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PASTEURELLA MULTOCIDA TYPE B IN MALAYSIA: CHARACTERISATION, IMMUNE RESPONSE AND PROTECTION IN VACCINATED BUFFALOES

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By

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LIST OF ABBREVIATIONS

APV	Alum Precipitated Vaccine
BB	Broth Bacterin
BWC	Boiled Whole Cells
cfu	Colony Forming Units
CMI	Cell-mediated Immune Response
DEV	Double Emulsion Vaccine
DTH	Delayed-type Hyprsensitivity Reaction
ed	Edition
ELISA	Enzyme-linked Immunosorbent Assay
FAO	Food and Agticultural Organisation
FKWC	Formalin-killed Whole Cells
GDPT	Gel Diffusion Precipitation Test
HCHO	Formaldehyde
HS	Haemorrhagic Septicaemia
H_2S	Hydrogen Sulphide
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHA	Indirect Haemmagglutination Test
LPS	Lipopolisaccharide
M	Months
n	Numbers
NAp	Not Applicable
NAv	Not Available
ND	Not Done
OAV	Oil Adjuvant Vaccine
OD	Optical Density
PAGE	Poliacrylamide Gel Electrophoresis
PBS	Phosphate-buffered Saline
PMPT	Passive Mouse Protection Test
pp	Pages
PreChall	Pre-challenge
PreVacc	Pre-vaccination
SEM	Standard Error of the Mean
SDS	Sodium Dodecyl Sulphate
Vs	Versus
W	Weeks

Abstract of the thesis presented to the Senate of Universiti Pertanian Malaysia as fulfilment of the requirements for the degree of Master of Science.

PASTEURELLA MULTOCIDA TYPE B IN MALAYSIA: CHARACTERISATION, IMMUNE RESPONSE AND PROTECTION IN VACCINATED BUFFALOES

By

CHANDRASEKARAN SUBRAMANIAM

June, 1993

Chairman : Associate Professor Abdul Rani Bahaman, Ph.D.
Faculty : Veterinary Medicine and Animal Science

A study on the characteristics of the Malaysian field and vaccine strains of *Pasteurella multocida* type B was conducted. In addition, the immunogenic potential of haemorrhagic septicaemia (HS) vaccines was evaluated in buffaloes.

A total of 48 *P.multocida* isolates including the five HS vaccine strains were included in the study. Initial studies showed significant cross-protection in mice amongst the five vaccine strains. The 48 isolates, when subjected to biochemical tests had very little or no variability amongst them.

Type-specific hyperimmune sera were prepared in rabbits and buffaloes. The sera were then absorbed using antigens prepared from the 48 isolates. A series of serological tests were conducted using the pre- and post-absorbed sera and the antigen preparations. The protein profile of the five vaccine and five randomly selected field strains of this organism revealed that there appeared to be no variation amongst the field and vaccine strains.

The antibody responses of buffaloes immunised with three conventional HS vaccines viz., broth bacterin (BB), alum-precipitated vaccine (APV) and oil adjuvant vaccine (OAV) and one experimental double emulsion vaccine (DEV) were determined by an enzyme-linked immunosorbent assay (ELISA). Antibody levels were significantly higher in buffaloes immunised with the adjuvanted vaccines (APV, OAV,DEV). Buffaloes immunised with BB alone were protected at 6 weeks but not at 12 weeks post-immunisation. Buffaloes immunised with APV were protected at 24 weeks but only partially at 52 weeks post-immunisation. On the other hand, buffaloes immunised with OAV or DEV were fully protected at 52 weeks but only partially at 76 weeks post-immunisation. There was also a positive relationship between the ELISA titres and protection in buffaloes.

All vaccinated buffaloes (with the exception of the BB group at three months post-immunisation) developed substantial to significant cutaneous delayed-type hypersensitivity (DTH) reactions. However, its role was not clear at this stage.

There also appeared to be no relationship between active protection in buffaloes, PMPT and the IHA. Vaccinated buffaloes had elevated ELISA antibody titres, and, those that survived challenge had high pre-challenge titres in comparison with the buffaloes that succumbed to challenge. Similarly, a positive relationship between the active protection and ELISA antibody titre in the homologous mouse system was observed. Further, a dose response relationship was also evident in the homologous mouse system.

