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RESEARCH ARTICLE

Expression and Purification of Recombinant Multi-epitope Protein of *Rhipicephalus microplus* Tick and its Antigenicity in the rabbit model

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A B S T R A C T

Rhipicephalus microplus, hard tick causes babesiosis and anaplasmosis in cattle. For decades, ticks control has been depending on synthetic acaricide use, however, acaricide-resistant ticks led to alternative effective and eco-friendly tick control approach such as vaccination. Currently, vaccine design using immuno-informatics is a promising method in the vaccination field. Here, we proposed a multi-epitope-based (ME) vaccine against *R. microplus* tick, comprising potential immunodominant B-cells, Helper-T Lymphocytes and Cytotoxic-T Lymphocytes epitopes. Then, designing of ME vaccine construct, containing sequences of Bm86, Subolesin and Bm95, was cloned into pET28a a prokaryotic expression vector. The recombinant ME protein was expressed in the *Escherichia coli* BL21 strain. After that protein purification was done by Nickle-NTA affinity chromatography. Evaluation of expression of recombinant ME based protein by SDS-PAGE analysis showed that optimal expression in LB medium for 8 h at 37 °C post-induction by 0.7 mM IPTG. The quality of purified recombinant protein was validated by Western blot analysis. 100 µg/2ml of purified ME protein with adjuvant Montanide ISA 50V was used in rabbits to evaluate the antigenicity of ME protein. Our study finding revealed that the expressed recombinant ME protein's molecular weight was 42kDa. The anti-recombinant protein antibodies raised in immunized rabbit's sera was detected through western blotting and ELISA. This study results showed that recombinant ME protein is a potential vaccine candidate against *Rhipicephalus microplus* tick infestations.

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INTRODUCTION

Ticks are small blood-sucking ectoparasites of both animals and humans, and the estimated annual losses due to tick infestation are US $20 to 30 billion globally (Pegram *et al*., 1993; Lew-Tabor and Valle, 2016). Among the many tick species, *Rhipicephalus microplus*, cattle tick, is found in tropical and subtropical regions of the world. It infests the cattle and causing significant economic losses to the livestock industry (García-García *et al*., 2000; Estrada-Peña *et al.,* 2008). *Rhipicephalus microplus* infestation results in direct economic losses in term of decreased meat and milk production and as a vector of *Babesia spp.*, *Anaplasma spp.* and *Ehrlichia spp.* (Marques *et al*., 2020).

The most common conventional tick control method is treating the animals with synthetic acaricides (Graf *et al*., 2004). However, this method has several drawbacks such as the contamination of milk, meat, and the environment with its chemical residues and the selection of tick-resistant strains (Graf *et al*., 2004; de la Fuente and Contreras, 2015). Therefore, the alternative measure to control tick infestation is the application of biological agents and vaccination. Vaccination is a sustainable method and has various advantages over chemical methods such as it doesn't induce bioaccumulation and is safe for the

environment (Henrioud, 2011; Kiss *et al.,* 2012). In the 1990s, the recombinant glycoprotein-based Bm86 vaccine was developed to control *R. microplus* and commercialized with the name of TickGARD® and GAVAC® in Australia and Cuba respectively (Rand *et al*., 1989; Willadsen 2004). But these vaccines show a varying level of efficacy in various geographical locations of the world, which may be due to strain-to-strain variation (Guerrero *et al.,* 2012). The expressed Bm95 was used as a chimeric protein with major surface protein 1a (MSPa1) for the control of *R. microplus* infestation and were reaching an efficacy of 64% (de la Fuente *et al*., 2005; Almazán *et al*., 2012). Subolesin protein was identified in insects and ticks as it is involved in signal transduction and immune response and also its role in pathogen infection and tissue development (de la Fuente *et al.,* 2006; Galindo *et al*., 2009). It was observed that subolesin gene was expressed in all the stages of the tick and was conserved in the vertebrate and invertebrate host (Almazán *et al*., 2003). The recombinant subolesin protein was used as vaccination in cattle and provides 51% efficacy against ticks (Almazán *et al*., 2010). Subolesin and Bm86 antigens were used as a combined vaccine but no significant variation in results was found (Hope *et al*., 2010; Schetters and Jansen, 2017). Up till now, several antigens were identified and used as vaccines in controlling ticks and tick-borne pathogens. There is a need to identify such antigens that could provide long-lasting and effective immunity in the host. With the advancement of the reverse vaccinology approach, there is a possibility of developing an effective vaccine by using immunogenic and bioinformatics tools (Lew-Tabor and Valle, 2016). This approach has been successful in the development of a vaccine against Onchocerciasis, *leishmania*, *plasmodium falciparum, Ebola virus,* and ticks (Damfo *et al.,* 2017; Khatoon *et al.,* 2017; Shey *et al*., 2019; Ullah *et al.,* 2020). Identification of target antigens using reverse vaccinology is possible that decrease the tick reproduction by damaging the tick feeding and activating the specific antibodies. Reverse vaccinology approach has several benefits over conventional approach of vaccine development such as safe, sustainable and cost-effective in vaccine production. Furthermore, bioinformatics tools were helpful in identification of antigenic T-cell and B-cell epitopes in development of epitope based vaccine (Bahrami *et al.,* 2019). Recently, researchers have used the antigenic epitopes of different vaccine candidates for production of vaccine that could be more sustainable and safer in hosts (Gaafar *et al.,* 2019). First step in construction of immunogenic vaccine is the identification and prediction of immune protective antigens using immunoinformatics approach. In our recent study (unpublished data), authors have analyzed the T and B-cell epitopes of three proteins (Bm86, Subolesin and Bm95) of *R. microplus* by using various immunoinformatic tools. In this study, we used the antigenic protein sequences of *R. microplus* for construction of multi-epitope (ME) DNA fragment. After that, DNA fragment was cloned in prokaryotic expression vector pET28*a* through commercial service provided by GeneScript®. The plasmid "pET28*a*-ME" was transformed in competent *E. coli* BL21 cells. The synthetic DNA fragment was verified by restriction analysis using *XhoI* and *NdeI.* The expression of recombinant ME protein was induced by adding 0.7 mM IPTG for 8h at 37°C and analyzed on SDS-PAGE and Western blot. The purified protein was evaluated for its antigenicity in rabbit using adjuvant Montanide ISA 50V.

