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Pasteurellosis in Production Animals

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Editors: B.E. Patten, T.L. Spencer, R.B. Johnson, D. Hoffmann and L. Lehane

Organised by:

Department of Agriculture, Regional Veterinary Laboratory, Benalla, Australia
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Barry Patten BVSc, PhD
Convener, Pasteurella Workshop

OPENING SESSION

Pasteurellosis in Production Animals: A Review

M.C.L. De Alwis[1]

Abstract

A historical review is made of the nomenclature of the genus *Pasteurella*, and the early and currently accepted classification of the species, with special attention to *P. multocida* and *P. haemolytica*. The different systems of serotyping and serotype designation are compared and reviewed against a background of the diseases caused. The ubiquitous nature of the organism, and the wide range of disease syndromes caused in a variety of host species, are discussed.

A general review is made of specific diseases of economic importance in production animals, dealing in depth with those diseases and aspects of them not covered by other plenary speakers. The diseases reviewed are haemorrhagic septicaemia, bovine pneumonic pasteurellosis, pasteurellosis of sheep and goats, and, briefly, atrophic rhinitis.

Dr Rothschild, Director, Australian Centre for International Agricultural Research (ACIAR), Dr Soetatwo, Director AARD, Department of Agriculture, Indonesia, Dr Barry Patten, Workshop Coordinator, distinguished guests, it is indeed my pleasure and privilege to address this august gathering at the opening session of the International Workshop on Pasteurellosis in Production Animals. Being the opening session of the workshop, the gathering here will consist not only of scientists, highly specialised in the field of pasteurellosis, but also lay personnel. Each technical session will contain a keynote address on the specific theme of the session. Hence, I will make this address as general as possible and give a broad overview of the subject, rather than dealing in specific areas in depth.

Today, more than 80 scientists from 17 countries are gathered in Bali to deliberate over this tiny microorganism belonging to the genus *Pasteurella* — a rod-shaped organism, 1–2 μm by 0.3–1 μm in size. As its name implies, the organism was named after Louis Pasteur, who in 1880 was the first person to show that it caused the disease fowl cholera.

The first description of the bacterium dates back to the period 1887–89, when Rivolta and Rivolee described an outbreak of disease in fowl. A disease in cattle was described by Bollinger in Germany in 1878. Its causative organism was isolated by Kitt in 1885. Gaffky in 1881 described a septicaemia in rabbits and Loeffler, in 1886, a swine plague. The German pathologist Hueppe in 1886 noted similarities between all of the above diseases in various species of host animals, and also similarities in the bacterium isolated from the conditions. Later, in 1887, Oreste and Armani described a disease called barbone, in buffalo, caused by a similar organism. Hueppe proposed the name *Bacillus septicaemia* for this bacterium. It was Trevisan who first suggested in 1887 the name *Pasteurella* for the organism, to commemorate the work of Louis Pasteur on the elucidation of the aetiology of fowl cholera.

Human infections caused by *Pasteurella* were not described until 1920. Since bacteria belonging to the genus *Pasteurella* caused disease in many species of animals, in the early years species names were given according to the host animal. The isolates from cattle were called boviseptica; from pigs, suiseptica; from poultry, aviseptica; and so on. After several name changes, Rosenbach and Merchant (1939) proposed the name *Pasteurella multocida*.

In subsequent years, new species were added to the list. *Pasteurella haemolytica* was recognised in 1932, *P. pneumotropica* in 1950, *P. gallinarum* in 1955, *P. ureae* in 1962, and the gas-producing *P. aerogenes* in 1974.

More recent classification of the genus *Pasteurella* is based on DNA hybridisation techniques. On this basis, 11 species were identified as belonging to the genus *Pasteurella*. These were, *P. multocida* (3

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subspecies), *P. dagmatis, P. gallinarum, P. volantiu, P. canis, P. stomatis, P. avium, P. langaa, P. anatis, Pasteurella* sp. A and *Pasteurella* sp. B. (Mutters et al. 1985). Several previously recognised serotypes, *Pasteurella ureae, Pasteurella haemolytica* biotypes A and T, *P. testudinis*, and *P. pneumotropica* were excluded from the genus *Pasteurella*, as they were found to be more closely related to the *Actinobacillus* group.

Today, more than 100 years after the original description of the organism by Louis Pasteur, *Pasteurella* remains an ubiquitous organism with a worldwide distribution, which causes a wide range of diseases in animals and man.

Some common diseases caused by *Pasteurella multocida* and *Pasteurella haemolytica* in agricultural and domestic animals are listed in Table 1.

Table 1. Diseases caused by pasteurella species in production animals.

Species	Disease
Cattle	Haemorrhagic septicaemia
	Bovine pneumonic pasteurellosis
Buffalo	Haemorrhagic septicaemia
Sheep and goats	Pneumonic pasteurellosis
Pigs	Atrophic rhinitis
	Pneumonia
	Septicaemia (rare)
Poultry/turkeys	Fowl cholera
Rabbits	Snuffles

In addition to the diseases outlined in Table 1 — in cattle, buffalo, sheep, goats, pigs, poultry and laboratory animals — pasteurella infections have been recorded in other species (Table 2). As far back as 1959, Carter recorded pasteurellae associated with disease in deer, cats, dogs, horses, mink and monkeys. More recent reports of disease in donkeys and horses (Carter 1959; Pavri and Apte 1967) and deer (Jones and Hussaini 1982; Carrigan et al. 1991) are available. Disease has also been recorded in elephants in Sri Lanka, bison in the USA, camels in Sudan, and in a snow leopard in the Himalayas (Carter 1957; Carter 1959; De Alwis and Thambithurai 1965; Bain et al. 1982; De Alwis 1982a; Wickremasuriya and Kendaragama 1982; Chaudhuri et al. 1992).

The types of infections that have been recorded are also highly varied and range from septicaemias and respiratory infections, which are the most common forms, to wound infections, skin abscesses, mastitis, peritonitis, encephalitis and tooth abscesses.

Table 2. Pasteurella infections in other species.

