Original Article

A preliminary evaluation of tick cement-cone protein extract for a vaccine against *Hyalomma* infestation

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Abstract

Background: Vaccines have been widely exploited to prevent tick-borne infections in cattle. Most vaccines have faced failure in the field because of inconsistency in an immune response. It is presumed that the cement-cone proteins of ticks that participate in the acquisition of blood meal for ticks possess strong immune-stimulating properties and, hence, could be a useful candidate in vaccine development. **Aims:** We evaluated cement-cone proteins of tick *Hyalomma anatolicum* as a vaccine candidate against infestations of *H. anatolicum* and *H. aegyptium* in cattle. **Methods:** The cement-cone proteins were extracted from *H. anatolicum* to develop stage-reactive and immunogenic cross-reactive vaccine against the infestation of two species of ticks *H. anatolicum* and *H. aegyptium*. The immune response of the vaccine was tested against cement-cone proteins starved, partially fed, and richly fed ticks. **Results:** The findings of the present study demonstrated the cross-reactivity among the two species of ticks that belonged to the same genus (*Hyalomma*). The antigenic similarity between the two ticks species suggests that a common antigen may possibly be suitable for a vaccine against the two different species of ticks. The results have also indicated that the 23 kDa cement-cone protein of *H. anatolicum* and *H. aegyptium* may be responsible for the induction, or elicitation of immunogenic, common stage reactive, and cross-reactive host immune responses with consistent intensity throughout the life stages of ticks. **Conclusion:** The vaccine based upon cement-cone proteins of ticks may be a useful deterrent against tick-borne infections in cattle in countries like Pakistan.

Key words: Cattle, Cement-cone, Cross-reactive, Tick, Vaccine

Introduction

The tick has been considered as one of the most important parasites of cattle with greater economic consequences in tropical and subtropical countries of the world (Musa *et al*., 2014). Ticks of the family Ixodidae are ectoparasites of cattle. Numerous studies have witnessed their role in various human and animal infections. Ticks have been shown to play a vital role in various haematological (Babesiosis, Rocky Mountain fever, Feline Haemobartonellosis) (Walker *et al*., 1983; Demma *et al*., 2005; Akel and Mobarakai, 2017; Lappin *et al*., 2020) and immunological complications (rheumatoid arthritis, autoimmune thyroid disease, and vasculitides) (Rodríguez *et al*., 2018). Additionally, ticks also serve as a vector for various bacterial, protozoa, and viral infections in meat-producing animals. Tick infestations in meat-producing animals have resulted in greater economic losses worldwide especially in South Asian countries including Pakistan (Rafique *et al*., 2015; Karim *et al*., 2017;Guglielmone and Robbins, 2018; Roy *et al*., 2018; Wikel, 2018; Ramzan *et al*., 2020). Ticks of genera *Amblyomma*, *Dermacentor*, *Hyalomma*, *Haemaphysalis*, *Ixodes*, and *Rhipicephalus* have great importance both from medical and veterinary points of view (Balinandi *et al*., 2020). Negative consequences of tick-borne infestations in the livestock industry have urged stakeholders to take effective measures to completely eradicate the tick-borne infestations. It has been estimated that around 80% of the world's cattle (1,298 billion) are plagued with ticks accounting for $7,500 million losses for cattle (De La Fuente and Contreras, 2015). The organophosphates are amongst the most widely used acaricides followed by organochlorines, pyrethroids, and carbamatesto control tick-born infestation in cattle. The frequent use of acaricide compounds has resulted in the emergence of resistance in ticks (Awumbila, 1996; Elhachimi *et al*.,

2022).

A plethora of studies have reported the growing interest of researchers towards the development of vaccines as a favourable method in controlling tick-borne infections (Agbede and Kemp, 1986; Ghosh *et al.*, 1998; Olds *et al.*, 2016; Rodríguez-Mallon, 2016; Schetters *et al.*, 2016; Kamran *et al*., 2020). Two prime objectives of developing vaccines are to control diseases and to prevent their transmission to healthy animals (Rodriguez *et al*., 1995; Rego *et al*., 2019). Vaccines based upon multiple antigens have been found to show greater neutralizing capability and cross-reactivity against multiple species of a pathogen than monovalent vaccines (De La Fuente and Contreras, 2015; Iqbal *et al*., 2016; Mi *et al*., 2017; Knorr *et al*., 2018; Chmelař *et al*., 2019; Kamran *et al*., 2020). A vaccine based upon a combination of multiple antigens on some occasions exhibits failure probably due to the competitive inhibition of antigens. Such a phenomenon was reported by researchers against the infestation of *Rhipicephalus appendiculatus* in cattle (Olds *et al*., 2016). Vaccination with a defined protein antigen can induce a strong immunity against tick infestation (Iqbal *et al*., 2016).

