Antimicrobial Susceptibility of *Pasteurella multocida* Isolates

P. Abeynayake,¹ T.G. Wijewardana² and S.A. Thalagoda²

Abstract

The sensitivity to antimicrobial agents of 27 *Pasteurella multocida* isolates was assayed in terms of minimum inhibitory concentration, employing a serial broth dilution technique. Prepared microtitre plates used in the study contained 17 drugs in four or eight dilutions, with four controls. Isolates of *P. multocida* were considered in four groups, depending on their origin (from field outbreaks; from carrier animals; from species other than cattle and buffalo; and national reference strains). Despite diverse geographical and host origins, the isolates exhibited uniformity in sensitivity to a majority of the antibacterial agents. For example, all *P. multocida* isolates were highly sensitive to penicillin, ampicillin, cephalothin, enrofloxacin, chloramphenicol and nitrofurantoin; and all isolates except three were highly sensitive to streptomycin. High resistance to streptomycin was observed in one isolate from a field outbreak; in the 'streptomycin-resistant' marker strain 335 used in Sri Lanka; and in the Thai reference strain. A considerable number of isolates were resistant to fusidin, sulphamethaxozole, spiramycin and clindamycin. On the basis of these results, it is suggested that the field practice of administering sulphadimidine to clinically affected animals be discouraged.

*Pasteurella multocida* causes haemorrhagic septicaemia (HS) — a highly fatal, septicaemic disease of cattle and buffalo. Since the disease has a sudden onset and a short course, leaving little opportunity for treatment and recovery (De Alwis 1984), attempts to control HS in endemic countries are based on vaccination programs.

For chemotherapy to be effective, drugs need to be administered during the early phase of the disease, before specific clinical signs appear (Prescott and Baggot 1988). In HS, this phase is characterised by an elevated temperature and inappetence, increased salivation, and respiratory distress. Treatment may also be of some value in eliminating ‘active carriers’ that shed pasteurellae intermittently.

This study was aimed at determining the susceptibility of *P. multocida* isolates, expressed in terms of minimum inhibitory concentration (MIC), to antibacterial agents. Since antibacterial susceptibilities of bacteria are not constant, but vary from time to time and in different environments, it was decided that the *P. multocida* group from field outbreaks should cover isolates over a 5-year period from different geographical areas of Sri Lanka.

Materials and Methods

Twenty-seven isolates of *P. multocida* were tested against 17 antimicrobial agents. All the isolates assayed were held in the freeze-dried collection at Veterinary Research Institute, Peradeniya, Sri Lanka.

The four groups of *P. multocida* comprised:

- isolates from field outbreaks of HS from different districts of Sri Lanka over 5 years (1985–1990);
- isolates from ‘active’ and ‘latent’ carriers of HS collected at abattoirs;
- field isolates from species other than cattle and buffalo;
- national HS reference strains.

The microtitre assay plates (VETMIC +/−, National Veterinary Institute, Uppsala, Sweden) contained 17 antimicrobial agents in eight or four dilution steps, along with four control wells containing trisodium citrate (2), phosphate-buffered saline (1),

¹ Faculty of Veterinary Medicine and Animal Science, University of Peradeniya, Peradeniya, Sri Lanka
² Veterinary Research Institute, PO Box 28, Peradeniya, Sri Lanka
and distilled water (I). Each time an assay was carried out, a reference strain of Staphylococcus aureus (ATCC 25923) was tested to ensure the stability of the antibacterial agents.

P. multocida, being a rapidly growing bacterium, was propagated by inoculating 5 mL brain–heart infusion broth (BHI) with three to five colonies. The inoculum was incubated for 6 hours at 37°C. A 10-μL volume of the bacterial suspension was transferred to 10 mL of BHI to obtain an inoculum density of 10³–10⁴ colony-forming units (CFU) per 50 μL. Each well of the assay plate was inoculated with 50 μL of broth culture. After inoculation, plates were sealed with transparent adhesive tape and incubated at 37°C for 18 hours, or longer when required. The plates were placed on a viewer and the opacity in the wells detected in the mirror of the viewer.

**Results**

In spite of the different sources from which isolates were obtained, there was a great uniformity of response to the antibacterial agents tested. The sensitivity pattern is summarised in Table 1.

All *P. multocida* isolates were sensitive to penicillin (MIC = 0.12 μg/mL), ampicillin (0.25 μg/mL), cephalothin (4 μg/mL), enrofloxacin (0.25 μg/mL), chloramphenicol (2 μg/mL) and nitrofurantoin (4 μg/mL). Further, a majority of isolates were sensitive to neomycin (26/27, 1–4 μg/mL), gentamicin (26/27, 1–4 μg/mL), and oxycycline (25/26, 0.5 μg/mL).

Variable sensitivity was observed to oxacillin, erythromycin, spiramycin, clindamycin, and a trimethoprim-sulfamethoxazole combination. A considerable number of isolates were resistant to fusidin (7/27, >8 μg/mL), sulfamethoxazole (15/27, >128 μg/mL), spiramycin (11/27, >16 μg/mL) and clindamycin (10/27, >4 μg/mL).

Only three isolates were resistant to streptomycin: one isolate from a field outbreak; 'streptomycin resistant' marker strain (33S) of Sri Lanka; and the Thai strain.

**Discussion**

The determination of antibiotic sensitivity in vitro of particular *P. multocida* isolates would certainly increase the chances of successful therapy for HS. However, MIC test conditions are strictly artificial; results of such experiments cannot be extrapolated directly to the field situation. Prescott and Baggot (1988) suggested a safety factor of three to five times the MIC as a likely minimum therapeutic concentration within the tissues of clinically affected animals.

**Table 1. Sensitivity of 27 isolates of *P. multocida* to 17 antimicrobial agents.**

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Number of isolates responded (27)</th>
<th>Range of concentration (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recommended for cattle and buffalo:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penicillin</td>
<td>27</td>
<td>—</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>27</td>
<td>—</td>
</tr>
<tr>
<td>Oxacillin</td>
<td>14</td>
<td>3</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>27</td>
<td>—</td>
</tr>
<tr>
<td>Enrofloxacin</td>
<td>27</td>
<td>—</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>15</td>
<td>9</td>
</tr>
<tr>
<td>Spiramycin</td>
<td>14</td>
<td>2</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>9</td>
<td>8</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>25</td>
<td>1</td>
</tr>
<tr>
<td>Trim-sulpha</td>
<td>23</td>
<td>2</td>
</tr>
<tr>
<td>Sulfamethoxazole</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Not recommended for parenteral use:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythromycin</td>
<td>8</td>
<td>17</td>
</tr>
<tr>
<td>Neomycin</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>Fusidin</td>
<td>7</td>
<td>—</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>27</td>
<td>—</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>27</td>
<td>—</td>
</tr>
</tbody>
</table>

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Table 2. Antibacterial sensitivity pattern of *P. multocida* isolates from cattle and buffalo.

<table>
<thead>
<tr>
<th>Antimicrobial agent</th>
<th>Number of isolates sensitive (%)</th>
<th>Concentration of drug (µg/mL)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin</td>
<td>5/10</td>
<td>0.4 iu/mL</td>
<td>Chang and Carter 1976</td>
</tr>
<tr>
<td></td>
<td>112/144 (77.7)</td>
<td></td>
<td>Fales et al. 1982</td>
</tr>
<tr>
<td></td>
<td>4/7</td>
<td></td>
<td>Allan et al. 1985</td>
</tr>
<tr>
<td></td>
<td>(80)</td>
<td>≤2.0</td>
<td>Karlsson and Nystrom 1962</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td></td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>116/144 (80.5)</td>
<td></td>
<td>Fales et al. 1982</td>
</tr>
<tr>
<td></td>
<td>(96.7)</td>
<td></td>
<td>Cox et al. 1981</td>
</tr>
<tr>
<td></td>
<td>6/7</td>
<td></td>
<td>Allan et al. 1985</td>
</tr>
<tr>
<td></td>
<td>(80)</td>
<td></td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>(81.3)</td>
<td>0.4-100</td>
<td>Chang and Carter 1976</td>
</tr>
<tr>
<td></td>
<td>61/145 (42)</td>
<td></td>
<td>Fales et al. 1982</td>
</tr>
<tr>
<td></td>
<td>5/7</td>
<td></td>
<td>Allan et al. 1985</td>
</tr>
<tr>
<td></td>
<td>(44)</td>
<td>≤16</td>
<td>Karlsson and Nystrom 1962</td>
</tr>
<tr>
<td></td>
<td>(37.5)</td>
<td></td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td>Cephalothin</td>
<td>(96.7)</td>
<td>1.0</td>
<td>Cox et al. 1981</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td></td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td>Chlorotetracycline</td>
<td>(25.1)</td>
<td>0.4-50</td>
<td>Chang and Carter 1976</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>5/7</td>
<td>1</td>
<td>Karlsson and Nystrom 1962</td>
</tr>
<tr>
<td></td>
<td>(94)</td>
<td>0.4-1.6</td>
<td>Chang and Carter 1976</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>≤0.5</td>
<td>Cox et al. 1981</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>7/7</td>
<td></td>
<td>Allan et al. 1985</td>
</tr>
<tr>
<td></td>
<td>(92)</td>
<td>≤4</td>
<td>Karlsson and Nystrom 1962</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td></td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td>Triple-sulpha</td>
<td>(12.5)</td>
<td></td>
<td>Fales et al. 1982</td>
</tr>
<tr>
<td>Trim-sulpha</td>
<td>4/7</td>
<td>0.5</td>
<td>Cox et al. 1981</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td></td>
<td>Allan et al. 1985</td>
</tr>
<tr>
<td></td>
<td>(12.5)</td>
<td></td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td>Sulphonamide</td>
<td>(12.5)</td>
<td></td>
<td>Fales et al. 1982</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>119/140 (85)</td>
<td></td>
<td>Karlsson and Nystrom 1962</td>
</tr>
<tr>
<td></td>
<td>(52)</td>
<td>≤5</td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td></td>
<td>Fales et al. 1982</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>142/145 (97)</td>
<td></td>
<td>Cox et al. 1981</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>1.0</td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td></td>
<td>Fales et al. 1982</td>
</tr>
<tr>
<td>Nitrofurantoin</td>
<td>138/144 (96)</td>
<td></td>
<td>Cox et al. 1981</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>≤64</td>
<td>Karlsson and Nystrom 1962</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>≤10</td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td>Neomycin</td>
<td>90/140 (64)</td>
<td></td>
<td>Fales et al. 1982</td>
</tr>
<tr>
<td></td>
<td>(78)</td>
<td>≤16</td>
<td>Karlsson and Nystrom 1962</td>
</tr>
<tr>
<td></td>
<td>(93.75)</td>
<td></td>
<td>Bandopadhyay et al. 1991</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>32/142 (22.5)</td>
<td></td>
<td>Fales et al. 1982</td>
</tr>
</tbody>
</table>
Only 11/17 antimicrobial agents used in this study are recommended and suitable for parenteral administration in cattle and buffalo. Of the drugs recommended in cattle and buffalo, *P. multocida* is highly sensitive to penicillin, ampicillin, cephalothin, enrofloxacin and oxytetracycline. *P. multocida* also exhibited a high sensitivity to chloramphenicol and neomycin, although these compounds are not recommended for parenteral use in the two species. Our observations are generally in agreement with previous reports by different investigators (Table 2).