Animal	Country	Serotypes
Bison	USA	B
Yak	China	B?
Deer	England, Australia	B, A
Elephants	Sri Lanka	B
Camels	Sudan	
Horses	India, Egypt, USA	B, D
Snow leopard	India	F
Cat	USA, France	
Mink	USA	A, D
Monkey	USA	A

The most common form of pasteurella infection in humans is that of animal bite-wound infections. Dogs and cats frequently harbour pasteurellae as commensals. Infected bite wounds can lead to septicaemia and localisation at certain specific sites. A chronic respiratory tract infection has also been recorded in man, where pasteurellae play a role together with other bacteria. Most rarely, nervous system infections, peritonitis, appendicitis and urogenital infections are recorded (Miller 1966; Hubbert and Rossen 1970; Anon. 1972).

This workshop will be dealing with pasteurellosis in production animals, and is, therefore, confined to diseases caused by *P. multocida* and *P. haemolytica*.

Pasteurella multocida

The numerous strains of *P. multocida* associated with a wide range of diseases in many species of animals cannot be differentiated morphologically or by cultural methods. Various attempts were therefore made to classify this heterogeneous group of bacteria.

The earliest attempts were to classify according to the host species. This did not meet with success, since strains isolated from different host species and from different disease conditions were indistinguishable in other respects. For nearly half a century, from the 1920s to the 1970s, numerous attempts were made to classify the group on a biochemical basis. Carter (1976) proposed five biotypes, based on a combination of cultural and biochemical properties and pathogenicity.

Serological classification

The classifications that gained widespread acceptance were based on serological properties of the organism. Early attempts at serological classification date back to the 1920s, and include the work of Cornelius

Table 3. Early attempts at classification of *P. multocida*.

Authors	Tests	Classification
Cornelius (1929)	Agglutination absorption	Groups I II III & IV
Yusef (1935)	Precipitation	Groups I II III & IV
Rosenbusch and Merchant (1939)	Agglutination fermentation	Groups I II & III
Little and Lyon (1943)	Slide agglutination	Types 1, 2 & 3
Roberts (1947)	Passive mouse protection	Types I II III & IV

Table 4. Current systems of classification of *P. multocida*.

Authors	Tests	Classification
'Capsular'		
Carter (1955)	IHA	Types A B C D
Carter (1961)	IHA	Type E
Carter (1963)	IHA & PMPT	Excluded type C
Namioka and Murata (1961a)	Slide agglutination	Types A B C & D
Rimler and Rhoades (1987)	IHA	Type F
'Somatic'		
Namioka and Murata (1961b)	Agglutination of HCl-treated cells	Types 1–11
Namioka and Bruner (1963)		
Namioka and Murata (1964)		
Heddleston et al. (1972)	AGPT	Types 1–16

(1929), Yusef (1935) and Little and Lyon (1943) (Table 3). The earliest serological classification was that of Roberts (1947), which was based on passive mouse protection tests. He demonstrated four types, designated I, II, III and IV. Hudson (1954) added a fifth serotype. Carter, using precipitin tests (1952) and indirect haemagglutination tests (1955), recognised four serological types and designated them A, B, C and D. In 1961, Carter added a new serotype (E) and, in 1963, he deleted type C. Rimler and Rhoades (1987) added a new capsular type F. This typing system was based on the specificity of the surface polysaccharide, and is commonly referred to as 'capsular typing' (Table 4).

In the early 1960s, the Japanese workers Namioka and Murata described what they called 'somatic typing' using an agglutination test with HCl-treated cells. They recognised 11 'somatic' serotypes (Namioka and Murata 1961b,c). Heddleston et al. (1972) developed an agar gel precipitin test using supernatants of heat-treated cells, and identified 16 serotypes. Today, the popular method of designating serotypes is to combine Carter's system of 'capsular' typing and Heddleston's system of 'somatic' typing. Occasionally the Namioka designation is also used, particularly within the capsular type B, where only two 'somatic' types have been identified, and only one of them is associated with disease (Tables 5 and 6).

Once the specificity of the serotypes was defined, it became possible to link specific serotypes with specific diseases. The production of specific vaccines also became a reality. The association of serotypes with disease is shown in Table 7.

The serology of *P. multocida* is complicated by the complexity of antigens. Brogden and Packer (1979) compared the different methods of classification and found that, while each method was reproducible and was in agreement with the results published, the serotypes determined by one method did not directly correlate with those determined by another. It was evident that there were common antigenic components as well as specific antigenic components, and that a serotype identified by a particular method represented an antigen complex consisting of common and specific components. Similar observations were made by De Alwis (1987) in comparing Carter's IHA test, Heddleston's AGPT and Namioka's HCl-treated cell agglutination test for the haemorrhagic septicaemia serotypes. He observed that what was described as a 'somatic' antigen in one classification was different to that in another, and that there was considerable overlap between the so called 'capsular' and 'somatic' antigens.

Table 5. Designation of serotypes of *P. multocida* by the Carter-Heddleston method.

Capsular type	Somatic type	Serotype	Disease
A	1,3,4	A:1	Fowl cholera
		A:3	
		A:4	
	5,6	A:5	Fowl cholera
		A:6	(less common)
	7–10	A:7,A:8,A:9,	
	12–15	A:10,A:12,A:13,	
		A:14,A:15,	
	16	A:16	Fowl cholera (turkeys)
B	2	B:2	Haemorrhagic septicaemia
D	11	D:11	Atrophic rhinitis (pigs)
E	2	E:2	Haemorrhagic septicaemia

Table 6. Designation of serotypes of *P. multocida* by the Namioka-Carter method.

Capsular Type	Somatic Type	Serotype	Disease
A	1,3,5,7 & 9	1:A	Pneumonia (cattle, sheep, pigs)
		3:A	Pneumonia (pigs)
		5:A	Fowl cholera
		7:A	Septicaemia (cattle)
		8:A & 9:A	Fowl cholera
B	6 & 11	6:B	Haemorrhagic septicaemia
		11:B	Wound infection (bovine)
D	1,2,3,4 & 10	1:D,2:D and 10:D	Pneumonia (pigs)
		3:D	Pneumonia (cats)
		4:D	Pneumonia (sheep and pigs)
E	6	6:E	Haemorrhagic septicaemia

Table 7. Diseases caused by pasteurella host species (serotype association).