It has been reported that repeated exposure of cattle to tick infestation may result in the development of an adaptive immune response against the ticks' salivary and cement-cone proteins. Such an immune response has been found to interfere with the functions of tick salivary and cement-cone proteins leading to poor feeding in ticks and, ultimately, to high mortality rate of ticks. These findings formed the basis for using cement-cone proteins of ticks as an antigenic source for vaccine development (Wikel, 1999; Ribeiro and Francischetti, 2003; Bowman and Sauer, 2004; Valenzuela, 2004; Olds *et al.*, 2016).The vaccine based on the single-conserved protein antigen must be able to exert cross-immunity to various kinds of tick species (De La Fuente and Contreras, 2015; Iqbal *et al.*, 2016). During the preliminary studies for vaccine development, it is a common practice to use crude-protein extracts as a source of immunogen. After getting satisfactory results, purification and sequencing of these proteins are performed (Knorr *et al.*, 2018). In the present study, we performed primary screening of cement-cone proteins of tick *Hyalomma anatolicum* as a vaccine candidate against infestations of *H. anatolicum* and *Hyalomma aegyptium* in cattle. The efficacy of immunization with the cement-cone proteins of tick *H. anatolicum* was evaluated in cattle through immunization experiments and the tick's morphology evaluation.

Materials and Methods

Study area and sampling of ticks

The present study was designed to develop a vaccine from cement-cone proteins of tick ixodid *H. anatolicum*. The given study was reviewed and approved by the "Institutional Ethics Committee for Animal Care and Use" and the Advanced Studies and Research Board, University of Balochistan Quetta, Pakistan (UOB/Reg/ GSO/938). Animal handling was done by following a protocol specified by "Pakistan's Prevention of Cruelty to Animal Act, 1890". Specimens of ticks were collected from the female domestic cows of breed *Bos primigenius* reared on animal farms in Quetta district of Balochistan province, Pakistan. For ticks collection, regular visits (Nikpay and Nabian, 2016) of three times a month were made from April 2019 to March 2020. A total of 450 specimens were detached from the body of the animal host. Ticks were collected from several body parts of cows including ears, legs, and interdigital skin folds (Kakar and Kakarsulemankhel, 2008).

Morpho-taxonomic identification of ticks

Tick identification was done by the tick identification key based on the taxonomic and morphological features of ticks reported by the researchers (Kaiser and Hoogstraal, 1964; McCarthy, 1967; Keirans and Litwak, 1989; Bischof, 2022) using a microscope and a visual inspection (Olympus-4, Japan).

Extraction of proteins from cement-cone of tick

The cement-cone of hard body tick *H. anatolicum* was used as a source of antigenic protein to develop a vaccine. For cross-reactivity experiments, cement-cone proteins of *H. aegyptium* were also extracted. The extraction of proteins from the cement-cone was performed following a published method (Walker *et al*., 1984). Briefly, the cement-cones were isolated from the mouthpart of different developmental stages of ticks using a dissection microscope. The cones were crushed and washed with 10 mM phosphate-buffer saline solution pH 7.4. The resultant mixture was vortex and it was suspended in cold PBS [10 mM phosphate, 140 mM NaClpH 7.2) followed by sonication using a probe sonicator (Soniprep-150 PLUS, MSE, UK) under a cold condition. The sonicated suspension was mixed with buffer solution (0.5 M tris glycine pH 6.8) containing 10% SDS, b-mercaptoethanol 3%, and glycerol 30% in the ratio of 2:1. The mixture was heated up to 40°C for 5 min, centrifuged 8000 × g (Clifton 000 series, Nickel Electro Co., England) for 10 min and the supernatant was decanted through floatation method (Rodríguez-Mallon, 2016), filtered through a 0.45 µM filter (Sartorius) and stored at -20°C in the presence of protease inhibitor cocktail (Sigma-Aldrich, Germany) (Nuttall *et al*., 2006) for further experiments. The total protein contents of the supernatant were determined by Bradford method (Bradford, 1976).

Fractionation of cement-cone proteins by SDS-PAGE

The SDS-PAGE technique was employed to resolve crude protein extracts of cement-cones into individual fractions based upon the molecular weight. The discontinuous SDS-PAGE (BDH, Poole, England) method was used for this purpose as described by researchers in their study (Laemmli, 1970). After the completion of electrophoresis, the isolated bands were stained with Coomassie brilliant blue. Pre-stained molecular markers were also run parallel to the samples

for the comparison of molecular weights. The standard pre-stained molecular markers used in SDS-PAGE were carbonic hydrase: 29 kDa, oval albumin: 45 kDa, BSA: 66 kDa, phosphorylase: 92 kDa, and β galactosidase 23 kDa (Bio-Rad) were used for SDS-PAGE Western blotting (Towbin *et al*., 1979). Band intensities were determined by ChemiDoc gel imaging system (Bio-Rad).

