VETERINARSKI ARHIV 74 (2), 129-140, 2004

Evaluation of Saponin and Montanide ISA 50 adjuvants for their immunopotency and effect on humoral immune response of calves to purified midgut antigen of *Boophilus microplus*

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RAMAN, M., P. RAMADASS, G. RAJAVELU: Evaluation of Saponin and Montanide ISA 50 adjuvants for their immunopotency and effect on humoral immune response of calves to purified midgut antigen of *Boophilus microplus*. Vet. arhiv 74, 129-140, 2004.

ABSTRACT

An immunization experimental study was conducted on eighteen cross-bred calves (*Bos taurus x Bos indicus*) using a gel chromatography purified midgut antigen of *Boophilus microplus* with two adjuvants, namely Saponin and Montanide ISA 50. Of the two adjuvants, a pronounced humoral immune response was observed by ELISA (1.428±0.061) with a significantly ($P<0.01$) higher rejection of ticks (48.03%) and low egg rate conversion efficacy (21.13) in the Saponin group of calves than in the Montanide group. Performance of the two adjuvants was assessed using various feeding and reproductive parameters of ticks collected from immunized and control animals. Nevertheless, the level of protection offered by Montanide ISA 50 was almost equal to the Saponin group, but with development of a mild local inflammatory reaction in immunized calves.

Key words: *Boophilus microplus,* midgut antigen, Saponin, Montanide ISA 50, immunopotentiation effect, calves

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ISSN 0372-5480
Printed in Croatia

Introduction

The tropical cattle tick *Boophilus microplus* continues to be a major threat to cattle throughout the tropics, both as a debilitating agent and as a vector of Babesiosis and Anaplasmosis (LODOS et al., 2000), in addition to causing damage to hides and skin resulting in deterioration of hide quality (LAHKAR et al., 1997). Recently, SANGWAN et al. (2000) observed a progressive displacement of the *Hyalomma* tick by *B. microplus* due to drastic changes in microclimate in favour of *B. microplus*, mainly due to the efforts taken by the authorities to improve the plastering of cattle sheds in Haryana, India. The chances of competitive exclusion of *Hyalomma* spp. by *B. microplus* operated through some form of cross immunity are also not completely ruled out, thus indicating the historic spread of *B. microplus* over the last three decades from being a rare tick to being the most prevalent.

As the control by use of chemical acaricides led to serious drawbacks, such as toxicity, high cost and difficulties with disposal (WILLADSEN and KEMP, 1988), alternative approaches involving immunoprophylaxis had emerged against *B. microplus* (JOHNSTON et al., 1986). The series of elegant studies on the immunization against *B. microplus* had led to identification of a protective antigen (WILLADSEN et al., 1989) leading to development of recombinant Bm 86 vaccine (RODRIGUEZ et al., 1994). However, the use of a more appropriate adjuvant even, for recombinant Bm 86 vaccine (LEE and OPDEBEECK, 1999), resulted in the highest level of antibodies and increased protective immunity (JACKSON and OPDEBEECK, 1995). Inclusion of an appropriate adjuvant in a vaccine reduces the amount of antigen and the number of doses necessary to induce immunity (EDELMAN, 1980) and therefore choice of a suitable adjuvant is an essential aspect in the development of any effective vaccine. In the present study the effect of Saponin (Sigma, U.S.A.) and Montanide ISA 50 (Seppic, France) on the humoral immune response of calves immunized with purified midgut antigen of *B. microplus* was investigated. These adjuvants were selected as they were widely accepted for use in food animals and are relatively non-toxic when used in appropriate doses (ANONYM., 1976; DALSGAARD, 1987).

Materials and methods

Experimental animals. Eighteen cross-bred male calves (*Bos taurus* x *Bos indicus*) aged 3-6 months purchased from a tick-free herd maintained at the Livestock Research Station, Kattupakkam, were used. Infection-free *B. microplus* larval colonies maintained using cross-bred calves were used in the study.

