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29-kDa: A Potential Candidate for Anti-Tick Vaccine Antigen Source as Immunogenic and Stage Reactive Targeting Hard-Bodied *Hyalomma* Ticks (Ixodidae)

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ABSTRACT

The objective of present study is to develop a vaccine against *Hyalomma* hard-bodied tick and to analyze relevant experimental data on immunized indigenous horse breed Morna. Montanide (ISA-50) adjuvant-based vaccine induced significantly ($p < 0.02$) higher antibody titre through intradermal route with 99.98% vaccine efficacy. Humoral response was determined though indirect ELISA; where peak level of serum antibody was recorded after six weeks interval of post-immunization. Most of hematology and biochemical parameters remained consistent to normal reference values. Our report also indicates a significant percentage decline in the numbers of engorged ticks, eggs mass, eggs number and increased tick rejection. The animal travel history can promote tick burden and is a potential risk factor. Based on biological and hematological parameters, it is possible to conclude that 29-kDa antigen can be used as an effective antigen vaccine candidate to control tick infestation detected through humoral response.

Key words: Adjuvant, Anti-tick vaccine, ELISA, Risk factor, 29-kDa.

INTRODUCTION

Pakistan economy is mainly focused in agriculture. Currently its gross domestic product (GDP) growth rate correspond to 4.71%, with an approximate 11.39% livestock contributions as per reports of Ministry of Finance, Pakistan (2016-2017). According to livestock census for 1999 and 2006; horses occupies third place in abundance, 18%, in second place are camels (41%) and sheep (48%) are the most abundant. Horse population in Pakistan have increased by 3.1% over a period of eight years from 0.33 million to 0.34 million and their role is continuously shifting from transport to sports and in recreational activities (Rehman *et al*. 2017) both in the rural as well in urban areas (Javed *et al*. 2014).

Pakistan lies in the subtropical zone of South Asia, where the hard-bodied ticks genus *Hyalomma* (*Ixodida: Ixodidae*) is widely distributed in its different ecological zones. Ticks are non-permanent and obligate hematophagous arthropod. Elevated temperature and the scarcity of water contribute to the rapid growth of tick species, particularly in developing countries including Pakistan. Several *Hyalomma* ticks are capable of transmitting grievous pathogens such as viral disease and rickettsias (Kernif *et al*. 2012; England *et al*. 2016) and have drastic effects both on animal and human health due to their high infestation rate (Asmaa *et al*. 2014). Special precautionary measures are needed in collection of *Hyalomma* species because they are responsible for spreading of Crimean-Congo hemorrhagic fever virus in animals and humans (Gargili *et al*. 2017). Hard-bodied ticks infesting equines in Pakistan has been reported previously. The only tick reported belong to the genus *Hyalomma* include *Hyalomma anatolicum* (Kaiser and Hoogstraal 1962; Javed, 2013) and *Rhipicephalus turanicus* (Ali *et al*. 2019). Very few studies have been conducted on these species, which were also confined to restricted sites. These studies did not focus on risk factors associated with tick's infestation (Bisen *et al*. 2011; Jabbar *et al*. 2015).

The use of acaricides in different geographical regions is on decline because of its short shelf life, high cost and repeated applications. As ticks have also developed resistance against acaricides, particularly in *Hyalomma* genus. This has resulted in a decline in the purchase of current acaricides among farmers (Olmeda *et al*. 2008; Pradeep *et al*. 2012; Singh *et al*. 2015). Therefore, there is a need to develop new effective vaccine dealing with ticks.

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The use of an *anti-tick "vaccine"* is more practical approach to overcome on the issue of tick resistance.