Western blotting

After fractionation of cement-cone protein extracts by SDS-PAGE, the isolated bands were transferred to a nitrocellulose membrane through Western blotting using the mini trans-blot electrophoresis cell (Bio-Rad: 170-3940, USA). The Western blotting was performed by following a procedure reported by researchers in their study (Towbin *et al.*, 1979). Briefly, gel having fractionated protein bands was packed in gel cassette and closed with a latch. The electrophoresis bath was filled with Towbin transfer buffer. The electrophoresis cell was operated at 30 V and 90 mA at 4°C for 1 h. The membrane was removed in a sandwich box, rinsed multiple times with double distilled water, and finally dried. The dried membrane was blocked in 5% skimmed milk and then incubated with the serum of cattle immunized against infestation by *H. anatolicum* for a period of 24 h at 4°C followed by washing with buffer. After being washed, the immune reactive bands of the cement-cone protein were visualized by using rabbit-anti-bovine IgG HRP conjugated secondary antibody (Bischof, 2022). Images were taken by Bio-Rad chemidoc XRS system (Bio-Rad, Richmond, CA, USA).

Purification of fractionated proteins

After localization of individual protein fractions of the cement-cone, each protein band in the gel was cut out with a sharp blade and washed with a buffer of pH 7.4 (250 mM EDTA/250 mMTris) followed by three times washing with double distilled water. Water was removed and the gel was crushed with a fine spatula into small pieces. Gel pieces were then suspended in 20 mMTris buffer of pH 7.2. The mixture was sonicated with a probe sonicator for 3 min at a low temperature. The gel debris was removed by centrifugation and supernatant containing a particular protein fraction was passed through the Sephadex G-25 resin column to remove the non-protein trace elements. The purified protein fractions were stored at -20°C until further use (Retamal *et al*., 1999). Finally, the purified protein isolates were utilized for the determination of cross-reactivity and feed-stage reactivity.

Vaccination of cattle with the cement-cone proteins

In the present study, a total of 30 cattle of the domestic breed *Bos primigenius* of age group 6 months to 3 years were selected. The cows were divided into control and treatment groups. Ten animals were included in the control group (n=10) and twenty animals (n=20) were included in the treatment group. Animals were randomly selected from five different animal farms in district Quetta, Pakistan. The animals were housed in tick-controlled conditions and serological tests were performed before vaccination to assure they were free of tick-borne infections. The vaccines were formulated by reconstituting 50 µg of crude cement-cone proteins of tick *H. anatolicum* in 1 ml of adjuvant Montanide ISA-50. Control formulation was prepared by reconstituting PBS in 1 ml of the adjuvant. The immunization protocol consisted of 3 doses (days 0, 28, and 56) so each animal of the treatment groups received 150 µg of the cement-cone proteins. The vaccine was injected intramuscularly. One animal from the control and one from the immunized group died at 3-5 weeks post-immunization due to some unknown reasong.

Tick challenge

Control and immunized cow were housed in tick-proof sheds on wire mesh floors with one-inch stagnant water to minimize accidental escape of ticks (Agbede and Kemp, 1986). Seven days after immunization, animals were infested with ticks of *H. anatolicum*. A total of 630 ticks (larval, nymphs and adult forms, each morphological form in 210 numbers) were used to infest each cow of both the control and immunized groups. The infestation was done through a locally made neoprene chamber (Opdebeeck *et al*., 1988b). The chamber was tied up on the flank and ear region of the animal for thirty days (Allen, 1973; Waladde and Gichuhi, 1991). The ticks were scored into normal, damaged, discolored, unengorged, partially engorged, fully engorged, % dead, % drop, % live, and unidentifiable categories of male and female adults (Opdebeeck *et al*., 1988a). Live ticks recovered from immunized and control animals were maintained in a humidity-controlled incubator (BINDER -UK) at 35°C+60% humidity to find tick challenge reproductive responses like egg mass, non-viable eggs, mortality, oviposition period, egg incubation periods, molting and larval/nymphal weights. Ticks captured from control cows were also observed and dealt with in the same manner for comparison.

Tick behavior

Tick behavior was observed after the tick challenge to observe the changes that take place in biology. The ticks' egg weight, oviposition period, egg incubation period, and non-viable eggs count were recorded to determine significant disparity in the development of eggs among control and immunized cows. Variation in the percentage of molted ticks from larva to nymph was also noted. Tick engorgement was examined using a stereoscopic microscope (Wild Heerbrug M1, Switzerland). Partly fed, unfed, and fully fed tick counts were verified. Tick weight (adult, larva, and nymph) was calculated using a digital balance (KERN.EW, West Germany). Deferred attachment mean feeding time was recorded. Mean tick counts, tick damage, attached and % drops, mortality, and fertility were also monitored.

Evaluation of immune response

Immune response of the host was assessed by the

measurements of humoral immunity and cellular response.

