IMMUNE RESPONSE OF SALIVARY GLAND VACCINE AGAINST HYALOMMA ANATOLICUM TICKS IN RABBITS

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ABSTRACT

In the present study, attempts were made to prepare Hyalomma tick salivary gland vaccine and to evaluate its potency in rabbits. There was reactivity amongst salivary gland vaccine prepared from this hard tick. It was found that, the vaccine dose containing 5 mg antigen did not induce anti-tick salivary gland vaccine antibodies while the vaccine containing 7.5mg or more antigen induced detectable level of anti-tick salivary gland vaccine antibodies. In rabbits, experiments have shown that, montanide has act as a good adjuvant in inducing better sero-conversion. As compared to unvaccinated rabbits the antibody level in vaccinated rabbit reached to peak level on day 28 post priming and started declining thereafter. It is concluded that tick salivary gland vaccine with more than 7.5mg protein per dose is effective in inducing antibodies in the rabbits.

KEYWORDS: Ticks, Immune response, Salivary gland vaccine, Hyalomma anatolicum

INTRODUCTION

Ticks are responsible for huge economic losses all over the world but especially in tropical and subtropical countries. Their bites are debilitating, annoying and responsible for depletion of hide quality. Tick bite wounds can become infected and cause tick pyaemia or predispose to screw worm myiasis. Heavy tick infestation can result in significant blood loss, reduced productivity and weight gain. Tick bites may cause toxicosis and paralysis and is an important cause of death in sheep, goat and cattle (Blood et al., 1994).

The use of tick vaccines in mammalian hosts has been shown to be the most promising alternative tick control method to current use of acaricides, which suffers from a number of limitations. However, the success of this method is dependent on the identification, cloning, and in vitro expression of tick saliva molecule involved in the mediation of key physiological roles with respect to the biological success of a tick as a vector and pest. Tick saliva molecule protein is expressed in both unfed and fed immature
and mature *H. longicornis* ticks. Immunization with the recombinant p29 conferred a significant protective immunity in rabbits, resulting in reduced engorgement weight for adult ticks and up to 40 and 56% mortality in larvae and nymphs that fed on the immunized rabbits. (Riding *et al.*, 2007).

Different attempts have been made to immunize animals against ticks by using crude extract (Sangwan *et al.*, 1998) but information about the potency or efficacy of such crude extract is scanty. Moreover, very little work has been done in this connection in Pakistan (Ali *et al.*, 2009) and limited research has been reported in this connection in Sindh. Therefore, this study was planned to evaluate the antibody response of *Hyalomma* tick salivary gland vaccine and to study the immune response produced by host against tick salivary gland extract to check the potential vaccine production possibilities against tick.

**Materials and Methods**

Partially engorged female *H. anatolicum* ticks, previously fed only on rabbits, were used as a source of an antigen. Salivary glands of the ticks were collected and processed for preparation of vaccine. Vaccine was tried in different groups of experimental rabbits and then was challenged with ticks.

**Rearing and maintenance of tick colony:**

*H. anatolicum* ticks were collected from cattle and buffalo hosts and identified under low power magnification in dissecting microscope. The rabbits were artificially infested with 10 adult female and equal numbers of male ticks. For infestation, the rabbit ear was shaved with electric shaver, and bio-contaminations were removed by cotton swab soaked in 70 percent alcohol. Infestation socks were placed on each ear and the base was tied with cotton adhesive tape. 20 (10 male and 10 female) partially fed ticks were gently placed on ear base and the socks were sealed with cotton tape. The rabbits were left in cages and fed on barley and provided water *ad libitum*. After 4 days, the socks were opened to see if the ticks have engorged. The engorged ticks were collected and kept in petri dishes covered with muslin cloth and placed growth chamber with fix temperature i.e. 37 centigrade and 70 percent relative humidity. Ticks were checked every day to see the oviposition and larval hatching. The larvae were then subjected to grow and continue the life cycle.

*H. anatolicum* ticks were collected from the tick colony exclusively maintained for this purpose at the Department of Veterinary Parasitology, Faculty of Animal
Husbandry and Veterinary Sciences, Sindh Agriculture University Tando Jam. Ticks were reared on rabbit ears in tightly fitted bags. Partially engorged adult female ticks were collected from the bags. These ticks were carefully removed so as to prevent any damage to their mouthparts. Ticks were maintained at 24°C in Petri dishes in growth chamber.