MATERIALS AND METHODS

Designing and construction of synthetic ME DNA fragment of *Rhipicephalus microplus:* We designed the ME-based synthetic DNA fragment using various immunoinformatic tools. Firstly, we tested the antigenicity of protein constructed from selected vaccine candidates through online Scratch Protein Predictor (https://scratch.proteomics.ics.uci.edu/). After that, prediction of T-cell epitopes were done using IEDB server and B-cell epitopes were predicted through ABCPred server. Finally, the ME based gene fragment was constructed by combining the fourteen sequences of T cell epitope (HTL and CTL) and eight B-cell epitopes. Sequences were displayed in results section Fig. 1 (B). Using suitable linkers as reported in previous studies (Kalita *et al.,* 2019; Yadav *et al*., 2020). The ME based gene fragment was synthetized and cloned into pET-28a vector commercially (Genscript Biotech Corp). The preparation of competent *E. coli* BL21 (DE3) cells were performed as described in our previous studies (Akbar *et al.,* 2021). The digestion was done by using 1µg of plasmid with 3 U of enzymes and 10X compatible Buffer and digestion was completed at 37°C in 60 minutes.

Expression and purification: Transformed *E. coli* was cultured on LB plates containing (50 µg/ml) kanamycin at 37°C for overnight incubation. Transformed cells were further cultured in 10ml LB broth medium incubated at 37°C 220 rpm until the OD_{600}: 2.0. Then 100µl of LB medium containing transformed bacteria into fresh 60ml LB medium and allowed growing at 37°C in shaker, 220 RPM until OD_{600} reached 0.4-0.6. Culture ($OD_{600:}$ 0.6) was induced for 8 h at 37°C in shaker, 220 RPM with 0.7 mM isopropyl-$_{\beta}$-D-thio-galactopyranoside (IPTG). HisPur nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Thermo Fischer Scientific, Cat#. 88228) was used to purify the expressed recombinant protein according to the manufacturer's protocols. After that collected lysate was centrifuged at (8000g, 30 minutes, at 4°C) to pellet the cellular debris and transfer the supernatant to 1.5 ml micro tube. The BCA (bicinchoninic acid) protein assay kit (G-Biosciences, Cat# 786-570) was used to estimate the protein concentration (Walker, 2009).

SDS-PAGE and western blotting: Expression and purification of recombinant ME protein was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The 30ul of samples were suspended in 30 ul of 10x Tris-Glycine SDS buffer (Invitrogen™, Cat#LC2675) and boiled at 95°C for 5 minutes. SDS-PAGE gel was visualized with Coomassie blue staining. SDS-PAGE gel was transferred into a nitrocellulose membrane (NCM) for western blotting using Mini Trans-Blot® Turbo™ Transfer System (BIO-RAD) (Goldman *et al.,* 2015). Further protocol was done by following the steps as reported in our previous study (Akbar *et al*., 2021).