Disease	Host species	Serotypes
Haemorrhagic septicaemia	Buffalo, cattle	*P. multocida* B:2 and E:2
Bovine pneumonic pasteurellosis	Cattle	*P. multocida*
		P. haemolytica 'A'1
Pneumonic pasteurellosis	Sheep, goats	*P. haemolytica* 'A'2
Septicaemia Pasteurellosis	Sheep, goats	*P. haemolytica* 'T'
Atrophic rhinitis	Pigs	*P. multocida* D (rarely A)
Fowl cholera	Poultry, turkey	*P. multocida* A (also D and F)

Pasteurella haemolytica

A pneumonic syndrome in cattle associated with stress and transport has been recorded since the 1880s. It was in 1921 that bipolar-staining organisms were isolated from the lungs of these animals. It was found later than one group of organisms associated with this condition was distinctly different from *P. multocida*. This group was designated *P. haemolytica*. Early work on the group (Jones 1921; Newson and Cross 1932) helped to establish firmly its antigenic distinctness from *P. multocida*. Much light was thrown on the identity of this group of organisms during the late 1950s and 1960s (Carter 1956; Biberstein et al. 1958, 1960; Smith 1959, 1961).

Two distinct biotypes were identified. They were designated A and T types on the basis of their ability to ferment arabinose and not trehalose and vice versa. Using the indirect haemagglutination test, to date 16 different serotypes have been identified. Strains of *P. haemolytica* are now differentiated by a combination of biotype and serotype. This is shown in Table 8.

The association between biotype, serotype and disease caused became apparent. Type A1 for instance was associated with septicaemia in young lambs and epidemic pneumonia in cattle. Type A2, which was a common serotype, was associated with pneumonic pasteurellosis in sheep and goats. The T type consistently gave rise to a distinct septicaemia syndrome in sheep.

Specific diseases caused by pasteurellae

Pasteurella species may be primary pathogens or may play a secondary role in the pathogenesis of various diseases. Examples of diseases where pasteurella plays a primary role are haemorrhagic septicaemia and fowl cholera. In such situations, the disease is reproducible using pure cultures of the organism. Treatment with appropriate antibacterial agents can cure the disease. Vaccines produced using the specific bacteria give protection against the disease. Where pasteurellae play a secondary role, reproduction of the disease is not always possible. Interaction with other physical and biological agents is required.

I shall now deal with some specific diseases of economic importance caused by *Pasteurella* spp., giving greater emphasis to diseases or aspects of disease not dealt with by other plenary speakers.

Haemorrhagic Septicaemia

The term haemorrhagic septicaemia (HS) is used to describe an acute, fatal disease principally affecting water buffalo and cattle, and caused by two specific serotypes of *P. multocida*. It is a primary pasteurellosis. It is thus a specific form of pasteurellosis, as much as typhoid and pullorum disease are specific forms of salmonellosis caused by specific serotypes of salmonellae in specific host species. Two serotypes are associated with the disease. The Asian serotype is designated B:2 and the African serotype E:2 by the Carter–Heddleston system, and 6:B and 6:E respectively by the Namioka–Carter system.

Distribution

HS occurs in southern and Southeast Asia, including Indonesia, the Philippines, Thailand and Malaysia. It occurs in the Near and Middle East, and in northern, eastern, central and southern Africa. It is not reported in Western Europe, Australia, Oceania, and Canada (FAO 1991). The disease was recognised in Japan in 1923, but no epidemics occurred, and it did not become endemic (Carter 1982). It was reported in bison in the National Parks of the USA in the years 1912, 1922 and 1967, and there was one report in dairy cattle in the USA in 1969 (Carter 1982). Low sporadic incidence is reported in many southern European states. Reports very suggestive of HS emerge from Italy and many southern and central American States, where the types of cattle reared and husbandry practices are similar to those prevailing in the HS-endemic countries. Nevertheless, no confirmed laboratory reports based on serotype identification are available. In Africa, the presence of disease is definitely confirmed, but little information is available on actual incidence and economic losses. Table 6 summarises the current status of HS on a global basis.

Equally important is the distribution of the two serotypes. Asian countries have recorded only serotype B:2, and the African countries only E:2. A few countries, notably Egypt and Sudan, have recorded both serotypes (Shigidi and Mustafa 1979; Farid et al. 1980). The USA isolate was the Asian type (Carter 1982). There are still grey areas in the global picture of the occurrence of HS. This presumably arises from some countries not clearly differentiating between HS as a specific disease entity and other forms of non-specific pasteurelloses.

Table 8. Designation of types of *P. haemolytica*.

Biotype	Serotype	Designation	Source/Disease
A	1,2,5-9 11-14 and 16	A1	Septicaemia (lambs) Pneumonia (cattle)
		A2	Pneumonia (sheep, goats)
		A5-A9	Pneumonia
		A11-A14, A16	Inhabitant of URT of normal sheep, goats and cattle
T	3,4,10 & 15	T3, T4, T10, T15	Septicaemia (sheep)

Economic losses

The greatest economic losses caused by HS are recorded in Asia. The water buffalo is particularly susceptible, and it is important both as a dairy animal and for draught power in the rice fields. Quantitative estimates of economic losses are, however, scarce. It must also be noted that HS occurs mainly in regions where husbandry practices are poor. In such situations, disease reporting and surveillance systems are also naturally poor. Reported losses may therefore reflect no more than the trend, the actual losses being greater. Epidemiological studies have shown that, while sporadic flash epidemics attract the attention of the authorities, in endemic areas the disease smoulders in young adult animals and losses, though economically more significant, escape unrecorded.

There is a dearth of information on epidemiology in general, and economic losses in particular, from Africa. The trends, however, appear to be similar. For instance, the reports of the 1955 epidemics in Sri Lanka (Dassanayake 1957; Perumalpillai and Thambiyiah 1957) show striking similarities to the 1978 epidemic in Zambia (Francis et al. 1980).

The disease

The first reports of outbreaks among free-range animals were of sudden deaths. On closer observation, animals may be found with elevated temperature, submandibular oedema (sometimes spreading to the brisket region), and respiratory distress with profuse nasal discharge. Most cases lead to recumbency and death. The duration of illness is shorter in buffalo than in cattle, and may range from less than 24 hours in peracute cases to 2–5 days. Symptoms usually occur after an incubation period of 2–5 days. Typically the clinical picture displays three phases — one of temperature elevation, a phase of respiratory distress, and a terminal phase of recumbency. Septicaemia is in most instances a terminal event. Varying degrees of overlap occur between phases, depending on the duration of the disease.