Ticks have salivary glands in their mouth, which are the source of transmission of tick-borne diseases to certain viable livestock (Rizzoli *et al*. 2014). Salivary glands secrete a proteinaceous matrix called cement cone protein, which helps in blood feeding and immunomodulation of the host responses to the ectoparasite infestation (Sangwan and Sangwan 2016). This unique class of protein could bind with vertebrate immunoglobulins and is crucial for survival of parasite (Steen *et al*. 2006). In the present work the relationship between the incidences of multi-host tick (*i.e*. *Hyalomma anatolicum anatolicum*) and its associated risk factors is explored. Moreover antigen cement cone was inoculated as immunogenic protein emulsified with Montanide (ISA-50) adjuvant, based on this procedure the intensity of humoral responses in terms of hematology and biochemical parameters was quantified. These results are discussed in terms of the zoosanitary pattern of *Hyalomma* hard-bodied tick infestation and the potential use of tick resistance inducers as vaccines.

MATERIALS AND METHODS

Study and sampling

To investigate the population density of *Hyalomma* ticks, a cross-sectional study was designed based on the random three districts namely Quetta, Qilla Abdullah and Nushki (Fig 1). Ticks were identified using taxonomic descriptions (Walker *et al*. 2014).

In vitro rearing and tick testing

A standard artificial feeding device for ixodid ticks was used in a modified version previously developed by other researchers (Kröber and Guerin 2007). Ten weeks after immunization, each horse was experimentally challenged with total 500 laboratory reared female *Hyalomma* ticks

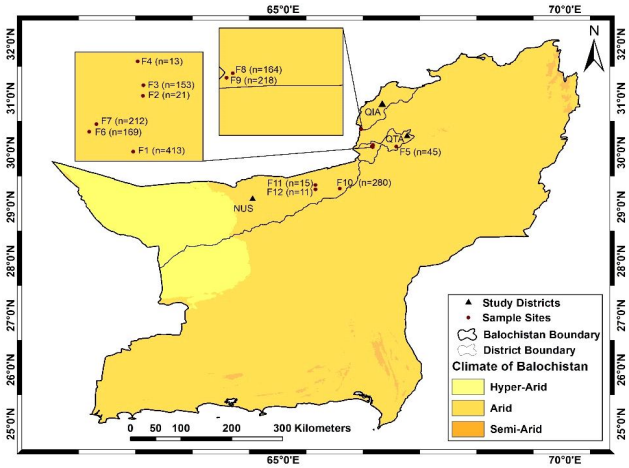

Fig 1: Map showing geographical distribution of *Hyalomma anatolicum anatolicum* in selected sample sites of Balochistan province of Pakistan. Abbreviation: F, Farm; QTA, Quetta; NUS, Nushki; QIS, Qilla Abdulla.

which were exposed on the flank and ear regions within locally made neoprene chambers. Standard formula was used to calculate the immunization efficacy (Andreotti 2006a).

Antigen preparation and purification

Dorsal integument of adult ticks were removed and extracted protein was rinsed and triturated in 0.01 M phosphate-buffered saline solution to avoid desiccation of salivary glands. The mixture vortexes and suspended in 150 µL cold PBS mixture with 1% protease inhibitor. Extracted material was homogenized and sonicated for tissue disruption for 40 s three times on ice bath 40 W. The mixture was then centrifuged at 15000 x g for 20 minutes at 4°C in order to collect the supernatant and decanted the pellet. Centrifuged material was filtered through 0.22 µm non-pyrogenic filter membrane and stored at -20°C.

SDS and western blotting

Molecular weight of crude extract protein as well as fractions were determined by discontinuous SDS-PAGE (BDH, Poole. England) as per standard procedure (Lämmli 1970). Total protein concentration for extracted acarine saliva was also estimated (Brad, 1976). Electroblotted protein bands obtained from SDS-PAGE were transferred to a nitrocellulose paper using a mini blotter (Bio-Rad). Band intensities were analyzed in ChemiDoc Gel Imaging System (Bio-Rad).

Sensitivity and specificity of antigen

Specific diagnostic Indirect-Hemagglutination-Assay (IHA) was also performed taking horse red blood cells with 1 mL sample in EDTA tubes, sensitized with 300 µg/ 2 mL antigen (Rehman *et al*. 1994). Red cell agglutination at ≥ 1:25 was considered positive for the presence of antibody (Harris *et al*. 2009). White Rabbits weighing 3-4 kg were selected for positive and negative testing groups for the developed vaccine for its possible immunogenic reaction in horses (Kesdangsakonwut *et al*. 2014).