Humoral immune response

The indirect ELISA was performed to determine the humoral immunoresponse (Galay *et al.*, 2014) of the cattle. Humoral immunoresponse was determined for five weeks post-immunizations. Blood samples were taken from the ear vein of the cattle. The blood was left for 2 h at room temperature to clot (Canals *et al*., 1990). Serum was separated by centrifugation at 800 × g for 10 min. After separation, the serum was stored at -20°C (Ontario ovens, 15AF Benchtop freezer). The antibody titer was determined against the isolated proteins including 23 kDa antigenic proteins of the ticks. Negative, positive, and reference sera from non-infested and infested cows were used to normalize the ELISA. All sera were analyzed in triplicate. Polystyrene 96 well plates (Dynex, Billing Hurst, UK) were coated overnight at 4°C with protein in carbonate coating buffer (0.1 M, pH 9.6). Wells were risen at least 5 times with PBS containing 0.05% polysorbate (Tween-20: SC-29113) and blocked with 5% skimmed milk in PBST for 1 h at room temperature. About 100 µL of sera from cows vaccinated by cement-cone proteins of *H. anatolicum* (serially diluted 1:500) was added to each well and incubated for 2 h at room temperature. 100 µL (1:10000) rabbit-anti-bovine IgG HRP (Sigma, USA) conjugated polyclonal secondary antibodies solution was added to each well and incubated at room temperature for another 2 h followed by the addition of 100 µL 3,3', 5,5'-Tetramethylbenziline peroxidase substrate (T0440, Merk, USA) to develop a color reaction. After sufficient color development, the absorbance (450 nm) was taken using a microplate reader (Bio-Rad 680, 168-1000, USA) within 30 min at 1.0 optimal density (OD) with standard error. Antibody titer was estimated as compared to that of the control group (Merino *et al.*, 2011; Galay *et al*., 2014).

Cellular response

Twenty different skin (dermis and epidermis) segments of immunized cows were selected to estimate cellular response by counting infiltrating basophils, eosinophils, and leucocytes. Skin samples were collected by minor incision of ear and flank regions through a sterile scalpel blade. Each sample size was 4 × 4. Sampling area was disinfected before sample collection (Hill *et al*., 2007). Segments were fixed in 10% neutral formalin and the tissues were processed through tissue-processing techniques. Then the tissues were embedded in paraffin wax blocks and thin sections of 5 µm were cut with the help of a microtome. Haematoxylin-eosin staining was performed, tissues were observed under an Olympus compound microscope (Leica, Germany), and representative images were taken. The total count of cells present either in the epidermis or dermis was summated at 540 mm deep and 180 mm wide sweeps in the dermis. Each segment sweep was done in triplicate (Allen, 1973). The diameters of inoculation sites (Ear and Flank) were measured for several weeks with an interval of a week interval after skin testing using a skin caliper to detect local hypersensitivity and to record delayed hypersensitivity.

Hematological and biochemical analysis

Hematological and biochemical analysis were performed for both control and immunized cows using an automatic hematology analyzer (XS-500i-Sysmex Europe GmBH) and was also compared with standard hematological parameters described in Schalm's Bovines Hematology (Sattar and Mirza, 2009; Bedenicki *et al.*, 2014). The hematological response was observed by counting RBCs, WBCs and by measuring hematocrit and hemoglobin levels which were detected through Schalm's hemoglobin meter and Neubauer's chamber. To obtain these parameters, the study followed the standard operating procedures described by the researchers (Bimerew *et al*., 2018). For the microhematocrit method, blood was centrifuged for 10 min at 14,000 × g. Then the tube was placed in the microhematocrit reader to take hematocrit readings according to the manufacturer's instructions.

Statistical analysis

Both the descriptive statistics and the one-way ANOVA were applied to analyze the data statistically using (Stat view 5.0, SPSS and MS-Excel 2007).

Results

Purification of antigenic protein and immunization of cow

The cement-cone proteins of the larva of tick *H. anatolicum* were used as a source of antigen for the development of anti-tick vaccine. The reasons for using cement-cone proteins in vaccine development included the strong immunogenic properties, cost-effectiveness, easy extraction, and purification. Results of SDS-PAGE Western blotting revealed the resolution of cement-cone protein extracts into multiple protein fractions. Immunological screenings have shown that fractions 23 kDa and 45 kDa showed immune reactivity against the serum of cows immunized with cement-cone proteins extract of *H. anatolicum* (Supplementary Table 1 (ST1)). The immune response against the 23 kDa protein fraction was pronounced while the immune response against 45 kDa was of low intensity as reflected by the Fig. 1A. The serum antibodies induced against *H. anatolicum* expressed cross-reactivity against *H. aegyptium* and also exhibited life stage-immune reactivity (Tables 1 and 2). The intensity of the immune response against the 23 kDa protein extracted from all life stages of individual tick species was invariable. The results indicated that the cement-cone protein (23 kDa) of the two tick species may be responsible for the induction, elicitation of immunogenic, common stage reactive, and cross-reactive host immune responses with consistent intensity throughout the life stages of ticks.