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PASTEURELLA MULTOCIDA JENIS B DI MALAYSIA: PENCIRIAN, TINDAKBALAS IMUN DAN PERLINDUNGAN DALAM KERBAU YANG TELAH DILALIKAN

Oleh

CHANDRASEKARAN SUBRAMANIAM

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Suatu kajian mengenai pencirian strain kuman bakteria *Pasteurella multocida* jenis B dari lapangan dan yang digunakan sebagai benih vaksin di Malaysia telah dijalankan. Selain dari itu, keupayaan keimunogenan vaksin hawar berdarah (HS) telah dinilai dalam kerbau.

Sejumlah 48 isolat *P. multocida* termasuk kelima-lima strain benih vaksin HS digunakan dalam kajian. Kajian awal menunjukkan terdapat perlindungan silang yang bererti di kalangan isolat vaksin. Kesemua 48 isolat itu, yang dibuat ujian biokimia menunjukkan keadaan berubah-ubah yang tak bererti di kalangannya.

Serum hiperimun khusus-tip telah disediakan dalam arnab dan kerbau. Serum tersebut (pra- dan pasca-terserap) dan antigen yang disediakan dari 48 isolat digunakan di dalam ujian serologi. Profil protein bagi lima strain vaksin dan strain luar yang terpilih secara rawak telah dikaji, dan didapati bahawa tiada perbezaan di kalangan isolat lapangan dan vaksin.

Gerakbalas antibodi serum dari kerbau terimun dengan tiga vaksin HS lazim iaitu bakterin broth (BB), vaksin alum-termendak (APV) dan vaksin adjuvan minyak (OAV); dan satu vaksin emulsi dedua (DEV) telah ditentukan dengan assai imunoerap terangkai enzim (ELISA). Paras antibodi kerbau terimun dengan vaksin adjuvan (APV, OAV, DEV) didapati lebih tinggi.

Kerbau terimun dengan BB terlindung pada 6 minggu dan tidak pada 12 minggu pasca-pengimunan. Kerbau terimun dengan APV terlindung pada 24 minggu tetapi dengan separa sahaja pada 52 minggu. Sebaliknya kerbau terimun dengan OAV dan DEV adalah terlindung sepenuhnya pada 52 minggu tetapi hanya separa sahaja pada 76 minggu pasca- pengimunan. Pertalian di antara titer ELISA dan perlindungan dalam kerbau juga dapat dipastikan.

Kesemua kerbau disuntik (kecuali kumpulan BB pada tiga bulan pasca-pengimunan) membentuk tindakbalas kehiperpekaan tip tertangguh kutis (DTH) yang teguh hingga bererti. Walau bagaimanapun, peranan DTH tidak jelas pada peringkat ini.

Didapati juga tiada pertalian di antara perlindungan aktif dalam kerbau, PMPT dan IHA itu. Kerbau yang disuntik mempunyai titer antibodi ELISA ternaik dan kerbau yang terselamat dari cabaran mempunyai titer pra-cabar yang tinggi berbanding dengan kerbau yang tewas pada cabaran. Adalah terdapat juga pertalian positif di antara perlindungan aktif dan titer antibodi ELISA dalam sistem mencit homologus. Seterusnya suatu pertalian gerakbalas dos adalah juga ternyata dalam sistem mencit homologus.

CHAPTER 1

INTRODUCTION

Malaysia with a land area of 331,520 square kilometers is composed of three regions, i.e., Peninsular Malaysia, Sabah and Sarawak. The country is in the wet tropical region with an average annual rainfall of about 254 cm, most of which is precipitated between the months of October and January.

Based on the Department of Veterinary Services livestock census the cattle and buffalo population ranged from about 680,000 to 743,000 heads for the last ten-year period (Table 1)

Table 1

Cattle and Buffalo Population in Peninsular Malaysia : 1981 - 1990

Year	Cattle	Buffalo
1981	498,000	180,000
1982	503,000	179,000
1983	518,316	163,051
1984	556,420	166,626
1985	552,695	163,359
1986	559,582	141,934
1987	NAv	NAv
1988	586,408	139,674
1989	607,414	135,260
1990	613,689	129,515

NAv - Not available

Pasteurellosis is a disease affecting man and animals. It is caused by a Gram-negative bacillus grouped under the genus pasteurellae.