At necropsy in a typical case of HS, the first obvious lesion is subcutaneous oedema, with serogelatinous fluid particularly in the submandibular, throat and brisket regions. The subcutaneous connective tissue may be dotted with petechial haemorrhages, and the lymph nodes may be swollen. In the thoracic cavity, varying degrees of lung involvement are evident, ranging from generalised congestion to extensive consolidation with thickening of the interlobular septae, giving rise to a lobulated appearance. Marked pleurisy and pericarditis may be seen with thickening of the pericardium and a collection of serosanguinous fluid in the pleural cavity and pericardial sac. Pleural and pericardial adhesions may be present. Petechial and ecchymotic haemorrhages will invariably be seen on the heart, particularly in the auricular region and at the base of the ventricles. The gastrointestinal tract will show signs of hyperaemia, with petechial and sometimes massive ecchymotic haemorrhages on the abomasum.

In an experimental transmission study (De Alwis et al. 1975), it was shown that the extent of the lesions depended on the duration of the disease. In peracute cases, where death occurred in 24–36 hours, no more than a few scattered petechial haemorrhages were seen.

Epidemiology

The species most commonly affected by HS are cattle and buffalo, and there is general agreement that buffalo are more susceptible than cattle. Several epidemiological studies in Sri Lanka have provided evidence to support this view (De Alwis 1981). In Malaysia, 73% of all recorded losses due to HS during 1970–79 and 90% in the period 1980–89 were among buffalo, despite the fact that the buffalo population is half that of cattle (FAO 1991). The HS serotypes occasionally cause disease in other species. Reports are available of disease in pigs in Thailand, Malaysia (FAO 1991), India (Murthy and Kaushik 1965), and Sri Lanka (De Alwis unpublished). The HS serotypes have been associated with goats in India and Malaysia, but experiments conducted in Sri Lanka have shown goats to be highly resistant (Wijewardana et al. 1986).

Morbidity, mortality and case fatality

Morbidity and mortality are affected by a number of factors and their interactions. Age, endemicity of the region, previous exposure and immunity arising therefrom, and herd immunity levels are all important factors. The high susceptibility of young animals has been established in several studies in Sri Lanka (De Alwis et al. 1976). Different morbidity patterns in endemic and non-endemic areas, and the immunological basis for this situation, have been established (De Alwis 1981).

Immune carriers

The fact that a small percentage of animals harbour pasteurellae in their nasopharynx has been known for a very long time, and these animals have been labelled as carriers (Singh 1948). Later observations gave rise to the suspicion that the carrier rate may be related to recent exposure to HS (Gupta 1962; Mustafa et al. 1978; Hiramune and De Alwis 1982). More recent work has shown that a large proportion of cattle and buffalo in endemic areas harbour

HS-causing pasteurellae in their tonsils, and the organisms make their appearance intermittently in the nasopharynx. Such animals also have high antibody levels.

It is postulated that stress may play a role in causing pasteurellae in the tonsils to proliferate and invade the nasopharynx, and be disseminated through nasal secretions, infecting in-contact susceptible animals.

Diagnosis

Farmers in endemic areas recognise the disease. A clinical diagnosis in the field is usually based on history, symptoms, and lesions observed at necropsy. Blood from fresh carcases, or a longbone in old carcases, serve as good culture material. Since septicaemia is usually terminal, blood from clinical cases taken prior to death may not always yield organisms on culture. Mouse inoculation serves as a biological screen to obtain pure cultures.

Confirmation of diagnosis is by serotyping. The rapid slide agglutination test (Namioka and Murata 1961a), indirect haemagglutination test (Carter 1955), and the AGPT (Heddleston et al. 1972) serve as useful techniques. For rapid diagnosis, a counter immuno-electrophoresis test has been described (Carter and Chengappa 1981). Non-serological methods, such as the production of hyaluronidase by type B strains, have also been developed (Carter and Chengappa 1980).

Control

The accepted method of control is by vaccination. Many types of vaccines are used. Plain bacterins provide immunity for only a few weeks. The most commonly used vaccine is the alum-precipitated vaccine. This vaccine has to be used twice yearly. Many countries use the oil adjuvant vaccine (OAV), which gives both a higher level and a longer duration (1 year) of immunity, and thus remains the best established vaccine. Since this vaccine was first described by Bain and Jones (1955), research has been done on a wide variety of vaccines, but none has become established.

The OAV is the principal prophylactic agent used in countries such as Malaysia, Indonesia, Egypt, Iraq and Sri Lanka (De Alwis 1984; FAO 1991). Vaccination coverage is low in most countries, and ranges between 20 and 50%. High coverage may be attained in restricted areas, such as in the island of Lombok in Indonesia, and in certain endemic areas in Sri Lanka (FAO 1991).

Vaccines at the experimental stage include a double emulsion vaccine with low viscosity and a milky consistency (Gupta et al. 1979; Yadev and Ahooja 1983; Chandrasekeran et al. 1991), sodium alginate adjuvant vaccine (Bhatty 1973), and live vaccines using variant or related strains (De Alwis and Carter 1980; Myint et al. 1987; Myint and Carter 1989, 1990). The live vaccine based on an antigenically related deer strain of *P. multocida* (serotype B:3,4) developed by the latter workers is now widely used in Myanmar, although some reservations have been made on its use in young buffalo calves for primary vaccination.

The search for a better vaccine continues. Many papers on new vaccines will be presented at these sessions. It has been established in epidemiological studies that in vivo antigens are important immunogens and are responsible for the high level of immunity following arrested infections (De Alwis 1982b; De Alwis and Sumanadasa 1982). Thus, scope for development of new vaccines lies in the following three broad areas:

- development of a safe, avirulent, stable variant strain that will produce the important immunogens in vivo when administered as a live vaccine;
- identification of the important immunogens and development of artificial media in which these are optimally expressed; and
- use of adjuvants, including the new-generation adjuvants, which will give rise to an easily injectable vaccine.

A fact that must be borne in mind is that the production technology must be one that is sustainable in developing countries, where the disease is endemic. Effective control depends not only on a good vaccine, but also on strategic vaccination programs. These must be based on a sound knowledge of HS epidemiology in the particular country, and on a recognition of the true magnitude of the problem.