Immunogen preparation

Crude protein was emulsified in equal amounts (1:1 ratio) with 1 mL Montanide (ISA-50) and mixed thoroughly on homogenizer before giving it to treatment groups. Three study groups were made comprising of two animals in each group. Group I (IA, IB) received percutaneous, group II (IIA, IIB) intradermal and group III (IIIA, IIIB) for intraperitoneal adjuvants vaccine respectively. Second dose of 500 µg/mL per animal was given after five weeks as described previously (Iqbal *et al*. 2016). Preparation was made for the second control group by taking 1 mL of PBS (pH 7.4) emulsified in 1 mL of Montanide (ISA-50) adjuvant and administered through intraperitoneal injection.

Dynamics of humoral response through ELISA

Salivary extract (2 µg/mL) antigen was diluted in carbonate coating buffer (0.1M, pH 9.2). About 100 µL (serially diluted 1:2000) rabbit anti-goat IgG (F9012 Merck, USA) conjugated polyclonal secondary antibodies was added per well and

incubated at room temperature for 1 hour. Later on, 100 mL of 3,3′, 5,5′ -Tetramethylbenzidine peroxidase substrate (T0440, Merck, USA) including horseradish peroxidase (Sigma, H1009) and hydrogen peroxide (0.3%) was dispensed and added on titre plate. After sufficient colour development, the absorbance (450 nm) was monitored using microplate reader (Bio-Rad 680, 168-1000) within 30 minutes at 1.0 Optimal Density (OD) with standard error.

Hematological and biochemical parameters

We ensured to follow the standard operating procedures for current experiment (Bimerew *et al*. 2018). Hematological analysis was carried out using automatic hematology analyzer (XS-500i-Sysmex Europe GmBH) and were also compared with standard hematological parameters described in Schalm's Equine Hematology (Walton 2013). Biochemical levels were determined using OLYMPUS AU 680 analyzer (Beckman Coulter, Japan) and were compared with earlier reported values (Aros *et al*. 2017; Cywińska *et al*. 2015).

Statistical analysis

ArcGIS software was used to map tick collection sites. Data were analyzed in three ways: (i) Data related to the vaccine efficacy were analyzed by applying Student t-test (*p* (one tail) <0.05, considered significant), using PAST 3.2 (Øyvind Hammer, Natural History Museum, University of Oslo) (ii) Data on possible risk factors of hard-bodied tick burden and associated aspects in farmland were assessed by applying Mantel-Haenszel analysis based on odds ratio (OR) at 95% confidence intervals using WinEpi-info® 7.0 (CSELS) (iii) Mean values of hematological and biochemical parameters were evaluated on ANOVA using SPSS® 20.0 (Inc., Chicago, Illinois, USA).

RESULTS AND DISCUSSION

Purification of antigen

A broad protein profile shows seven immunopositively bands of molecular weight (Fig 2) with few impurities. Among these immunogenic fractions, only 29-kDa remained consistent throughout feeding stages showing common stage reactivity. Crude cement cone protein was selected due to its easy handling, cost effectiveness and the fact that its purification allow it to be used as source of vaccine. It also helps to run anti-tick vaccination campaigns in underdeveloped countries (Iqbal *et al.* 2016). It is difficult to draw any conclusion for this entire work due to the distribution of ticks over the

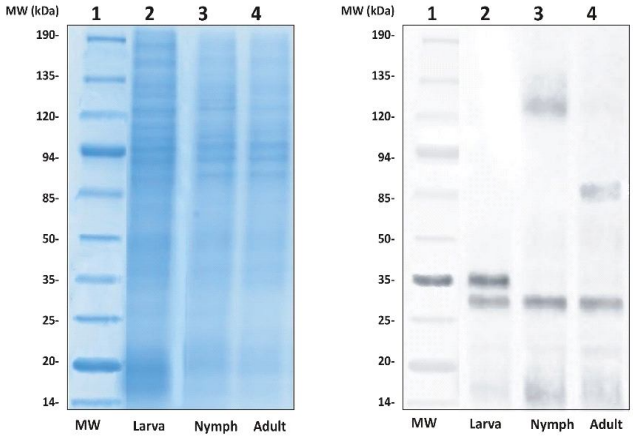

Fig 2: Identification of antigens in the salivary extraction of *Hyalomma anatolicum anatolicum* during artificial feeding. (A) SDS-PAGE, (B) Western Blot (Bovine serum albumin at dilution ratio of 1:50). Lane 1: indicate pertained protein molecular marker ranged (14-190 kDa), Lane 2: nymph salivary extraction, Lane 3: larval salivary extraction, Lane 4: adult female salivary extraction.

