Protective Efficacy of Subunit Antigens Isolated from
*In vitro* Mid Gut Cells of *Boophilus microplus*

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**Abstract:** Protein subunit (PS) and lipopolysaccharides complex subunit (LPCS) antigens were extracted from the *in vitro* cultured mid gut cells of *Boophilus (B.) microplus*. PS and LPCS vaccines were injected in buffaloes. Their efficacy was evaluated based on cellular, humoral, and challenge responses. There was a significant difference (P < 0.05) in cellular, humoral and challenge responses between the two subunit vaccines. The vaccines with adjuvant gave significantly higher cellular, humoral and challenge responses compared to those without adjuvant. The per cent protection and damage of *B. microplus* in PS vaccines with and without adjuvant was 75.79 and 61.56, and 44.56 and 42.75 per cent, respectively, while in LPCS vaccines with and without adjuvant was 85.25 and 75.79; and 76.25 and 64.16 per cent, respectively. The results of the current study confirm the role of protein and carbohydrates as a protective antigen or as a factor for specificity of these antigens.

**Key words:** *Boophilus microplus* mid gut cells, lipopolysaccharide, protein, cellular response, humoral response

**Introduction**

Currently tick control relies heavily on the use of chemicals. There are a number of problems with this strategy. Ticks have a striking ability to become resistant to the toxic effects of the chemicals (Nolan and Schnirzerling, 1986; Nolan et al., 1989; Schnirzerling et al., 1989). This rapidly developing problem of tick resistance would lead animal population completely susceptible to both the ticks and the diseases they transmit, with disastrous consequences. Thus, there is an urgent need for new tick control strategies.

The ability of manipulating organisms on molecular level and advancement in immunological procedures have provided an alternative for tick control. The possibility of vaccinating against ticks has been investigated in the laboratory for many years. Much of the emphasis has been given on mimicking the immunity which a host would acquire following natural tick infestation, or through vaccination with tick saliva glands (Gill and Walker, 1987; Girardin and Brossard, 1989; Nyindo et al., 1989; Walker and Fletcher, 1980) as the presumed source of protective antigen in such immunity or by isolating the antigens recognized by antibodies produced following natural tick infestation. Although this approach seems reasonable but it has met with a disappointing lack of success. However, antigens from partially engorged female *B. microplus* have been used with variable success to immunize the animals against tick infestation (Johnston et al., 1986). Likewise, midgut of the ticks have also been used as a candidate immunogen (Oepbeek et al., 1986, 1988a; Jackson and Oepbeek, 1990). The feasibility of producing midgut vaccines depends upon sufficient quantity of antigenic material either through maintenance of tick colonies and/or *in vitro* propagation of the midgut cells. Successful attempts have been made for laboratory maintenance (Akhtar and Hayat, 1993) and *in vitro* propagation of midgut cells (Akhtar et al., 1992); and the protective effects of *in vitro* mid gut cells (Akhtar, 1995). Present study reports the immunogenic and protective effects of lipopolysaccharide complex and protein subunit component isolated from *in vitro* cultured mid gut cell of *B. microplus*.

**Materials and Methods**

**Isolation of midgut of *B. microplus*:** Ticks were surface sterilized by washing in 0.5 per cent benzalkonium chloride (Sigma Laboratories, St., Louis, USA) and 70 per cent ethanol; and finally in three changes of sterile distilled water. The washed ticks were dissected and midguts were separated and properly cleaned from the malpighian tubules, rectum, epimera, appendages, muscles, trachea and central ganglia (Akhtar et al., 1992).