Pneumonic Pasteurellosis of Cattle

Unlike haemorrhagic septicaemia, which is caused by very specific serotypes of *P. multocida*, pneumonic pasteurellosis is often associated with more than one species and serotype. Pasteurellae play a secondary role in the disease process, and a number of viruses have been incriminated. The disease is variously named as 'shipping fever' in North America and 'transit fever' in the UK and Europe. It is also referred to as 'bovine epizootic pneumonia'. All of these conditions constitute different syndromes that occur under different circumstances, and pasteurellae have a definite role to play in this bovine respiratory disease (BRD) complex.

Aetiology

The pasteurellae associated with pneumonic pasteurellosis are *P. haemolytica*, predominantly

type A1 (Biberstein et al. 1960; Frank 1983) and *P. multocida* capsular type A (Carter 1973). There is no consistency in the somatic antigens of the latter (Blackburn et al. 1975).

Economic losses

Next to HS, pneumonic pasteurellosis has been rated as the most economically important form of pasteurellosis affecting the beef and dairy industries. It is believed that, in the USA, losses due to the BRD complex are greater than the losses due to all other diseases of cattle put together.

Epidemiology and pathogenesis

The pasteurellae causing pneumonic pasteurellosis are carried in the upper respiratory tract (URT) of healthy calves. In the case of *P. haemolytica* type A1, the bacterium is not easily detectable in the URT of healthy cattle, but is shed and isolated with greater frequency in stressed or otherwise diseased cattle. The URT of unstressed, healthy cattle is not easily colonised with *P. haemolytica*. In contrast, in calves that are clinically ill with respiratory viral infections or in calves otherwise stressed, the URT will be easily colonised with A1. In the case of *P. multocida*, however, no such relationship between ability to colonise and stress has been demonstrated.

Stress or viral infection results in an explosive proliferation of A1 strains of *P. haemolytica* from low or undetectable levels. Such proliferation leads to two processes — invasion of the lungs resulting in pneumonia, and shedding in the nasal secretions leading to dissemination of infection to other animals. Thus, explosive outbreaks can occur in batches of stressed calves, as seen in the enzootic pneumonia syndrome. The mechanisms that are involved in the rapid proliferation process are incompletely understood. Much work has been done on the development of experimental models to reproduce the disease, using bacterial cultures alone or in combination with other agents.

Prevention of pneumonic pasteurellosis

Prevention is effected by skilful management and by the use of vaccines. The avoidance of stressful management practices is of primary importance.

Vaccines have been prepared against certain respiratory viruses, as well as pasteurellae. Pasteurella vaccines are important for two reasons: first, a multiplicity of viruses are involved, as against specific serotypes of pasteurella; and second, pasteurella infection is the most common sequel to BRD that leads to fatality. The response to the traditional whole-cell bacterin is disappointing. Newer vaccines include those containing bacterial extracts, chemically altered strains, and live vaccines. Adjuvants are widely used.

The effect of most vaccines has been a reduction in the severity of the clinical disease and extent of lesions, rather than complete prevention of the disease. Much research into development of better vaccines is in progress.

Diagnosis

Diagnosis of pneumonic pasteurellosis is by culture from the pneumonic lung or from nasal secretions. Serotyping is important, since only some serotypes are associated with disease. A rapid plate agglutination test is available for *P. haemolytica*; and the hyaluronic acid decapsulation test is used for *P. multocida* type A.

Treatment

Early treatment is important for more complete recovery. A knowledge of the antibiotic sensitivity pattern of prevalent strains is also important. Antibiotic resistance among strains is increasing. In individual outbreaks, the sensitivity pattern of isolates from nasal secretions will give early information, but this may not reflect the status of strains from the lungs. Thus, antibiotic sensitivity determination of strains isolated from the lungs at necropsy should not be missed.

Pasteurellosis of Sheep and Goats

Pasteurellosis is one of the most common bacterial diseases of sheep and goats. The earliest reports of pneumonia in sheep were from Iceland. Outbreaks of 'enzootic pneumonia' were reported in England and Wales by Montgomerie et al. 1938. A septicaemia syndrome caused by *P. haemolytica* was reported by Stamp et al. (1955). It was the advent of biotyping and serotyping in the 1950s and 1960s that led to a better understanding of the disease.

Aetiology, clinical syndromes and serotypes

In temperate climates, pasteurellosis in sheep and goats is caused mainly by *P. haemolytica*, and rarely by *P. multocida*. The biotype A causes pneumonia in all ages of sheep, and septicaemia in young lambs. Biotype T causes a distinct septicaemia syndrome in young adult sheep. Outbreaks occur sporadically and are unpredictable. A syndrome referred to as atypical pneumonia associated with *P. haemolytica* and *Mycoplasma* spp. has also been described.

Pasteurella haemolytica is carried in the nasopharynx and tonsils of apparently healthy sheep. Lambs acquire infection soon after birth, presumably by contact (Shreeve and Thompson 1970). The

carrier rate is low in normal healthy flocks and an assortment of serotypes is present. In flocks undergoing outbreaks, on the other hand, the carrier rate is high, and a few specific serotypes dominate (Biberstein and Thompson 1966). Thus a high carrier rate is indicative of prevalent infection in the vicinity. This carrier status has been found to display seasonal variations (Biberstein et al. 1970).

Reports on the disease and associated serotypes from countries other than the UK are scarce. In the USA and New Zealand, serotype A2 is common. In Kenya, all known serotypes have been recorded in sheep and goats, with serotypes 3, 4, 6, 8 and 10 being associated with pneumonia (Mwangota et al. 1978). In Sri Lanka, serotype 2 was found to be associated predominantly with pneumonia in goats, while serotypes 1, 6, 7, 8, 9, 11 and 12 have also been recorded (Hordagoda et al. 1981).

P. haemolytica has been associated with goat pneumonia in Malaysia and the Philippines, but no typing of the organism is recorded (Jasni et al. 1991).

Predisposing factors

Predisposing factors play a role in most outbreaks of disease. Climatic changes and stressful management practices such as movement, dipping, worming etc. have been associated with outbreaks.

Vaccines and vaccination

Many predisposing factors are normal environmental changes or routine management practices that cannot be avoided. Outbreaks are sporadic and unpredictable. Thus vaccines should seek to achieve round-the-year protection in areas with a high prevalence.

A wide range of vaccines are available. Combined vaccines with pasteurella and clostridial antigens are popular.