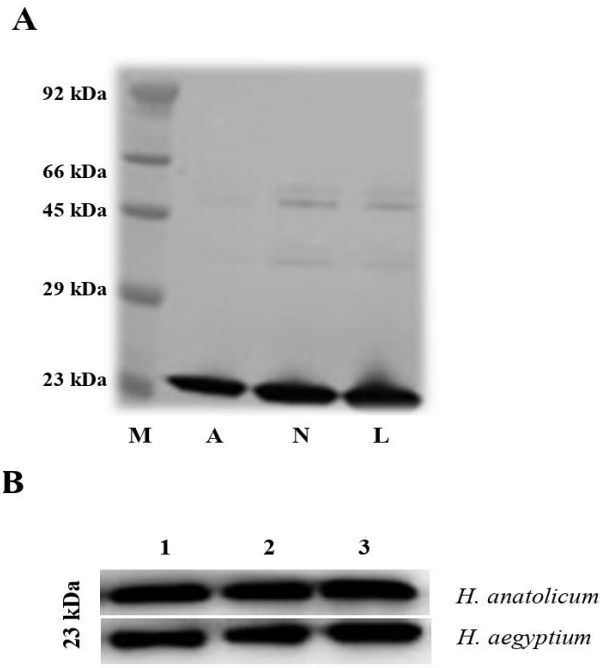

Fig. 1: SDS-PAGE Western blotting of immune reactivity of tick cement-cone proteins with the serum of cattle *Bos primigenius* immunized against cement-cone protein extract of *H. anatolicum*. (**A**) Recognition of 23 kDa (intense) and 45 kDa (weak intensity) proteins in cement-cone protein extracts of tick *H. anatolicum* by bovine antisera. M: Pre-stained markers, A: Adult, N: Nymph, and L: Larva of tick, and (**B**) Represents the feed stage cross reactivity of 23 kDa cement-cone protein of larva of *H. aegyptium* tick against the serum of cattle immunized by cement-cone protein extracts of the larva of *H. anatolicum*. 1: Unfed, 2: Partially fed, and 3: Fully fed ticks

Table 1: Cross reactivity of cement-cone protein fraction 23 kDa with the serum of cow immunized against the cement-cone proteins

Cow serum	*H. anatolicum*			*H. aegyptium*		
Anti-*Hyalomma anatolicum*	L	N	A	L	N	A
Reactivity	+	+	+	+	+	+

L: Larvel, N: Nymph, A: Adult, and + Cross reactive reactions

Table 2: Life stage reactivity of cement-cone protein fraction 23 kDa with the serum of cow immunized by crude cement-cone protein. Fraction 23 kDa was isolated from *H. anatolicum*

Protein	kDa	Larvae	Nymphs	Adults
Cement cone	23.0	+++	+++	+++

+++ Intense immune reactivity

Feed stage antiserum reactivity

The antiserum reactivity against cement-cone protein fraction of 23 kDa was found from unfed, partially fed, and fully fed larvae of the ticks. An intense but similar immune reactivity of serum of the immunized cow against the protein fraction 23 kDa isolated from all the feed stages of larvae of *H. anatolicum* and *H. aegyptium* was observed. Data is presented in Fig. 1B and Supplementary Table 2 (ST2).

Antibody titer

The results of antibody titer against cement-cone proteins are given in Supplementary Table 3 (ST3). A gradual increase in antibody titer was observed post-infestation. The larval response was comparatively greater than adult stage antigen (Fig. 2) and this could indicate a first-line of defense. A positive correlation (r=0.75) was observed between antibody titer (OD) and the mean number of reproduced and developed adult ticks per animal (Supplementary Fig. 1 (SF1). These findings reflect that the developed vaccine is more effective in highly infested cows. Many eosinophils were clustered under the attached mouthparts (Supplementary Fig. 2 (SF2)). The epithelium was thickened and appeared to grow around the leukocyte mass and cutaneous tissue along with edematous pathological changes. Attachment sites were shown to have degranulated mast cells (Supplementary Fig. 3 (SF3)). The results were consistent with those reported by the researchers (Tatchell and Moorhouse, 1968). Figure 3 represents the eosinophil stimulation index in cows in response to antigen actions.

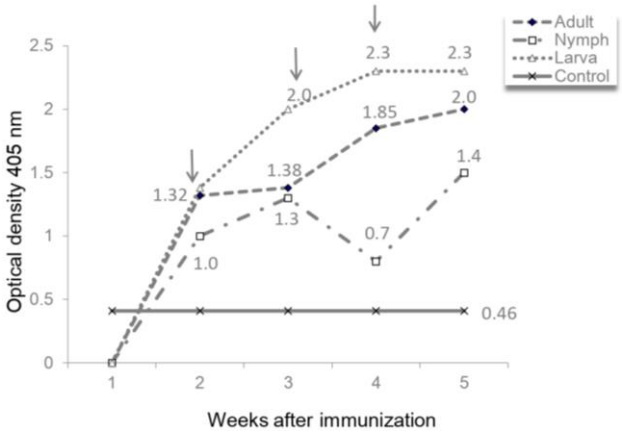

Fig. 2: Serum anti-body response of cows against cement-cone proteins of tick *H. anatolicum*. The anti-body response was evaluated through ELISA. The cows of both control and vaccinated groups were infested with equal numbers (100/instar) of ticks (adults, larvae, nymphs). The secondary response was three times greater than control. Arrow signs indicate primary, secondary, and tertiary responses

Delayed type hypersensitivity reactions

The results of delayed-type hypersensitivity reactions revealed that the diameter of skin at the site of injection (ears and flanks) was higher in vaccinated cows than in cows of the control group (Fig. 4). Nymphs showed intense cutaneous hypersensitivity response (ear thickness) in vaccinated cows post-immunization when compared to the control. Control animals showed mild reactions (Fig. 5).