Rabbit immunization and immunoassay: To produce the anti-ME antibodies, two 5-8 month healthy New Zealand white rabbits (*Oryctolagus cuniculus*) were immunized with 100 µg/ml (2ml) of purified protein combined with adjuvant (Montanide ISA 50 V; France) injected subcutaneously at (0, 4 and 8 weeks) interval in treated group. After 14 days, blood samples were collected to evaluate the presence of anti-rME antibodies in the rabbit's sera. Serum sample was analyzed by using rME protein based Indirect enzyme-linked immunosorbent assay (ELISA) was performed to detect IgG antibodies as described previously (Akbar *et al*., 2021). The 100µl purified rMEP (0.2µg/ml) diluted in coating buffer (50mM sodium carbonate buffer) pH 9.6 used to coat ELISA plates and incubated overnight at 4°C. Blocking the plate with 200µl of 4% BSA (Bovine serum albumin) and 100µl diluted sera (1:100, 1:50 and 1:25) were added to the plates incubated for 1 h at 37°C. After five times washing, 100µl of AP-conjugated Goat anti-Rabbit IgG (H+L) secondary Antibody diluted at a ratio of 1:1000 (Invitrogen, Cat# 65-6122) was added to plate and incubated at 37°C for 2 h. Then, 100µl/well of substrate p-Nitrophenyl Phosphate, Disodium Salt (pNPP) (Thermo Fischer Scientific Cat#34045) was added with substrate buffer DAE (1mg/ml) (Thermo Scientific, Cat#34064) and incubated at 37°C for 10 minutes. The optical density (OD) values were taken three times at 405 nm using Microplate ELISA Reader (ELX-800, Tennessee, BioTek, USA).

RESULTS

Designing of recombinant ME based synthetic construct: *Rhipicephalus microplus* ME based synthetic construct was designed by combining the predicted immunodominant epitopes. Six Helper T-cell epitopes, eight cytotoxic T-cell and B-cells were selected and used in construct based on their scoring. The epitopes were linked together with suitable linkers such as HTL epitopes combined with each other using GPGPG linker, AAY linker combined the CTL epitopes and B-cell epitopes linked with each other by KK linker. The 50S ribosomal protein L7/L12 was used as an adjuvant and linked with remaining epitopes by EAAAK linker as shown in Fig. 1 (A). The final novel ME-based vaccine construct length was 490 amino acids as shown in Fig.1 (B).

Cloning and restriction analysis of pET28α-ME construct: The recombinant pET28α-ME construct was successfully transformed, and colonies appeared white and clear as shown in Fig. 2A. The map for pET28α-ME was constructed to verify the gene fragment ME within the plasmid Fig. 2B. Restriction analysis of pET28α-ME was done by double digestion with *XhoI* and *NdeI* and two fragments of 5400 bp and 1482 bp were observed in Fig. 2B.

SDS-PAGE analysis: The recombinant ME based protein is composed of potential T and B-cell epitopes of three proteins of *R. microlpus*. Evaluation of recombinant ME protein using SDS-PAGE analysis showed that transformed *E. coli* strains BL21 had maximum expression in LB medium after 8 h induction with 0.7 mM IPTG in a shaker incubator at 37°C. The rME protein was expressed at approximately 42kDa and band was observed through Coomassie-blue staining. The results are shown in Fig. 3A.

Western blot analysis: His-tagged rME protein was purified Ni-NTA column affinity chromatography and in western blot analysis, rME protein was expressed and confirmed with immunized rabbits' sera. Based on the immune reaction of antigen-antibody interaction and 42kDa band was observed as shown in Fig. 3B.

Evaluation of antigenicity of rME protein in rabbits: The purified rME protein was mixed with adjuvant Montanide ISA 50 V, and injected into two rabbits to evaluate its antigenicity. The blood samples were collected pre and post-immunization from both groups immunized and control group. The reactivity of rabbits' sera with the rME protein coated to ELISA plate is shown in Fig. 4. It was observed that the immunized rabbit sera showed high reactivity as compared to the control group with anti-rabbit antibodies that confirmed the antigenicity of rME protein in eliciting the immune response. These results showed the high antibody response in a rabbit immunized with rME protein and agree with the analysis of western blotting results.

DISCUSSION

Ticks infestation and tick-borne pathogens still remains a constraint in the livestock industry. Acaricide resistance against ticks has led to adopt alternative tick control approach such as vaccination (Willadsen, 2006). Currently, using bioinformatics seems an auspicious method for designing a successful protective *R. microplus* vaccine. ME-based vaccine has various advantages over others such as specificity, stability, and safety, and has been developed against various infectious diseases including schistosomiasis, toxoplasmosis, and coronavirus (Enayatkhani *et al*., 2021; Forouharmehr, 2021; Sanches *et al*., 2021). In this study, we developed and expressed a ME-based vaccine against *Rhipicephalus microplus* and evaluate its antigenicity in the rabbit model. The first step in designing an effective vaccine is the identification of protective epitopes which can be fulfilled by bioinformatics (Naveed *et al*., 2022). The development of an effective multiepitope-based vaccine is based on the selection of potentially appropriate immunoprotective antigens which can be doable by using bioinformatics. In this regard, the selection of antigens is the major factor that can activate the host immune system against tick infestations. We selected the three proteins Bm86, subolesin, and Bm95 based on their vaccine efficacy against *Rhipicephalus microplus* tick as reported in previous studies (Kumar *et al.,* 2009; Mendoza-Martínez *et al.,* 2021). The Bm86 a glycoprotein is present in the gut of ticks and is known as a protective antigen in cattle by activating the antibody-mediated immune response (Willadsen and Kemp, 1988). The efficacy of recombinant Bm86-based vaccine varies from 50 to 90% in various regions of the world (Parizi *et al.,* 2009) due to variation in the sequences of Bm86 that are present in different regions of the world (García-García *et al*., 2000). Similarly,

