THE RESPONSE OF ANIMALS TO *PASTEURELLA MULTOCIDA* VACCINATION AS MEASURED BY PMPT AND ELISA

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ABSTRACT

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The passive mouse protection test (PMPT) is often used during investigations of haemorrhagic septicaemia (HS). Although this test has been shown to give a good indication of the immunity of individual animals, it is none-the-less expensive, time-consuming and difficult to standardize. Several experiments were conducted during which the PMPT was compared to ELISA as an alternative methods for investigating the response of cattle to vaccination with *Pasteurella multocida*. A group of cattle were vaccinated with one or two doses of a commercial HS vaccine and the ELISA antibody response was compared with the protective response observed in the PMPT. The PMPT utilised the virulent Katha strain of *P. multocida* as the challenge strain and the ELISA used a lipopolysaccharide extracted from formalin-killed bacteria of the same strain as the antigen. The use of one or two doses of vaccine as a booster dose made no significant difference in the subsequent antibody response. The correlation (R^2 = 0.740, n = 718) between PMPT and ELISA was significant ($p < 0.01$). A group of cattle and buffalo were also vaccinated with various strains of *P. multocida* isolated from cattle or bison. All strains produced positive PMPT protective responses and the animals were resistant to challenge with a known HS-causing strain of *P. multocida* (strain M1404). The ELISA titres of most of the animals were, however, low at the time of challenge and were not a reliable indicator of resistance to infection. The results indicate that both the PMPT and the HS-antibody ELISA are not specific to HS-causing strains of *P. multocida* but may only indicate previous exposure to *Pasteurella* antigen.

Key words: *Pasteurella multocida,* vaccination, PMPT, ELISA

ABSTRAK

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Uji proteksi pasif pada mencit (PMPT) sudah sering digunakan dalam penyidikan penyakit *Septicaemia Epizootica* (SE). Meskipun uji ini telah mampu memperlihatkan petunjuk yang baik dari kekebalan individu hewan, uji ini ternyata cukup mahal, memerlukan waktu yang lama dan sukar distandardisasi. Beberapa percobaan dilakukan untuk membandingkan PMPT dan ELISA yang merupakan uji alternatif untuk menyelediki respon sapi terhadap vaksinasi dengan *Pasteurella multocida*. Sekelompok sapi telah divaksinasi dengan satu atau dua dosis vaksin SE komersial dan respon antibodinya diukur dengan ELISA dan PMPT. Hasil kedua pengujian kemudian dibandingkan. PMPT dilakukan dengan menggunakan galur Katha ganas dari *P. multocida* sebagai galur tantang. ELISA menggunakan ekstrak lipopolysakarida dari bakteri galur sama yang dibunuh dengan formalin sebagai antigen. Penggunaan satu atau dua dosis vaksin sebagai booster ternyata tidak memberikan respon antibodi yang berbeda nyata. Korelasi (r^2 = 0,740; n = 718) antara PMPT dan ELISA adalah nyata ($p < 0,01$). Sekelompok sapi dan kerbau juga telah divaksinasi dengan berbagai galur *P. multocida* yang diisolasi dari sapi atau bison. Semua galur memberikan respon PMPT protektif dan hewan dapat bertahan terhadap tantangan oleh galur *P. multocida* penyebab S.E. (galur M1404). Titer ELISA dari hampir semua hewan meskipun rendah pada waktu ditantang, bukan merupakan indikator untuk dapat bertahan terhadap infeksi. Hasil ini menunjukkan bahwa PMPT dan ELISA antibodi untuk S.E. tidak spesifik terhadap galur *P. multocida* penyebab S.E., tetapi hanya menunjukkan adanya sejarah pernah terekspos oleh antigen *Pasteurella*.

Kata kunci: *Pasteurella multocida,* vaksinasi, PMPT, ELISA

INTRODUCTION

Haemorrhagic septicaemia (HS) is an acute infectious bacterial disease of cattle and buffaloes caused by certain type B or type E strains of *Pasteurella multocida* (Bain *et al.*, 1982). HS has spread to almost all provinces of Indonesia (Darmadi, 1991) and with the movement of livestock in conjunction with transmigration programs it would not be surprising if the disease is also now in provinces previously reported free of HS. The economic loss in Indonesia due to HS has been estimated at US$8.64 million (Winrock, 1985). Vaccination programs using a Katha strain oil adjuvant, lanolin based vaccine, have been implemented to control, and in some cases try and eradicate, HS in Indonesia. The vaccine appears to offer relatively long protection (>6 months) however it is difficult to inject due to the oil content of the vaccine.

