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Analysis of Immune Response in Goats *Capra hircus lehri* Against Different Doses of Cement Cone Extract Antigen Taken from Ticks (Ixodidae) Emulsified with Different Adjuvants

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ABSTRACT

Immunological studies against ixodid ticks, *Hyalomma anatolicum anatolicum (*Linnaeus, 1758) were conducted on six-month old domestic goat breed *Capra hircus lehri*, randomly selected from different farms of districts of Balochistan, Pakistan. Goats were allocated into four groups of twenty animals each for immunization experiments. Each group was used to assess the effect of immunization against ticks *Hyalomma a. anatolicum* by using extracts of attachment cones made up of cement proteins taken from mouthparts of tick whole instars as source of antigen. Effects of different antigen doses emulsified with different adjuvants were analyzed in four groups, each cohort comprising twenty goats. First group was immunized with alum adjuvant, second with Freund's complete adjuvant (FCA), third with Incomplete Freund's adjuvant (IFA) and fourth with Montanide ISA-50 (Incomplete Seppic) adjuvant. Goats administered with different doses (5-500 µg) of tick cement antigen emulsified with Montanide adjuvant showed effective immune response through reduction in tick engorgement (decreased weight) and increased percentage tick rejection which proved that Montanide adjuvant is the most potent adjuvant. Results also showed that lower doses of tick cement antigen (5 µg of antigen/ml of adjuvant/animal) emulsified with Montanide ISA-50 adjuvant induced a better stimulatory effect on percentage tick rejection.

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Authors' Contribution
AI and AK conceived and designed the study. AI, SI and SG executed the experimental work. AK, MAP and MR analyzed the data.

Key words
Ticks, goat, anti-tick vaccine, adjuvant selection, tick control.

INTRODUCTION

Ixodid ticks (Acari: Ixodidae) are the most important haematophagous arthropod vectors of infectious agent to domestic livestock and wild animals, attack a broad range of vertebrates and transmit a great diversity of pathogenic microorganisms (Yu *et al*., 2015). They are responsible for great economic losses in cattle farming worldwide (Perveen *et al*., 2010; Cunha *et al*., 2013).The ixodid tick, *Hyalomma anatolicum anatolicum* L. maintains a range of economically important infections transmissible to domestic animals and humans (Latif *et al*., 2004). Pakistan being a tropical country provides optimal climatic conditions for growth and multiplication of ticks. Tick fauna of Pakistan is rich in number of genera and species (Rasul and Akhtar, 1975; Hussain and Kumar, 1985), and the prevalence of *Hyalomma* species is significantly higher than *Haemaphysalis, Amblyomma* and *Boophilus* species of hard tick (Ali *et al*., 2009).

Surveillance studies of ticks as vector in transmission of the disease in livestock was done by Durani *et al.* (2008) in Rawalpindi, Multan and Lahore Districts, Pakistan reported highest prevalence (86.6%) for *Hyalomma a. anatolicum* followed by *Boophilus* (8.1%), *Haemaphysalis* (5%) and *Rhipicephalus (*3.1%), respectively. Balochistan (Pakistan) has a multitude of tick species that is *Hyalomma a. anatolicum* is an important carrier of Crimean-Congo hemorrhagic fever (CCHF) virus reported in the former USSR, Pakistan and Nigeria (Hoogstraal, 1979). They parasitize among domestic goat *Capra hircus* breed affecting the health of livestock and serious economic losses through impairing productivity (Ahmad *et al*., 2011; Bente *et al*., 2013).

Immunological control of ticks is currently the only sustainable and practical alternate method as compared to the use of acaricides (Allen, 1973, 1989). Developing countries like Pakistan needs cost effective solutions for the control. The best choice for the sources of antigen today for developing vaccine is unpurified crude antigens from cement proteins of attachment cones taken from tick's mouth parts (Willadsen *et al*., 1989). It has

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received the most attention as a source of ammunogen for the induction of host protective response against *Hyalomma anatolicum excavatum* cement extracts (Nutall *et al*., 2006). Tick attachment cone cement is among the initial exposed adhesive chemical compound that all ticks studied to date secrete and inject into the feeding site with five to nine minutes after the proboscis of tick penetrates into the dermis of the host, making a cone shape attachment apparatus (Fig. 1) which help ticks to remain anchored on to the host skin.