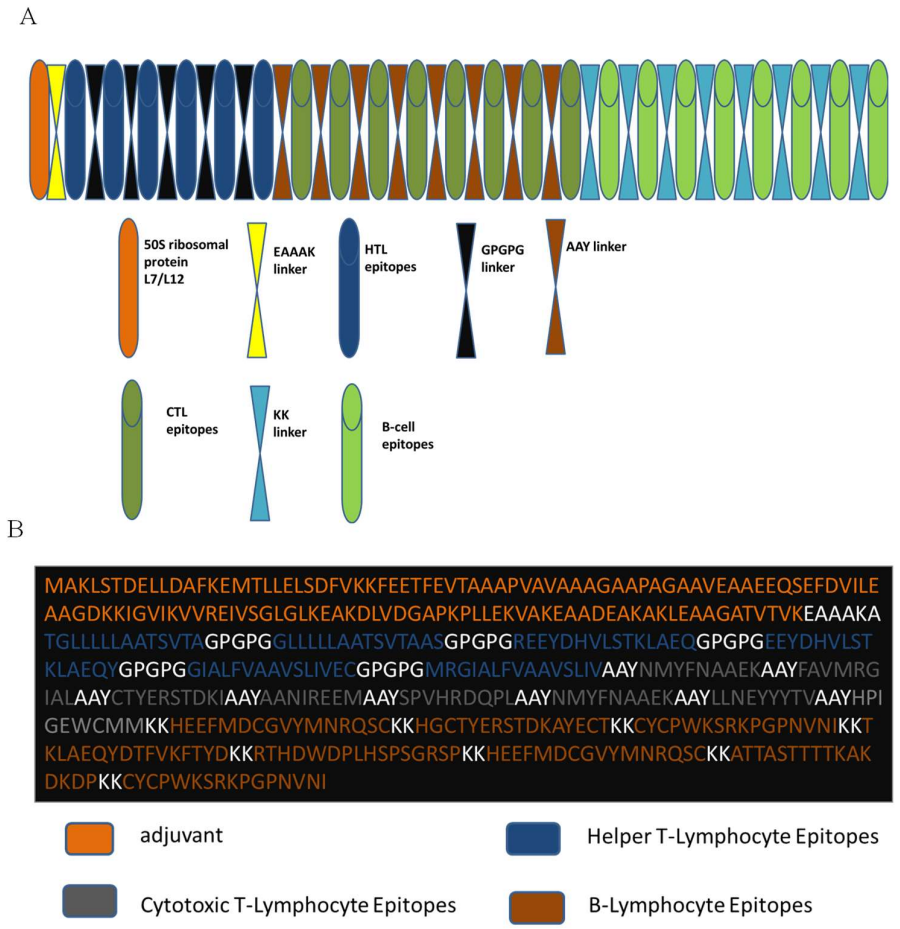

Fig. 1: (A) A graphical image of a final ME vaccine construct. The final vaccine construct is 490 amino acid residues long, with 130 amino acids adjuvant and remaining residues representing HTL, CTL, and B-cell epitopes. (B) Systematic representation of different epitopes. Different colored highlights various epitopes, Adjuvant, Helper T-lymphocyte, Cytotoxic T-lymphocyte, and B-cell epitopes.