The passive mouse protection test (PMPT) has been used in Indonesia to measure the antibody response of animals to HS vaccine and to determine if the animals have protective antibodies to HS. The test however has a number of disadvantages in that it is expensive to perform, requires the use of a large volume of serum and a large number of mice. An ELISA has been developed to

measure antibodies to the lipopolysaccharide complex of *P. multocida*. The ELISA is easier and quicker to use than the PMPT, requires a lower volume of serum and does not require the use of live animals.

The PMPT and ELISA were compared in groups of vaccinated cattle and buffalo to determine the suitability of the ELISA for routine diagnostic use. The antibody response of animals to some different vaccination schedules for the prevention of HS was also compared.

MATERIALS AND METHODS

Experimental animals

Two experimental groups of animals were used during the course of this study. Group I animals originated from the Kupang district of the Province of East Nusa Tenggara. They consisted of Bali cattle which were kept in small groups under traditional rearing systems. Animals from a nearby research station were used as the control group. Group II consisted of eight Friesian cows which had been reared from six months of age at Balitvet, plus three cattle (S82, S107, S108) and two buffalo which had been obtained from the IPB farm Jonggol. The animals had no history of prior vaccination against HS.

Vaccination

Five different vaccines were used during the course of these studies, one commercial HS vaccine and four other vaccines. Details are as follows:

(i) HS oil adjuvant vaccine produced by Pusat Veterinaria Farma, Surabaya, Indonesia (Pusvetma). The vaccine is prepared from a formalin killed *P. multocida*, Katha strain, bacterin which is emulsified with liquid paraffin and lanolin in the proportion of 5:4:1. The vaccine is reported to contain at least 2.0 mg of dry bacterial mass per dose of vaccine. The recommended dose of vaccine for cattle and buffalo is 3 mL injected intramuscular once per year.

(ii) Double emulsion oil adjuvant vaccines were produced from strains of *P. multocida* which had been isolated from field cases of pasteurellosis. The strains used and their histories were: 989A (cattle; Carter B, Heddleston 11), P2225 (cattle; Carter A, Heddleston type strain 14), P1255 (cattle; Carter A, Heddleston 10; Namioka type strain 7A) and M1404 (Bison; Carter type strain B, Heddleston 2). All except M1404 were negative to the HS-Antigen ELISA (Dawkins *et al.*, 1990). The organisms were grown overnight, as a lawn culture, on 15 cm diameter sheep blood agar plates, and harvested in 0.3% formol saline at the rate of 0.5 mL of saline per agar plate. The suspension was emulsified with an equal volume of Freund's incomplete adjuvant (Sigma Chemical, St Louis, USA) and then with an equal volume of 2% Tween 20 in normal saline. The vaccines were injected subcutaneously at a dose rate of 4-5 mL per animal.

Experimental schedule

Experimental Group I

Animals were randomly divided into three vaccination and one control group. All of the vaccination groups received 3 mL of HS vaccine (Pusvetma) at the time of collection one. One month latter (collection two) one group of animals received a second 3 mL dose of vaccine. The control group were not vaccinated during the course of the trial and, as far as could be determined, had not received any HS vaccination for at least 1 year prior to this study.

Experimental Group II

Two animals were vaccinated with each of the four *P. multocida* vaccines. The animals were vaccinated at week one, week five, week 15 and week 53. The animals were challenged with the HS-causing *P. multocida* M1404 strain at week 88 to week 96.

Sample collections

Samples were collected from all animals in a similar method. Blood samples were collected by venipuncture of the medial coccygeal vein into sterile silicon coated vacuum evacuated tubes (Vacutainers, Becton-Dickinson, USA). The blood was allowed to clot at room temperature and then placed on wet ice or refrigerated at 4°C until the serum was removed from the clot. Serum samples were stored at -20°C until required for testing.

Group I

Blood samples were collected from all animals at the time of the first vaccination (pre-vaccination sample), 1 month after the first vaccination, at which time one animal group was re-vaccinated, and 3 months after first vaccination.

Group II

Blood samples were collected at approximately weekly intervals for 80 weeks.

