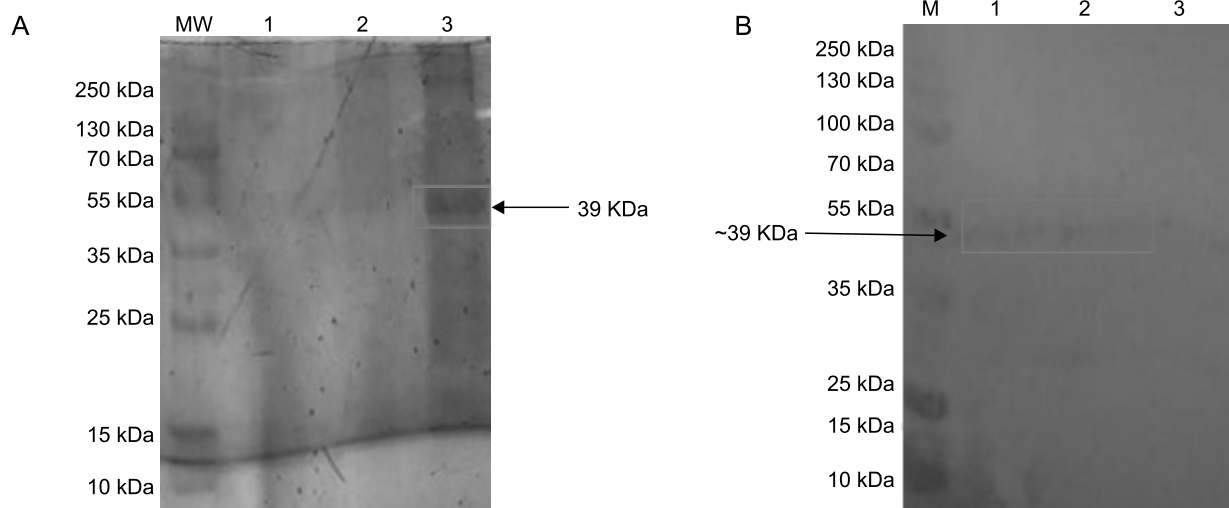bases to design vaccines (Kumar et al. 2021). Along

Fig. 4. (A) SDS-PAGE results: Lane (M) shows Protein molecular weight marker (Thermo Scientific Cat# 26619). L1 shows BL21 culture without induction after 7 hours, L2 shows BL21 culture without induction after 24 hours, and L3 shows a prominent band of purified rec-gpME between 39 kDa. (B) Western Blot results: Lane (M) shows a Protein molecular weight marker (Bio-Hilex Catalogue # PM007-0500). L1 and 2 show a band of 39 kDa and L1 shows no specific band in non-induced culture.
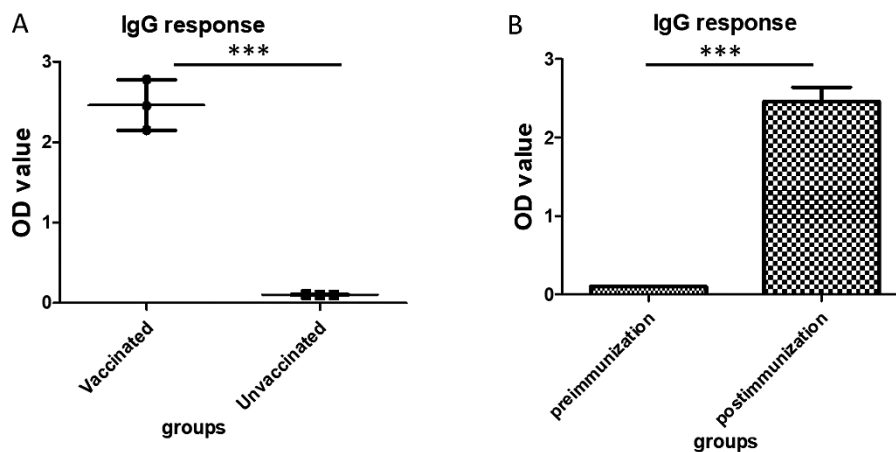

Fig. 5. ELISA plate was coated with rec-gp45 protein for indirect ELISA. Specific IgG response is, (A) between immunized and non-immunized groups, (B) between before and after vaccination in the immunized group.