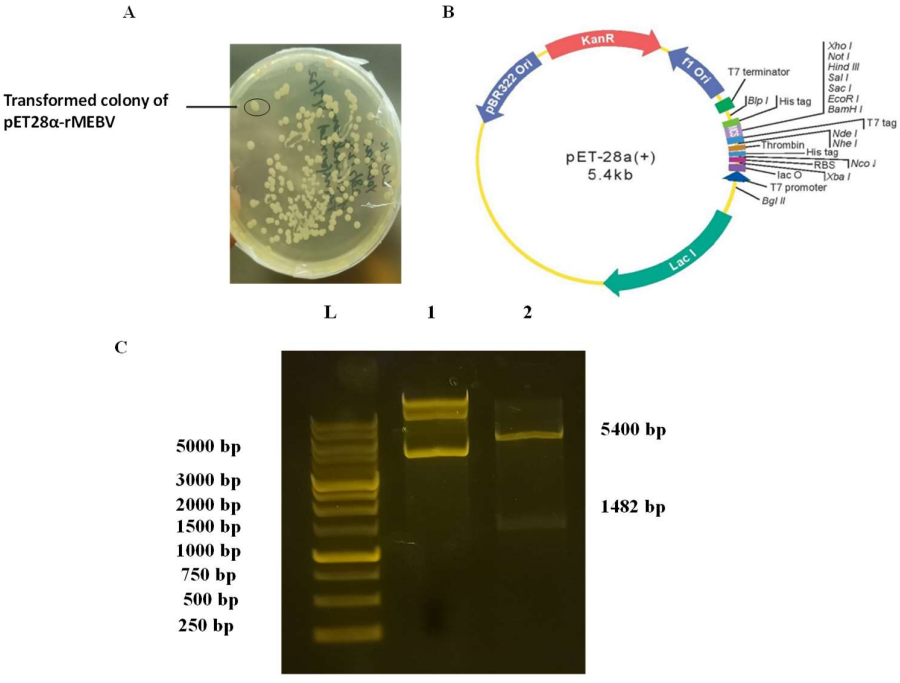

Fig. 2: The transformed colonies of bacteria are spherical and clear white (A). The restriction map of pET28α-ME construct and restriction sites of *XhoI* and *NdeI* are in the MCS of vector (B). Lane L shows the 1 kb DNA ladder (Thermo Scientific™ GeneRuler 1 kb DNA Ladder, catalog number: SM0313), Lane 1 show plasmid and 2 shows two fragments of 5400 bp and 1482 bp double digested with *XhoI* and *NdeI* (C).

cattle were vaccinated with recombinant Bm95 to protect against tick infestations and it achieved an efficacy range from 81 to 89% in India and Argentina respectively (Kumar *et al*., 2009). Subolesin is known as tick protective antigen as it is involved in the regulation of gene expression and its vaccine efficacy was described as 83% against tick infestations (Shakya *et al.,* 2014). Based on these protein vaccine efficacies, in the current study, the combination of three proteins as a multi-antigenic vaccine was developed. In this study, the ME-based vaccine was designed by using

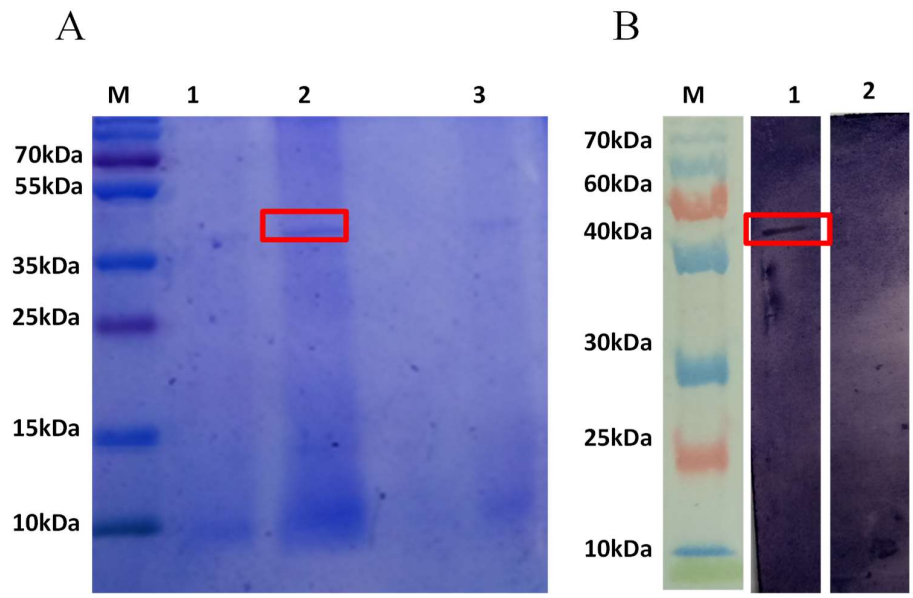

Fig. 3: SDS-PAGE analysis(A). Lane M: Pre-stained protein molecular marker (Thermo scientific PageRuler™ Plus Pre-stained Protein Ladder, Catalog number 26619) Lane 1: non-induced *E. coli* BL21, Lane 2: *E. coli* BL21 show expression 8 h after induction, Lane 3: non-transformed *E. coli* BL21. (B) Western Blot, using AP-conjugated Goat anti-Rabbit IgG (H+L) Cat# 65-6122). Lane M: Pre-stained Protein marker (Thermo Scientific Cat# 10747-012), Lane 1: Purified rME protein about 42kDa, Lane 2: control culture of a cell without ME fragment.