Preparation of midgut antigen. Partially fed, 17-19-day-old apparently pathogen-free female ticks collected from experimental calves were used for antigen preparation. The dissected midgut diverticula were harvested in 0.15 M PBS pH 7.2 and homogenized at 1500 cycles per minute for 15 minutes in Potter's homogenizer (B-Braun Bio-tech International, Germany) and sonicated at a standard probe of 6 constant points for a total period of 10 minutes, each cycle lasting for 2.5 minutes with an interval of one minute each in a sonicator (Branson Sonifer-400, Germany) in an ice bath (JOHNSTON et al., 1986).

The sonicated homogenates were centrifuged at 30000 X g for 30 minutes at 4 ^{0}C. Sediments (pellets) were discarded and the supernatant was pipetted out and filtered through a 0.45ěm millipore disposable membrane filter (Millex-HV). Phenyl methyl sulfonyl fluoride (PMSF) was added ≅ 1ěl/ml of antigen (3 mM) and stored at -20 ^{0}C; protein concentration of the samples was determined (LOWRY et al., 1951).

Purification of midgut antigen. The midgut antigen of *B. microplus* was purified by gel filtration chromatography using Sephadex G-200 column (GHOSH and KHAN, 1997). About 2 ml of partially purified midgut antigen in 0.15 M PBS was applied to a 100 × 2 cm column of evenly packed Sephadex G-200 (Sigma, U.S.A.). The fractions were eluted in 12 ml/h. using 0.15 M PBS pH 7.2 containing 0.2% sodium azide. At least 90 fractions of each 3 ml were collected and the optical density (OD) value was arrived at for all samples in a spectrophotometer (UV-180 spectrophotometer, U.K.) at 280 nm.

The elution profile of the midgut antigen was arrived at by plotting a graph based on the OD value of fractions. Fractions from each peak were pooled, concentrated by dialysis against polyethylene glycol 6000 and immunogenic fractions were identified by immunodiffusion

(OUCHTERLONY, 1958) and immunoelectrophoresis (GHOSH and KHAN, 1997) and were stored at -20 ^{0}C for further study.

Immunization trial

Trial protocol. The immunization trial was carried out on eighteen cross-bred male calves (*Bos indicus* × *Bos Taurus*) aged 3-6 months with no previous exposure to ticks, in three groups as follows:

Group I: Six calves were immunized using 1 mg of purified midgut antigen with 0.1% Saponin (Sigma, U.S.A.)

Group II: Six calves were immunized using 1 mg of purified midgut antigen with Montanide ISA 50 (Seppic, France).

Group III: Six calves, three calves each for Saponin and Montanide, were given 0.15 M phosphate buffered saline with respective adjuvant.

The calves were immunized on day 0, 14 and 28 through sub-cutaneous route at multiple sites. Blood samples were collected at weekly intervals until 182 days post-immunization (DPI) for assessment of humoral antibody response.

Challenge infestations. All calves were challenged, each with 200 laboratory-bred pathogen-free *B. microplus* larvae (100 in each ear) on 35 DPI. Ear bags were regularly checked to assess the number of ticks attached, or which had dropped off, until the end of the observation period.

Adjuvants. Two adjuvants were used in the immunization trial and their efficacy rate with antigen was assessed.

Saponin. Saponin powder (Sigma, U.S.A.) from *Quillaja* bark was used as a 0.1 per cent solution with midgut antigen in the experimental trials (THAKUR et al., 1992)

Montanide ISA 50. Mannide oleate derivative in a mineral oil solution (Montanide ISA 50, Seppic SA, France) was used at a level of 25 per cent, with 75 per cent of antigen (ANANDA RAO et al., 1993; MARTINEZ et al., 1996).

Dose rate. Each calf received 2 ml of antigen adjuvant mixture at multiples sites, both subcutaneously and intramuscularly. In the Saponin group of calves, 1 ml of antigen was mixed with an equal volume of 0.1

per cent Saponin, while the Montanide adjuvant group received 1.5 ml of antigen diluted in PBS with 0.5 ml of Montanide ISA 50. The dose rate was arrived at so as to contain 1 mg of protein per ml of antigen for both adjuvant groups. Phosphate-buffered saline pH 7.2 was used instead of antigen in unimmunized control calves.