with the tick control strategy, the development of a vaccine is inevitable to control bovine babesiosis through an integrated approach. In the previous studies, the animals were immunized with recombinant Merozoite Surface Antigens (MSA) of *B. canis* and *B. divergens* and infections entrenched glycosylphosphatidylinositol (GPI)-tied up MSAs (Debierre-Grockiego et al. 2010) to get protection in cattle (Delbecq et al. 2006) and dogs (Moubri et al. 2018). Thus, it is pertinent to develop a vaccine against bovine babesiosis. Similarly, gp45 is a GPI- anchored MSA in *B. bigemina* which is a polymorphic protein (Fisher et al. 2001). Variable Merozoite Surface protein like gp45 has been characterized in *B. bigemina* and it has not been recognized *B. bovis* (Fisher et al. 2001). Immunization with native gp45 from the Mexico strain instigates preservation against homologous challenge in cattle (Mishra et al. 1992).

To overcome the variability of the protein, we first obtained the consensus sequence out of all 27 sequences which were retrieved from the NCBI database using Geneious Prime software and then epitopes were chosen to construct a multi-epitope protein (Ezediuno et al. 2021). Adjuvant 50S L7/L12 of ribosomes (Uniprot ID: P9WHE3) was reckoned to the N terminal of multi-epitope construct to make a promising vaccine candidate against *B. bigemina* infection in cattle. The multi-epitope vaccine was built as previously described (Khatoon et al. 2018, Kalita et al. 2020, Yadav et al. 2020). There is a need for epitopes of B-cell, HTL, and CTL in the proteins to develop of Th1 and Th2 arms of immunity for protection against blood parasites like babesia (Djokic et al. 2018, Debierre-Grockiego et al. 2019). Recently, conserved B-cell epitopes in gp45 were found in geographically

distinct isolates of *B. bigemina* in Mexico (Mercado-Uriostegui et al. 2022).

Epitope-based diagnosis (Mucci et al. 2017) and vaccination (Patarroyo et al. 2008, Hajissa et al. 2019) have been developed against parasitic infections. Mucci et al. (2017) carried out a serological evaluation of 27 epitopes in a different multi-peptide employed for the diagnostic method. A union of these seven peptides was finally assessed with ELISA against a panelof 199 sera (Chagas-positive and negative, adding sera from Leishmaniasis-positive subjects). Multi-peptide formulation expressed a high diagnostic performance, with a sensitivity of 96.3% and a specificity of 99.15% (Mucci et al. 2017). Immunization of cattle against *B. bovis* using a multi-epitope modified vaccinia Ankara virus induced strong Th1 cell responses but failed to trigger neutralizing antibodies required for protection (Rauf et al. 2020). While we observed humoral response in the form of IgG, the difference with our work might be due to the use of a viral vector which induced Th1 type of immunity while we used TLR-2 triggering adjuvant in our multi-epitope protein.

The sera were tested with rec-gp45. The sequence of gp45 was also cloned in pET-28α and expressed in a prokaryotic system (results not shown), similar kinds of expression we had already done in our laboratory to express rec-protein like rec-ROP18 (Nabi et al. 2017) and rec- SAG1 (Naeem et al. 2018, Rahman et al. 2021). In our previous work, cattle calves vaccinated with attenuated *B. bigeminal*-infected RBCs showed IgG response against crude antigens as 1.881±0.085 (Kumar et al. 2021) which is less than the response that we achieved in the current study. The elevated level of IgG response (2.460±0.3151) with the same dilution i-e 1:50 is due to the use of recombinant protein.

Further studies are required to evaluate the efficacy of rec-gpME as a vaccine against both homologous and heterologous challenges of *B. bigemina* and *B. bovis*. These studies are crucial for the development and optimization of a viable vaccine that can effectively control the spread of these diseases.

Conclusions

There is an urgent need to develop a recombinant-based vaccine against *B. bigemina* to control bovine babesiosis, which has become increasingly difficult to treat due to the development of drug resistance. Although a live attenuated vaccine against *B. bigemina* is available in the form of infected RBCs in Australia and Israel, its use poses a risk of disseminating exotic DNA to other countries. A recombinant protein-based vaccine is a safer and more viable option, but only

a limited number of immunogens have been explored thus far for launching vaccination against *B. bigemina* elicits an immunogenic response in calves on inoculation. This multi-epitope-based recombinant protein produced a specific IgG response.

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