**Dissection and harvesting of salivary glands:**
Ticks were brought to the room temperature and were surface sterilized by submersion and then intermittent agitation in 0.5 % benzalkonium chloride solution followed by washing with 70% alcohol until they were cleared of benzalkonium chloride, and then were rinsed thrice in sterilized distilled water. Partially sterilized engorged female were dissected individually to harvest salivary glands. Salivary glands were separately washed with three changes of phosphate buffer saline (PBS) by centrifuging at 1000 rounds per minute for 5 minutes. The supernatant were removed and 5 ml PBS was added to each test tube. The samples then were sonicated for 2 minutes and subsequently centrifuged at 5000 rpm for 30 minutes at 4°C. The supernatant were collected and pellets discarded. Total protein contents of the salivary gland extracts was determined by Nanodrop 1000 Spectrophotometer Thermo scientific USA. The samples were reconstituted in PBS (pH 7.2) to achieve a final protein concentration of 10 mg mL⁻¹. Antibiotics (Potassium Penicillin 100 IU / ml and Streptomycin Sulphate 100 µg /ml) were added to samples to prevent bacterial growth.

**Preparation of vaccine:**
The vaccine was prepared by adding oil-based adjuvant to crude sample. One part of sample was mixed with four parts of oil-base adjuvant composed of liquid paraffin, Span-80 and Tween- 80.

**Experimental design:**
Twelve adult rabbits were used in the study and were divided into two groups, each comprising of six animals. The rabbits of first group were injected with salivary gland vaccine (SGV). The animals of second group were acted as control (CTR) and received only PBS along with adjuvant in parallel to the immunization schedule.
Immunization:

The first dose of 1 ml of SGV (containing different dilutions of 5mg, 7.5mg, and 10mg protein) was given sub-cutaneously on day-0 and booster doses (1 ml each) of SGV were given sub-cutaneously on days 14 and 21.

Challenging of rabbits with ticks:

After seven days of last dose, animals of each group were challenged with ticks. Each animal was infested with 10 pairs of adult *H. anatolicum*. The number of dead ticks was recorded regularly. The surviving ticks were collected and maintained in the laboratory to monitor the effect of immunization on ticks.

Measurement of antibody titers:

Blood samples were collected from rabbits after 7 days of last injection and sera were obtained. The antibody levels were measured by agar gel precipitation test. Antigen sample was put in the central well and the serum samples were poured in the surrounding wells with two-fold dilutions of serum (1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:128) in sequence. These plates were incubated at 37ºC and the results were recorded after 48 hours.

Statistical analysis:

The data were analyzed using One-Way Analysis of Variance (ANOVA) and statistical difference among the immunized and non-immunized groups were determined by Least Significant Difference (LSD). Analysis was made with the help of SPSS software.

Results:

Ticks are important ectoparasites of domestic and wild animals, and tick infestations economically impact cattle production world-wide. Control of cattle tick infestations has been primarily by application of acaricides which has resulted in selection of resistant ticks and environmental pollution. Since development of tick vaccines for cattle based on the *Boophilus microplus* Bm86 gut antigen, which have proven to be a feasible tick control method and offers a cost-effective, environmentally friendly alternative to the use of acaricides, have impressed worker all around the globe to develop broad-based tick vaccines.
to reduce, if not eliminate the tick infestations on cattle and the intensity of acaricide usage, as well as increasing animal production and reducing transmission of some tick-borne pathogens.

Partially engorged ticks were left on adult male rabbit(s) and left till engorged. The engorged ticks were collected as they dropped from the rabbit body. The ticks were subjected to micro dissection for obtaining the salivary glands.

Each of the protein suspension when admixed with Montanide (ISA-70) resulted into milky white homogenous suspension. Each of the salivary gland vaccine prepared from *Hyalomma anatolium* when injected into rabbits (1ml: sub-cutaneously) induced detectable level of anti-salivary gland protein antibodies.

Table-1 is numerical expression of Antiserum containing tick salivary gland antibodies reaction with that of tick specific homogenate protein antigen. The proteins of *Hyalomma anatolium* hard ticks were found antigenically similar. Similarly, vaccine prepared from salivary gland of *Hyalomma anatolicum* ticks was found to induce a detectable anti-tick organ specific protein agar gel precipitating antibodies (Table-2). The vaccine prepared from salivary gland induced 0, 3.2 and 6.5 geometric mean titer of salivary gland vaccine antibodies on 0, 14 and 21 days post priming, respectively.