Cutaneous pathological response

Cutaneous pathological response of immunized cows infested by *H. anatolicum* was measured. Skin sections from the infestation site were processed through a microtome. The sections were stained with haemotoxylin

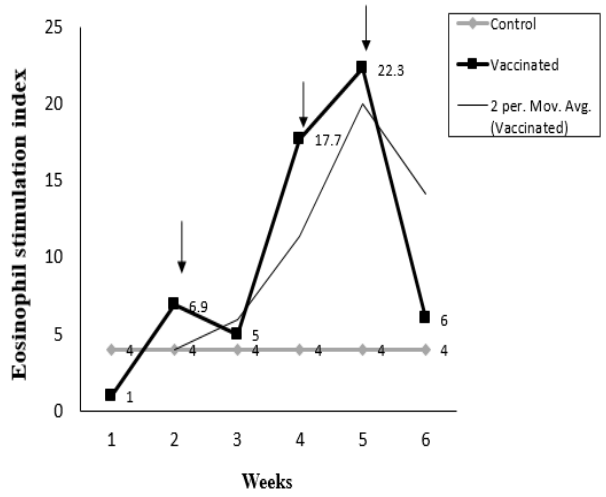

Fig. 3: Eosinophil stimulation index as a measure of antigen-specific response in cows. Eosinophils index was measured against cement-cone proteins of tick *H. anatolicum* during the course of infestation with ticks *H. anatolicum*. Arrows indicate primary, secondary, and tertiary infestations. Controls were only given Montanide ISA-50 adjuvant

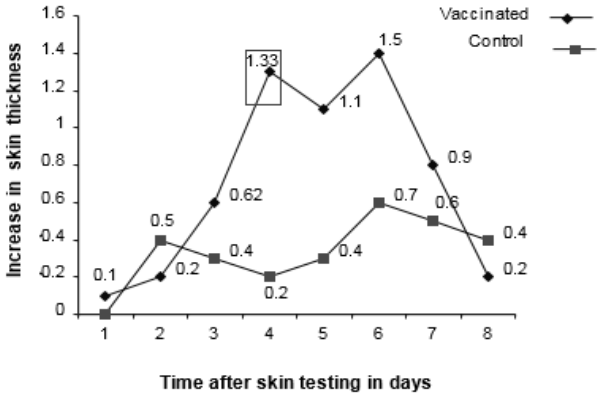

Fig. 4: Mean skin reactivity of cows injected by crude cement-cone proteins of tick *H. anatolicum*. Measurement was done at the injection site

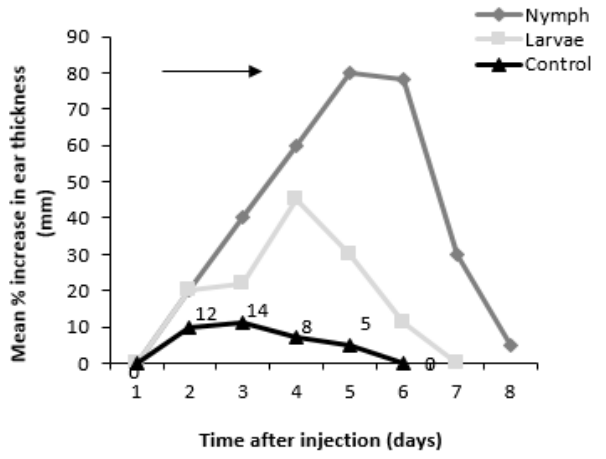

Fig. 5: Post immunization cutaneous-hypersensitivity induced by cement-cone proteins. Cows were sensitized through infestation with larvae and nymphs on the ear. The arrow indicates a significant difference between nymphs and control means (P<0.05)

and eosin and observed for epidermal hyperplasia, vesiculation packed with basophils, erythematous maculae, inflammation, ulcerated nodule formation, oedema, lymphadenopathy, changes in hair formation, hair growth, and eosinophil infiltration (Supplementary Table 4 (ST4)) which substantiates the previous study (Rubaire-Akiki and Mutinga, 1980).

Egg laying capability of tick *H. anatolicum*

Mean egg mass, mean egg number, egg-laying capability, and % age hatchability were the readouts observed in ticks fed on vaccinated and control cattle. The results are summarized in Supplementary Table 5 (ST5) and represented graphically in Fig. 6. The results of tick's encouragement on immunized cow and mottling performance are presented in Supplementary Tables 6 and 7 (ST6 and ST7). The careful observation of our results revealed a significant reduction in egg number, egg mass, egg laying capacity, and % age hatchability in ticks fed on the vaccinated cattle than in ticks fed on cattle of the control group.