Haemorrhagic septicaemia (HS) which is caused by *Pasteurella multocida* Carter's Type B is an enzootic disease of cattle and buffaloes in most countries in South East Asia including Malaysia. Epizootics still continue to occur with the loss of susceptible bovines. Bain (1957) estimated the figure to be 100,000 susceptible animals in Asia alone.

No accurate figures are available as to the number of animals that die annually due to HS in Malaysia though the disease has been known to occur for more than 50 years. In a survey conducted by Joseph (1979b), for a ten-year period from 1967 to 1976, there were a total of 287 outbreaks averaging out to 28.7 outbreaks per year. Outbreaks occurred in all the states in Peninsular Malaysia except the state of Perlis in the northern region. Terengganu with at least one outbreak each year had the largest number of outbreaks. However, in the state of Perak, which had the next most number of outbreaks, there were no incidents in the years 1971,1972 and 1975.

Kelantan, which had the third most number of outbreaks, and Pahang had them every year for the ten-year period. In Kedah there were no outbreaks in 1969 to 1973 and 1975. Negeri Sembilan had a total of 21 outbreaks for the review period with no outbreak in 1967, 1968 and 1970. These states are the enzootic areas of Peninsular Malaysia with prevalence being low in other states such as Johor, Malacca, Selangor and Penang.

It has been categorically stated that the incidence of the disease has dropped significantly as a result of better control measures, including the use of better vaccines (adjuvanted) and larger vaccination coverage of susceptible population in enzootic areas.

The vaccine initially used, viz., the broth bacterin (BB), was subsequently discontinued and replaced by the alum precipitated vaccine (APV). APV was a proven prophylactic agent (Iyer and Rao, 1959; Venkatesh *et al.*, 1991) and used widely in many Asian countries. The BB and APV were used in an outbreak area under the assumption that they would initiate rapid immunity so that animals in-contact with diseased animals would be protected. The oil adjuvant vaccine (OAV) was used for prophylaxis based on previous trials when it was claimed to protect animals upto 11 months post-vaccination (Thomas *et al.* 1969).

Although considerable reduction in deaths due to this disease had been achieved by immunisation with the currently available vaccines, there are still some major problems including adverse reactions and other drawbacks encountered in the use of these vaccines. Briefly, some common ones include the lack of readily available methods to evaluate the potency of different vaccine batches, occasional breakdowns in the immunity in areas covered by vaccination, undesirable side effects such as post-vaccination shock and the difficulty in injectability of the oil adjuvant vaccine. To overcome the high viscocity of OAV an experimental vaccine called the HS double emulsion vaccine (DEV) was produced and evaluated together with the existing vaccines.

Epidemiological studies carried out in West Malaysia suggested that the occurrence, extent and duration of an outbreak were dependant on several factors. At the present time the only means of effective control would seem to be by vaccination. Vaccination against HS has been carried out for many years. There have been occasions where vaccinated animals have come down with the disease. This has prompted speculations in the antigenic diversity of the strains of *P. multocida* included in the vaccine preparations and those causing outbreaks in the field.

To assess the immunogenic potential of the strain of *P.multocida* chosen (C82) it was considered necessary to gain information on the duration of protection and immune response generated as a result of immunising buffaloes with these vaccines.

The passive mouse protection test (PMPT) has been recommended for evaluating immunity against HS in either vaccinated ruminants or those with naturally acquired immunity (Bain, *et al.*, 1982). The relationship between vaccinated buffaloes and the PMPT, and the relationship of the antibody titres measured by indirect haemagglutination (IHA) and the enzyme-linked immunosorbent assay (ELISA) to active protection in buffaloes were also studied.

Experiments carried out in this investigation were designed to achieve the following objectives :

1 To characterise the strains of *P.multocida* isolated from HS outbreaks in Malaysia and compared with those used in vaccine preparations.
2 To assess the immunogenicity and the duration of protection elicited by three conventional and one experimental HS vaccine prepared from selected strain/strains.
3 To determine the duration of antibody titres in buffaloes vaccinated with such vaccines.
4 To determine the class of antibody isotypes generated in buffaloes vaccinated with HS vaccines.
5 To determine if there is cell-mediated immune response generated in buffaloes vaccinated with HS vaccines.
6 To assess if the PMPT is a reliable indicator of the protective status of vaccinated buffaloes.
7 To assess if the IHA has a relationship with protection in buffaloes, and,
8 To determine the relationship of ELISA antibody titres with protection in vaccinated buffaloes.