PMPT

The PMPT as described by Bain *et al.* (1982) and modified by Dawkins *et al.* (1991) was used in this study. Groups of 7 mice per test sample were injected intra-peritoneally with 0.2 mL of test serum. Twenty four hours later each mouse was challenged with 100 LD_{50}, or approx. 300 colony forming units (cfu), of rapidly growing log phase *P. multocida,* strain M1404. A challenge control group consisting of 5 mice were injected with the *P. multocida* challenge dose only. All mice were observed for a period of 7 days and mortalities recorded on a daily basis. The test was regarded as valid and results recorded only if all mice in the challenge control group and none of the serum control mice died. The protective response of the test serum was recorded as the percentage of mice surviving the challenge.

HS-antibody ELISA

An ELISA to detect antibody to a lipopolysaccharide (LPS) extract of *P. multocida* as described by Johnson *et al.* (1988) was used in this study. The LPS 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 hour in a constant boiling water bath. The supernatant was collected by centrifugation at 7000 *g* for 15 minutes at 4°C. The supernatant was stored at -20°C until required.

The ELISA test was performed in round bottom 96 well microtitre plates which were coated with a 1:200 dilution of antigen in phosphate buffered saline 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 Ig (heavy and light chain specific) horseradish peroxidase conjugate (Silenus Laboratories, Victoria, Australia) was diluted to working strength in PBS-Tween with 0.2% casein (Sigma Chemical, St Louis, USA). The same conjugate was used for buffalo serum but at a 1-2 two-fold dilution lower than the dilution for cattle serum. ABTS (1 mM;Sigma Chemical Co, St.Louis, USA) and H_2O_2, in the form of urea-peroxide (2.5 mM; Fluka Chemicals, Switzerland), in 0.1 M citrate buffer pH 4.2 was used as the substrate and the optical densities (OD) were 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 ELISA OD readings from Group II animals were further analysed using a computer program (Platereader program V3.2, Regional Veterinary Laboratory, Benalla, Victoria, Australia). This program compared the OD of the test sample to that of the positive control serum dilutions on the same plate to produce an ELISA Unit (EU) value for each serum. The positive control was arbitrarily assigned a value of 1024 EU for the lowest dilution (1:200) and 16 EU for the highest dilution (1:6400).

RESULTS

Group I

The control group, which consisted of a total of 15 animals, had a mean ELISA Unit value of 34 ±18 and the PMPT protection level was zero at the pre-bleed sampling (results not shown). The animals had, as far as could be determined, no vaccination for HS for at least one year, and more likely for much longer.

A total of 275 samples were collected from the test group at the time of the first vaccination of which 113 (41%) were ELISA negative, 9 (3%) were ELISA suspect and 153 (56%) were ELISA positive (Table 1). In the PMPT a total of 66 (24%) of animals showed no protective antibodies, and 209 (76%) had a protective PMPT titre (Table 2). Thirty eight animals (14%) had a protection level of 20%, 37 (13%) a level of 40%, 31 (11%) a level of 60%, 29 (11%) a level of 80% and 74 (27%) a protection level of 100%.

Two hundred and twenty six samples were collected from the test group 1 month after the first vaccination. Nineteen animals (8%) were ELISA negative, 11 animals (5%) were ELISA suspect, and 196 animals (87%) were ELISA positive (Table 1). Seven animals (3%) were negative in the PMPT and 219 animals (97%) were positive in the PMPT (Table 2). Eleven animals (5%) had a protection level of 20%, 18 animals (8%) a level of 40%, 26 animals (12%) a level of 60%, 14 animals (6%) a level of 80%, and 150 animals (66%) a level of 100%. At the time of the second collection those animals in the two vaccine group were given a second dose of vaccine.

Of the 156 samples collected from the test group 3 months after the first vaccination, 95 animals had received one dose of vaccine and 61 had received two doses of vaccine. Eight seven animals (92%) which received one dose of vaccine were ELISA positive and 55 animals (90%) which received two doses of vaccine were ELISA positive (Table 1). In the one dose vaccine group, six animals (6%) were ELISA negative and two (2%) were ELISA suspect. In the two dose vaccine group, four animals (7%) were ELISA negative and two (3%) were ELISA suspect. In the one dose vaccine group, only one animal (1%) was PMPT negative and 94 animals (99%) were PMPT positive (Table 2). Three animals (31%) had a protection level of 20%, one animal (1%) a level of 40%, five animals (5%) a level of 60%; eight animals (9%) a level of 80%, and 77 animals (81%) a level of 100%. In the two dose vaccine group, none of the animals had a protection level less than 20%. Three animals (5%) had a protection level of 40%, two animals (3%) a level of 60%, seven (11%) a level of 80%, and 50 animals (81%) a level of 100%. At 3 months after the first vaccination there was no significant difference between vaccinal antibody responses to one dose or two doses of vaccine ($P < 0.01$).