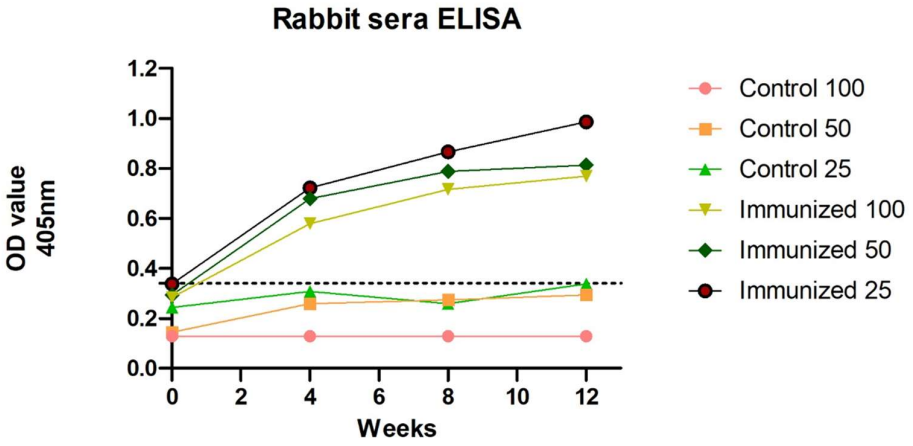

Fig. 4: Antibody IgG level was determined using ELISA assay. 0, 4 and 8 are the weeks of immunization. Two week after immunization antibody was assessed. The sample absorbance was determined at 405 nm. The reaction of rME protein with rabbit's sera in three dilutions 1:100, 1:50 and 1:25 was determined.

the potential HTL, CTL, and B-cell epitopes of three proteins (Bm86, subolesin, and Bm95). HTL, CTL, and B-cell epitopes were linked together with GPGPG, AAY, and KK linkers respectively as reported previously (Kalita *et al*., 2019; Yadav *et al*., 2020). The aim of using linkers is to improve the biological activity and expression of the designed vaccine. These linkers are also helpful in increasing the immunogenicity of the designed vaccine and inducing the humoral immune response by presenting epitopes (Chen *et al.,* 2013). The GPGPG linker is used to conjugate the HTL epitopes as it prevents the changes in the final vaccine construct and enhances the immunogenicity of the vaccine (Saadi *et al.,* 2017). The CTL epitopes were joined together by AAY linker by providing the cleavage site, influencing the protein stability, and enhancing the epitope presentation. The KK (bi-lysine) linker was used to link the B-cell epitopes and maintain the epitope's immunogenic properties (Khan *et al*., 2019). The EAAAK linker is rigid and was used to combine an adjuvant with the remaining epitopes. Here, we used 50S ribosomal proteins L7/L12 as an adjuvant, as an adjuvant enhances the antigen immune response and vaccine longevity as compared to a vaccine without using an adjuvant (Lee and Nguyen, 2015). The final ME-vaccine construct was composed of 490 amino acid residues. In this study, we developed the ME construct and pET-28*a* was chosen to construct a recombinant ME protein expression plasmid. The restriction analysis was performed to clone the ME vaccine fragment in the prokaryotic expression vector. We selected *XhoI* and *NdeI* enzymes at the 5′ end and 3′ end for cloning respectively, as double digestion was done. The sequence of the ME construct was 1482 bp, it was used to produce recombinant ME protein. The ME protein was produced after the transformation of pET-28a-ME into BL21 cells by heat shock method and the induction of protein was done with 0.7 mM IPTG. SDS-PAGE was done with 12 % gel to evaluate the expression of rME protein. A band of 42kDa of rME protein was achieved. It was observed that in bacterial cell lysate, the protein was expressed. A similar result from the prokaryotic expression system was also achieved in a previous study (Asadollahi *et al.,* 2021). In our study, His-tagged rME protein was purified Ni-NTA column affinity chromatography, and in western blot analysis, rME protein was identified by Rabbit anti-6-His tag polyclonal HRP conjugated antibody (Bethyl Laboratories Cat# A190-114P). Western blotting analysis validates the protein and the expected 42kDa band was observed as shown in Fig. 3 B. The reactivity of rabbits sera with the rME protein coated to ELISA plate is shown in Figure 4. It was observed

that the immunized rabbit sera showed high reactivity as compared to the control group with anti-rabbit antibodies that confirmed the antigenicity of rME protein in eliciting the immune response. These results showed the high antibody response in a rabbit immunized with rME protein and agree with the analysis of western blotting results. As the rabbit is not the natural host for *R. microplus* tick and rabbit antibody response was higher in an immunized group as compared to control showed that developed rME protein is immunogenic and these results are supported by previous studies (Canales *et al.,* 2009; Lee *et al*., 2020). In this study, we used recombinant ME protein in rabbit to raise the antibodies and confirmed through ELISA assay. However, based on these results, future study should be focused on the efficacy of this recombinant ME based vaccine against *R. microplus* in calves.

Conclusions: In current study, a novel anti-*Rhipicephalus microplus* tick ME based vaccine construct was designed using immunoinformatics approach. The designed vaccine construct was successfully expressed in the laboratory and estimated molecular weight of 42kDa. The purified protein was evaluated for its antigenicity in rabbits and this protein will be evaluated for its vaccine potential in cattle in future study.