CHAPTER 2

LITERATURE REVIEW

Pasteurellosis affects a wide range of animals and avians leading to diseases which are manifested mainly in the pneumonic and/or septicaemic forms.

The disease has been given different terms depending on the species of animals, viz., haemorrhagic septicaemia (cattle and buffaloes), fowl cholera (chickens), duck plague (ducks), pneumonic pasteurellosis (sheep and goats), swine pasteurellosis (swine), snuffles (rabbit) and bubonic plague (human beings). Many of these diseases were clinically similar but were caused by antigenically different species of organisms belonging to the genus Pasteurella.

Hueppe in 1886 (Bain *et al.*, 1982), noting similarities in the disease and the organism proposed a collective name of haemorrhagic septicaemia (HS) and *Bacillus septicaemiae haemorrhagicae.* In 1900, Lignieres (Merchant and Packer, 1961) proposed the name pasteurellosis for all septicaemic diseases

caused by this organism. As the organism was isolated by different individuals from different hosts different names were given to the disease (Merchant and Packer, 1961).

The first report of an organism of this group was made by Bollinger in 1878 when he investigated a fatal disease amongst wild animals and cattle (Bain *et al.*, 1982). The fowl cholera organism was described by Rivolta in 1877, Perrincito in 1879 and Toussaint in 1879 (Merchant and Packer, 1961).

A detailed and complete description of fowl cholera was made by Pasteur in 1880 (Merchant and Packer, 1961). Rabbit septicaemia organism was described by Davaine in 1880 and Gaffky in 1881 (Merchant and Packer, 1961). In 1882, Loeffler and Schutz described the swine septicaemia organism (Merchant and Packer, 1961). In 1885, Kitt (Merchant and Packer, 1961) made a comparative study of the organism in the disease manifested in fowl, rabbit, swine and cattle and found similarities in many aspects. He described them as *Bacterium bipolare multocidum.* Oreste and Armanni (1887) (Merchant and Packer, 1961) studied and reported on the strain which caused the disease in buffaloes.

Trevison in 1887, (Merchant and Packer, 1961) called the bipolar organisms as Pasteurella and listed three species, viz., *Pasteurella cholerae gallinarum, Pasteurella davainai* and *Pasteurella suilla.* Early classifications had a tendency to name a species based on the host affected. Following this procedure Lignieres (Merchant and Packer, 1961) listed the organism causing pasteurellosis as follows :

P. aviaire – producing infection in birds

P. bovine – producing HS in wild animals, cattle, buffaloes and pleuropneumonia in cattle.

P. ovine – producing HS, pneumoenteritis and enzootic pneumonia in sheep

P. porcine – producing swine plague

P. equine – producing contagious pleuropneumonia in horses

P. canine – producing various forms of disease in dogs

Lignieres, in 1901, introduced the generic name Pasteurella for the whole group of these organisms in honour of

Louis Pasteur (Bain, *et al.*, 1982). It consisted of *P. septica*, *P. pestis*, *P. pseudotuberculosis* and *P. tularensis*. Though they were grouped together, biochemical differences were seen amongst these species. *Pasteurella septica* could not grow on media containing sodium taurocholate like the McConkey agar. However, it was able to produce indole and ferment saccharose. *Pasteurella pestis*, on the other hand, produced opposite reactions to these. Motility, a characteristic not exhibited by pasteurellae was seen in *P. pseudotuberculosis*. It also had the property of an alkalising action on milk (Browning and Mackie, 1949). *Pasteurella tularensis* could only grow when cystine was present (Kelser and Schoening, 1948). This was not the case where the other species were concerned. Based on these differences, Carter (1979) separated them into three genera, viz. *Yersinia*, *Francisella* and *Pasteurella*. *Pasteurella pestis* and *P. pseudotoberculosis* were placed in the genus *Yersinia*, *P. tularensis* in genus *Francisella* and *P. septica* in the genus *Pasteurella* (Duguid, *et al.*, 1984; Carter, 1979). Hence the former were called *Yersinia pestis* and *Yersinia pseudotuberculosis* and *P. tularensis* as *Francisella tularensis*. *Pasteurella septica* has been named according to the host that it affected. In 1899, Lechmann and