In this experiment, the effect of salivary gland protein amount per dose of the vaccine on the antibody response of rabbits was monitored (Table 3). The vaccine containing 5 mg protein/dose did not induce antibody response in rabbits. However, the vaccine containing 7.5 mg protein/ dose induced 32, 39.4 and 13.0 GMT of salivary gland vaccine antibodies on 0, 14 and 21 days post priming, respectively (Table 4). The vaccine containing 10 mg protein/dose induced 32, 64 and 19.7 GMT of salivary gland vaccine antibodies on 0, 14 and 21 days post priming, respectively (Table 5). It was further noted that antibody response of rabbits to vaccine containing 7.5mg protein was not significantly higher than that of vaccine dose containing 10.0 mg protein (P<0.05). On the basis of these observations, it may be concluded that a low dose of salivary gland concentration may not boost the titer development, however the salivary gland vaccine containing more than 7.5 mg protein per dose can be used to induce detectable level of salivary gland vaccine antibodies.
**Table-1** Immunization schedule of rabbits.

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Group</th>
<th>0 day</th>
<th>14 day</th>
<th>21 day</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Experimental</td>
<td>5mg/ml</td>
<td>7.5mg/ml</td>
<td>10mg/ml</td>
</tr>
<tr>
<td>02</td>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Group I, oil based vaccine of the tick salivary gland protein
Group II, oil based vaccine of the phosphate buffered saline without tick protein

**Table-2** Antibody response of rabbits to oil based vaccine prepared from salivary glands of the *Hyalomma anatolicum*.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pre Immunization</th>
<th>5mg/ml</th>
<th>7.5mg/ml</th>
<th>10mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>0</td>
<td>6.5</td>
<td>13</td>
<td>19.7</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table-2 Parenthesis indicates geometric mean titers (GMT) and mean cumulative geometric mean titer CGMT.
The values with similar letter are not significantly different ($P<0.05$).

**Figure-1** Antibody titer of salivary gland vaccine against *H. anatolicum* with a dose rate of 5 mg/ ml.

**Figure-2** Antibody titer of salivary gland vaccine against *H. anatolicum* with a dose rate of 7.5 mg/ ml.
**Figure-3** Antibody titer of salivary gland vaccine against *H. anatolicum* with a dose rate of 10 mg/ml.

**Figure-4** Antibody titer of salivary gland vaccine against *H. anatolicum* in whole immunization.

**Table No: 3.** *In-vivo* response of Salivary Glands Vaccine of *H. anatolicum*

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Group</th>
<th>Rabbits</th>
<th>Ticks</th>
<th>Alive</th>
<th>Dead</th>
<th>Mortality rate %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Experimental</td>
<td>6</td>
<td>120</td>
<td>9</td>
<td>111</td>
<td>92.5</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>6</td>
<td>120</td>
<td>109</td>
<td>11</td>
<td>9.17</td>
</tr>
</tbody>
</table>

**Figure-5** Mortality rate of Ticks in Rabbits using salivary gland vaccine against *H. anatolicum* in whole immunization.
Discussion:

Salivary glands of ticks have been focus of research to understand the feeding behavior and life cycle success of ticks. The salivary glands are the organs of osmoregulation in ticks and, as such, are critical to the biological success of ticks both during the extended period off the host and also during the feeding period on the host. Absorption of water vapors from unsaturated air into hygroscopic fluid produced by the salivary glands permit the tick to remain hydrated and viable during the many months between blood-meals. During feeding, the tick is able to return about 70% of the fluid and ion content of the blood-meal into the host by salivation into the feeding site. This saliva also contains many bioactive protein and lipid components that aid acquisition of the blood-meal. The salivary glands are the site of pathogen development and the saliva the route of transmission.

The importance of the multi-functional salivary glands to tick survival and vector competency makes the glands a potential target for intervention. This is why this research was conducted to try and see if the salivary glands may be used as vaccine candidate for controlling ticks. Present study will open avenues for studies on cell biology of tick salivary glands and discuss the application of new approaches.

The multifunctional salivary glands are essential to the biological success of ticks and are intricately involved in the transmission of pathogens. They are innervated, and there is convincing evidence that, dopamine is a neurotransmitter at the neuro-effector junction controlling fluid secretion. As feeding progresses, the rate of salivary fluid secretion increases greatly, enabling the tick to concentrate the blood meal by returning excess water and ions to the host. Saliva in feeding ticks is rich in bioactive components and exhibits a range of pharmacological properties. Factors identified in saliva or salivary glands include cement to help anchor the mouthparts to the host, various enzymes and inhibitors, histamine agonists and antagonists, prostaglandins, anti-hemostatic factors, and immuno-modulating factors. A secretion from the salivary glands allows ticks to absorb water from the air during the lengthy periods off their hosts. The physiology of this remarkable organ (salivary gland) provides a
striking example of strategies that have evolved to meet the challenge of a unique parasitic life style.