**Midgut cell culture:** The cleaned guts were washed and suspended in sufficient quantity of Hank's Balanced Salt Solutions (Flow Laboratories, Irvine, Scotland) (HBSS; 0.5 g of gut tissue in 5 mL). The gut tissue was dissociated into cells by simple teasing method with a sterilized scissor (Guru et al., 1976). The cell suspensions were inoculated into Grace's Insect Medium (GIM) (Sigma Laboratories, St., Louis, USA) supplemented with heat inactivated (56°C for 30 minutes) 5 per cent FCS (Sigma Laboratories, St., Louis, USA), and antibiotics (Penicillin 100 IU/mL, Streptomycin 100 µg/mL and fungizone 50 IU/mL) and maintained at 28°C (Akhtar et al., 1992). The cells adherent to the flask's surface were harvested by a rubber policeman and media were transferred to the sterile centrifuge tubes. The flasks were rinsed two to three times with 5 mL sterile phosphate buffered saline (pH 7.2) and added to the tubes containing harvested cells in media. Finally, pooled cell suspension was centrifuged (6000 rpm/30 minutes/4°C) and the supernatant was removed. Sedimented cells were repeatedly washed in fresh phosphate buffered saline (PBS) by centrifugation as described above. Pellet was collected and cells were reconstituted in minimum volume of PBS and homogenized by sonication and stored at -20°C.

The homogenized cell suspensions were used for the preparation of protein and lipopolysaccharide subunit vaccines.

**Preparation of vaccines:**

**Protein subunit (PS) vaccines:** Protein was extracted from homogenized cell suspension following the method described by Herbert (1974). Protein concentration in the extracted material was assayed by Bradford (1976) method and adjusted at 250 µg/mL of PBS for the preparation of following PS vaccines.

a. One part of extracted protein plus two parts of adjuvant (Friend's complete adjuvant).

b. One part of extracted protein plus two parts of PBS.

The vaccine preparations were homogenized through sonication.

**Lipopolysaccharide complex subunit (LPCS) vaccines:** The LPCS was extracted from the homogenized cell suspensions
following the method of Broggen and Rebers (1978). Glucose was considered as a representative of all carbohydrate moieties; therefore, its concentration in the extracted LPCS was measured by colorimetric method (Kolmer et al., 1969). The final concentration of glucose (representing LPCS) was adjusted at 160 μg/mL of PBS for the preparation of following LPCS vaccines.

Safety tests for vaccine preparations: Sterility and toxicity tests were performed for all the vaccines (Collee et al., 1982). All vaccines were sterile and toxic free as no growth of microorganisms on inoculated media and in vitro inoculation symptoms/mortality in rabbits were noted. Hyperimmune sera was raised in rabbits against PS and LPCS antigens as described by Collee et al. (1982).

Evaluation of vaccines: Fifty buffaloes were selected from a private livestock farm and divided into seven equal groups, each used for the evaluation of a different vaccine. All the vaccines were homogenized through sonication and extracted with PBS. The vaccines were assigned to different groups of animals as follows:

Group 1: PS with adjuvant.
Group 2: PS without adjuvant.
Group 3: LPCS with adjuvant.
Group 4: LPCS without adjuvant.
Group 5: One part of PBS plus two parts of adjuvant.

Blood samples (25 mL) were collected from all animals before and after three and six weeks of vaccination. Each blood sample was divided into two aliquots of 15 and 10 mL with and without an anticoagulant, respectively. Serum was separated from the clotted blood and stored at -20°C. Heparized blood and serum were used to detect by cellular and humoral responses, respectively.

Immunological studies: The cellular response to vaccines was assayed by erythrocyte rosetting formation (Akhtar et al., 1998) and leucocyte adherence inhibition (Akhtar, 1996) tests. The humoral response to vaccines was assayed by agar gel precipitation (Akhtar, 1998b), indirect hemagglutination (Akhtar and Hayat, 2000) and single radial hemolysis tests (Akhtar et al., 2001).

Challenge response: Six buffaloes from each group of 10 vaccinated (group 1 to 4) and control (group 5) were used for challenge infection. The animals were infected (day 7 post boosting) with 10-days-old B. microplus larvae (20,000) following the method described by Opdebeeck et al. (1989). Protection was calculated by comparing the weight of eggs laid by ticks dropped from vaccinated animals to the same mean value for a group of control animals in the same experiment.

The data obtained from the experiments were analyzed by Randomized Complete Block Design with analysis of variance using MSTAT-C (Crop and Soil Sciences Department, Michigan State University, USA) and means were compared at 5 per cent level of probability by Duncan’s Multiple Range test.

**Results**

**Protein subunit (PS) vaccines:** The specific erythrocyte rosette enhancement in response to PS with and without adjuvant was 19.48 and 20.54 per cent; and 53.78 and 49.80 per cent in week 3 and 6 postvaccination (pv), respectively (Table 1). There was no difference (P > 0.05) in response (specific erythrocyte rosette enhancement) between PS with and without adjuvant in week 3 pv. However, the response was significantly higher (P < 0.05) to PS with adjuvant as compared to that without adjuvant in week 6 pv; in week 6 pv compared to week 3 pv to both PS with and without adjuvant.

The specific leukocyte adherence inhibition in response to PS with and without adjuvant was 8.97 and 7.88 per cent; and 22.38 and 16.88 per cent in week 3 and 6 pv, respectively (Table 2). There was no difference in response (specific leukocyte adherence inhibition) between PS with and without adjuvant in week 3 pv. However, the response was significantly higher (P < 0.05) response to PS with adjuvant compared to that without adjuvant in 6 pv; in week 6 pv compared to week 3 pv to both PS with and without adjuvant.

The results of AGP test revealed presence of precipitating antibodies produced in response to both PS with and without adjuvant. However, single line was a sign of the vaccine contains a specific antigen. In contrast to it, no precipitin lines were observed in samples from adjuvant control animals.

The IHA antibody titres (titre range (geometric titre)) in response to PS with and without adjuvant were 1:4-1:64 (28.18) and 1:4-1:64 (28.18); and 1:19-1:266 (74.13) and 1:8-1:256 (24.25) in week 3 and 6 pv, respectively. There was a significantly higher (P < 0.05) response (antibody titre) to PS with adjuvant compared to that without adjuvant both in week 3 and 6 pv; in week 6 pv compared to week 3 pv to both PS with and without adjuvant.

The hemolytic zone (mean ± standard deviation) in response to PS with and without adjuvant was 6.8 ± 9.50 (5.80 ± 1.08) and 6.6 ± 8.00 (5.90 ± 3.15); and 7.6 ± 13.00 (10.07 ± 2.06) and 7.00-10.5 (8.10 ± 1.20) in week 3 and 6 pv, respectively. There was no difference (P > 0.05) in response (average hemolytic zone) between PS with and without adjuvant both in week 3 and 6 pv; and between week 6 and 3 pv to both PS with and without adjuvant.

The protection and damage of B. microplus in PS with and without adjuvant was 75.79 and 61.56 per cent; and 44.56 and 42.78 per cent, respectively (Table 3). There was a significantly higher (P < 0.05) protection against ticks using PS with adjuvant compared to that without adjuvant.

**Lipopolysaccharide (LPS) vaccines:** The specific erythrocyte rosette enhancement in response to LPCS with and without adjuvant was 36.06 and 19.24 per cent; and 73.82 and 27.67 per cent in week 3 and 6 pv, respectively (Table 1). There was a significantly higher (P < 0.05) response (specific erythrocyte rosette enhancement) to LPCS with adjuvant compared to that without adjuvant both in week 3 and 6 pv; in week 6 pv compared to week 3 pv to both LPCS with and without adjuvant.

The specific leukocyte adherence inhibition in response to LPCS with and without adjuvant was 12.88 and 8.66 per cent; and 31.70 and 18.93 per cent in week 3 and 6 pv, respectively (Table 2). There was a significantly higher (P < 0.05) response (specific leukocyte adherence inhibition) to LPCS with adjuvant compared to that without adjuvant both in week 3 and 6 pv; in week 6 pv compared to week 3 pv to both LPCS with and without adjuvant.

The results of AGP test revealed presence of precipitating antibodies produced in response to both LPCS with and
Table 1: Erythrocyte rosette formation in immunized and control animals

<table>
<thead>
<tr>
<th></th>
<th>Erythrocyte rosette formed (%) ± sd</th>
<th>Rosettes enhancement (%)</th>
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<tbody>
<tr>
<td></td>
<td>Pre vaccination</td>
<td>3 weeks post vaccination</td>
</tr>
<tr>
<td>LPCS with adj</td>
<td>32.4 ± 3.6</td>
<td>43.76 ± 1.03</td>
</tr>
<tr>
<td>LPCS without adj</td>
<td>29.7 ± 5.2</td>
<td>36.42 ± 1.82</td>
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<tr>
<td>PS with adj</td>
<td>33.5 ± 2.8</td>
<td>40.01 ± 1.33</td>
</tr>
<tr>
<td>PS without adj</td>
<td>36.1 ± 4.4</td>
<td>43.52 ± 1.07</td>
</tr>
<tr>
<td>Adj control</td>
<td>30.8 ± 3.5</td>
<td>30.9 ± 0.9</td>
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Table 2: Leukocyte adherence inhibition in immunized and control animals

<table>
<thead>
<tr>
<th></th>
<th>Leukocyte adherence inhibition (%) ± sd</th>
<th>Specific adherence inhibition (%) ± sd</th>
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<tbody>
<tr>
<td></td>
<td>Pre vaccination</td>
<td>3 weeks post vaccination</td>
</tr>
<tr>
<td>LPCS with adj</td>
<td>42.40 ± 5.60</td>
<td>55.28 ± 2.29</td>
</tr>
<tr>
<td>LPCS without adj</td>
<td>42.50 ± 7.70</td>
<td>51.86 ± 2.12</td>
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<tr>
<td>PS with adj</td>
<td>43.90 ± 9.50</td>
<td>62.87 ± 2.49</td>
</tr>
<tr>
<td>PS without adj</td>
<td>41.70 ± 4.60</td>
<td>48.58 ± 1.45</td>
</tr>
<tr>
<td>Adj control</td>
<td>46.70 ± 6.60</td>
<td>47.90 ± 4.90</td>
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Table 3: Protective effects of immunized animals without adjuvants

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Buffalo</th>
<th>Normal</th>
<th>Damaged</th>
<th>Partially damaged</th>
<th>Unimmunized</th>
<th>Total</th>
<th>No. of eggs</th>
<th>Eggs vs.</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td>LPCS with adj</td>
<td>6</td>
<td>179±17</td>
<td>319±12</td>
<td>63±8</td>
<td>56±16</td>
<td>119±20</td>
<td>220±43</td>
<td>75±16</td>
<td>65±35</td>
</tr>
<tr>
<td>LPCS without adj</td>
<td>6</td>
<td>191±78</td>
<td>580±68</td>
<td>702±6</td>
<td>57±8</td>
<td>904±101</td>
<td>523±47</td>
<td>35±2±50</td>
<td>76±20</td>
</tr>
<tr>
<td>PS with adj</td>
<td>6</td>
<td>232±53</td>
<td>795±26</td>
<td>101±4</td>
<td>99±53</td>
<td>1239±54</td>
<td>417±50</td>
<td>25±5±70</td>
<td>75±79</td>
</tr>
<tr>
<td>PS without adj</td>
<td>6</td>
<td>396±95</td>
<td>980±77</td>
<td>179±6</td>
<td>85±9</td>
<td>1122±188</td>
<td>1204±704</td>
<td>77±0±80</td>
<td>44±66</td>
</tr>
<tr>
<td>Adj control</td>
<td>6</td>
<td>1312±64</td>
<td>98±40</td>
<td>36±5</td>
<td>96±12</td>
<td>1542±715</td>
<td>2159±161</td>
<td>126±1±87</td>
<td>126±1±87</td>
</tr>
</tbody>
</table>

without adjuvant. However, the precipitating lines were faint and not that prominent as in case of other vaccines. Like other vaccines, presence of only a single line indicated that this vaccine contains a specific antigen. In contrast to it, no precipitin lines were observed in samples from adjuvant control animals. The antibody titre (titre range [gemoean titre]) of response to LPCS with and without adjuvant were 1:8-1:266 (46.67) and 1:8-1:128 (26.70); and 1:32-1:512 (120.23) and 1:16-1:256 (60.16), respectively. There was no difference (P > 0.05) in response (antibody titre) between LPCS with and adjuvant in week 3 and 6 pv, respectively. There was a significant higher (P < 0.05) response to LPCS with adjuvant compared to that without adjuvant in week 6 pv; in week 6 pv compared to week 3 pv to LPCS with adjuvant. However, there was no difference (P > 0.05) in response between week 6 and 3 pv to LPCS with adjuvant.

The hemolytic zone (range [mean ± sd]) in response to LPCS with and without adjuvant was 7.0-12.30 (9.08 ± 1.50) and 7.0-10.50 (8.28 ± 1.22); and 9.00-14.5 (10.86 ± 2.07) and 7.60-10.5 (9.46 ± 1.42) in week 3 and 6 pv, respectively. There was no difference (P > 0.05) in response (average hemolytic zone) between LPCS with and without adjuvant both in week 3 and 6 pv; between week 6 and 3 pv to LPCS with adjuvant. However, there was a significantly higher (P < 0.05) response in week 6 pv compared to week 3 pv to LPCS without adjuvant.

The protection and damage of B. microplus in LPCS with and without adjuvant was 69.86 and 73.29 per cent; and 76.28 and 64.18 per cent, respectively (Table 3). There was a significantly higher (P < 0.05) protection against ticks LPCS with adjuvant compared to that without adjuvant.

Discussion
A variety of antigens have been used with variable success for immunization of animals against ticks. These include whole tick (Dhadialla et al., 1990), salivary glands (Nyindo et al., 1989), salivary gland plus digestive system (Kohler et al., 1967), midgut plus reproductive organs (Allen and Humphreys, 1979), midguts (Jackson and Opdebeeck, 1980), and axillary, prescapular or cervical lymph nodes (Wikel and Allen, 1978). These workers have used crude antigen extracts for immunization. It has been suggested without controversy that midgut contains the protective antigen that is able to confer immunity against ticks (Jackson and Opdebeeck, 1980). Midgut has been used as a crude antigen or as membrane glycoproteins isolated either from gut wall (Willadsen and Kemp, 1989) or luminal surface of the plasma membrane of tick gut epithelial cells (Rand et al., 1989). It was investigated earlier (Akhtar et al., 1999) that cultured midgut cells had high proportion of membrane associated component compared to soluble component. The gut membrane associated component has been reported to be a better immunogen than the soluble component (Jackson and Opdebeeck, 1989).

Protein and lipopolysaccharide complex subunit vaccines were prepared and evaluated to identify the more immunogenic part of gut membrane associated component. It was interesting to note that LPCS vaccines had significantly higher (P < 0.05) level of protection than to PS vaccines. This observation was contrary to the understanding that proteins are most commonly the best antigens. Research has been focused
recently on the carbohydrate moieties to investigate their role as antigens/immunogens. It has been reported that carbohydrate antigens play a major, often dominant part in the immunological profile of infectious organisms and, therefore, cannot be overlooked for this purpose in parasites (Furguson and Romans, 1985). Investigations have been conducted on this aspect in parasites. For example, glycoconjugates including the phosphoinositolglycan of trypanosome (Furguson et al., 1988); lipophosphoglycan and glycolipids of leishmania (Turco, 1985; Handman and Mitchell, 1986); phosphorylcholine-bearing macro molecules of filaria (Maizels et al., 1986) without adjuvant. The results of schistosoma (Dissous et al., 1986) have been used as protective immunogens.

The results of the current study confirmed the role of carbohydrates as protective antigen or as a factor for specificity of these antigens (Lee et al., 1991; Willadsen and McKenna, 1991). It has been reported that LPCS extract from bacteria contains proteins (RNA, DNA) in addition to polysaccharides. It was found that LPCS vaccines may, therefore, be due to the cumulative activity of both carbohydrate and integrated protein components. This assumption is supported by Rebers et al. (1980) who stated that protein integrated in the basic LPS structure of bacteria may be essential for immunogenicity.

It was observed that the vaccines with adjuvant provided a significantly higher (P<0.05) protection compared to those without adjuvant. This indicated the compatibility of Freund’s Complete Adjuvant with all vaccines. The results of cellular and humoral assays, however, did not always support the better response with adjuvanted compared to non-adjuvanted vaccines. For example, the cellular response to all vaccines with adjuvant was higher (P<0.05) compared to those without adjuvant. The results of humoral response were only partially consistent to those of cellular response. IHA indicated that PS and LPCS vaccines with adjuvant had higher response (P<0.05) compared to their non-adjuvanted counterparts. SRH indicated that there was no difference between the adjuvanted and non-adjuvanted vaccines. The inconsistency in cellular and humoral responses may be attributed to a variety of factors. For example, the level of humoral response is not essentially comparable and proportionately dependent on cellular response. Secondly, differences in the sensitivity and specificity of IHA and SRH may also influence the results.

The recent studies in the immunological processes may play role in resolving the differences in humoral response which well existed in cellular response between adjuvanted and non-adjuvanted vaccines.

The best results obtained with LPCS vaccine are supposed due to its better immunogenicity and structural similarity with Freund’s complete adjuvant. Chemically, LPCS is a hydrophilic heteropolysaccharide component and a covalently linked hydrophobic lipid portion (Riettschel et al., 1989) having adjuvant activity (Imoto et al., 1987). The monophosphoryl lipid A of LPCS has structural similarity with Trehalis dimycolate (Mycobacterial component in FCA). Both these components have a link between two glucosamine (LPCs) and glucose (FCA) molecules which are in turn linked to β-hydroxy fatty acids, β-hydroxy myristic acid and β-hydroxy mycolic acid, respectively (Ribi, 1986). The exact mode of action of LPCS has not so far been investigated. However, it is believed that LPCS and its derivatives act as T-cell independent polyclonal B-cell mitogens (Seppal and Makela, 1982; Gery et al., 1972). The experimental data (Tomai and Johnson, 1989; Johnson and Tomai, 1990) suggests that monophosphoryl lipid “A” in LPCS acts directly upon T-cells to stimulate IFN (but not IL-2) resulting in stimulation of macrophages and production of IL-1 which ultimately influence the proliferation of lymphocytes.

A prominent cellular response to the vaccines used in current study is another favorable characteristic. It is speculated that such a high cellular response would be helpful in maintaining persistency in the immune response through T-helper cells which regulate humoral immunity to produce specific antibodies (Tizzard, 1992). It was observed that the gut of affected ticks was damaged probably due to high level of specific antibodies as reported earlier (Jackson and Opiebeek, 1989; Wong and Opiebeek, 1989). Extensive damage to the gut kills the tick resulting not only in disruption of life cycle but also in blocking the transmission of pathogens to other potential hosts. The resistance induced by such type of vaccines may be called as a kind of “blocking immunity” because the ticks acting as potential vectors would be prevented from feeding on another animal after obtaining a blood meal from a vaccinated host.

Ticks from vaccinated animals had less weight due to affected feeding and inhibition of blood meal endocytotic activity/intracellular digestion by digest cells of gut attributable to specific antibodies (Agbede and Kemp, 1985; Coons et al., 1982). The lower number of eggs produced by affected ticks may be explained on the basis of lowered oviposition due to damage of basophilic cells involved in synthesis of vitellogenins required for egg formation as reported for R. sanguineus (Coons et al., 1982).

Further investigations on the characterization and evaluation of specific antigens and cross immunogenicity experiments are under way.

References
Akhtar and Hayat: Protective efficacy of subunit antigens of Boophilus microplus