Resistance of some isolates of *P. multocida* to oxacillin has been reported by A. Franklin of the National Veterinary Institute, Uppsala, Sweden (pers. comm.). Similar studies carried out by Karlsson and Nystrom (1962) revealed a variable sensitivity of *P. multocida* to erythromycin. Streptomycin resistance of the Thai strain observed in this study is in agreement with a previous report (De Alwis 1984). Streptomycin resistance (>32 µg/mL) of the marker strain of Sri Lanka (33S) is highly specific. This specific resistance to streptomycin is evident by the high sensitivity of the same isolate to neomycin (MIC = <1.0 µg/mL) and gentamicin (<1.0 µg/mL), the other two aminoglycoside antibiotics included in the study.

A remarkable observation in this study is the resistance shown by a high proportion of isolates to sulphamethoxazole (15/27, >128 µg/mL). A similar study carried out in the USA reported 88% (84/145) of *P. multocida* isolates from cattle resistant to triple sulphonamide (a combination of sulphadiazine, sulphamerazine and sulphamethazine). The observed resistance of field isolates to sulphonamides could possibly be attributable to the accepted practice of intravenous administration of sulphadimidine in the treatment of animals clinically affected with HS (Bain et al. 1982).

The results of this study confirm the high sensitivity of *P. multocida* to enrofloxacin, one of the new drugs recommended for parenteral administration to cattle and buffalo. However, based on the resistance pattern, the existing field practice of using sulphonamide in the treatment of clinically affected animals should be discouraged. As a prerequisite to any authoritative statement on treatment strategy for HS, the effectiveness of the currently studied antimicrobial agents needs to be evaluated by controlled clinical trials.

**Acknowledgments**

This study was financed by a research grant (SAREC/09/BF-31) from the Swedish Agency for Research Cooperation with Developing Countries. The collaboration of Dr M.C.L. De Alwis is highly appreciated.

**References**


SESSION 4: PASTEURELLA:
LABORATORY TECHNIQUES FOR
TYPING AND DIAGNOSIS OF
INFECTION
**Pasteurella: Laboratory Techniques for Typing and Diagnosis of Infection**

R.B. Rimler

**Abstract**

Various immunologic and non-immunologic tests for systematic characterisation and diagnosis of *Pasteurella multocida* and *Pasteurella haemolytica* are described. Some of the immunologic tests, e.g. indirect haemagglutination and passive serum transfer, can be adapted to determine immune responses in vaccinated and non-vaccinated animals. Newer tests, such as DNA fingerprinting and ribotyping, are becoming important tools for determining epidemiology of infections caused by these bacterial species.

*Pasteurella multocida* and *P. haemolytica* are bacterial pathogens that produce septicemic or respiratory diseases in domestic and wild animals. Both species can be primary or secondary pathogens and can exist as commensals in clinically normal animals. Particular groups or types tend to be associated with specific diseases and virulence for certain animals. Various tests used to differentiate these particular groups or types can sometimes be used to measure immune responses in vaccinated and non-vaccinated animals, as well as to diagnose specific diseases and determine their epidemiology.

**Fermentation Tests and Biotyping**

Early studies attempted to categorise different varieties of *P. multocida* and *P. haemolytica* into biotypes, based on their reactions in carbohydrate fermentation tests, and to relate these biotypes to specific diseases. For *P. multocida*, biotyping by these tests has failed on a practical basis. Definite associations between a pattern of fermentation reactions and the ability of the bacteria to be specific or virulent for any particular animal or disease have not been shown. With *P. haemolytica*, biotyping has been more successful. Two biotypes are recognised, based on different varieties being able to ferment arabinose or trehalose (Smith 1961). There is a correlation between *P. haemolytica* biotype, serotype, and certain diseases (Biberstein 1978).

The main use of carbohydrate fermentation reactions is for confirmed identification of an isolated organism. Table 1 shows laboratory tests for differentiating between cultures of *P. multocida* and *P. haemolytica*.

**Table 1. Biochemical tests for the differentiation of *P. multocida* and *P. haemolytica***

<table>
<thead>
<tr>
<th>Test</th>
<th><em>P. multocida</em></th>
<th><em>P. haemolytica</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Fermentation (glucose)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MacConkey agar growth</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Haemolysis</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Indole</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Urease</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Mannitol</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>Arabinose</td>
<td>–/+</td>
<td>+/–</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

**Capsulation, Colonial Morphology and Virulence**

Both capsulated and noncapsulated *P. multocida* and *P. haemolytica* can be isolated from animals. Capsulation or noncapsulation can be recognised when young colonies on transparent agar media are viewed...
in oblique transmitted light. Colonies appear blue or iridescent. The cells that make up blue colonies are noncapsulated, do not contain a certain specific antigen on their cell surface, and show reduced virulence or are avirulent. The iridescent colonies are made up of capsulated cells, and when first isolated they are usually virulent. There is a relationship between certain kinds of specific capsule antigens and diseases of specific animals. For example, the serogroup B or E capsule antigens of *P. multocida* are associated mainly with strains that cause haemorrhagic septicaemia (HS) in cattle or buffalo.

### Immunologic Tests

The main antigens of *P. multocida* and *P. haemolytica* involved in laboratory diagnosis, serotyping, and protection against disease seem to be carbohydrate-containing capsule and somatic antigens and protein toxins. Antibody protection against these bacteria and their toxins can be shown by passive immunisation and neutralisation tests in mice and other animals.

### Capsule typing of *P. multocida*

*P. multocida* strains can be grouped on antigenic differences of their capsules. Recognition of these groups is by passive immune protection of mice (Roberts 1947) or by indirect haemagglutination (IHA) tests (Carter 1955). These typing systems sometimes show a direct correlation between capsule group, specific disease, and immune protection in different animals.

The Roberts system recognises five immunological groups of *P. multocida*, which are designated I–V. Specific antibodies for testing are usually made in rabbits. The Roberts system has not been extended in recent times to recognise newer immunological groups, and is now used mainly for recognition of HS strains within localised areas.

Adaptations of Roberts' methods with known reference strains have been used to detect naturally occurring antibodies in animals (Sawada et al. 1985) and immune protection relationships between unknown strains. A particular problem is that unknown strains must be virulent for mice. Weakly virulent or avirulent strains are sometimes encountered.

The IHA test has become the standard for capsule typing. There are now five capsule groups in the Carter system. They are designated A, B, D, E and F (Rimler and Rhoades 1988). The IHA test can be adapted to measure antibodies in vaccinated and unvaccinated animals.

There is some relationship between antigens recognised by the Carter and Roberts systems; e.g., Roberts' group I is equivalent to Carter's group B. A common obstacle to both typing systems is the difficulty in preparing antibodies to all the specific capsule antigens. Antibody preparation against B and E capsule antigens is usually simple, as rabbits usually respond well to inoculation. Preparation of antibodies against D and F capsule antigens is especially difficult, for unknown reasons.

### Capsule typing of *P. haemolytica*

This is done by IHA tests as described by Biberstein (1978). Modifications such as microtitre technique and use of glutaraldehyde-fixed erythrocytes have been described (Fraser et al. 1983). Sixteen capsule serotypes designated 1–16 are recognised, and a correlation exists between biotype and serotype. Serotypes 3, 4, 10 and 15 are T biotypes, whereas all others belong to the A biotype. Some strains of *P. haemolytica* are untypeable by IHA tests. Seemingly, these untypeable strains lack specific capsule antigens that can affix to erythrocytes and they usually belong to biotype A (Biberstein 1978).

### Somatic antigen typing of *P. multocida*

Somatic antigen typing has been done as described by Namioka and Murata (1964) and Hedlestone et al. (1972). The Hedlestone system is now used almost exclusively and will be dealt with here. Immune protection for some animals correlates with somatic antigens recognised by this system. Typing is done by a gel diffusion precipitin test (GDPT), and both capsulated and noncapsulated strains can be typed. The specific somatic antigens are extracted from agar-grown cells with formalised saline and heating at 100°C for 1 hour. Antiserums for typing are made in chickens, and recent findings indicate that antibodies in eggs from vaccinated hens can also be used (Rimler, unpublished). Sixteen somatic serotypes of *P. multocida*, numbered 1–16, are recognised (Rimler and Rhoades 1988). However, recent studies suggest only minor differences occur between serotypes 2 and 5 (Rimler 1990). These two serotypes may eventually be combined as a single type.

The heat-stable type antigens that react in the GDPT are heterogeneous complexes. The heterogeneity is due in part to lipopolysaccharide (LPS) and part to LPS complexed with protein. Antigenic
specificity of a somatic serotype is due to the LPS portion of the complexes (RimIer and Rhoades 1988).

**Rapid serologic tests for diagnosis of *P. multocida***

Most immunologic tests proposed for diagnosing *P. multocida* recognise either specific capsule antigen or somatic antigen, not both. Therefore, they should be considered presumptive tests.

Two tests for diagnosis of group B and E HS strains of *P. multocida* are co-agglutination (RimIer 1978) and counterimmuno-electrophoresis (Carter and Chengappa 1981). The former is a rapid slide test that uses heat-killed *Staphylococcus aureus* as a carrier particle for anti-capsule IgG. Both soluble and cell-bound capsule antigens are detected in cultures, tissue fluids and extracts by this test. In principle, a latex agglutination test may have a similar application.

Counterimmuno-electrophoresis can detect group D, B and E capsule antigens. The test can be done in 30 min – 1 hour, and capsule antigen of B and E strains can be detected in tissue fluids.

An enzyme-linked immunosorbent assay (ELISA) for recognition of HS strains has been described (Dawkins et al. 1990). Antibodies for the test are made using heat-stable antigens prepared like those for the Heddleston somatic typing test. The test is reported to be highly sensitive and specific. Presumably, the specificity is due to antibodies recognising LPS or LPS-protein complexes.

A variation of the ELISA test is used routinely in the United States to detect and quantify antibodies in turkeys vaccinated against *P. multocida*. Some workers have shown a correlation between antibody titre and protection against fowl cholera, whereas others have not.

Magyar and Rimler (1991) developed a colony-lift membrane assay for detecting protein toxin that occurs in some group A and D strains of *P. multocida*. Recent work suggests that this test may be adapted to identify HS strains. The test can be done directly with colonies lifted from agar plates after primary isolation from a clinical specimen. Colonies near the source of hyaluronidase are reduced in diameter and appear blue in oblique transmitted light.

Flocculation of a broth culture in the presence of acriflavine dye has been used to recognise serogroup D *P. multocida*. Use of the acriflavine test as a screening test is helpful. However, flocculation also occurs with some group F strains (Rimler and Rhoades 1988).

The serotype B:2 HS strains of *P. multocida* are unique in that they can be differentiated from other A, B, D, E and F serotypes based on an ability to produce an enzyme with hyaluronidase and chondroitinase activity (Carter and Chengappa 1980; Rimler, unpublished). Chondroitinase and hyaluronidase production can be tested as described by Smith and Willett (1968).

**Non-Immunologic Typing Tests**

The A and D serogroups of *P. multocida* occur in many different animals and are isolated most often around the world. Serogroup A strains are unique, because hyaluronic acid of the capsule makes agar colonies larger and very mucoid. These colonies can be identified after growth in the presence of a paper disk containing hyaluronidase, or in the presence of a hyaluronidase-producing strain of *S. aureus*. Colonies near the source of hyaluronidase are reduced in diameter and appear blue in oblique transmitted light.

Molecular Epidemiology Tests

Recent advances in molecular biology have produced techniques that have significantly increased understanding of the epidemiology of pasteurella diseases. These techniques, DNA fingerprinting and ribotyping, are not based on expression of phenotypic characteristics by the bacteria, as are other tests. Rather, they are based on the unique properties of the bacterial chromosomal DNA.

DNA fingerprinting was used with avian and swine isolates for epidemiologic purposes and to differentiate field isolates and live attenuated vaccine strains of the same serotype (Harrel et al. 1990; Snipes et al. 1990a). Wilson et al. (1992) used the technique to differentiate HS strains of *P. multocida*. Thirteen unique profiles were found among 54 HS strains of
the B:2 serotype using HhaI endonuclease; and five fingerprint profiles were found among 13 strains of the E:2 serotype with HpaII endonuclease.

Ribotyping is a technique that uses labelled rRNA probes to highlight specific profiles generated by restriction endonucleases. This results in recognition of a lesser number of bands than are recognised with DNA fingerprinting. Snipes et al. (1990b) used the technique to study the epidemiology of fowl cholera in turkeys and found that up to three different strains of *P. multocida* could be involved in a single fowl cholera outbreak.

**Conclusions**

Many immunologic and non-immunologic tests can be used to differentiate varieties of *P. multocida* and *P. haemolytica*. Because certain varieties of these bacteria tend to be associated with specific diseases, sometimes these tests can be used for disease diagnosis and measuring immune responses in vaccinated animals.

**References**


Characterisation of *Pasteurella multocida* (Haemorrhagic Septicaemia) Isolates from the Philippines


Abstract

Haemorrhagic septicaemia (HS) is one of the most economically important livestock diseases in the Philippines and is reported to have affected 20,337 cattle and 20,896 buffalo during 1989 (Bureau of Animal Industry 1989). However, at the molecular level, relatively little is known of the causative organism, *Pasteurella multocida*, or of differences between HS isolates from various geographic regions. The aim of this study was to characterise isolates from the Philippines and compare them with those from other countries.

Six field strains isolated from clinically affected cattle and buffalo in the Philippines were identified as *P. multocida* Carter serotype B and were positive in the disease-specific HS-Antigen ELISA. The protein profiles of the isolates were analysed using polyacrylamide gel electrophoresis and were found to be similar to those of other Asian strains. Antibodies raised against the Philippine isolates showed evidence of strong cross-reaction with other classical HS strains of the Carter serogroup B. Serum from cattle vaccinated with HEMOBAC (a HS vaccine prepared from the six isolates) reacted with antigen extracts of the Indonesian vaccine strain, Katha (0132), in both ELISA and immunoblotting assays. This serum also protected mice from challenge with the reference strain, Buffalo B (0332, M1404). Together these results provide evidence of biochemical and antigenic homogeneity among the Asian HS isolates.

HAEMORRHAGIC septicaemia (HS) is one of the most economically important livestock diseases in Southeast Asia, affecting both draught and production animals. In the Philippines, the disease is reported to have affected 20,337 cattle, 20,896 buffalo, 845 horses and 492 goats during 1989 (Bureau of Animal Industry 1989).

HS is caused by infection with certain strains of *Pasteurella multocida* (Bain et al. 1982). Although there are occasional reports of differences between HS isolates (Bain et al. 1982), vaccination programs in many countries utilise local isolates with little appreciation of the relative composition, antigenicity or virulence of either the vaccinal or field strains.

Traditionally, *P. multocida* isolates from animals with HS have been typed as Roberts type 1, Heddleston types 2 and 5, Carter types B and E, and Namioka types 6:B and 6:E according to their serological properties (Brogden and Packer 1979; Bain et al. 1982). Recently, an enzyme-linked immunosorbent assay (ELISA) has been developed for the identification of strains causing HS (Dawkins et al. 1990). The assay is highly specific (>99%) and is based on the reactivity of a rabbit antiserum raised against a crude lipopolysaccharide (LPS) extract. Electrophoretic analysis of soluble proteins has also been used to classify various bacterial species (Lema and Brown 1983), including those of *P. multocida* (Lugtenberg et al. 1984; Choi et al. 1989; Knights et al. 1990; Johnson et al. 1991).

The aims of this study were to characterise six HS isolates from the Philippines and compare them with those from other countries. These isolates were originally isolated from clinically affected animals and are now used in a commercial HS vaccine.

Materials and Methods

Bacteria

Six field isolates (described in Table 1) were obtained from clinically sick Philippine cattle and buffalo...
suspected of having HS. Katha (0132) is a well known HS isolate from Myanmar, while Buffalo B (M1404/0332) is a reference strain isolated in North America from a bison with HS. Identification of the isolates was confirmed using the Microbact System 24E (Disposable Products, Adelaide, Australia).

Table 1. Origins of the Philippine *P. multocida* strains and their relevant properties.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>History, location</th>
<th>Serotype</th>
<th>HS ELISA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0432</td>
<td>HS, Central Luzon</td>
<td>B</td>
<td>209 (+)</td>
</tr>
<tr>
<td>0433</td>
<td>HS, Cagayan Valley</td>
<td>B</td>
<td>201 (+)</td>
</tr>
<tr>
<td>0434</td>
<td>HS, Cagayan de Oro</td>
<td>B</td>
<td>199 (+)</td>
</tr>
<tr>
<td>0435</td>
<td>HS, Negros Occidental</td>
<td>B</td>
<td>198 (+)</td>
</tr>
<tr>
<td>0436</td>
<td>HS, Bicol Region</td>
<td>B</td>
<td>206 (+)</td>
</tr>
<tr>
<td>0437</td>
<td>HS, Ilocos Region</td>
<td>B</td>
<td>193 (+)</td>
</tr>
</tbody>
</table>

* The HS-antigen ELISA results are expressed as ELISA units; i.e. optical density at 414 nm x 100.

HEMOBAC vaccine was produced by Riverdale Biological Laboratories Inc. from local isolates (0432-0437). It contains $4 \times 10^9$ formalin-killed *P. multocida* organisms/mL in aluminium hydroxide adjuvant and is registered with the Bureau of Animal Industry in the Philippines.

**Animals**

Cross-bred Brahman cattle (Tryco-Riverdale Farm, San Rafael) were bled prior to (NAIVE) and after receiving five doses of HEMOBAC vaccine during a 392-day period (IMMUNE). The cattle were injected intramuscularly with 5 mL of HEMOBAC at 6 months of age, followed by a second injection 4 weeks later. Booster injections were given at approximately 4-monthly intervals. All serum samples were gamma-irradiated (6 megarads) before importation into Australia for testing.

BALB/c mice, 8–10 weeks of age, were used in the passive protection studies.

![Coomassie blue-stained PAGE profiles of lysates of eight *P. Multocida* HS isolates. From the left, the lanes contain lysates from the following strains: 0132, 0332, 0432, 0433, 0435, 0436, 0437. Low molecular weight standards are listed to the left of the figure (Pharmacia: lactalbumin [14 400]; trypsin inhibitor [20 100]; carbonic anhydrase [30 000]; ovalbumin [43 000]; albumin [67 000] and phosphorylase b [94 000]).](image-url)
HS-antigen ELISA
Details of the assay have been previously published (Dawkins et al. 1990).

Serotyping
The serotype of the isolates was determined with a haemagglutination assay (Sawada et al. 1982) using human erythrocytes (Carter 1955).

Electrophoresis and immunoblotting
The electrophoretic methods for analysing Pasteurella antigens have been described (Johnson et al. 1989; Johnson et al. 1991). Bacterial lysates were prepared according to the method of Lema and Brown (1983). The gels were stained with either Coomassie Blue R250 or silver (Tsai and Frasch 1982) to reveal protein and LPS components respectively.

HS-antibody ELISA
Serum antibody levels were measured using an ELISA (Johnson et al. 1989) in which the wells of microtitre plates were coated with heat-stable antigen from Katha (Heddleston et al. 1972) to which dilutions of test sera were added followed by horseradish peroxidase-labelled anti-cow immunoglobulin (Silenus, Melbourne, Australia) as the conjugate, and 2,2'-Azino-di-[3-ethylbenzthiazolin sulphonate(6)] (ABTS) as the substrate.