Ticks have diversification in terms of population dynamics. Prevalence of *Hyalomma* spp. is significantly higher than *Haemaphysalis, Amblyomma* and *Boophilus* spp. of hard ticks (p>0.05) (Shahnaz et al., 2012). *Hyalomma* ticks are member of *Ixodidae* (hard ticks) and are potential vector for the tropical Theileriosis in buffalo, cattle and other wild animals in countries of Europe, Africa, South Asia and Middle East (Dumanli et al., 2005). There is rational use of chemotherapeutic agents to treat the tick infested animals. Repeated use of such agents results in induction of drug resistance that necessitated the investigation of alternate methods of tick control (De la Fuente et al., 2003). In some animal species, innate immunity factors mediate non specific resistance to tick infestation (Kashino, 2005).

Histological structure of hard ticks is mainly composed of chitinous coverings, legs, mouth parts, and internal organs like salivary tissues and intestinal epithelium lining the body cavity. Muslin cloth filtrate of whole tick homogenate contained mainly extract of intestinal epithelium and salivary gland. Such crude extract prepared from *Hyalomma*, induced detectable level of anti-salivary gland tick protein agar gel precipitating antibodies in rabbits.

Present study (Table-1 & 2) confirms that, salivary gland extract may be used as anti-tick vaccine. On the basis of present study (Table 4 & 5), it may be further concluded that a low dose 2mg of salivary gland concentration may not boost the titer development, however, the salivary gland vaccine containing more than 7.5 mg protein per dose can be used to induce detectable level of salivary gland vaccine antibodies. Similar results were obtained by Zakir et al., (2009) who reported that a dose higher than that of 7mg may be enough to help develop antibody titer.

There are many published reports regarding use of the rabbit SGH -AGP antibodies against *Hyalomma* tick homogenate which have demonstrated reaction with salivary gland homogenate of *Amblyomma* and *Boophilus* spp. indicating that, the immunity against one species of hard tick can induce immunity in the vaccination against other species of the ticks. De Vos et al. (2001) found that, tick GARD vaccine, which was *B. microplus* derived recombinant Bm86 vaccine, was also effective against *Hyalomma, Rhipicephalus* and *Amblyomma* species of ticks. Similarly, Trimnel et al., (2005) concluded that anti-sera rose against 64 truncated recombinant proteins (TRPs), a secreted cement protein from salivary gland of *Rhipicephalus appendiculatus* showed cross reactivity with crude extract proteins of
Ixodes recinus and Rhipicephalus sanguineus. Cross reaction was also observed by Singh and Ghosh, (2003) among un-fed larval glycoproteins (34 and 29 kDa) of Hyalomma anatolicum and Boophilus microplus. Only Hyalomma sp. of hard ticks are incriminated to transfer Theileria sp. in Asian countries (Dumanli et al., 2005) while other species of the hard tick do not play any role in its transmission. Vaccination of dairy animals with salivary gland vaccine from field isolates of Hyalomma sp. may impose threat of transferring the Theileria sp. In such areas, immunization of the susceptible animals with salivary gland vaccine from species other than Hyalomma may control all above mentioned species of ticks and support the control of Theileria sp. as well.

The findings of present studies reveal that, the vaccine prepared from salivary glands induced detectable level of anti tick organ specific protein agar gel precipitating antibodies. Wiladsen, (1980) also saw antibody response in dairy animals infested with hard ticks immunized with crude extract of hard ticks Preparation of WTH vaccine. It was found economical and less laborious. It contained multiple immunogens of intestinal as well as salivary gland origin of the hard ticks. Such crude extract vaccine might have better protective effect as compared to a single component vaccine (Mulenga et al., 2000).

Many methods have been tried for the control of ticks aimed at eliminating or at least reducing the tick burden on a dairy host for better results in terms of increasing milk and meat production. Chemical control has raised objections as in some parts of the world ticks have developed resistance. Release of transgenic ticks may also be an idea to control tick population, but it may be highly time consuming target. Use of vaccine has potential as small scale results have already been obtained in terms of tick control using Tick-Guard vaccine. The present study opens the avenues for development of vaccine from tick salivary glands. Before embarking up on practical application of such vaccine, huge base line work is required to generate data to address questions and quarries that may develop. Besides, in vivo application is yet another parameter to be tested through small and large scale studies before reaching final conclusion.

Bibliography:


