Serological evaluation of *Pasteurella multocida* antigens associated with protection in buffalo calves

M. AFZAL, R. MUNEER and S. AKHTAR *

**Summary:** Different antigens of *Pasteurella multocida* Carter’s type 6:B including whole bacterium, antigen heated at 56°C, antigen heated at 100°C, sonicated antigen, capsular antigen, potassium thiocyanate extract, lipopolysaccharide and sodium salicylate extract were evaluated to assess protection in buffalo calves against haemorrhagic septicemia. Sera from calves with known protection status in experimental challenge were titrated by enzyme-linked immunosorbent assay (ELISA) against all antigens. Capsular antigen extracted with 2.5% sodium chloride was superior to other antigens for assessing protection status of buffalo calves against *P. multocida* by ELISA. This capsular antigen was able to differentiate clearly between well protected, protected and unprotected animals.

**KEYWORDS:** Antigens - Buffalo - Enzyme-linked immunosorbent assay - *Pasteurella multocida* - Protection Serology.

**INTRODUCTION**

Haemorrhagic septicemia is the most important bacterial infection of cattle and buffaloes in many countries of Asia. The disease is caused by *Pasteurella multocida* Carter’s type 6:B. Vaccination has been proved to be effective for the control of this infection (4). The haemagglutination inhibition test has been used extensively to monitor antibody response to *P. multocida* (9, 15). However, these titres cannot be directly correlated with levels of protection (6).

Enzyme-linked immunosorbent assay (ELISA) is currently considered as the serological test of choice due to its speed, sensitivity and ease of automation. ELISA has been used previously in serology of *P. multocida* Carter’s type 6:B employing sonicated antigen (1, 14). The present study compares a variety of antigens extracted from *P. multocida* for suitability in measuring antibodies associated with protection against haemorrhagic septicaemia.

**MATERIALS AND METHODS**

**Preparation of antigens**

*P. multocida* Carter’s type 6:B was grown in buffered medium containing casein hydrolysate, sucrose and yeast extract in one-litre flasks at 37°C for 24 hours (2). Growth

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was harvested by centrifugation. Bacteria were suspended in normal saline, aliquoted and stored at 4°C until use. Different antigens were prepared as detailed below:

a) Whole bacteria: Pasteurella suspended in normal saline was killed by the addition of 0.5% formalin and left at 4°C overnight.

b) Antigen heated at 100°C: Pasteurella suspended in normal saline was heated at 100°C for 1 hour in a shaking water bath. The suspension was centrifuged at 4,000 r.p.m. for 1.5 hours and the supernatant used as antigen (18).

c) Antigen heated at 56°C: Pasteurella suspended in normal saline was heated at 56°C for 1 hour in a shaking water bath. The suspension was centrifuged at 4,000 r.p.m. for 1.5 hours and the supernatant used as antigen.

d) Sonicated antigen: Pasteurella suspended in normal saline was sonicated at 50 watts for 30 min using a 3/4 inch probe. The suspension was centrifuged at 4,000 r.p.m. for 1.5 hours and the supernatant used as antigen.

e) Capsular antigen: Pasteurella was suspended in 2.5% sodium chloride and shaken in a water bath at 37°C for 4.5 hours at 80 r.p.m. (19). The suspension was centrifuged at 4,000 r.p.m. for 1 hour. Supernatant dialysed against normal saline at 4°C for 72 hours served as antigen.

f) Potassium thiocyanate extract (KSCN): This antigen was prepared in a similar manner to capsular antigen but extraction was carried out in a solution of 0.5 M potassium thiocyanate in 2.5% sodium chloride (13).

g) Lipopolysaccharide (LPS): LPS was extracted following the method described by Rebers and Heddlestone (16). Briefly, dried Pasteurella was extracted with phenol water at 68°C for 30 min. The aqueous layer was collected after centrifugation at 7,000 r.p.m. at 10°C for 45 min and dialysed for 3 days against distilled water.

h) Sodium salicylate extract: Pasteurella suspended in a solution of 1 M sodium salicylate (pharmaceutical grade) in 0.85% sodium chloride was extracted at 37°C for 3 hours in a shaking water bath (8). The suspension was centrifuged at 4,000 r.p.m. for 1.5 hours and the supernatant dialysed against normal saline for 3 days.

Buffalo sera

Serum samples were collected from ten buffalo calves. These animals belonged to a vaccine study and were challenged with live P. multocida on the day of serum collection. Based on their response to experimental challenge, the animals were divided into three groups. Group 1 consisted of six vaccinated animals. These animals remained normal and did not show any oedema at the site of injection or any increase in temperature and were classified as "well protected". Group 2 consisted of two vaccinated animals. These animals showed a rise in temperature and oedema at the site of injection but recovered from the challenge and were classified as "protected". Group 3 consisted of two unvaccinated calves. These showed a rise in temperature and oedema at the site of inoculation and succumbed to experimental challenge. Animals in this third group were classified as "unprotected".

Enzyme-linked immunosorbent assay

An indirect ELISA was performed as described previously (14) to measure antibodies against P. multocida. Briefly, antigen was coated on microtitre plates in carbonate buffer and left overnight at room temperature. Plates were washed three
times with phosphate buffered saline containing 0.5% Tween 20. Test serum was added and plates incubated at 37°C for 1 hour. Plates were washed five times and rabbit anti-bovine IgG peroxidase conjugate added. The plates were incubated at 37°C for 1 hour. After washing five times, substrate (o Phenylenediamine containing 0.01% hydrogen peroxide) was added and the plates allowed to develop in the dark. Colour development was stopped with 2 N sulphuric acid and plates read at 492 nm in an ELISA reader. Results were expressed as ELISA absorbance at serum dilution of 1:125 and titre in log₅.

Chemical analysis

Protein was assayed using bovine serum albumin as standard (12). Carbohydrates were assayed by the phenol sulphuric acid method (5) and 2 Keto 3 deoxyoctonate (KDO) was analysed by thiobarbiturate reaction (17).

RESULTS

Mean ELISA titres (log₅) of sera against different antigens extracted from *P. multocida* are shown in Figure 1.

![Mean ELISA titres (log₅) of sera against different antigens extracted from *P. multocida*](image_url)

**FIG. 1**

Mean ELISA titres (log₅) of sera from buffalo calves against different antigens of *P. multocida*
Whole bacteria, antigen heated at 100°C and capsular antigen were able to differentiate between well protected, protected and unprotected animals. Antigens other than LPS, although able to differentiate well-protected animals from the other two groups, were unable to distinguish between protected and unprotected animals.

With a single dilution (1:125) of sera, only capsular antigen was able to differentiate clearly between the three types of animals (Fig. 2). Some differences in ELISA absorbance readings were too small to distinguish between well-protected, protected and unprotected animals. Other antigens failed to differentiate between protected and unprotected animals.

![Mean ELISA absorbance of sera from buffalo calves at single serum dilution (1:125) against different antigens of *P. multocida*](image)

Analysis of the chemical composition of the antigens indicated that only LPS antigen contained KDO (Table 1). Other antigens did not contain detectable quantities of KDO and therefore probably did not contain a significant amount of LPS. More proteins than carbohydrate were extracted from antigen heated at 56°C, sonicated antigen, KSCN and sodium salicylate extract, while the other antigens contained more carbohydrates than protein.
TABLE I

Chemical composition of different antigens of Pasteurella multocida

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Protein (µg/ml)</th>
<th>Carbohydrate (µg/ml)</th>
<th>KDO (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen heated at 100°C</td>
<td>300</td>
<td>540</td>
<td>ND</td>
</tr>
<tr>
<td>Antigen heated at 56°C</td>
<td>620</td>
<td>350</td>
<td>ND</td>
</tr>
<tr>
<td>Sonicated antigen</td>
<td>560</td>
<td>350</td>
<td>ND</td>
</tr>
<tr>
<td>Capsular antigen</td>
<td>120</td>
<td>200</td>
<td>ND</td>
</tr>
<tr>
<td>Potassium thiocyanate extract</td>
<td>212</td>
<td>110</td>
<td>ND</td>
</tr>
<tr>
<td>Lipopolysaccharide</td>
<td>78</td>
<td>660</td>
<td>17.6</td>
</tr>
<tr>
<td>Sodium salicylate extract</td>
<td>56</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

KDO 2-Keto 3-deoxyoctonate
ND not detected

DISCUSSION

Some antigens have been extracted and characterised from Pasteurella multocida associated with haemorrhagic septicaemia in cattle and buffaloes in South-East Asia (9, 13, 15). These antigens have also been used in various studies to evaluate antibody response through a haemagglutination inhibition test conducted following infection or vaccination. However, antibody titres have not always been correlated with protection (6). In the present study, eight antigens were evaluated for their diagnostic abilities. Three antigens (namely, capsular antigen, antigen heated at 100°C and whole cell antigen) were able to differentiate between the three groups of animals (i.e. well-protected, protected and unprotected), while the remaining five antigens were unable to differentiate between the three groups. LPS antigen was least able to make this differentiation. Previous studies have also shown poor immunogenicity of LPS antigen (3, 7, 13). The poor ability of these five antigens to differentiate between the three groups of animals might be due to cross reactivity with non-specific antibodies.

Of the three antigens which were able to make the differentiation, capsular antigen was more specific than whole cell antigen and antigen heated at 100°C, which probably indicates that capsular material elicits more specific and protective antibodies against Pasteurella multocida. Furthermore, during the preparation process, the antigenic configuration of capsular antigen might have remained intact compared to the two other antigens. Previous studies have also reported capsular antigen to be stronger than other known antigens for these organisms (10, 11, 14).

The authors demonstrated that capsular material of Pasteurella multocida represents an improvement in diagnostic antigen and could be used to efficiently monitor the immune status of vaccinated dairy herds by ELISA. The results of the present study should be examined carefully, however, due to the small and unequal numbers of animals in the three groups. The authors suggest that the properties of capsular material as diagnostic antigen be evaluated using a substantial number of animals in different groups.
ÉTUDE SÉROLOGIQUE DES ANTIGÈNES DE PASTEURELLA MULTOCIDA LIÉS À LA PROTECTION CHEZ LES BUFFLONS. — M. Afzal, R. Muneer et S. Akhtar.

Résumé : Pour évaluer la protection contre la septicémie hémorragique chez les bufflons, les auteurs ont testé différents antigènes de Pasteurella multocida (type 6:B de Carter) : bactérie entière, antigène chauffé à 56 °C, antigène chauffé à 100 °C, antigène traité par ultrasons, antigène capsulaire, extrait au thiocyanate de potassium, lipopolysaccharide et extrait au salicylate de sodium. Ils ont déterminé, par la méthode ELISA, les titres des anticorps dirigés contre tous ces antigènes, chez des animaux dont la protection avait été contrôlée par épreuve expérimentale. L'antigène capsulaire extrait par du chlorure de sodium à 2,5% s'est révélé supérieur aux autres antigènes pour évaluer, par la technique ELISA, la protection des bufflons contre P. multocida. Cet antigène capsulaire a permis de différencier clairement entre les animaux protégés, bien protégés et non protégés.

MOTS-CLÉS : Antigènes Buffle Epreuve immuno-enzymatique Pasteurella multocida - Protection Sérologie.


Resumen: Se evaluaron diferentes antígenos de Pasteurella multocida del tipo Carter 6:B incluidos bacteria entera, antígeno calentado a 56°C, antígeno calentado a 100°C, antígeno expuesto a altas frecuencias sónicas, antígeno capsular, extracto de trocianato de potasio, lipopolisacárido y extracto de salcicaldo de sodio para determinar el grado de protección de las búfalas contra la septicemia hemorrágica. Los sueros de animales con protección determinada por pruebas experimentales fueron evaluados por la prueba de dosificación inmuno enzimática (ELISA) contra todos los antígenos. El antígeno capsular extraído con el 2,5% de cloruro de sodio fue superior a los otros antígenos para evaluar el estado de protección de los terneros de búfalos contra P. multocida por ELISA. Dicho antígeno capsular resultó capaz de diferenciar claramente entre los animales con buena protección, poca protección y sin protección.


REFERENCES