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Authors contribution: All author's made equal contributions to the study. MY, MIR and KA designed the experiment and supervised. MY, MI, SUR conducted the experiments and data analyzed. MIR, KA, MS and TAC edited the manuscript. All authors reviewed and approved the manuscript.

REFERENCES

Akbar H, Shabbir MZ, Ullah U, *et al.,* 2021. Development of Human Toxo IgG ELISA Kit, and False-Positivity of Latex Agglutination Test for the diagnosis of Toxoplasmosis. Pathogens 10(9):1111.

Almazán C, Kocan KM, Bergman DK, *et al.,* 2003. Identification of protective antigens for the control of Ixodes scapularis infestations using cDNA expression library immunization. Vaccine 21(13-14):1492-501.

Almazán C, Lagunes R, Villar M, *et al.,* 2010. Identification and characterization of Rhipicephalus (Boophilus) microplus candidate protective antigens for the control of cattle tick infestations. Parasitol Res 106:471-9.

Almazán C, Moreno-Cantú O, Moreno-Cid JA, *et al.,* 2012. Control of tick infestations in cattle vaccinated with bacterial membranes containing surface-exposed tick protective antigens. Vaccine 30(2):265-72.

Asadollahi Z, Nabian S, Taheri M, *et al.,* 2021. *Introducing a new anti-Rhipicephalus (Boophilus) microplus tick recombinant vaccine candidate using cathepsin and tropomyosin ME gene.* Vet Res Forum 12(4):445

Bahrami AA, Payandeh Z, Khalili S, *et al.,* 2019. Immunoinformatics: in silico approaches and computational design of a ME, immunogenic protein. Int Rev Immunol 38(6):307-22.

Canales M, Labruna MB, Soares JF, *et al.,* 2009. Protective efficacy of bacterial membranes containing surface-exposed BM95 antigenic peptides for the control of cattle tick infestations. Vaccine 27(52):7244-8.

Chen X, Zaro JL and Shen W-C, 2013. Fusion protein linkers: property, design and functionality. Adv Drug Deliv Rev 65(10):1357-69.

Damfo SA, Reche P, Gatherer D, *et al.,* 2017. In silico design of knowledge-based Plasmodium falciparum epitope ensemble vaccines. J Mol Graph Model 78:195-205.

de la Fuente J, Almazán C, Blouin EF, *et al.,* 2006. Reduction of tick infections with Anaplasma marginale and A. phagocytophilum by targeting the tick protective antigen subolesin. Parasitol Res 100:85-91.

de la Fuente J and Contreras M, 2015. Tick vaccines: current status and future directions. Expert Rev. Vaccines 14(10):1367-76.

de la Fuente J, Lew A, Lutz H, *et al.,* 2005. Genetic diversity of Anaplasma species major surface proteins and implications for anaplasmosis serodiagnosis and vaccine development. Anim. Health Res Rev 6(1):75-89.

Enayatkhani M, Hasaniazad M, Faezi S, *et al.,* 2021. Reverse vaccinology approach to design a novel ME vaccine candidate against COVID-19: an in silico study. J Biomol Struct Dyn 39(8):2857-72.

Estrada-Peña A, Venzal JM, Kocan KM, *et al.,* 2008. Overview: ticks as vectors of pathogens that cause disease in humans and animals. Front Biosci 13:6938-46.

Forouharmehr A, 2021. Engineering an efficient poly-epitope vaccine against Toxoplasma gondii infection: A computational vaccinology study. Microb Pathog 152: 104646.

Gaafar B, Ali SA, Abd-Elrahman KA, *et al.,* 2019. Immunoinformatics approach for multiepitope vaccine prediction from H, M, F, and N proteins of Peste des Petits ruminants virus. J Immunol Res 2019:1-18.

Galindo RC, Doncel-Perez E, Zivkovic Z, *et al.,* 2009. Tick subolesin is an ortholog of the akirins described in insects and vertebrates. Dev Comp Immunol 33(4): 612-7.

García-García JC, Montero C, Redondo M, *et al.,* 2000. Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, Boophilus microplus. Vaccine 18(21):2275-87.

Goldman A, Ursitti JA, Mozdzanowski J, *et al.,* 2015. Electroblotting from polyacrylamide gels. Curr Protoc Protein Sci 82(1): 10.17. 11-10.17. 16.

Graf J-F, Gogolewski R, Leach-Bing N, *et al.,* 2004. Tick control: an industry point of view. Parasitology 129(S1):S427-42.

Guerrero FD, Miller RJ and de León AAP, 2012. Cattle tick vaccines: many candidate antigens, but will a commercially viable product emerge? Int J Parasitol 42(5):421-7.

Henrioud AN, 2011. Towards sustainable parasite control practices in livestock production with emphasis in Latin America. Vet Parasitol 180(1-2):2-11.