Pasteurellosis of Pigs

Besides occasional sporadic outbreaks caused by *P. multocida* type B, pasteurellosis in pigs is associated with types A and D. Two well-defined syndromes are atrophic rhinitis and pneumonia.

Atrophic rhinitis

Atrophic rhinitis is a disease associated with intensive pig breeding in most parts of the world. It was originally described in Germany nearly 160 years ago, but it was only during the past decade that its complex aetiology and pathogenesis were revealed.

Economic losses have been variously estimated and are due not only to deaths, but more significantly to reduced weight gains in affected herds. The disease is characterised by atrophy of the nasal turbinates, resulting in a shortening and twisting of the snout, sneezing and epistaxis.

Aetiology

Two bacterial organisms have been incriminated with the disease.

- *Bordetella bronchiseptica* — a normal inhabitant of the URT of pigs (This bacterium can cause turbinate hypoplasia, but not all the changes associated with atrophic rhinitis.)
- toxigenic strains of *P. multocida* — these strains usually belong to type D.

Turbinate atrophy is preceded by rapid proliferation and colonisation by *P. multocida*. Concurrent infection with *B. bronchoseptica*, or the action of certain irritants, create an environment favourable for such proliferation and colonisation.

Vaccines and vaccination

Earlier vaccines contained *B. bronchiseptica* and *P. multocida* bacterins. These vaccines had limited effects. Modern vaccines are a combination of *B. bronchiseptica* bacterin, which prevents initial proliferation of the organisms, and pasteurella toxoids derived from specific toxigenic strains. Both components play a role in the protection.

Conclusions

What I have endeavoured to do in this opening keynote address is to give you a broad overview of pasteurellosis in production animals. The keynote speakers will be dealing with specific subject areas in depth. The large number of papers to be presented at these sessions will provide an excellent updating on the subject to all workers in the field present here. It also bears testimony to the importance of pasteurellae as pathogens of production animals.

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SESSION 1: THE ORGANISM

The Biology of *Pasteurella multocida* and *Pasteurella haemolytica*

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Abstract

The importance of *Pasteurella multocida* and *P. haemolytica* in the production of disease in a wide range of animal species has been long recognised. However, despite continuing investigation of both the organisms and the affected animal hosts, they still contribute to significant losses in animal production worldwide. Understanding of the nature of the organisms and their pathogenic potential has increased considerably over the past decade, and a much greater appreciation of the nature of the pathogens has come with the development of improved techniques for examining microorganisms and their component parts.

This review will consider the structure and cellular components of *P. multocida* and *P. haemolytica*. It will focus on aspects of the organisms' composition that may contribute to disease pathogenesis or to stimulation of the host's immune response. Emphasis will be given to advances made in the past 5 years.

Pasteurella multocida

Growth

Pasteurella multocida is a facultative anaerobe that presents no peculiar demands for its isolation. Although it will grow on basic media such as nutrient agar, in common with most mucosally related bacteria, growth of *P. multocida* improves with enrichment. Its longevity in ageing cultures, as observed by Pasteur, was confirmed by Watko (1966), who recovered viable organisms from a chemically defined medium after 45 days of static incubation.

The many studies that have been performed on the growth requirements of *P. multocida* have had the predominant aim of improving cell yield. This has been the primary motivation for vaccine production, and is logical if the premise is true that more cells equals better vaccine. Certainly this was likely to hold when whole bacterial cells were the basis of vaccine composition. However, as antibacterial immunity became more targeted to specific cellular products rather than whole cells, the equation of high cell numbers with improved vaccines became less secure. Unless those higher numbers of cells are producing the appropriate protective antigen(s), then nothing is gained by sheer growth increase.

This organism has long been considered somewhat fragile, requiring regular subcultures in the laboratory. However, toxigenic *P. multocida* has recently been shown to survive for several months in Bacto Tryptose broth at 15 and 37 °C (Thomson et al. 1992). This same study showed that the organism can survive the stresses of aerosolisation very well.

Capsules

The antigenic specificity of the capsule of *P. multocida* determines its serogroup — either A, B, D, E or F. Within each serogroup strains may be further classified by somatic antigen type, and more recently by DNA fingerprinting (Wilson et al. 1992).

The capsule of type A is composed of hyaluronic acid, but intimately associated with it are other polysaccharides, proteins and lipids (Carter 1967). By itself, the hyaluronic acid does not exert antiphagocytic activity, but saline-extractable capsular material contains a factor (about 300 kDa) capable of inhibiting the functions of bovine polymorphonuclear leucocytes (Ryu et al. 1984). The capsule of avian strains provides protection from the action of

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complement (Snipes and Hirsh 1986), but has no influence on the association of the organism with phagocytic cells (Snipes et al. 1987). Removal of the hyaluronic acid capsule increases both the adhesiveness of the organism to animal cell surfaces (Glorioso et al. 1982) and its susceptibility to phagocytosis (Harmon et al. 1991). The production of capsular material, as well as the morphology of *P. multocida*, is affected by subminimal inhibitory concentrations of antibiotics (Jacques et al. 1991). Capsular antigens can be detected in the crypt lumen of porcine tonsils colonised by type D strains (Ackerman et al. 1991).

Fimbriae

Fimbriae have been demonstrated by electron microscopy on rabbit type A strains capable of adhering to isolated pharyngeal cells (Glorioso et al. 1982). Strains belonging to other serotypes (B, D and E) showed much less adhesive capacity in the same system. The attachment could be inhibited by N-acetyl-D-glucosamine, which may suggest that this aminosugar could be the animal cell receptor to which the fimbriae adhere. Adhesiveness is reduced following treatment of the organism with pronase, heat or homogenisation, all of which would reasonably affect fimbrial function. Porcine type A strains similarly adhere more readily than type D strains to porcine tracheal epithelial cells (Parent et al. 1987), but the organism seems to lack the ability to colonise normal swine nasal mucosa (Nakai et al. 1988). There is a poor correlation between fimbriae and experimental production of turbinate atrophy in pigs when different isolates are tested (Trigo and Pijoan 1988). Indeed toxigenic strains of *P. multocida* may have no detectable fimbriae or flagella (iDali et al. 1991) and yet can colonise the procine tonsil and respiratory tract, both alone or concurrently with *Streptococcus suis* (Ackermann et al. 1991).