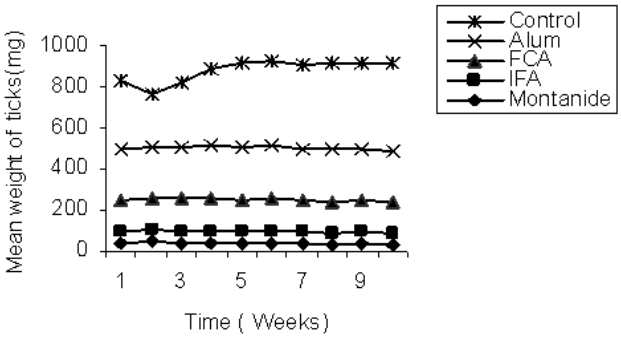

Fig. 1. Mean weights of engorged female adult ticks *Hyalomma anatolicum anatolicum* from domestic goat breed *Capra hircus lehri* vaccinated with Montanide (ISA-50), IFA, FCA, and Alum adjuvant separately**.as compared with ticks fed on control. While controlled were only given antigen without any adjuvant.
IFA, Incomplete Freund's adjuvant; FCA, Freund's complete adjuvant; All animal potency studies were performed in accordance with National Institutes of Veterinary Health guidelines and under the auspices of an animal care approval protocol.

Today is the era of host specific regional vaccine development because of specific cross reactivity among regional tick combinations (Trimnell *et al*., 2005), efficacy, seasonal variations, cross protection, dosage variations, environmental factors (Nutritional stress), cost effectiveness, Adjuvant selection, price, host resistance versus dosage (Brown, 1985), host selection pressures and industrial constraints of vaccine development (Mulenga *et al*., 2000; Sing and Ghosh, 2003) Progress in vaccine development is related with these ground realities.

The aim of current study was to detect the best suitable antigen-adjuvant combinations as well as to identify the effect of different doses of antigen taken as the adjuvant selection is the critical area for the success and efficacy of vaccine.

MATERIALS AND METHODS

Antigen preparation

Unfed, partially fed and fully fed larvae, nymphs and adult ticks were placed in a tube separately for a period of 1-2 days to allow the cement cone to dislodge from the mouth parts, and remaining cement cones were dissected out under a dissecting microscope (Wild M1A, Switzerland). The cement cones were rinsed, triturated in 10mM Phosphate buffered saline (PBS [pH 7.4]), homogenized and vortexed (Barnstead/Thermolyne Maxi Mix II, IOWA USA) in cold PBS (10 mM Phosphate, 140 mM NaCl, pH 7.2) (Walker *et al*., 1984). These were sonicated in a sonicator (9 mm Probe, Soniprep-150 PLUS, MSE,UK) under cooling on ice and mixed with sample buffer (0.5 M tris glycine, pH 6.8) containing 10 % SDS, 1% β-mercaptoethanol and 30 % glycerol in ratio of two volumes of cement suspension plus one volume of sample buffer. The mixture was boiled for five minutes, spun at 8000x g in a micro centrifuge (Clifton 000 series, Nickel Electro Co, England) for ten minutes and supernatant was decanted, filtered through 0.45 µm filter (Sartorius) and stored at 2°C in the absence of protease inhibitor for further experimentation.

Preparation of immunogen

Antigen isolated from cement cone of ticks *Hyalomma anatolicum anatolicum* feeding on domestic goat *Capra hercus lehri* was emulsified with equal amounts of Freund's complete adjuvant (FCA), incomplete Freund's adjuvant (IFA), Montanide ISA-50 and Alum adjuvant (1ml of each adjuvant separately mixed with 5-500 µg/ml/animal), injected to four cohort of goats and after tick challenge, engorged tick performance was recorded for ten weeks.