Assessment of humoral immune response by ELISA. Serum samples collected from immunized and un-immunized control calves were assessed for humoral immune response by ELISA (NJAU and NYINDO, 1987) with some modifications (GHOSH and KHAN, 1997) using optimum dilutions (1/100 antigen, 1/200 known positive serum and 1/2000 conjugate as determined by chequerboard titration). The plates were read at 492 nm in a Multiscan ELISA reader (Flow lab, U.K.) and the OD values were recorded for analysis.

Assessment of immunization by observing feeding and reproductive performance of ticks. Fifteen adult replete female ticks were collected from each calf and the effect of immunization on various feeding parameters, such as engorgement weight, percentage of rejection of ticks (MOMIN et al., 1991), and reproductive parameters, such as weight of egg mass, egg-rate conversion efficacy (CHILTON, 1992), were observed in all three groups.

Results

In the present study the purified midgut antigen of *B. microplus* combined with Saponin adjuvant worked out well, inducing better humoral immune response in calves as the ELISA absorbance values were maintained at a moderately higher level during the entire study period than in the group of calves that received antigen with Montanide ISA 50 (Table 1). In the Saponin group, values rose sharply from 28 DPI (1.012 ± 0.005), reaching their peak value on 49 DPI (1.428 ± 0.061) and which persisted for up to 119 DPI (1.109 ± 0.009), while the peak values returned early on 91 DPI (0.966 ± 0.033) in the Montanide group of calves. Since there was no significant difference between Saponin and Montanide control groups, the control values were pooled and analysed.

Table 1. Humoral immune response in experimental calves to immunization with purified midgut antigen of *Boophilus microplus* (n = 18) (Mean ELISA absorbance value ± SE)

Days (post immunization)	Immunized animals		Unimmunized controls
	Saponin group	Montanide group	
0	0.191 ± 0.006	0.214 ± 0.026	0.181 ± 0.003
7	0.283 ± 0.004	0.271 ± 0.004	0.185 ± 0.002
14	0.587 ± 0.003	0.574 ± 0.007	0.188 ± 0.001
21	0.775 ± 0.012	0.912. ± 0.006	0.198 ± 0.004
28	1.012 ± 0.005	0.933 ± 0.005	0.195 ± 0.003
35	1.016 ± 0.030	0.922 ± 0.020	0.188 ± 0.003
42	1.0201 ± 0.022	1.0165 ± 0.053	0.194 ± 0.005
49	1.428 ± 0.061	1.333 ± 0.203	0.202 ± 0.005
56	1.289 ± 0.041	1.269 ± 0.035	0.185 ± 0.005
63	1.242 ± 0.031	1.198 ± 0.012	0.191 ± 0.009
70	1.226 ± 0.040	1.147 ± 0.029	0.179 ± 0.005
77	1.170 ± 0.023	1.090 ± 0.034	0.193 ± 0.007
84	1.153 ± 0.036	1.129 ± 0.028	0.188 ± 0.004
91	1.149 ± 0.018	0.966 ± 0.033	0.175 ± 0.003
98	1.136 ± 0.011	0.902 ± 0.011	0.175 ± 0.003
105	1.128 ± 0.012	0.919 ± 0.006	0.184 ± 0.004
112	1.120 ± 0.010	0.898 ± 0.009	0.181 ± 0.001
119	1.109 ± 0.009	0.798 ± 0.019	0.177 ± 0.004
126	0.920 ± 0.017	0.714 ± 0.008	0.175 ± 0.002
133	0.866 ± 0.015	0.587 ± 0.019	0.184 ± 0.003
140	0.749 ± 0.013	0.449 ± 0.011	0.177 ± 0.004
147	0.580 ± 0.018	0.321 ± 0.014	0.182 ± 0.003
154	0.340 ± 0.023	0.224 ± 0.024	0.184 ± 0.006
161	0.287 ± 0.008	0.177 ± 0.003	0.182 ± 0.005
168	0.272 ± 0.004	0.153 ± 0.023	0.175 ± 0.004
175	0.245 ± 0.006	0.179 ± 0.008	0.181 ± 0.004
182	0.212 ± 0.004	0.182 ± 0.002	0.182 ± 0.022