Outer membrane (envelope) proteins (OMP)

Strains from atrophic rhinitis exhibit three different types of cell-envelope protein patterns: I, II and III (Lugtenberg et al. 1984). The different types are based on the mobility of heavy (H) and weak (W) protein bands between 28 000 and 40 000 Da. Protein H has a number of properties that are shared by pore proteins of members of the Enterobacteriaceae; e.g. surface location, affinity for peptidoglycan, trypsin resistance, resistance to SDS solubilisation (Lugtenberg et al. 1986). Furthermore protein H forms an immunogenic complex with lipopolysaccharide (LPS), which may be the same as the α antigenic complex described 20 years previously (Prince and Smith 1966). It was not possible to detect a protein in cell envelopes that correlated absolutely with pathogenic strains, but it was observed that cell-envelope type I strains were pathogenic while type II were nonpathogenic (Lugtenberg et al. 1984).

Bovine strains of type A, examined for their OMP patterns, appear similar, although the four major proteins (a–d) show sufficient variation in molecular mass to allow arrangement into different groups (Abdullahi et al. 1990). Protein a in this study would appear to be the same as the 37.5 kDa OMP described by Lu et al. (1988) in *P. multocida* 3:A from rabbits. These latter workers showed that OMP demonstrated by immune serum directed against live cells and against cell components were virtually identical, which indicated that they were expressed in vivo.

Johnson et al. (1989) demonstrated a complex protein profile of more than 40 bands in a strain of *P. multocida* from haemorrhagic septicaemia. When this study was extended to include 14 isolates originating from haemorrhagic septicaemia in various animal species from different locations, the same pattern emerged (Johnson et al. 1991). The profiles, which were relatively homogeneous, allowed two groups to be distinguished, according to country of origin. Significantly, electrophoretic pattern correlated with serotypic properties, but the molecular weight of the somatic antigen did not correlate with serotype. No protein could be identified that was unique to strains from haemorrhagic septicaemia, but one major protein band (27 kDa) was common to all isolates, regardless of serotype. Serum from vaccinated animals contains antibodies that react with a small number of the protein bands in this organism and also with LPS (Johnson et al. 1989).

A 50 kDa OMP of an avian *P. multocida* strain has been found to inhibit the phagocytic capacity of avian mononuclear phagocytes (Truscott and Hirsh 1988). Differences between isolates of the one serotype from fowl cholera can be recognised by the position of one of the major proteins in the 34–38 kDa region (Ireland et al. 1991). The 34 kDa protein, but not LPS, reacted with serum from chickens experimentally infected with *P. multocida* of the same serotype.

Lipopolysaccharide (LPS)

Examination of strains from a variety of animal sources confirms that LPS from *P. multocida* are similar to semirough LPS of Enterobacteriaceae (Manning et al. 1986). A more recent examination of two avian strains, one a vaccine strain and the other a virulent field isolate, indicated a typical rough LPS that was readily released by complement (Lee et al. 1992). The LPS of rabbit isolates contained

either a non-serospecific antigen, a serospecific antigen, or both. The LPS is responsible for somatic serotype specificity (Brogden and Rebers 1978), and when examined electrophoretically the LPS is of low molecular weight. It would seem that the LPS molecule of *P. multocida* is much shorter than those of *Escherichia coli* and *Salmonella typhimurium* for example, and is more likely a lipo-oligosaccharide with a heterogeneous core (Lugtenberg et al. 1984).

Thirty-four strains from atrophic rhinitis (type D) showed at least six electrophoretic types of LPS and these frequently coincided both with a certain cell envelope protein type and with the presence or absence of the pathogenic character of the strain (Lugtenberg et al. 1984). Antibody reacting with the LPS of type A strains has given protection against murine infection (Wijewardana et al. 1990). However, LPS appears to play a subordinate role in protection against infection with *P. multocida* type B, at least in laboratory animals (Ramdani and Adler 1991). Avian strains from fowl cholera show some degree of resistance to complement (Lee et al. 1991) and serum resistance is an indicator of virulence of *P. multocida* for turkeys (Morishita et al. 1990). Clinical isolates of both *P. multocida* and *P. haemolytica* from cattle exhibit serum resistance, while isolates from asymptomatic cattle vary in serum susceptibility (Blau et al. 1987). Endotoxin can stimulate TNF-α release from bovine alveolar macrophages (Bienhoff et al. 1992).

Iron acquisition

Iron is an essential element for growth of *P. multocida*. When grown in conditions of iron deprivation, the organism is able to secrete a growth-enhancing factor that functions as a siderophore (Hu et al. 1986; Lee et al. 1991). This factor has been called multocidin and is neither a phenolate nor a hydroxymate. The organism is also capable of obtaining iron for growth by a non-siderophore mediated mechanism. Such acquisition is associated with the production of a number of high-molecular-mass, iron-regulated OMP including an 82 kDa receptor protein for transferrin-bound iron (Ogunnariwo et al. 1991). This capacity was observed in bovine strains and not in avian strains, and was restricted to acquisition from bovine transferrin.

Toxins

The production of protein toxins by *P. multocida* has been recognised for some time, although it is only in the last decade that some definition has been given to the nature of these products following the discovery of toxigenic strains. Some strains of *P. multocida*, in particular those of capsular serotype D, produce a factor designated dermonecrotic toxin (DNT) (Sawata et al. 1984). Purified DNT is a protein of estimated molecular weight ranging from 112 to 160 kDa and can be recovered from sonic and hypotonic extracts and culture fluids. It can be further dissociated into three fragments that are individually non-toxic (Nakai and Kume 1987).

Dermonecrotising activity is but one of the manifestations of the toxic action of the DNT. Others include cytotoxicity for embryonic bovine lung cells, lethality, mucoid diarrhoea or splenic atrophy in mice, turbinate atrophy in a range of animals including pigs, rats, rabbits and goats, lymphopaenia and vascular endothelial damage in the liver of pigs and rats. The toxin gene has been cloned into *E. coli* (Petersen and Foged 1989; Kamps et al. 1990) and the recombinant toxin used to reproduce atrophic rhinitis in pigs (Lax and Chanter 1990).