Experimental design

The experiment was based on randomized experimental design and took place from January 2009 to December 2010 on cohorts comprising 80 animals in four replicates of (20x4) domestic goat breed (*Capra hircus lehri)* at randomly selected sixteen native farms from five randomize districts of Balochistan and same (20x4) in four replicates goats were selected as control. Sample size was based on economic and practical considerations of the region. The domestic goat breed were maintained according to laws of cruelty to animals at temperature ranges from 24°C to 35°C with a twelve hour photoperiod and were fed *ad libitum* with conventional fodder (Chara and water) in combination of 1Kg/hay/day under natural environment as well as in controlled pens in some cases due to handling constraint (website: Health science centre, University of Tennessee,

USA). All the selected domestic goat breeds were about six months old having weight ranges from 15-35 Kg/ animal.

Stored antigen was thawed and homogenized in a sterile pestle and mortar kept on ice and rehydrated, emulsified with selected adjuvant (FCA, IFA, Alum and Montanide ISA-50) in combinations of 5-500 µg of antigen/ml of adjuvant. Before challenge, the domestic goat breeds were shaved to make observation of ticks easier and disinfected also. Immunization protocols followed each of 20 domestic goat breeds from five districts, separately received aseptically 5–500 µg antigen mixed with 1 ml separately with Montanide ISA-50 adjuvant (Seppic, France) (Martinez *et al.*, 1996), incomplete Freund's adjuvant (IFA), Freund's complete adjuvant (FCA) (Difco labs, Detroit, MI, USA) and with Alum adjuvant in to the shaved flank and ear of experimental goats. Similarly control native goats were only given adjuvants. Three control and one immunized domestic goats were died after 3-5 weeks of post immunization due to some unknown reasons which needs to be studied further.

Dose ranging experiments were made to establish a suitable range of antigen doses to be evaluated in a dose titration studies (Table I). Rejection %age was calculated as:

$$\% \text{ Recovery} = \frac{\text{No of ticks dropped}}{\text{No of total ticks parasitized}} \times 100$$

The percent rejection was calculated as hundred percentage recovery following Singh and Ghosh (2003).

Tick challenge

The immunized and control goats were subjected to challenge infestations seven days after the last immunization, with ticks (*Hyalomma a. anatolicum* larvae, nymph and adult) males and females in approximate amounts of 120 of each instar per goat. During experimentation environmental factors of each pen was also recorded. Neoprene (Polychloroprene latex chamber, DuPont) containing tick larvae, nymphs and adults were tied on each goat for thirty days (Fig. 2). The immunized and control goats were housed in tick proof sheds. Domestic goat breeds were kept in sheds with wire mesh floors with one inch stagnant water to minimize accidental escape of ticks (Allen, 1973).

Visual observations

Daily visual observations of the ticks parasitizing on each animal were performed. Ticks were visually examined using a stereoscopic dissecting microscope, washed and stored into normal, partially engorged and fully engorged categories of male and female adults (Opdebeeck *et al.*, 1988). Ticks collected from control goat were also observed and recorded in the same manner for comparison.

Table I.- Results of vaccine trials related with dosage of vaccine against ticks *Hyalomma a. anatolicum* feeding on experimental domestic goat breed *Capra h. lehri* (n=80) Immunized with putative cement cone antigen emulsified with different doses of Montanide ISA-50 adjuvant.

Antigen Dosage**	Performance in Vaccinated goats (n=80) Mean (% Tick rejection) ±SD
1 ml Montanide ISA-50 adjuvant +500 µg antigen (Anatolin) /ml/animal	34±13
1 ml Montanide ISA-50 adjuvant +50 µg antigen (Anatolin)/ml/animal	52.3±12
1 ml Montanide ISA-50 + 20 µg antige(Anatolin) n/ml/animal	61±12
1 ml Montanide ISA-50 + 10µg antigen(Anatolin) /ml/animal	73±12
1 ml Montanide ISA-50 + 5µg antigen(Anatolin) /ml/animal*	71±12

*Lower doses consistently induced a better stimulatory effect on % tick rejection reactivity from tick resistant domestic goat breed.