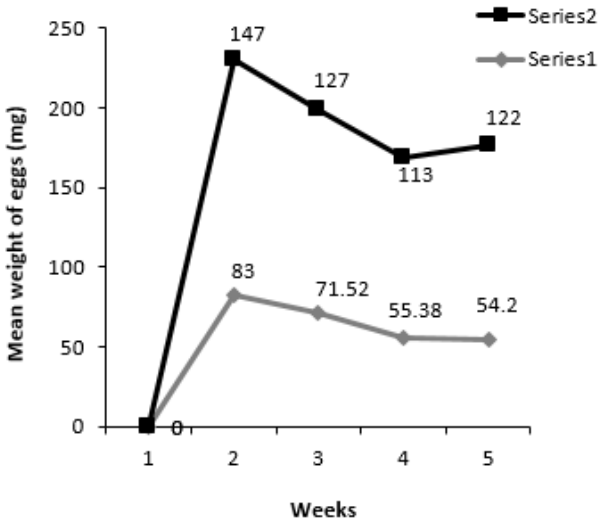

Fig. 6: Mean weight of eggs produced by tick *H. anatolicum* in weeks. Ticks were reared on cows vaccinated with crude cement-cone protein extracts. Series 1 vaccinated cows and series 2 control cows (P<0.01)

Discussion

Ticks obtain the blood meal from the host through a series of events. The process initiates with the attachment of tick to the host skin, selection of bite site, creation of feeding niche, and secretion of the salivary molecules and it finally ends up with the feeding of blood. The secretary molecules from ticks' saliva include cement molecules, polypeptides, heat shock proteins and transporting peptides. These molecules help the ticks in blood-feeding by suppressing the host immune response, complement fixation, blood coagulation, platelet aggregation, and by inducing vasodilatation. Amongst these, cement-cone protein molecules are especially known for their immune-modulating effects. The immune-modulating role of cement-cone protein

molecule including 29 kDa protein has been reported in the literature (Mulenga *et al*., 1999; Sonenshine and Roe, 2014; Šimo *et al*., 2017; Neelakanta and Sultana, 2022). Immune system activation in animals against the tick's cement-cone proteins could be a useful preventive strategy against tick infestation. In the present study, the cement-cone proteins of the tick were considered antigenic materials for vaccine production. During the developmental cycle, cement-cone proteins are generated during the larval stage of the tick. They help the tick to remain attached to the skin of the host. Potentially, it could be assumed to be the best source of vaccine since the immune response produced by the host against cement-cone proteins is quite strong and could reduce the infestation of the host by both larval and post-larval stages of the tick. A plausible finding in this regard indicates that antibodies generated in the host against the cement-cone proteins could possibly interfere with the ability of a tick to maintain constant adherence to the host skin, hence the possibility of a parasitic infestation being minimized or completely stopped (Iqbal *et al.*, 2016). Practically, cement-cone proteins are easy and simple to isolate and are cost-effective immunogens to produce. Serum antibodies recognize cement-cone protein-antigens effectively and studies demonstrate that these antigenic proteins are secreted when the tick is attached to the host, hence playing an important role in the attachment and intake as reported by researchers in their study (Bullard *et al.*, 2016). In a similar context, another study has also reported that the cement proteins from *Amblyomma americanum* tick are shown to be potent immunogen which is composed of proteins of around 20,000 Da (Iqbal *et al*., 2016). For further evidence strengthening our findings, there are previous studies that have reported the ability of cement-cone proteins of larval and adult ticks of *H. anatolicum* to induce an immune response in rabbits (Ghosh *et al*., 1998).

The cement-cone proteins of *H. anatolicum* and *H. aegyptium* utilized in vaccine development and testing in our experiments were detached from the tick during the larval stage. The efficiency of protein extracted from unfed, partially and fully fed ticks was identical. However, antigen from larval stages was found to be most efficient as it was protective against infestation. Our study indicated that unfed, partially and fully fed larvae provided an easier source of antigenic material for the development of a vaccine. The present study is the first to reveal that cement-cone antigens of larval tick *H. anatolicum* are the most probable source of stage reactive common immunogen, cross-reactive and sufficiently enough immune protective for making an anti-tick vaccine that can be cost-effective against the tick species *H. anatolicum* and *H. aegyptium.* We assumed that purified 23 kDa protein fraction from cement-cone could provide better immune response than the cement-cone protein extract utilized in the present study. The first step in the purification of protein fraction is its identification through amino acid sequencing. This information could be utilized for complete purification of a specific protein. This fact needs to be proven in future molecular and purification studies. The present study has revealed that using tick cement-cone larval antigens provided the elevated 47% ($P<0.05$) protective response against the tick species of *H. anatolicum* and *H. aegyptium*. The protective potential of the tick's antigen evaluated in the present study was comparable to the published studies of the protective response of tick cement-cone proteins concerning antibody reactivity, tick engorgement, tick mortality rate, and tick feeding success of ticks on animals (Mulenga *et al*., 1999; Knorr *et al*., 2018; Valle and Guerrero, 2018) but different from midgut glycoprotein Bm86 isolated and purified from *Rhipicephalus* (*Boophilus*) *microplus* tick (Rodríguez-Mallon, 2016).