Table 2. Effect of purified midgut antigen immunization on the feeding performance of *Boophilus microplus* in experimental calves (n=18) (Mean value ± SE)

Parameter	Saponin		t-value	Montanide		t-value
	Immunized	Control		Immunized	Control	
Engorgement Weight (mg)	114.00 ± 0.00	138.50 ± 0.00	-21.2868**	115.00 ±0.00	141.50 ± 0.00	9.3133**
Engorgement Period (days)	33.20 ± 0.40	24.2 ± 0.21	8.8629**	31.83 ± 0.02	24.7 ± 0.31	11.6250**
Feeding efficacy Index (FEI)	3.40 ± 0.06	6.60 ± 0.69	19.8406**	3.70 ± 0.17	5.80 ± 0.40	15.9454**
Rejection of Ticks (percent)	48.03 ± 0.71	27.10 ± 0.40	-6.6313	46.23 ± 0.70	28.85 ± 0.451	-6.8224**

** Highly significant

Immunization with purified midgut antigen significantly ($P<0.01$) affected all feeding performance parameters in ticks fed on immunized calves than on controls (Table 2). The effect was moderately higher in the Saponin group than in the Montanide group. A significantly ($P<0.01$) higher rate of rejection of ticks (48.03%) with reduced feeding efficacy index (3.40) was observed in the Saponin group than in the Montanide group. Statistical analysis of this feeding performance by Student's t-test revealed

Table 3. Effect of purified midgut antigen immunization on the reproductive performance of *Boophilus microplus* in experimental calves (n=18) (Mean value ± SE)

Parameter	Saponin		t-value	Montanide		t-value
	Immunized	Control		Immunized	Control	
Egg mass weight(mg)	23.83 ± 0.86	53.00 ± 4.10	-10.2556**	27.95 ± 0.88	59.00 ± 0.00	-23.6026**
Preoviposition period (days)	8.90 ± 0.57	3.05 ± 0.25	19.7566**	7.80 ± 0.10	2.85 ± 0.15	28.1425**
Oviposition period (days)	26.20 ± 0.57	16.20 ± 0.00	8,6387**	23.90 ± 0.45	16.75 ± 0.35	13.2693**
Incubation period (days)	22.70 ± 0.40	15.50 ± 0.80	9.3243**	24.33 ± 1.12	14.60 ± 0.70	5.6240**
Egg rate conversion efficacy (ERCE)	21.13 ± 1.03	38.3 ± 2.90	-7.2845**	24.73 ± 0.79	41.75 ± 1.15	-12.3762**

** Highly significant

highly significant ($P<0.01$) differences between immunized and controls in their value, as shown by a significant 't' value for the Saponin and Montanide groups of calves.

Likewise, immunization with purified midgut antigen severely affected the reproductive performance of ticks, with a significant ($P<0.01$) reduction in egg mass weight and egg-rate conversion efficacy, with a more extended preoviposition, oviposition and incubation period in immunized calves than in controls (Table 3). The effect was more pronounced in the Saponin group than in the Montanide group, as shown by the Student's t-test analysis of the reproductive performance parameters of ticks fed on immunized calves.

Discussion

Previously, vaccination of cattle against *B. microplus* demonstrated that a high degree of protective immunity could be induced using midgut antigen together with Freund's adjuvant (OPDEBEECK et al., 1988a) although it is of limited use and unsuitable for commercial application in food animals in veterinary practice (EDELMAN, 1980). This was followed by a series of investigations which proposed Quil A (BOMFORD, 1980) as the adjuvant of choice for vaccines containing tick antigens rather than FCA and aluminium hydroxide. (JACKSON and OPDEBEECK, 1995).

In the present study, among the two adjuvants used a significant ($P<0.01$) protective immunity was induced in calves that received Saponin with purified midgut antigen, as evidenced by a pronounced humoral immune response (1.428 ± 0.061), than in the Montanide group of calves (1.333 ± 0.203), with a significantly ($P<0.01$) higher rejection of ticks (48.03%) and low egg-rate conversion efficacy (21.13). This could be attributed to binding and interaction of Saponin with cholesterol in the membrane of the antigen-presenting cell, and also formation of insoluble complexes with free cholesterol, as observed by BOMFORD (1980). The formation of these insoluble complexes may have enhanced the level of protective immunity of the midgut antigens (BANGHAM and HORNE, 1962) in the Saponin group.