** All animal potency studies were performed in accordance with National Institutes of veterinary health guidelines and under the auspices of animal care approval protocols.

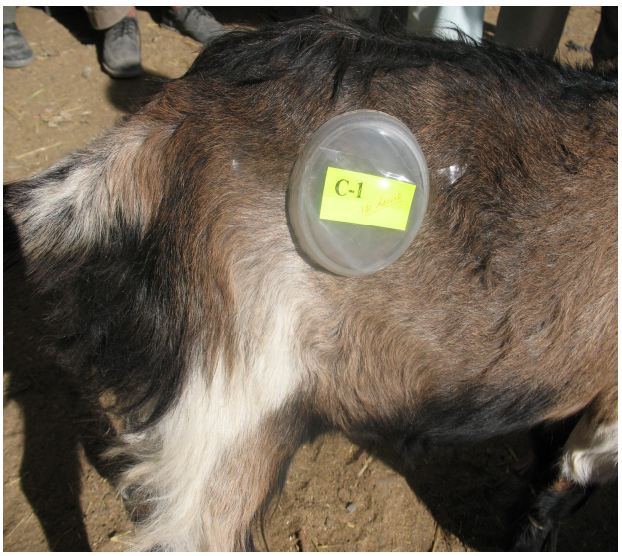

Fig. 2. Neoprene chamber attached on shaven part of challenged goats showing tick challenge.

Tick response

To test the host protective potential, certain parameters were devised to detect the immune response. Tick engorgement was visually examined, using a stereoscopic microscope (Wild Heerbrug M1, Switzerland). Unfed, partially fed and fully fed tick counts were done. Tick weight (Adult, Larvae, and Nymphs) were measured on digital balance (KERN.EW, West Germany).

Statistical analysis

All the observations were recorded on overall behaviors of ticks, parasitized on vaccinated and control goats. The experiments were conducted in replicates of three. Experimental designs and analyses were performed using a standard statistical package (Stat view, 5.0 for windows; SAS institute Inc) and MSTAT-C (Russell, 1992), SPSS and MS-Excel 2003). Statistical differences in mean values from immunized and control animals were determined by using 'z' test (Sokal and Rolph, 1973; Snedecor and Cochran, 1968; Bernard, 2000).

Fig. 3. Comparison of fully fed and unengorged tick clusters before (Upper) and after (Lower) immunization. Low engorgement in female tick mean weight ($p < 0.01$) parasitized on goats, which were given antigen emulsified with Montanide ISA-50 adjuvant as compared to controls.

RESULTS

There was significantly low engorgement in female tick mean weights ($p<0.01$) in goats which were given antigens emulsified with Montanide ISA-50 adjuvant (Fig.3). Montanide ISA was effective as compared to FCA and other adjuvant (Fig. 1). It induced high level of tick rejection comparable to domestic goat breeds immunized with FCA, IFA and Alum. Mean weight of female engorged ticks was 48 mg parasitized on goats which were immunized with Montanide ISA-50. The antigenic dose of 5-500 µg/ml per animal emulsified with Montanide ISA-50 adjuvant was used successfully against tick *Hyalomma a. anatolicum* challenge infestations (Fig. 1). Mean weight of ticks recovered from immunized and control groups shows contrasting values and there was positive correlation (Correlation coefficient r = 0.8) between vaccinated goats with their % resistance against ticks (Table II).