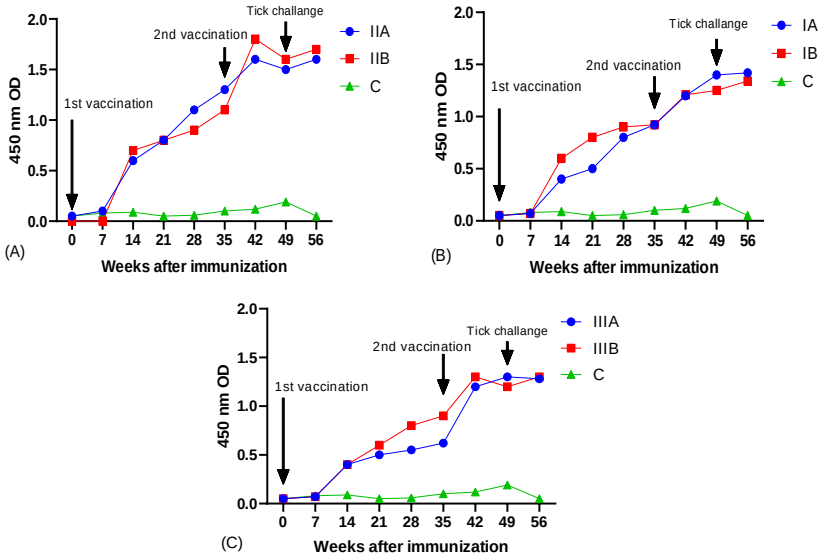

Fig 3: Serum antibody titers of immunized horse were determined by indirect ELISA compared to adjuvant control. Antibody titre was determined for the serum dilution 1:2000 at 450nm O.D administrated via (A) intradermal, (B) percutaneous, (C) intraperitoneal routes. First, second vaccinations and tick challenge are indicated (arrows). Optimal density below 0.5 cut off value was considered negative for antibody titre ($p<0.05$).

different host species, differences in extraction preparation salivary protein and variation in effectiveness of the developed vaccine. Therefore, it is justified to say that currently developed vaccine can specifically work on horse ticks.

Vaccine efficacy

An overall 99.98% vaccine efficacy was achieved after 15 days of *Hyalomma* tick test. The current findings show that engorged ticks were detached after 13 days from post tick infestation in all vaccinated groups. All these observations show a direct effect on the fertility index and subsequently an increase in the overall vaccine efficacy (Table 1). A significant increase in the tick rejection was indicated by our entomological data. Longer feeding period, oviposition and reduced reproductive performance were observed in those ticks that were recovered from immunized host. It was observed that the results were independent of the weights of the vaccinated horses. This suggests that there are no obvious negative effects of vaccination on the target host.

Our sample of animals in this study was small owing to cost constraints. It was not possible to use a large sample in the absence of any financial support. The experiments involving vaccine were initially conducted on rabbits to minimize the cost of animals under test *i.e.* horse. The rabbits used in preliminary trials did not show any clinical symptoms. During the study no animal died as a result of ticks and tick-borne diseases. The results of current research were in agreements with results of other researchers (Andreotti *et al.* 2013b; Miller *et al.* 2012).

Clinical aspect of experimental animals

Six horses were used and none of them showed any clinical symptoms throughout the experiments except that a slight increase in temperature in one horse inoculated through intraperitoneal route. This increase in temperature lasted only for a maximum of 4 days on second vaccination dose due to antigenic response. It is evident from the results of Table 2 that total leucocyte counts was higher in all experimental groups than in control group. No specific change in any other hematological parameters was observed except neutrophil. All biochemical parameters remained fairly in the normal range as reported by other researchers (Cywińska *et al.* 2015; Aros *et al.* 2017).