Saponin, a surface-active agent isolated from the South American tree *Quillaja saponaria* (Molina), was found to be clearly superior to Freund's adjuvant and aluminium hydroxide (JACKSON and OPDEBEECK, 1995). The Purified midgut antigens combined with Saponin induced a significantly higher immune response (HELLER-HAUPT et al., 1988) in the current study, which is in agreement with the works of JACKSON and OPDEBEECK (1995), who observed that Quil A - a semi-purified substance derived from Saponin, was considerably superior to Freund's incomplete adjuvant and aluminium hydroxide (OPDEBEECK et al., 1988b) and also when combined with midgut antigens of *Hyalomma anatolicum anatolicum* (THAKUR et al., 1992) in rabbits.

Likewise, the level of protection and host immune response offered in calves administered with Montanide ISA 50 was almost equal to that of the Saponin group of calves, indicating the consistent immunopotentiation effect of Montanide (COOK et al., 1990). Evidence of the establishment of a long-lasting immunologic memory with Montanide ISA 50 adjuvant was provided by a significant humoral antibody response lasting up to 154 DPI, with a high rejection of ticks (46.23%) and low egg-rate conversion efficacy (24.73) in immunized animals. This powerful immunostimulatory effect of w/o emulsion is mediated by the creation of a depot from where the antigen is slowly released, ensuring continuous stimulation of the immune system and the induction of local inflammatory reaction consisting mainly of oil-ingesting macrophages and lymphocytes (ALTMAN and DIXON, 1989). This local inflammatory reaction constitutes the major drawback in the use of oil adjuvant (MARTINEZ et al., 1996) as observed in the study of animals with development of sterile abscesses at the site of injection. However, reduction of immunizing doses (ANDERSON et al., 1971) and the use of the intramuscular route (PHAN THANH PHUONG, 1992) for immunization could help in solving this problem. Hence, Montanide ISA 50 cannot be accepted for use in vaccines until this drawback is overcome or rectified.

Therefore, among the various adjuvants available we propose Saponin as the adjuvant of choice for vaccines containing midgut antigens of *B. microplus.*

Acknowledgements

The senior author wishes to express his gratitude to Tamil Nadu Veterinary and Animal Sciences University, Chennai, for permitting him to pursue a doctoral thesis on part-time basis, and to the Dean, Madras Veterinary College, Chennai, for the facilities provided.

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Received: 25 July 2003
Accepted: 15 March 2004

RAMAN, M., P. RAMADASS, G. RAJAVELU: Vrednovanje adjuvanata saponina i montanida ISA 50 radi pojačanja imunosti i pojačanog učinka na humoralni imunosni odgovor teladi na pročišćeni antigen srednjeg crijeva krpelja *Boophilus microplus*. Vet. arhiv 74, 129-140, 2004.

SAŽETAK

Pokusna imunizacija provedena je na 18 križane teladi (*Bos taurus* x *Bos indicus*) upotrebom kromatografski pročišćenog antigena srednjeg crijeva krpelja *Boophilus microplus* s adjuvantima saponinom i montanidom ISA 50. Izražen humoralni imunosni odgovor bio je dokazan imunoenzimnim testom (1,428 ± 0,061) sa signifikantno (P<0,01) većim odbacivanjem krpelja (48,03%) i malom proizvodnjom jajašaca (21,13) u skupini teladi tretiranoj saponinom nego u skupini tretiranoj montanidom. Učinak dvaju adjuvanata bio je određen na osnovi različitih hranidbenih i reprodukcijskih pokazatelja krpelja prikupljenih s imuniziranih i kontrolnih životinja. Razina zaštite koju je pružio montanid ISA 50 bila je gotovo jednaka kao i u skupini teladi tretiranoj saponinom uz razvoj blage lokalne upalne reakcije u imunizirane teladi.

Ključne riječi: *Boophilus microplus*, antigen, saponin, montanid ISA 50, imunopojačivački učinak, telad