Fig. 4. *Hyalomma a. anatolicum* tick with intact cement cone.10x

DISCUSSION

The results of this study has revealed that the host reaction to tick feeding is a complex phenomenon and depends greatly on the species of tick and host concerned, the time post-attachment and whether or not the host is sensitized. Various adjuvants have been used in order to enhance the immune response against specific antigens (Audibert and Lise, 1993). A good adjuvant can allow a reduction of the dose or of the antigenic concentration, decreasing then the price of the vaccine and improve protection. The philosophy behind the adjuvant selection is that the adjuvant of choice allow for the induction of the correct antibody response that allows expression of a cutaneous (skin) cellular response that calls in particular effector cells, such as basophils and eosinophils required for rejection of ticks (Brown *et al.*, 1984) resulting reduction in engorgement duration. These findings suggest that basophils and eosinophils are involved in

Table II.- Results of vaccine trials related with engorgement performance of ticks *Hyalomma a. anatolicum* ticks feeding on domestic goat breed *Capra h. lehri*,(n = 80) immunized with putative tick cement cone antigen (10μg**/animal) as compared to control.**

Parameters	Performance in		% reduction	Level of significance
	Control***	Vaccinated		
Mean engorged female tick weight (mg (±SD)	203.7±100	84±105.7	58.7	P<0.05*
% resistance88	32	84	61.9	Correlation coefficient r=0.8
(% of adult ticks which did not engorged)				

*Mean engorged weights of adult female ticks that fed on goats, immunized with ticks *Hyalomma a. anatolicum* cement cone antigen were significantly less than mean engorged weight of female ticks collected from control.
** % Resistance = total attached ticks – total engorged ticks.
*** Controls were given only Montanide ISA-50 adjuvant.

caperine immunity to ticks Antigenic dose and its combination with specific adjuvant is the most important factor on which the success of immuno-protective measures stands (Gills and Walker,1985).

The success of Montanide ISA-50 adjuvant compared with IFA, FCA and Alum may be due to the type of immune-inflammatory response induced by respective adjuvant. Therefore, successful anti-tick immunization with tick cement cone antigen not only requires antigen recognition but also depends on the use of proper adjuvant with proper amounts. Montanide ISA have high oil content resulted delayed dissolution. The results are in conformance with Shapiro *et al*. (2005) suggested that montanide (ISA-50) has proved as a good adjuvant in inducing better sero-conversion and challenge infestation.

The reason for choice of "cement cone" selection (Latif *et al*., 2004) was that because its purification is cheapest as compared to recombinant vaccine development, so it is highly practical to prepare antigen from tick cement cone on a larger scale which is helpful in starting anti-tick vaccine campaign in deleveoping countries like Pakistan. It proved to be due to delayed dissolution ability of Montanide ISA adjuvant. Similar effects have been observed by Martinez *et al*. (1996) where they used Montanide ISA 50 oil adjuvant was shown to be similarly effective to the Freund's adjuvant preparation on laboratory challenge of immunised goats and sheep. The finding of the current study are also in line with Brown *et al*. (1984) where they applied incomplete Freund's adjuvant (IFA) containing tick (*Amblyomma americanum*) salivary gland extract antigens (SGA) from partially fed female ticks expressed a significant level of tick rejection when challenged 17 days later. This level of tick rejection was similar to animals (Guinea pigs) actively sensitized by tick feeding and challenged at the same time. SGA emulsified with complete Freund's adjuvant (CFA) or administered with saline was ineffective (Brown *et al*., 1984). However, ticks (Ixodes) that fed on animals immunized with SGA+IFA or SGA+CFA expressed significant reductions in engorgement weight (Aucouturier *et al*., 2001).

CONCLUSIONS

The adjuvant of choice let the induction of accurate antibody immune response The antigenic dose of 5-500 μg/ml per animal used against *Hyalomma a. anatolicum* showed maximum emulsification with Montanide adjuvant (500 μg) expressed high tick rejection (Table I). This reflects that proper timing of immunization can also help to maximize immune responses. From overall study we can conclude that anti-tick immunization with tick cement cone antigen against domestic goat breed be successful if timely usage of proper adjuvant with proper amounts is used.

Statement of conflict of interest

Authors have declared no conflict of interest.

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