Positive serum from a steer vaccinated against Katha was assigned 1024 ELISA units when diluted 1:400 in phosphate buffered saline containing 0.05% Tween 20 (PBST). Serial dilutions of the positive serum were used to construct a standard curve, relating optical density to ELISA units. All serum samples were diluted 1:400 in PBST and the antibody activity was expressed in ELISA units calculated from the standard curve.

Passive mouse protection test
Groups of at least five mice were injected intraperitoneally with 0.2 mL of test serum diluted 1:2 in sterile physiological saline. The mice were challenged with 100 viable Buffalo B organisms,

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Fig. 2. Silver-stained PAGE profiles of lysates of eight P. multocida HS isolates. From the left, the lanes contain lysates from the following strains: 0132, 0332, 0432, 0433, 0434, 0435, 0436, 0347. Low molecular weight standards are listed to the left of the figure (Pharmacia: lactalbumin [14 400]; trypsin inhibitor [20 100]; carbonic anhydrase [30 000]; ovalbumin [43 000]; albumin [67 000] and phosphorylase b [94 000]).
prepared from appropriately diluted log-phase broth cultures, 24 hours later. The challenge dose was estimated from optical density measurements (OD$_{600}$) and confirmed by plate counting procedures. Mice were observed for 7 days and the numbers surviving challenge recorded.

Results

Preliminary experiments, using conventional biochemical procedures, confirmed that the six Philippine field isolates were all *P. multocida*.

The Coomassie blue-stained electrophoretic profiles of the field isolates were similar to those of the two HS reference strains (Fig. 1). These protein patterns were also comparable with those observed for HS isolates from other Asian countries (Johnson et al. 1991). Each of the lysates contained more than 40 polypeptide species (reduced monomer), the majority of which were located in the mid-section of the gel between the 30 kDa and 94 kDa molecular weight markers. On the basis of stain intensity, the main protein bands had apparent molecular masses (M$_r$) of 27, 32, 45 and 47 kDa.

The LPS components of the bacterial lysates were examined using the silver staining procedure described by Tsai and Frasch (1982). All of the silver-stain positive material appeared as a broad smear in the lower part of the gel, with the major component having an apparent M$_r$ of approximately 14 kDa (Fig. 2). Similar patterns have been observed for other *P. multocida* strains (Johnson et al. 1991).

The serological properties of the organisms were examined using the HS-Antigen ELISA (Dawkins et al. 1990) as well as conventional typing methods. All six isolates were of the Carter B serotype and reacted positively in the disease-specific ELISA (Table 1). Antibodies raised against the Philippine isolates cross-reacted with antigens from the Katha strain (Figs 3 and 4). Serum from IMMUNE cattle, vaccinated with HEMOBAC, had elevated levels of antibody to the crude LPS extract (median titre 390 ELISA units), in comparison with those from NAIVE animals (median titre 13 ELISA units; P < 0.001, Wilcoxon Rank Sum Test). The specificity of the antibody response was studied by probing immunoblots of the SDS-PAGE fractionations of the Katha lysate. For brevity, only representative blots are shown in Fig. 4. Sera from the IMMUNE animals showed even labelling of the LPS region and several protein bands while sera from unimmunised NAIVE animals exhibited little, if any, activity. The pattern of reactivity was similar to that observed with cattle vaccinated with Indonesian and Malaysian strains (Johnson et al. 1989).

Finally, IMMUNE sera protected mice from challenge with Buffalo B, while NAIVE sera offered little protection (Fig. 3). However, serum samples from two of the NAIVE animals conferred partial protection (20% and 40%), which may reflect prior environmental exposure of those animals to *Pasteurella* species. In this study, there was a general correlation between the ELISA antibody levels and the passive mouse protection test (Pearson's Correlation r = 0.68, P < 0.01).

Discussion and Conclusion

The results of this study indicate that the six Philippine isolates are similar to other Asian strains that cause HS. The field isolates were serotyped as Carter B and reacted positively in the disease-specific ELISA (Table 1), which was consistent with both their clinical histories and Asian origins. The classical HS strains Katha and Buffalo B reacted similarly in both assays (Dawkins et al. 1990).

Fig. 3. Response of cattle to vaccination with HEMOBAC. Antibody levels to Katha heat-stable antigen were measured in NAIVE and IMMUNE bovine sera using the HS Antibody ELISA (○). The level of protection generated was assessed from the protection afforded to recipient mice by the passive transfer of 0.2 mL of bovine sera, diluted 1:2 in physiological saline (□).
Antibodies raised against the Philippine isolates showed evidence of strong cross-reaction with other classical HS strains of the Carter serogroup B. Serum from seven cattle vaccinated with HEMOBAC (prepared from a mixture of the six isolates) reacted with antigen extracts of the Indonesian vaccine strain, Katha, in both the HS-antibody ELISA (Fig. 3) and immunoblotting assays (Fig. 4). Such serum also protected mice from challenge with the reference strain, Buffalo B.

In this study, we used PAGE to examine the protein and LPS components in the six Philippine strains of *P. multocida*. The electrophoretic profiles of these field isolates (Figs 1 and 2) were similar to the patterns obtained with other HS strains of Asian origin (Johnson et al. 1991). Each isolate has a complex protein profile with more than 40 bands (Fig. 1) as well as an LPS component of low molecular weight (Fig. 2). However, we were unable to identify the distinct LPS bands found in both phenol-chloroform–petroleum ether extracts (Lugtenberg et al. 1984; Rimler 1990) and phenol-water extracts (Rimler 1990). Such discrepancies are hardly surprising, considering differences in the antigen preparations and gel systems employed.

In general, the electrophoretic profiles of HS strains appear to be surprisingly homogeneous, considering the wide geographic distribution of the isolates and the range of animal species from which they were originally obtained (Johnson et al. 1991). The protein profiles of such strains fell into two distinct groups, depending on whether the isolates were of African or Asian (and North American) origin. In contrast, 12 electrophoretic patterns have been reported for bovine strains of *P. multocida* type A (Abdullahi et al. 1990); while three protein patterns have been described for isolates from animals with atrophic rhinitis (Lugtenberg et al. 1984) and fowl cholera (Ireland et al. 1991).

One of the important issues arising from these and other recent studies concerns the apparent correlation between the electrophoretic pattern of the *Pasteurella* isolates and their serotypic properties. We have consistently noted differences between the capsular Carter B strains and the A, D and E serotypes (Johnson et al. 1991). The group B isolates contained

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Fig. 4. Comparison of the antibody specificities in NAIVE and IMMUNE bovine sera. Relevant controls include low molecular weight markers (Pharmacia) and Katha lysate fractionations on acrylamide gels stained with Coomassie blue and nitrocellulose strips stained with Indian ink (I) and amido black (A). The conjugate control is marked (B).
a major band at 32 kDa while the other serotypes had a major band in the 35–40 kDa region. These results are in agreement with those of Choi et al. (1989), who found that the B:2 reference strain (M1404/0332) expressed outer membrane proteins (OMP) in the 32 kDa and 36 kDa regions; while all but one of the remaining Heddleston strains expressed 34.5 kDa and 38 kDa OMP. Knights et al. (1990) also reported that the B serotype of Pasteurella multocida could be distinguished from the other serotypes by the location of a major protein band. Although the protein band in question had an apparent molecular weight of 38.5 kDa, such discrepancy may have reflected differences in the envelope extraction and solubilisation procedures employed.

In conclusion, these results provide further evidence of biochemical and antigenic homogeneity among the Asian HS strains. The results also illustrate the value of electrophoretic techniques to classify Pasteurella multocida isolates. Continuation of this work may provide further insights into the moieties responsible for the virulence and antigenic properties of the organisms causing haemorrhagic septicaemia.

Acknowledgments

The authors would like to thank Maureen Barton for confirming the identity of the isolates. The authors would also like to acknowledge the excellent technical assistance provided by Cresilda Tardio, Maria Zano, Joanne Sleep and Marilyn Houge. Carter typing sera was kindly provided by Dr M.C.L. De Alwis (Veterinary Research Institute, Peradeniya), while human O erythrocytes were supplied by the Red Cross Blood Bank (Melbourne). The work was supported by the Australian Centre for International Agricultural Research (project no. 8907).

References

Isolation and Characterisation and of *Pasteurella multocida* from Tonsils of Apparently Healthy Cattle

T.G. Wijewardana,¹ N.U. Horadagoda,² A.A. Vipulasiri¹ and S.A. Thalagoda¹

**Abstract**

A previous study on animals experimentally infected with haemorrhagic septicaemia (HS)-causing *Pasteurella multocida* showed that the tonsil was the most consistent site of persistence of the organism. The present study was aimed at investigating the role of tonsils under natural conditions. One-hundred-and-three pairs of tonsils were collected from cattle from a HS-endemic area. Forty-nine (24%) isolations of *P. multocida* were made — 10 of capsular type B and 23 of capsular type A. The capsular type of the other 16 isolates could not be determined — eight belonged to somatic type 3, three to type 6, and one each to types 4 and 12. The remaining isolate was untypeable by Heddeleston’s method. Analysis of protein profiles of whole cells of the latter 16 isolates by sodium dodecyl sulphate polyacrylamide-gel electrophoresis revealed that their electrophoretic pattern differed from that of the HS-type isolates. One isolate of somatic type 3 was used to infect two calves, one of which died showing signs typical of HS. The protein profile of the isolate did not alter following passage in the calf. The study confirmed that, under natural conditions, tonsils are an important site of persistence of HS-causing pasteurellae.

CARRIER animals occupy a vital position in the epidemiological cycle of haemorrhagic septicaemia, caused by serotypes B:2 and E:2 of *Pasteurella multocida*. Studies have shown that the carrier status is related to recent exposure to an outbreak (Hiramune and De Alwis 1982) and is transient in nature (De Alwis et al. 1986). The longest reported period of detection of pasteurellae in the nasopharynx is 215 days (De Alwis et al. 1990). There is a relationship between naturally acquired immunity and recent exposure to HS, and the percentage of animals developing antibodies is higher that the percentage of animals becoming carriers (De Alwis 1982; De Alwis and Sumanadasa 1982; De Alwis et al. 1990). Other than the nasopharynx, tonsils are the most common site of recovery of the HS-causing organism (De Alwis et al. 1990). The organism was found to lodge in the crypts of the tonsils (Horadagoda and Belak 1991), out of reach of antibiotics.

The observations on tonsils cited above were carried out in experimentally infected animals. The present study was undertaken to examine the role of tonsils as a site harbouring the HS-causing organism under natural conditions.

**Materials and Methods**

**Isolation of the organism**

One-hundred-and-three pairs of tonsils were collected from cattle of unknown history at an abattoir in an HS-endemic area. They were cleared of surrounding tissues, weighed individually, surface-sterilised and cut longitudinally to include a maximum number of crypts per portion. This part of each tonsil was then finely crushed and transferred to a bottle containing 3 mL sterile casein-sucrose-yeast (CSY) broth (Wijewardana et al. 1986) and mixed thoroughly. After direct culturing on CSY agar, 0.5 mL volumes of the suspension were inoculated subcutaneously into 6-week-old mice. The heart blood of mice collected at necropsy was plated on CSY agar and incubated at 37°C for 24-48 hours. Suspect colonies appearing on the plate were tested by the rapid slide agglutination test using *P. multocida* serotype B:2 hyperimmune rabbit antiserum. All isolates, irrespective whether they were positive, were subjected to conventional biochemical tests in order

¹ Veterinary Research Institute, Gannoruwa, Peradeniya, Sri Lanka
² Faculty of Veterinary Medicine, University of Peradeniya, Peradeniya, Sri Lanka
to confirm the identification as described by Wijewardana et al. (1986). These cultures were stored either freeze dried or in semi-solid agar for further studies.

**Hyaluronidase decapsulation test**

Hyaluronidase decapsulation of the isolates was detected using *Staphylococcus aureus* (Carter and Rundell 1975).

**Acriflavine flocculation test**

Isolates negative in both the rapid slide agglutination test with *P. multocida* B:2 antiserum and the hyaluronidase decapsulation test were subjected to the acriflavine flocculation test (Carter and Subronto 1973).

**Indirect haemagglutination test**

The indirect haemagglutination (IHA) test was carried out by the method described by Carter (1955) and modified by Wijewardana et al. (1986) using sheep erythrocytes. Capsular antigen of each isolate was tested against *P. multocida* B:2 hyperimmune rabbit antiserum.

**Somatic typing**

Somatic typing of the isolates was carried out by the original method of Heddleston et al. (1972). Type cultures 1-16 were kindly supplied by Dr R.B. Rimler (National Animal Disease Centre, Ames, Iowa, USA). Antiserum was raised in 12-16-week-old cross-bred local chickens by the method of Brogden and Rebers (1978).

**SDS-PAGE**

Whole cells were prepared by adjusting the harvest from a 24-h confluent growth off a dextrose starch agar plate to twice the concentration of Wellcome opacity tube no. 10. The whole-cell lysates were prepared by boiling the above suspension with an equal volume of the sample buffer (2×) for 3 min. The whole-cell lysates were electrophoresed in a vertical-slab gel apparatus (Hancock and Poxton 1988) incorporating molecular weight standards from 14 to 66 kDa (Sigma Chemicals, USA) by discontinuous SDS-PAGE (Laemmli 1970), using a 4% acrylamide stacking gel and 10% acrylamide resolving gel. They were stained with Coomassie blue using a sequential staining procedure (Hancock and Poxton 1988).

**ELISA**

The isolates were subjected to an antigen detection ELISA (Dawkins et al. 1990).

**Experimental infection of calves**

One of the 16 isolates that did not react in the capsular typing procedure was used to infect two 6-month-old calves. One calf (local cross-bred) received an inoculum of $1.27 \times 10^{10}$ organisms, and the other (Jersey) received an inoculum of $8.4 \times 10^{10}$ organisms, both subcutaneously. The animals were clinically examined and their rectal temperatures recorded twice daily.

**Results**

The tonsils varied in size and weight (4.25–15.3 g) but did not show any abnormality. From the 206 tonsils examined individually, 49 isolations (24%) were made. All these were confirmed as *P. multocida* by biochemical tests.

Ten of the isolates were typed as capsular type B by rapid slide agglutination against B:2 antiserum. The IHA titres of the capsular antigens (60°C, 30 min) of these isolates varied from 1/40 to 1/2560 when tested against B:2 hyperimmune antiserum (Table 1). In the HS ELISA, four isolates gave positive reactions, with an optical density (OD) greater than 1.0, four were negative (OD < 0.05), and two showed a weak reaction (Table 1). Of these 10 isolates, 7 were somatic type 2 and 1 was type 2,5 by Heddleston's method, and two others could not be typed (Table 1). As shown in Figure 1, all 10 B isolates demonstrated 22 and 47 kDa bands in the SDS-PAGE of whole-cell lysates. Six showed a band at 32 kDa and another five showed a band at 27 kDa. The known B:2 strain (Malaysian C82) gave only the common bands at 22 and 47 kDa.

**Table 1. Characterisation of capsular type B *P. multocida* isolates by IHA, ELISA and somatic typing by Heddleston's method.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>IHA reciprocal titre</th>
<th>ELISA* OD at 405 nm</th>
<th>Somatic type</th>
</tr>
</thead>
<tbody>
<tr>
<td>37a</td>
<td>2560</td>
<td>1.275</td>
<td>:2,5</td>
</tr>
<tr>
<td>37b</td>
<td>80</td>
<td>0.006</td>
<td>:2</td>
</tr>
<tr>
<td>38a</td>
<td>40</td>
<td>0.004</td>
<td>—</td>
</tr>
<tr>
<td>38b</td>
<td>2560</td>
<td>1.761</td>
<td>:2</td>
</tr>
<tr>
<td>39a</td>
<td>2560</td>
<td>1.102</td>
<td>:2</td>
</tr>
<tr>
<td>41b</td>
<td>40</td>
<td>0.748</td>
<td>:2</td>
</tr>
<tr>
<td>45b</td>
<td>40</td>
<td>1.081</td>
<td>:2</td>
</tr>
<tr>
<td>81a</td>
<td>2560</td>
<td>0.239</td>
<td>:2</td>
</tr>
<tr>
<td>88a</td>
<td>80</td>
<td>0.000</td>
<td>—</td>
</tr>
<tr>
<td>92b</td>
<td>160</td>
<td>0.000</td>
<td>:2</td>
</tr>
</tbody>
</table>

* Optical density of positive control was 0.604; negative control 0.001
A further 23 isolates were grouped as capsular type A by the hyaluronidase decapsulation test. Of these, 11 were typed as somatic type 3, 4 as type 1, 2 as type 6, and 1 as both type 1 and type 3, by Heddleston’s method. The somatic type of 5 of the isolates was not revealed by Heddleston’s method.

The remaining 16 isolates were negative in both hyaluronidase decapsulation and acriflavin flocculation tests for capsular typing. They also gave negative reactions against B:2 antiserum in the IHA test, indicating that they did not belong to any of the four capsular types (A, B, D or E). Of these, 8 were typed as somatic type 3, 3 as type 2, 2 as type 6, and 1 each as type 4 and type 12, by Heddleston’s method. The somatic type of the remaining isolate was not revealed by this method. These 16 isolates gave negative reactions in the HS ELISA. As shown in Figure 2, the protein profiles of these 16 isolates revealed that all 16 had an intensely stained protein band in the region 27-30 kDa.

**Experimental infection of calves**

The calf that received an inoculum of $8.4 \times 10^{10}$ organisms (isolate 120a, somatic type 3) died 48 hours post-infection after showing clinical signs and pathology similar to HS. The organism was re-isolated from heart blood. No serotype conversion or change in the protein profile (Figure 3) was observed following passage in the calf. (Details of this experiment will be published elsewhere.)
Fig. 2. Coomassie blue-stained SDS-PAGE profiles of whole-cell lysates of the 16 isolates of which capsular type was not determined (lane 1). Standards with molecular masses indicated in kDa. Protein profiles of isolates 41a, 48a, 48b, 50a, 53a, 67a, 69a, 71a, 72a, 74a, 103b, 107a, 119b and 120a are shown from lane 2 onwards.

Discussion

Previous studies have demonstrated the importance of tonsils in the pathogenesis and the epidemiology of HS under experimental conditions. This study examined the role of tonsils as a reservoir of HS-causing pasteurellae under natural conditions. The selected animals were from an HS-endemic area in Sri Lanka. Knowledge of their pre-exposure to HS and their vaccination histories were not available. The recovery rate of *P. multocida* in the present study (24%) was lower than that obtained from experimentally infected animals (66%). Of the 49 isolations made, only 10 were grouped as capsular type H, and only six of these were considered to be HS-causing pasteurellae. The recovery rate of HS-causing organisms from the tonsils of apparently healthy animals was therefore 12% under natural conditions.

The two H-type isolates that failed to reveal the somatic type by the Heddleston method were also negative in the HS-specific ELISA, despite showing IHA titres of 1/80 and 1/40 (isolates 38a and 88a respectively). Another isolate (37b), which belonged to B:2 (Table 1), considered HS-causing, was also negative in the HS-specific ELISA. There are reports of strains of the same serotype as those causing HS being negative in the antigen-specific ELISA (Dawkins et al. 1991) — the ELISA being very specific and sensitive, detects only those isolates that express the HS-associated epitope. Dawkins et al. (1991) showed, through immunoelectron microscopy, that ELISA-negative cultures are a mixture of phenotypes with less than 10%, and usually less than 2%, of the population expressing HS-causing epitopes. In the present study, since isolations were made after mouse inoculation, the loss of virulence factors could be discounted. The isolates were from the tonsils of healthy cattle and it could be hypothesised that, in order to exist in the crypts of the tonsils without being destroyed by the host's immune mechanism, the surface antigens would undergo some changes. Detailed studies on these isolates are warranted in order to arrive at a conclusion.

A majority of the isolates (47%) were of capsular type A. The study confirmed that this is a heterogeneous group having somatic types from 1–16. A large number of the type A isolates were of somatic type 3. *P. multocida* type A produces pneumonia in cattle (Carter 1967), which is considered a serious problem in countries of the western.
hemisphere. In Sri Lanka however, pneumonia with terminal septicaemia is not recognised as a problem. The question arises whether the latter condition is masked in the presence of acute and highly fatal diseases such as HS.

An interesting finding was the recovery of a group of isolates that were not identifiable with any of the capsular types A, B, D and E. As in type A isolates, the majority of these belonged to somatic type 3. Their electrophoretic profile was different from that of the typical HS-causing isolates from clinical cases of HS (Wijewardana et al., unpublished). These isolates displayed a major protein band within the 27-30 kDa range. It has been reported that Carter's B strains demonstrate a major band at 32 kDa (Johnson et al. 1991). Only six isolates of the B-type category displayed the 32 kDa protein (Fig. 1).

One of the calves infected with an isolate belonging to somatic type 3, the capsular type of which remained undetermined through hyaluronidase decapsulation and acriflavine flocculation tests, died exhibiting clinical signs and pathology similar to HS. It was assumed that the reason for the failure to identify the capsular type was that the isolate had undergone changes in its surface antigens while lodged in the tonsils. Therefore, it was further assumed that it would revert back to a HS-causing type following passage in the calf. No serotype conversion occurred, however, despite the production of a septicaemic disease similar to HS. There have been occasions where B:3,4 in fallow deer, A:3,4 in fallow deer, and F:3,4 in a snow leopard produced similar septicaemic diseases (Rimler et al. 1987; Carrigan et al. 1991; Chaudhuri et al. 1992). It is evident that, irrespective of the capsular antigen, a septi-

![Coomassie blue-stained SDS-PAGE profiles of whole-cell lysates of a P. multocida isolate used to infect calves (lane 1). Standards with molecular masses indicated in kDa. Protein profiles of 7908 (E:2), C82 (B:2 Malaysian), 120 (3 before passage through calf), 120 (3 after passage through calf), 1672 (type D) and W674 (type A) are shown from lane 2 onwards.](image-url)
caemic disease similar to HS could be caused by strains of the same somatic type. These aspects require further study.

In conclusion, this study confirms that, even under natural conditions, tonsils are an important site harbouring HS-causing Pasteurellae. It also highlights the importance of the capsular type A organism, which is one of the causative agents of pneumonia in cattle. This latter finding has led to further detailed studies that may in turn lead to a better understanding of the epidemiology of bovine pasteurellosis.

Acknowledgments
The authors appreciate the ELISA kit made available through IAEA (FAO). They are also grateful for advice from Dr M.C.L. De Alwis, Veterinary Research Institute, Sri Lanka.

References
Relationship Between Active Protection in Buffalo Vaccinated Against Haemorrhagic Septicaemia and Passive Mouse Protection and Serological Tests

S. Chandrasekaran, L. Kennett, P.C. Yeap, N. Muniandy, B. Rani and T.K.S. Mukkur

Abstract

The relationship was investigated between active protection in buffalo immunised with different types of haemorrhagic septicaemia bacterins and antibody measured by the standard passive mouse protection test (PMPT), the indirect haemagglutination (IHA) test, and enzyme-linked immunosorbent assay (ELISA). Groups of two to three buffalo were immunised with the bacterins currently in use in Asia — broth bacterin (BB), alum-precipitated vaccine (APV) and oil adjuvant vaccine (OAV) — either subcutaneously (BB, APV) or intramuscularly (OAV). They were then challenged subcutaneously with virulent organisms at different times post-immunisation. Although PMPT and indirect haemagglutination tests on the pre-challenge sera from vaccinated buffalo were not related to active protection, there was a relationship between ELISA antibody titres and protection. In contrast, a dose–response relationship was observed between the homologous active and passive mouse protection tests.

Materials and Methods

Preparation of vaccines and vaccination

Three types of vaccines prepared from P. multocida type 6:B strain C82 were used. The broth bacterin (BB) and oil adjuvant vaccine (OAV) were prepared as described previously (Cheah 1960; Thomas 1968). The alum-precipitated vaccine (APV) was prepared according to Iyer and Rao (1959) with a modification — broth culture produced by providing aeration and churning, contained 2.25 x 10^{10} colony forming units (CFU) per mL, was used.

Groups of two or three buffalo, presumed to be free of HS-specific antibodies, as judged by the absence of IHA titres Carter (1955) and negative PMPT (Bain et al. 1982), were immunised with 5 mL of one of the above vaccines containing approximately 2 mg dry bacterial weight per dose (equivalent to 2 x 10^{10} CFU). BB and APV were administered subcutaneously. OAV was injected by the intramuscular route.

Production of immune mouse serum

Thirteen mice were immunised with two doses (10^{6} CFU, 5 x 10^{8} CFU) of formalin-killed P. multocida type 6:B strain C82 administered 7 days apart by the intraperitoneal route. The mice were bled for serum collection 2 weeks after vaccination with the last dose. For evaluation of the dose–response relationship, serum from five immune mice was pooled and PMPT carried out with varying volumes (0.5–0.01 mL) of the pooled immune sera.
Challenge of buffalo

Immunised or non-immunised buffalo were challenged subcutaneously with \( 3 \times 10^6 \) CFU of \( P. \textit{multocida} \) strain C82. The challenged animals were observed for 14 days post-challenge, mortalities recorded, and the experiment terminated. Animals found moribund were euthanased and counted as mortalities.

Passive mouse protection test

The PMPT was carried out as described by Bain et al. (1982). Briefly, groups of four BALB/c mice per test sample were injected subcutaneously with 0.5 mL of neat or diluted buffalo serum and challenged 24 hours later with 100-200 CFU of homologous \( P. \textit{multocida} \) type 6:B. An equal number of untreated mice were used as controls. The challenged mice were observed for a period of 7 days, mortalities recorded, and the experiment terminated. Any mice found moribund were euthanased and counted as mortalities.

For comparative studies, PMPT was also carried out with pooled sera from a group of immune or nonimmune mice.

Determination of antibody levels

Antibody titres of pre-challenge bovine or mouse sera were determined using (a) ELISA (Engvall and Perlmann 1971), with formalin-killed \( P. \textit{multocida} \) type 6:B to coat the ELISA plates (M 129B); and (b) IHA (Carter 1955). ELISA titres were calculated by multiplying the optical density (adjusted for plate-to-plate variation) with the reciprocal of an appropriate serum dilution from the negative linear slope. IHA titres represent a reciprocal of the highest dilution yielding complete agglutination of antigen-coated red blood cells.

Statistical analysis

Student's \( t \) test was used to determine the significance of differences between the log\( _{10} \) mean values for ELISA titres.

Results

It was evident that there was no relationship between protection observed in immunised buffalo following challenge with virulent \( P. \textit{multocida} \) type 6:B and the PMPT (Table 1). For example, two buffalo vaccinated with BB and challenged at 6 weeks post-immunisation were protected, yet mice immunised

| Table 1. Relationship between active protection in buffalo and the PMPT, the IHA test and ELISA titres. |
|-------------------------------------------------|-------|-------|-------|-------|-------|-------|
| Type of vaccine | Challenge interval | Consequence of challenge in buffalo | ELISA titre | IHA titre | Consequence in the PMPT | PMPT results |
| BB               | 6W               | Protected          | 184*        | —        | Unprotected             | 0/4**       |
| BB               | 6W               | Protected          | 100         | —        | Unprotected             | 0/4         |
| BB               | 3M               | Unprotected        | 28          | —        | Protected              | 1/4         |
| BB               | 3M               | Unprotected        | 25          | —        | Unprotected             | 0/4         |
| APV              | 6W               | Protected          | 221         | —        | Unprotected             | 0/4         |
| APV              | 6W               | Protected          | ND          | ND       | Protected              | 2/4         |
| APV              | 3M               | Protected          | 84          | —        | Protected              | 1/4         |
| APV              | 3M               | Protected          | 719         | 20       | Unprotected             | 0/4         |
| APV              | 6M               | Protected          | 764         | 20       | Protected              | 3/4         |
| APV              | 6M               | Protected          | 165         | —        | Unprotected             | 0/4         |
| OAV              | 3M               | Protected          | 514         | —        | Unprotected             | 0/4         |
| OAV              | 3M               | Protected          | 426         | 640      | Protected              | 2/4         |
| OAV              | 6M               | Protected          | 403         | —        | Unprotected             | 0/4         |
| OAV              | 6M               | Protected          | 290         | —        | Unprotected             | 0/4         |
| NONE             | NA               | Unprotected        | 51          | —        | Unprotected             | 0/4         |
| NONE             | NA               | Unprotected        | 32          | —        | Unprotected             | 0/4         |
| NONE             | NA               | Unprotected        | 42          | —        | Unprotected             | 0/4         |
| NONE             | NA               | Unprotected        | 39          | —        | Unprotected             | 0/4         |
| NONE             | NA               | Unprotected        | 28          | —        | Unprotected             | 0/4         |

W, weeks; M, months; ND, not done; BB, broth bacterin; APV, alum-precipitated vaccine; OAV, oil adjuvant vaccine; NA, not applicable

* Represents difference between the pre-vaccination and pre-challenge antibody titres, except in the case of nonimmune buffalo, where it represents pre-challenge titres

** Number of mice surviving/total number challenged
passively with the pre-challenge sera of the above buffalo and experimentally challenged with the same organism were not protected. However, while mice passively immunised with pre-challenge sera of one of the two buffalo at 3 months post-immunisation with BB were unprotected (both succumbed to an experimental challenge infection), those passively immunised with serum from the second buffalo were protected. A similar pattern was seen in buffalo immunised with APV or OAV.

Only 3 of 14 vaccinated buffalo developed serum antibodies measurable by IHA. While there was no relationship between active protection in buffalo and IHA titres, all vaccinated buffalo developed serum antibody titres measurable by ELISA (Table 1). The titres of pre-challenged sera were significantly higher (P < 0.001) in buffalo that survived challenge than in those that succumbed to challenge (Table 2). However, if the antiserum used in the PMPT was from mice previously immunised using a protective immunisation regime, 100% of the passively immunised mice were protected. Further, a dose-response relationship was evident, and the protection observed appeared to depend on a certain threshold antibody level (54 arbitrary ELISA units) (Table 3).

**Discussion**

In addition to confirming the superior protective potential of the adjuvant vaccines (Bain et al. 1982), our data demonstrate the unreliability of the PMPT as an indicator of the protective status of buffalo immunised with different types of HS vaccines. Most immunised buffalo sera had no IHA titres, indicating a poor immune response to *P. multocida* type B capsular antigens. However, there appeared to be a relationship between the ELISA antibody titre and protection in buffalo. It was also clear that a certain threshold of antibody activity was necessary for the protection to occur.

In another investigation, however, we found that sera with high IHA activity obtained from cattle previously vaccinated with APV during an outbreak of HS passively protected mice in the PMPT, whereas those with low IHA activity did not. While it could be important to determine the basis of the observed IHA activity and its relationship to active protection in cattle, the fact that the sera from cattle with low IHA activity were negative to the PMPT clearly suggests that further work in cattle similar to that described for buffalo needs to be carried out for definitive determination of the value of the PMPT in predicting the immune status of cattle to HS.

Although the basis for the observed lack of relationship between the PMPT and active protection in buffalo is unclear at the present time, one possibility is the absence of specific receptors on the mouse phagocytic cells for the predominant buffalo antibody isotype(s). This suggestion is supported by the observation that sera from immune mice not only passively protected non-immune mice, but also exhibited a dose-response relationship.

**Acknowledgments**

The authors would like to thank the Director-General of the Department of Veterinary Services, Malaysia, for permission to present the paper; and the Director of the Veterinary Research Institute, Ipoh, for encouragement and facilities provided during the course of this trial. This project was supported by the Australian Centre for International Agricultural Research, Australia. It constitutes part of a submission by the senior author to the Agricultural University of Malaysia in fulfilment of the requirements for the award of a Master's degree in Veterinary Science.
Messrs S. Magendran and S.W. Liew are acknowledged for their assistance in the preparation of slides.

References


Evaluation of Bovine Antibody Responses to Haemorrhagic Septicaemia Vaccine Using ELISA and PMPT

L. Natalia¹, B. Patten² and A. Syamsudin¹

Abstract

The antibody response of cattle to one or two doses of an oil and lanolin-based haemorrhagic septicaemia bacterin vaccine was measured by ELISA using a lipopolysaccharide extract of formalin-killed Pasteurella multocida, Katha strain. ELISA antibody units were compared with the protective response in the passive mouse protection test (PMPT). There was a significant correlation between the HS antibody ELISA and PMPT results ($r = 0.76; P < 0.01$). The study suggests that ELISA may be preferable to PMPT for the evaluation of HS vaccination programs.

In 1984, haemorrhagic septicaemia (HS) was estimated to have resulted in an economic loss of around US$8.64 million in Indonesia (Winrock International Institute for Agricultural Development 1985). The disease has spread to almost all provinces (Darmadi 1991). Cases commonly occur from the middle to late dry season and extend into the rainy season. At these times cattle and buffalo are in poor condition and are further stressed by being used for draught power.

The effectiveness of vaccination programs that have been implemented in Indonesia to control and eradicate HS can be tested in two ways: the incidence of disease can be monitored after a vaccination program has been completed; and the post-vaccinal antibody titres of vaccinated animals can be monitored.

The passive mouse protection test (PMPT) (Bain et al. 1982), which has been used routinely in Indonesia to determine whether cattle and buffalo have protective antibody titres against HS-causing strains of Pasteurella multocida, has a number of limitations:

- the challenge dose of the organism needs to be standardised; and
- the test requires the use of large numbers of live animals, a practice that requires increasing justification.

An enzyme-linked immunosorbent assay (ELISA) has been used to detect antibodies to certain P. multocida antigens (Johnson et al. 1988). The ELISA has a number of advantages over PMPT in that it is relatively easy to standardise, can be used to test large numbers of samples quickly and easily, and eliminates the use of laboratory animals.

This study measured the antibody response of a group of cattle in the field to one or two doses of a commercial HS oil adjuvant vaccine using ELISA and PMPT, and compared the results.

Materials and Methods

Vaccine

A HS oil adjuvant vaccine, produced by the Centre for Veterinary Biological Products (Pusat Veterinaria Farma) and distributed by the Indonesian Government Livestock Services Directorate of Animal Health for routine use in Indonesia, was used in this study. The vaccine is prepared from a formalin-killed P. multocida Katha strain bacterin, which is emulsified with liquid paraffin and lanolin in the proportions 5:4:1. The vaccine contains at least 2.0 mg dry bacterial mass per dose (PusVetma, pers. comm.) and the recommended dose for cattle and buffalo is 3 mL injected intramuscularly once per year.

¹ Research Institute for Veterinary Science, PO Box 52, Bogor, Indonesia
² Present address: Regional Veterinary Laboratory, PO Box 388, Benalla, Victoria 3672, Australia
Animals and management system

Bali cattle from the Kupang district of the Province of East Nusa Tenggara were divided randomly into two vaccination groups and one control group. Vaccinated cattle were kept in small groups under traditional rearing systems using cut-and-carry feeding on small-holder farms. Cattle in the control group were kept at a research institute and managed using a cut-and-carry feeding system similar to that used on the small-holder farms.

Vaccination schedule

In the vaccination groups, one group of animals received one dose of vaccine only, and the other group received a second dose of vaccine 1 month after the first dose. The control group was not vaccinated during the course of the trial and, as far as could be determined, had not received HS vaccination for at least 1 year prior to the study.

Sample collections

Blood samples were collected from all cattle at the time of the first vaccination (pre-vaccination sample), 1 month after the first vaccination (at which time the two-vaccine group was revaccinated) and 3 months after the first vaccination. The samples were collected by venipuncture of the medial coccygeal vein into sterile, silicon-coated, vacuum-evacuated tubes. The blood was allowed to clot at room temperature and then placed on wet ice until the serum could be removed.

HS antibody ELISA

An ELISA to detect antibody to a lipopolysaccharide (LPS) extract of *P. multocida* (Johnson et al. 1988) was used. The LPS extract was prepared from *P. multocida* strain M1404, a type B:2 strain initially isolated from bison (Stein et al. 1949).

The organism was grown overnight on sheep blood agar and the bacterial cells were harvested into 0.3% formol saline (0.9% w/v NaCl) at a rate of 0.5 mL formol saline per blood agar plate. The cell suspension was heated for 1 h in a constant-boiling water bath. The supernatant was collected by centrifugation at 7000 x g for 15 min at 4 °C and stored at -20 °C until required.

The ELISA used round-bottom, 96-well microtitre plates that were coated with a 1:200 dilution of antigen in phosphate-buffered saline (PBS), pH 7.2, and left overnight at 4 °C. Test serums were diluted 1:200 in PBS containing 0.05% Tween 20 (Sigma Chemicals, Missouri, USA). An anti-bovine IgG (heavy- and light-chain specific) horseradish peroxidase conjugate (Silenus Laboratories, Victoria, Australia) was used at a dilution of 1:3500. ABTS (Sigma Chemical, Missouri, USA) in citrate buffer, pH 4.2, was used as substrate, and the test was read at 415 nm in a Titertek MCC340 plate reader (Flow Laboratories, USA). Each microplate contained a conjugate control, negative serum control, and a two-fold dilution series of a positive serum control.

The test was analysed using a computer program (Platereader Program Version 3.2, ACIAR Project No. 8907, Regional Veterinary Laboratory, Benalla, Victoria, Australia). This was employed to compare the absorbance of the test sample with that of the positive control dilution on the same plate in order to calculate an ELISA unit for each serum. The positive control was arbitrarily assigned a value of 1024 units for the lowest dilution (1:200) and 16 units for the highest dilution (1:6400).

Cattle were classed as either ELISA negative, ELISA suspect, or ELISA positive, depending on whether their ELISA unit value was less than the mean plus 2 standard deviations (SD) of the control sample; greater than the mean plus 2 SD, but less than the mean plus 3 SD of the control; or greater than the mean plus 3 SD of the control respectively.

Bain (1955) reported that no false positive PMPT protective values had been reported, so any protection was a presumptive indication of immunity. Bain et al. (1982) indicated that false positive PMPT reactions had been encountered in Asia, but were rare in Australia, where HS does not occur. Bain et al. (1982) also indicated that the survival of any mice in a group challenged with 100 LD₅₀ doses of virulent *P. multocida* was significant.

The challenge dose for PMPT has varied from 10 LD₅₀ (Bain 1955) to 100 LD₅₀ (Bain et al. 1982) and 10 LD₅₀ (100 CFU) (Dawkins et al. 1991), with little explanation except by Bain (1955), who indicated that the dose used guaranteed that all of the control mice died. Bain et al. (1982) cited the work of Roberts (1947) and Bain (1955): a 5 x 10¹ increase in injected serum required a 10⁴ increase in the challenge dose to produce a 40% protection level (Roberts 1947); whereas a 10² increase in challenge dose caused no change in the PMPT protection level from 9/11 cattle sera tested (Bain 1955).

In this study, a challenge dose of 100 LD₅₀ (approximately 300 CFU, data not presented) was used, and a PMPT protective value of 20% or greater was regarded as indicating immunity to infection. The Kupang district, where the study was undertaken, is a HS-endemic area (Dinas Peternakan 1991). HS cases are commonly reported in the middle of the dry season and up to the early rainy season. Cases begin in July, reach a peak in October, and then diminish to the extent that few are recorded after January (Darmadi 1991). While preventive vaccination for HS in this district is usually undertaken by the Government animal health service about June each year, vaccination may be undertaken at any
time in the face of an outbreak of HS. Due to the endemic nature of the disease and the use of vaccination in the area, it was not surprising that a number of the animals had detectable antibodies to *P. multocida* at the time of the first collection.

The antibody response of cattle following vaccination was in general very good. Only seven animals had no PMPT titre 1 month post-vaccination. Nineteen cattle were ELISA negative (and 19 ELISA suspect) 1 month post-vaccination. This number decreased to 6 animals after 3 months in the one-dose vaccine group, and 4 animals in the two-dose vaccine group. No significant difference was evident in the distribution of animals in the ELISA-negative or ELISA-positive groups after one or two doses of vaccine.

As would be expected, the pre-vaccination level of antibody influenced the level of post-vaccinal antibody. Cattle that were ELISA negative at the first collection (pre-vaccination) showed a proportionally greater increase in their antibody titres after vaccination.

PMPT has been used in Indonesia to determine the level of protective immunity of cattle or buffalo to HS. Problems are frequently experienced in performing the test in a standardised manner. This is due to lack of sufficient supplies of mice of a known genetic composition and difficulties in quantitating the challenge dose. These problems make it difficult to compare and interpret the results from different laboratories.

An alternative testing procedure to determine the protective antibody status of cattle and buffalo, either after vaccination in endemic HS areas or in monitoring areas where HS is considered eradicated, would be of value. The test would preferably be simple to standardise, it would be easy to interpret and therefore assist the comparison of results between laboratories, and it would not use live animal challenge. The results presented here suggest that the ELISA may fulfil some of these objectives and could be used to determine the immune status of large numbers of animals.

**Passive mouse protection test**

The PMPT described by Bain et al. (1982) and modified by Dawkins et al. (1991) was used in the study.

Groups of seven mice per test sample were injected intraperitoneally with 0.2 mL test serum. Five mice in each group were challenged with 100 LD₅₀ (approximately 300 colony-forming units, CFU) of rapidly growing log-phase *P. multocida* Katha strain 24 h later. The remaining two mice were left as the serum control mice. A challenge control group consisting of five mice was injected with the *P. multocida* challenge dose only. All mice were observed for 7 days, and mortalities were recorded on a daily basis.

The test was regarded as valid only if all mice in the challenge control group and none in the serum control group died. The protective response of the test serum was recorded as the percentage of mice surviving the challenge. The survival of 1/5 mice per group (20% protection) was recorded as PMPT positive.

**Results**

The results of the 15 cattle in the control group are given in Table 1. The distribution of ELISA units of samples from the first and second collections and the PMPT mean and SD from collections 1 and 2 are also shown in Table 1.

Two hundred and seventy-five samples were collected from the test group at the time of the first vaccination. The distribution of samples according to ELISA units and PMPT is shown in Tables 2(a) and 2(b).

**Table 1. Distribution of antibody response as measured by ELISA and PMPT.**

<table>
<thead>
<tr>
<th>Group (ELISA units)</th>
<th>Pre-vaccination Number/ELISA unit¹</th>
<th>Number/PMPT² (%)</th>
<th>1 month post-vaccination Number/ELISA unit¹</th>
<th>Number/PMPT² (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–&lt;70</td>
<td>110</td>
<td>110</td>
<td>87</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>37 ± 15</td>
<td>18 ± 23</td>
<td>306 ± 323</td>
<td>63 ± 35</td>
</tr>
<tr>
<td>70–88</td>
<td>14</td>
<td>12</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>76 ± 5</td>
<td>41 ± 31</td>
<td>481 ± 265</td>
<td>78 ± 23</td>
</tr>
<tr>
<td>&gt;88</td>
<td>151</td>
<td>151</td>
<td>130</td>
<td>128</td>
</tr>
<tr>
<td></td>
<td>440 ± 337</td>
<td>75 ± 30</td>
<td>701 ± 276</td>
<td>95 ± 13</td>
</tr>
<tr>
<td>Control</td>
<td>15</td>
<td>15</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>36 ± 20</td>
<td>0</td>
<td>103 ± 171</td>
<td>10 ± 17</td>
</tr>
</tbody>
</table>

¹ ELISA units mean and standard deviation.
² PMPT % protection, mean and standard deviation.
Table 2(a). Frequency distribution of ELISA units for collections 1, 2 and 3.

<table>
<thead>
<tr>
<th>ELISA units</th>
<th>Collection 1 Number (%)</th>
<th>Collection 2 Number (%)</th>
<th>Collection 3 1 vaccine dose Number (%)</th>
<th>2 vaccine dose Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-70</td>
<td>113 (41%)</td>
<td>19 (8%)</td>
<td>6 (6%)</td>
<td>4 (7%)</td>
</tr>
<tr>
<td>71-88</td>
<td>9 (3%)</td>
<td>11 (5%)</td>
<td>2 (2%)</td>
<td>2 (3%)</td>
</tr>
<tr>
<td>&gt;88</td>
<td>153 (56%)</td>
<td>196 (87%)</td>
<td>87 (92%)</td>
<td>55 (90%)</td>
</tr>
<tr>
<td>Total</td>
<td>275</td>
<td>226</td>
<td>95</td>
<td>61</td>
</tr>
</tbody>
</table>

Table 2(b). Frequency distribution of PMPT protective values for collections 1 and 2.

<table>
<thead>
<tr>
<th>PMPT protection value (%)</th>
<th>Collection 1 Number (%)</th>
<th>Collection 2 Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>66 (24%)</td>
<td>7 (3%)</td>
</tr>
<tr>
<td>20</td>
<td>38 (14%)</td>
<td>11 (5%)</td>
</tr>
<tr>
<td>40</td>
<td>37 (13%)</td>
<td>18 (8%)</td>
</tr>
<tr>
<td>60</td>
<td>31 (11%)</td>
<td>26 (12%)</td>
</tr>
<tr>
<td>80</td>
<td>29 (11%)</td>
<td>14 (6%)</td>
</tr>
<tr>
<td>100</td>
<td>74 (27%)</td>
<td>150 (66%)</td>
</tr>
<tr>
<td>Total</td>
<td>275 (100%)</td>
<td>226 (100%)</td>
</tr>
</tbody>
</table>

Table 3. Comparison of PMPT protection level and ELISA units.

<table>
<thead>
<tr>
<th>Number of samples</th>
<th>PMPT % protection</th>
<th>ELISA units¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>74</td>
<td>0</td>
<td>38 ± 28</td>
</tr>
<tr>
<td>48</td>
<td>20</td>
<td>62 ± 28</td>
</tr>
<tr>
<td>55</td>
<td>40</td>
<td>100 ± 48</td>
</tr>
<tr>
<td>57</td>
<td>60</td>
<td>182 ± 128</td>
</tr>
<tr>
<td>42</td>
<td>80</td>
<td>441 ± 269</td>
</tr>
<tr>
<td>223</td>
<td>100</td>
<td>663 ± 298</td>
</tr>
</tbody>
</table>

¹ ELISA units mean and standard deviation.

Discussion

The control cattle used in this study had received, as far as could be determined, no vaccination for HS for at least 1 year; and no animals on the same property, or in close contact, had died or shown clinical signs of HS.

The discrimination between ELISA sero-positive and ELISA sero-negative animals was based on whether the ELISA unit value of the sample was less than the mean of the control group plus two SD, or greater than the mean plus three SD (Tijssen 1985). Using these criteria, the test cattle were divided into three classes — ELISA negative (<70 ELISA units), ELISA suspect (70-88 ELISA units), and ELISA positive (>88 ELISA units). A similar discrimination is found if twice the mean value of the control samples (68 ELISA units) is used as the discrimination point.

Acknowledgments

The authors thank the Director of the Research Institute for Veterinary Science, Bogor, Indonesia, and the Director of the Australian Centre for Inter-
Table 4. Correlation of ELISA units and PMPT (% protection).

<table>
<thead>
<tr>
<th>ELISA group</th>
<th>PMPT</th>
<th>Passive mouse protection test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative (0%)</td>
<td>20</td>
</tr>
<tr>
<td>negative</td>
<td>67</td>
<td>35</td>
</tr>
<tr>
<td>suspect</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>positive</td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td>Total</td>
<td>73</td>
<td>50</td>
</tr>
</tbody>
</table>


Comparison of an Enzyme-Linked Immunosorbent Assay and a Passive Mouse Protection Test for Measuring Protective Antibodies Against Pasteurella multocida Serotype B in Cattle and Buffalo

P. Neramitmansook¹, W. Neramitmansook² and G.R. Carter³

Abstract

In an earlier study, an enzyme-linked immunosorbent assay (ELISA) used to measure antibodies against P. multocida serotype B in vaccinated cattle and buffalo gave results that correlated well with those of direct challenge (Neramitmansook et al. 1990). The present study compared the new ELISA with the passive mouse protection test (PMPT), which for many years has been the preferred test for measuring immunity to haemorrhagic septicaemia (HS) in either vaccinated or naturally immune animals (Bain 1954; Sawada et al. 1985; Neramitmansook et al. 1989).

Sera from 220 domestic cattle and 213 local water buffalo, which had been stored at -70°C since 1988, were thawed and subjected to the ELISA and the PMPT. The PMPT was carried out as described previously (Neramitmansook et al. 1989). Briefly, groups of four or five, 25–30 g, 6-week-old Albino-ICR mice (from the National Experimental Center, Nakornpathom, Thailand) were injected subcutaneously with 0.5 mL of cattle or buffalo serum. The following day each mouse was challenged intraperitoneally with 0.1 mL Trypticase soy broth containing 250–300 colony forming units or 1000 LD₅₀ of P. multocida isolate B-26679. Sera that protected any mice in the test groups up to 6 days after challenge were interpreted as positive or immune.

P. multocida isolate B-26679, which had been identified previously as serotype B (Neramitmansook et al. 1989), was used to prepare the boiled culture antigen in the ELISA, as described by Neramitmansook et al. (1990). Sera that produced an ELISA reading of greater than or equal to 0.25 optical density at a dilution of 1:160 (cattle) or 1:320 (buffalo) were designated as positive or immune (Neramitmansook et al. 1990).

Tests on cattle sera revealed 55% positive (or immune) results to either the ELISA at a titre of 1:160 or to the PMPT. At a titre of 1:320, 48.2% were positive to the ELISA. For either the ELISA at 1:160 or the PMPT, 46.0% were negative (or non-immune); and 51.8% were negative to the ELISA at a titre of 1:320. For buffalo sera, 62.4%, 67.1% and 58.2% were positive to the PMPT, the ELISA at 1:160, and the ELISA at 1:320 respectively; 37.6%, 32.9% and 41.8% were negative to the PMPT, the ELISA at 1:160, and the ELISA at 1:320 respectively. Only 15.5% (34/220) of cattle sera and 20.2% (43/213) of buffalo sera, at the ELISA titres used, did not show correlation in the tests.

The results indicate that the ELISA could be substituted for the more expensive in vivo PMPT currently used for determination of protective antibodies in cattle and buffalo. They further indicate that ELISA should be run with a serum titre of 1:160 for cattle and 1:320 for buffalo. This corresponds with the recommendation in our previous report (Neramitmansook et al. 1990).

¹ Northern Veterinary Research and Diagnostic Center, Hangchat, Lamphang 52190, Thailand
² National Animal Health and Production Institute, Kaset Klang, Bangkhen, Bangkok 10900, Thailand
³ Virginia — Maryland Regional College of Veterinary Medicine, Virginia Tech, Blacksburg, Virginia 24061-0442, USA
References