Indirect ELISA based humoral response

Antibody titre experiments showed predominantly higher antibody production in those animals that were inoculated intradermally. Intradermal vaccination has potential to induce more antibodies titre and is significantly more effective in elderly animals, which respond less to standard vaccination routes (Arnou *et al.* 2009). Elicited antibody titre results showed an increase in antibody level after one week of

Table 1: Vaccine efficacy (29-kDA antigen) on vaccinated horses, showing performance in the decline of number of life stages after successive infestation of *Hyalomma anatolicum anatolicum*.

Animal group	Total number of ticks (n)		Tick weight (mg)		Egg weight (mg)		Larvae hatchability (viability)	
	Control	Immunized	Control	Immunized	Control	Immunized	Control	Immunized
I A	396	135	345	112	211	37	91	22
I B	488	0	322	0	201	0	85	0
II A	475	0	399	55	212	0	88	0
II B	492	78	312	132	204	65	87	32
III A	398	0	355	39	198	0	84	0
III B	450	12	333	28	200	5	88	12
Athematic mean	449.80	42.60	346.60	67.60	205.20	20.40	87.00	10.80
Standard Deviation	39.69	51.70	28.22	46.56	5.38	24.89	2.27	12.42
CRT (%)	8.82	121.36	8.14	68.88	2.62	122.00	2.61	115.03
Reduction in variables (%)[b]	DT		DW		DO		DF	
	0.09		0.20		0.10		0.12	
t-test (p-value) [c]	13.96 (0.00001)		11.45 (0.00001)		16.22 (0.00001)		13.49 (0.00001)	
Efficacy © %[d]	100*(1-(449.80/42.60)*(346.60/67.60)*(205.20/20.40)*(87/10.80) = 99.98							

[a]Animal 1 belonged to control group, 2-5 belonged to vaccinated animals.

[b]Reduction was assessed in percentage to the unvaccinated control groups: DT is the adult engorged female ticks, DW is the weight of engorged tick, DO is the egg laying capacity and DF is the fertility.

D stands for difference and was calculated as

D (%) = 100 X (1- mean value of vaccinated group/control group).

[c]Performance of ticks analyzed by t-student independent test at 0.01 probability values (df = 3) to compare two means.

[d]Efficacy © % = 100[1–(CRT × CR0 x CRF)] was calculated to compare entomological data in both vaccinated and control groups, where; CRT: Coefficient of reduction in the number of engorged adult female ticks, CRO: Coefficient of reduction in oviposition, CRF: Coefficient of reduction in egg fertility.

immunization and maximum peak was achieved after seven weeks of testing (Fig 3). Raised antibody titre confidently reflects the failure of the attachment of ticks on horse skin during this course of research.

Potential risk factor assessment

Table 3 shows the results of Mantel-Haenszel test applied on given parameters. Prevalence of *Hyalomma* tick burden was found higher on those horses which were reared in

Table 2: Measurements of hematological and biochemical parameters of immunized horses on day-1 (first vaccination), day-36 (fist day after booster vaccination), day-50 (first days after tick challenge).