Hope M, Jiang X, Gough J, *et al.,* 2010. Experimental vaccination of sheep and cattle against tick infestation using recombinant 5′-nucleotidase. Parasite Immunol 32(2):135-42.

Kalita P, Lyngdoh DL, Padhi AK, *et al.,* 2019. Development of ME driven subunit vaccine against Fasciola gigantica using immunoinformatics approach. Int J Biol Macromol 138:224-33.

Khan M, Khan S, Ali A, *et al.,* 2019. Immunoinformatics approaches to explore Helicobacter Pylori proteome (Virulence Factors) to design B and T cell ME subunit vaccine. Sci Rep 9(1):1-13.

Khatoon N, Pandey RK and Prajapati VK, 2017. Exploring Leishmania secretory proteins to design B and T cell ME subunit vaccine using immunoinformatics approach. Sci Rep 7(1):1-12.

Kiss T, Cadar D and Spînu M, 2012. Tick prevention at a crossroad: new and renewed solutions. Vet. Parasitol 187(3-4):357-66.

Kumar A, Garg R, Yadav C, *et al.,* 2009. Immune responses against recombinant tick antigen, Bm95, for the control of Rhipicephalus (Boophilus) microplus ticks in cattle. Vet Parasitol 165(1-2):119-24.

Lee S-H, Li J, Moumouni PFA, *et al.,* 2020. Subolesin vaccination inhibits blood feeding and reproduction of Haemaphysalis longicornis in rabbits. Parasit Vectors 13(1): 1-10.

Lee S and Nguyen MT, 2015. Recent advances of vaccine adjuvants for infectious diseases. Immune Netw 15(2): 51-57.

Lew-Tabor A and Valle MR, 2016. A review of reverse vaccinology approaches for the development of vaccines against ticks and tick borne diseases. Ticks Tick Borne Dis 7(4):573-85.

Marques R, Krüger RF, Peterson AT, *et al.,* 2020. Climate change implications for the distribution of the babesiosis and anaplasmosis tick vector, Rhipicephalus (Boophilus) microplus. Vet Res 51:1-10.

Mendoza-Martínez N, Alonso-Díaz MA, Merino O, *et al.,* 2021. Protective efficacy of the peptide Subolesin antigen against the cattle tick Rhipicephalus microplus under natural infestation. Vet. Parasitol 299:109577.

Naveed M, Yaseen AR, Khalid H, *et al.,* 2022. Execution and Design of an Anti HPIV-1 Vaccine with Multiple Epitopes Triggering Innate and

Adaptive Immune Responses: An Immunoinformatic Approach. Vaccines 10(6):869.

Parizi LF, Pohl PC, Masuda A, *et al.,* 2009. New approaches toward anti-Rhipicephalus (Boophilus) microplus tick vaccine. Rev Bras Parasitol Vet 18:1-7.

Pegram R, Tatchell R, De Castro J, *et al.,* 1993. Tick control: new concepts. World Anim Rev 74(75):2-11.

Rand KN, Moore T, Sriskantha A, *et al.,* 1989. Cloning and expression of a protective antigen from the cattle tick Boophilus microplus. PNAS 86(24):9657-9661.

Saadi M, Karkhah A and Nouri HR, 2017. Development of a ME peptide vaccine inducing robust T cell responses against brucellosis using immunoinformatics based approaches. Infect Genet Evol 51:227-234.

Sanches RC, Tiwari S, Ferreira LC, *et al.,* 2021. Immunoinformatics design of ME peptide-based vaccine against Schistosoma mansoni using transmembrane proteins as a target. Front. Immunol 12:621706.

Schetters TPM and Jansen T, 2017. Vaccine against Rhipicephalus ticks. In: Google Patents.

Shakya M, Kumar B, Nagar G, *et al.,* 2014. Subolesin: A candidate vaccine antigen for the control of cattle tick infestations in Indian situation. Vaccine 32(28):3488-3494.

Shey RA, Ghogomu SM, Esoh KK, *et al.,* 2019. In-silico design of a ME vaccine candidate against onchocerciasis and related filarial diseases. Sci Rep 9(1):4409.

Ullah MA, Sarkar B and Islam SS, 2020. Exploiting the reverse vaccinology approach to design novel subunit vaccines against Ebola virus. Immunobiology 225(3):151949.

Walker JM, 2009. The bicinchoninic acid (BCA) assay for protein quantitation. The Protein Protocols Handbook, 11-15.

Willadsen P, 2006. Tick control: thoughts on a research agenda. Vet Parasitol 138(1-2):161-8.

Willadsen P and Kemp D, 1988. Vaccination with 'concealed'antigens for tick control. Parasitol Today 4(7):196-8.

Willadsen PJP, 2004. Anti-tick vaccines. *129*(S1): S367-87.

Yadav S, Prakash J, Shukla H, *et al.,* 2020. Design of a ME subunit vaccine for immune-protection against Leishmania parasite. Pathog Glob Health 114(8):471-81.