Table 1. Distribution of ELISA units for collections 1, 2 and 3

ELISA result	Collection number			
	1	2	3[1]	3[2]
Negative	113[3](41%)	19 (8%)	6 (6%)	4 (7%)
Suspect	9 (3%)	11 (5%)	2 (2%)	2 (3%)
Positive	153 (56%)	196 (87%)	87 (92%)	55 (90%)
Total	275	226	95	61

[1]One vaccine dose
[2]Two vaccine doses

Table 2. Distribution of PMPT protective values for collections 1, 2 and 3

PMPT (% protection)	Collection number			
	1	2	3[1]	3[2]
0	66 (24%)	7 (3%)	1 (1%)	0 (0%)
20	38 (14%)	11 (5%)	3 (3%)	0 (0%)
40	37 (13%)	18 (8%)	1 (1%)	3 (5%)
60	31 (11%)	26 (12%)	5 (5%)	2 (3%)
80	29 (11%)	14 (6%)	8 (9%)	7 (11%)
100	74 (27%)	150 (66%)	77 (81%)	50 (81%)
Total	275	226	95	62

[1]One vaccine dose
[2]Two vaccine doses

A comparison of the individual PMPT levels and the EU results for the data presented in Tables 1 and 2 is shown in Table 3. Although there was a relatively large range of EU for each of the PMPT protection levels, the trend was evident as indicated by a correlation coefficient of $R^2 = 0.740$ ($n = 718$). The calculated regression equation was $y = 6.481x - 36.510$. Based on their ELISA results, individual animals were categorized as either negative, suspect or positive. These results were then compared with the level of protection as measured by the PMPT (Table 4). Sixty eight samples were PMPT positive/ELISA negative whereas only four samples were PMPT negative/ELISA positive. A further 20 samples were PMPT positive/ELISA suspect and three were PMPT negative/ELISA suspect.

Table 3. Comparison of PMPT results and ELISA units

PMPT		ELISA Units
% protection	No. of samples	(meanSD)
0	86	37±26
20	51	66±33
40	59	104±80
60	64	197±137
80	57	408±255
100	350	646±294

Table 4. Correlation of ELISA result and % protection as measured by PMPT

ELISA result	PMPT result (% protection)						
	0	20	40	60	80	100	Total +ve[1]
Negative	78	35	22	7	1	3	68
Suspect	3	6	5	4	2	3	20
Positive	4	12	32	53	55	344	496
Total	85	53	59	64	58	350	584

[1] ≥ 20% protection

Group II

The PMPT protection levels of each of the vaccinated Balitvet cattle were 80% to 100% at the time of challenge, whereas the EU values were negative or suspect (25-88 EU) for six of the eight animals (Table 5). The remaining two animals had EU values of 130 and 383 EU. All animals survived challenge with 20-80 x 10^6 cfu of the known HS-causing *P. multocida* strain M1404. All cattle and buffalo from the IPB farm at Jonggol had PMPT protective values of 0%, and EU values of < 16 to

82 EU. The two non-vaccinated cattle and two non-vaccinated buffalo did not survive challenge whereas the single animal that had been vaccinated with the commercial HS vaccine survived challenge.

Table 5. The protective effect of various *P. multocida* strains against challenge with known HS-causing *P. multocida* strain M1404

Animal ID	Vaccine strain[1]	Parameters at time of challenge: ELISA units	Parameters at time of challenge: PMPT (% protection)	Survival time (hr)
S82[2]	Katha	82	0	> 60
S107	Nil	72	0	48 - 60
S108	Nil	68	0	48 - 60
Kb kec.	Nil	16	0	24 - 31
Kb bes.	Nil	16	0	24 - 31
S93	M1404	71	100	> 145
S284	M1404	25	100	> 145
S281	P1255	58	80	> 145
S476	P1255	64	100	> 145
S46	989A	88	80	> 145
S375	989A	130	100	> 145
S251	P2225	37	100	> 145
S266	P2225	383	100	> 145

[1]Full descriptions can be found in Materials and Methods section
[2]S = bovine, Kb = buffalo

DISCUSSION

The challenge dose for the PMPT has varied from 10 LD_{50} (Bain 1955) to 100 LD_{50} (Bain *et al.*, 1982; 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) where it was shown that a 5×10^1 increase in injected serum required a 10^4 increase in the challenge dose to produce a 40% protection level (Roberts 1947), whereas Bain (1955) demonstrated that a 10^2 increase in challenge dose caused no change in the PMPT protection level from 9 of 11 cattle sera tested. In this study a challenge dose of 100 LD_{50} (approx. 300 cfu) was used.