Variables (units)	Reference range	Day-1	Day-36	Day-50	*p*-value
	(min-max)	Mean ± SD	Mean ± SD	Mean ± SD	
RBC (x10^6/µL)	6.8-10 x 12.9	11.25±0.66	11.03±0.60	10.90±0.58	0.72
Hemoglobin (g/dL)	11.0-19.0	16.15±1.91	15.88±2.32	15.75±2.83	0.97
Hematocrit (%)	37-59	43.00±8.36	43.25±8.09	47.75±7.63	0.65
MCV (fL)	37-59	37.50±1.91	39.75±2.98	46.00±9.12	0.14
MCH (pg)	12-20	16.50±3.10	15.00±3.55	14.00±1.41	0.48
MCHC (g/dL)	31-39	34.25±2.87	37.25±2.06	31.75±0.95	0.02
Total Leukocytes count (µl)	5400-14,300	7479.57±33.57	16985.00±44.00	17031.00±48.58	4.19099E-08
Neutrophils (x10^9/l)	2260-8,580	6947.5±38.38	9389.75±32.86	4861.00±27.78	3.97337E-15
Segmented Lymphocytes/ x10^{3}µl	1500-14,300	3351±49.70	3978.99±45.99	5015±5.35	1.77E-12
Basophil / µl	0-290	0.00±0.00	0.00±0.00	0.00±0.00	0
Eosinophil / µl	0-1000	30.25±60.5	38.50±13.47	102.50±8.81	0.4
Monocytes / µl	0-1000	0.50±1.00	47.50±10.11	97.75±9.17	1.33E-06
Creatinine µmol/L	80-92	84.25±4.03	87.00±3.86	82.50±1.73	0.18
ALT (U/L)	310-320	314±3.55	313.00±3.74	316.00±3.82	0.53
AST (U/L)	350-400	357.00±10.13	371.25±14.82	368.50±19.12	0.39
Albumin (g/L)	25-40	24.75±2.06	28.25± 1.00	36.25±2.75	7.9518E-05
Globulin (g/L)	18-35	22.75±5.90	26.25±7.41	25.75±6.70	0.73
Total protein (g/L)	50-60	48.25±4.19	52.00±2.44	56.75±6.18	0.07

Table 3: Assessment of different potential risk factors or categorical variables associated with un-immunized horses using Mantel-Haenszel analysis.

Variables	Parameters	Present (%)	Absent (%)	OR[a] and 95% CI	*p*-value[b]
Tick Infestation/burden[c]	Yes	55	3	4.07 (0.59-27.87)	0.10
	No	9	2		
Government intervention	Yes	8	38	1.96 (0.47-8.07)	0.18
	No	3	28		
Vaccination treatment	Yes	3	36	1.16 (0.18-7.46)	0.44
	No	2	28		
Acaricidal treatment/applications	Yes	7	18	0.18 (0.65-5.46)	0.17
	No	2	42		
Adequate quarantine measures	Yes	2	42	0.34 (0.05-2.24)	0.15
	No	3	22		
Farmland type[d]	Concrete	3	45	3.52 (0.71-17.43)	0.07
	Muddy	4	17		
Animal travel history[e]	Yes	11	24	1.27 (0.44-0.36)	0.33
	Not	9	25		
Personal protection measures	Yes	6	25	2.80 (0.63-12.27)	0.09
	Not	3	35		

[a]Indicate Odds Ratio and 95% CI, in parenthesis range is given.

[b]All variables had a non-significant increasing incidence trend ($p < 0.05$, one tailed) on chi-square test, likely linked to the emergence of ticks in the selected localities, therefore multivariable model could not be adopted as the values of CI was recorded greater than 1.

[c]Complete information of 69 horses used for inclusion in the final model.

[d]Constructed in last 10 year, conformed through documentation record, muddy place had grassy floor.

[e]Dichotomous variable was applied in the 21 days prior to tick removal.

muddy farmland (OR = 4.07, CI = 0.59-27.87). It has been observed that animal travel history is another important factor in the possible spread of ticks (OR = 2.80, CI = 0.63-12.27) and has not been reported earlier from this region. In our analysis, high prevalence of ticks was strongly associated with the animals that were moving across the border. During handling of these animals, it is essential to adopt personal protective measures (Mead *et al.* 2018). It may be stressed that human behavior is directly linked with prevalence of ticks in domestic animals.

CONCLUSION

The 29-kDa antigen Montanide (ISA-50) emulsified vaccine was highly immunogenic and was successfully used for all life stages of hard-bodied ticks parasitizing horses. The intradermal route of vaccination provides a much superior immune response in comparison with conventional routes of standard vaccine delivery. Anti-tick vaccine is recommended to the livestock holders due to its high effectiveness and its low cost. No adverse effects of the vaccine tested were observed on the health of animals. It is further suggested that governmental agencies should consider the implementation of these kind of low cost preemptive measures.

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