Our study on 23 kDa protein fraction provided the intense immune response and showed cross-immune-reactivity for the ticks *H. anatolicum* and *H. aegyptium*. The 23kDa protein fraction also exhibited the intense consistent immune response with identical sensitivity across all the life-stages of ticks (Nymph, larva, and adult). The similar reactivity of sera obtained from the immunized cows (*Bos primigenius*) against the cement-cone protein fraction 23 kDa isolated from both species of ticks *H. aegyptium* and *H. anatolicum* suggested that antigenic molecule of *Hyalomma* species (Egyptian and Anatolian) may be immunologically identical and may share the common epitope. Researchers have already reported a similar phenomenon of stage-reactivity and cross-reactivity amongst salivary proteins of the ixodid ticks (Bernard *et al.*, 2016; Bullard *et al*., 2016; Šimo *et al*., 2017; Roy *et al.*, 2018). For instance, cross-reactivity has been reported against *H. anatolicum* and *R. microplus*in calves *Bostaurus*, *Bosindicus* (Šimo *et al.*, 2017). In another situation, the cross-reactivity against ticks *Dermacenter variabilis* and *Dermacenter ersoni* have been reported in domestic pigs (Bernard *et al*., 2016; Bullard *et al*., 2016). In most relevant scenario, Rego *et al*. (2019) have reported the cross reactivity of cement-cone protein among the *H. anatolicum* and *H. aegyptium* but they were unable to provide information regarding stage reactivity. In parallel, other researchers have reported that some salivary proteins demonstrated very efficient protection against the nymphal and larval ticks but weak response against the adults' ticks (Šimo *et al*., 2017). Reactivity of the serum of immunized cows against the unfed, partially fed and fully fed adult *H. anatolicum* ticks have also been investigated which showed consistency in the immune response across all the life stages of ticks. These findings are in accordance with a previously published study (Trentelman *et al*., 2017). Contrary to our findings, some researchers have reported the absence of cross-reactivity among *Haemaphysalis longicornis* and *Hyalomma dromedarii* and *R. microplus* ticks concerning the salivary-gland protein fraction 36 kDa (Tirloni *et al*., 2015). To the best of our knowledge, the present study is the first to report the cross-reactivity and stage reactivity of cement-cone protein 23 kDa among the two potential species of tick *H. anatolicum* and *H. aegyptium* and feed reactivity.

Furthermore, the cement-cone protein 23 kDa from the larva of ticks remained unvaried throughout the instars of feeding and it was found that stage reactive antigens helped in blocking infestation at first instar larval stage in addition to decreasing the overall chances of subsequent infestation. The compelling factor in the present study is the possibility of utilizing a relatively common antigen in developing an effective vaccine against two different species of the same genus ticks. However, further studies are required for the detailed characterization of cement cone immunogenic proteins. Two such types of characterizations recommended by the researchers are the scanning electron microscopy coupled with EDS analysis and the proteomics studies (Pacheco *et al.*, 2021).

The present study has also revealed that tick feeding tend to stimulate an amnestic response. The elevated antibody titer against the cement-cone protein 23 kDa of species *H. anatolicum* and *H. aegyptium* may be directly correlated with the failure of ticks to attach to the cows, increased number of tick death, poor tick development, and increased droppings. Additionally, the immunized cows were shown to elicit a local inflammatory response that involved a noteworthy increase in eosinophils, mast cells, and lymphocytes indicating an activated immune response of the animals. These findings based on cellular response were also found to be in accordance with a recently published study (Manjunathachar *et al*., 2019).

The histological profile of the skin of the immunized cows showed a highly active immune response. Hematology of the immune cow breeds also demonstrated an effectual immune response. There was a significant decrease in engorged tick egg masses and mean weight indicating efficient host immune response. Ticks fed on immunized cows showed greater weight loss than those fed on cows of control group indicating interference in the feeding process which can be attributed towards the immune response of the host. Noteworthy declines in the molting percentage was also observed in *H. anatolicum* tick which was consistent with the findings of Manjunathachar *et al*. (2019). Reduced development in tick, fertility, and feeding further authenticated the presence of immunogenic response of the cows to the tick's species of *H. anatolicum* and *H. aegyptium*. A direct correlation coefficient (r=0.6) could be observed among the increase in antibody titer and the increase in tick counts.

In conclusion, the Anatolian cement-cone protein fraction (23 kDa) is a unique secretary cross-reactive, stage reactive protein and is viewed as a significant candidate for developing anti-tick vaccine against infestations of *H. anatolicum* and *H. aegyptium* tick species in domestic cow breed *Bos primigenius* of a local area of Pakistan. The vaccine developed from crude cement-cone proteins of tick *H. anatolicum* has been found to induce both humoral immune response and cell-mediated immunity. Immunization of cows has resulted in the alteration of morphological parameters, poor development, and reduced egg-laying capability of ticks fed on cows. All the parameters evaluated in the present study were positively correlated with the success of the vaccine. It is highly recommended that prospective researchers perform amino acid sequencing of 23 kDa protein isolated from the cement-cone of tick in the present study before this vaccine is available for full-scale field trials.

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Conflict of interest

Authors declare non conflict of interest.

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