Bain *et al.* (1982) stated that the survival of any mice in a group challenged with 100 LD_{50} doses of virulent *P. multocida* was significant. They also indicated that false positive PMPT reactions had been encountered in Asia, but were rare in Australia where HS does not occur. In this study a PMPT protective value of 20% or greater, which represented survival of at least one of the five mice challenged, was used as an indicator of protection. This is consistent with the recommendation of Bain (1955) that any protection was a presumptive indication of immunity in the animals.

The discrimination between ELISA sero-positive and ELISA sero-negative animal was performed on the basis of whether the OD or EU value of the sample was greater than or less than the mean plus two standard deviations (SD) of the control group (Tijssen, 1985).

On the assumption that a PMPT protection level of 20% or greater indicated immunity to infection, the sensitivity of the ELISA was 88% and the specificity was 95% (Table 4). The sensitivity and specificity were calculated on the basis of exclusion of all suspect ELISA reactors from the calculations. If all the suspect reactors were regarded as negative then the sensitivity of the ELISA was 85% and the specificity 95%. Analysis also indicated that the relationship between EU and the PMPT results, as illustrated by the data in Table 3, was statistically significant with $P < 0.01$.

The antibody responses of the animals following vaccination were very good with only seven animals having a negative PMPT protective level 1 month post vaccination (Table 2). At 3 months after vaccination only one animal was negative in the PMPT in the one dose vaccination group and none of the animals had a PMPT protection level less than 40% in the two dose vaccine group. Nineteen animals were ELISA negative and 19 ELISA suspect one month post-vaccination (Table 1). This number decreased to six animals after 3 months, in the one dose vaccine group, and four animals, in the two dose vaccine group. No significant difference was evident in the distribution of animals in the ELISA negative or positive groups after one or two doses of vaccine. These results suggest that the use of two doses may be worth considering so as to eliminate the degree of variability seen in the response to vaccination and to ensure immunity to infection.

However, in the case of ELISA, low titres, especially in animals that survived in the vaccination/challenge experiments (Table 5), would question the predictive value of a negative EU reading. While conclusive data is not yet available, the study suggests that a positive ELISA titre indicated protection to infection whereas a negative EU value did not specifically indicate that the animal was susceptible to infection. Interpretations are complicated by the observation that animals had positive EU readings for most of the course of the study. However at the time of challenge, 30 weeks after the last vaccination, their EU values were negative or suspect (Table 5, results not shown). The PMPT levels of the animals which had been vaccinated using non-HS causing strains of *P. multocida*

were protective up to, and during, the time at which they underwent challenge (Table 5, results not shown).

The cross protective effect of different strains of *P. multocida* has been previously reported and is the basis of the recently suggested use of *P. multocida* strain 3,4, isolated from deer, as a live HS vaccine in buffalo and cattle (Myint and Carter, 1990). It appears, therefore, that both the PMPT and ELISA can only be used to indicate previous exposure to *P. multocida*, although a positive result may indicate protection to infection by HS-causing *P. multocida*. The results of this study also indicate that the PMPT and ELISA are not specific for HS-causing strains of *P. multocida* and so cannot be used to indicate previous exposure to HS-causing strains of the organism. Two animals, known not to have been vaccinated against HS, had EU which were in the suspect range (Table 5).

The PMPT has been used in Indonesia to determine the level of protective immunity of cattle or buffalo serum to HS. Difficulties are frequently experienced in performing the test in a standardised manner due to lack of sufficient supplies of mice of a known genetic composition and difficulties in standardisation of the challenge dose. These difficulties can make the comparison and interpretation of results from different laboratories difficult. 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, easy to interpret and to compare results between laboratories, and not use live animal challenge.

The results presented in this paper suggest that the ELISA may fulfil some of these objectives and that the HS antibody ELISA could be used to assist in determining the immune status of large numbers of animals.

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