Examination of the biochemical mechanism of action of the toxin on embryonic bovine lung cells did not reveal changes similar to those induced by other bacterial toxins; e.g. in cell ultrastructure, protein or nucleic acid turnover, intracellular concentrations of ATP and cAMP (Chanter et al. 1986). Furthermore, the toxin has the unusual feature of not being secreted by living intact cells (iDali et al. 1991). Commercial vaccines therefore incorporate formaldehyde-treated whole cells of toxigenic *P. multocida* or formaldehyde-detoxified crude bacterial extracts of toxigenic organisms (Kobisch and Pennings 1989). Both the toxin and its gene have been sequenced (Buys et al. 1990; Petersen 1990) and recombinant derivatives of the toxin have been assessed for the efficacy as vaccines (Petersen et al. 1991). Toxoid prepared from purified toxin is effective against the systemic effects of the toxin in rats (Thurston et al. 1991) and against turbinate atrophy in pigs (Foged et al. 1989).

Although serotype B of *P. multocida* has occasionally been reported as producing a protein toxin, there is no record of identification or characterisation of this product (Rimler and Rhoades 1989). Toxin production has only been reported for subsp. *P. multocida* and never for subsp. *gallicida* or *septica*, and not for the dog-type *P. multocida*, now *P. canis* (Donnio et al. 1991).

While DNT has been associated with the development of atrophic rhinitis in pigs, strains from other hosts also produce the toxin, including poultry, calves, cats and dogs (Nielsen et al. 1986) and rabbits (Chrisp and Foged 1991). When 94 clinical isolates of human origin (from respiratory tract and wounds inflicted by pets) were examined for DNT production, only six were toxigenic and these positive isolates were all from the respiratory tract (Donnio et al. 1991).

Extracellular enzymes

Avian strains do not have detectable extracellular enzymatic activity (Lee et al. 1991). On the other hand, examination of strains from carrier animals and from outbreaks of haemorrhagic septicaemia suggest a correlation of virulence with hyaluronidase production (Wijewardana et al. 1986). Hyaluronidase production appears to be limited to type B strains (Carter and Chengappa 1980). Neuraminidase activity is detectable in most serogroups, and high neuraminidase production seems to correlate with higher virulence of strains (Müller and Krasermann 1974).

Plasmids

Avian strains contain plasmids and this characteristic, together with a degree of complement resistance, are correlative markers of virulence (Lee et al. 1991). Plasmids have been recovered from *P. multocida* strains from various animal species (Haghour et al. 1987). Plasmids conferring antibiotic resistance have been isolated from fowl cholera and bovine pneumonia strains. *P. multocida* can be successfully transformed with plasmids, by electroporation, and this procedure could be used to develop a cloning system for studying virulence factors (Jablonski et al. 1992).

Bacteriocins

No bacteriocin activity has been shown in avian strains of *P. multocida* (Lee et al. 1991), although bacteriocin typing has been reported previously for avian and bovine strains.

Antibiotic resistance

Among isolates from fowl cholera, little evidence of antibiotic resistance could be demonstrated (Lee et al. 1991), although R-plasmids have been identified (Hirsh et al. 1985). Resistance to antibiotics is generally less frequent than is the case with *P. haemolytica* (Haghour et al. 1987).

Pasteurella haemolytica

Structures of adhesion

Two types of fimbriae have been demonstrated on serotype 1 grown in vitro (Morck et al. 1987). The large (12-nm wide) type are rigid, while the smaller (5-nm wide) type are flexible. In vivo similar structures have been seen, as in organisms recovered from lavage fluid from an experimentally infected calf (Morck et al. 1988) and in organisms adherent to tracheal epithelium in a naturally infected calf (Morck et al. 1989). The large rigid fimbriae are composed of 35 kDa subunits (Potter et al. 1988). Failure to demonstrate these structures (Gilmour et al. 1985) may reflect the method of preparation rather than a variable capacity of the organisms to produce them.

Capsules

The glycocalyx of *P. haemolytica* is polysaccharide in composition, is produced during the logarithmic phase of growth, and can be visualised in organisms grown in vitro (Gilmour et al. 1985) and in vivo (Morck et al. 1988). The same material is present in the alveoli of experimentally and naturally infected cattle (Morck et al. 1988, 1989; Whiteley et al. 1990) and affects adversely the interaction between the organism and bovine alveolar macrophages (Czuprynski et al. 1991a). Furthermore, the capsular material may complex with pulmonary surfactant, thereby facilitating local adherence of the organism (Brogden et al. 1989).

Serotypic differentiation is based on the sugar composition of the capsular material. This composition is known for some of the 16 serotypes and those examined all have complex polysaccharides (Adlam et al. 1986). This same material appears to be a means of attachment to epithelial cells (Morck et al. 1988) and of withstanding the activity of both phagocytes and complement (Chae et al. 1990). Because the serotype-specific capsular polysaccharide of serotype A2 is composed of sialic acid, commonly found in host membranes, this component is poorly immunogenic (Adlam et al. 1987). On the other hand, capsular polysaccharide material may be strongly antigenic if that material contains surface proteins (McVey et al. 1990) Different capsular extraction procedures affect the amount of protein extracted; e.g. saline extraction (Lessley et al. 1985) extracts fewer proteins than salicylate extraction (Donachie et al. 1984).

Capsular polysaccharide uncontaminated by LPS does not stimulate interleukin-1 and tumour necrosis factor release by bovine phagocytes (Czuprynski et al. 1991b).

Lipopolysaccharide (LPS)

The basic composition of LPS from *P. haemolytica* is similar to that of other Gram-negative bacteria, and this component constitutes from 12–25% of the dried cell wall (Keiss et al. 1964). LPS composition differs between in vivo grown bacteria and those grown in vitro, and these differences are associated with differences in relation to opsonophagocytosis and complement-dependent killing (Sutherland et al. 1990). The polysaccharide portion of the LPS moiety is composed of repeating disaccharide units and there are differences between serotypes (Leitch and

Richards 1988; Perry and Babiuk 1984) and even within serotypes (Utley et al. 1992).

There are also similarities as indicated by reactivity of monoclonal antibody to serotype 1 0 antigen with other serotypes (Durham et al. 1988). The LPS constitutes a major surface antigen and serotypes of biotype A possess rough-type LPS while those of biotype T contain smooth-type LPS (Adlam 1989). Recently smooth-type LPS has been found in some biotype A serotypes (Utley et al. 1992). Electrophoretic analysis of the LPS of two serotype A1 isolates showed O side chains with high molecular mass bands, and a low-mass, core-oligosaccharide region that differed between the isolates. An A2 isolate had only a core-oligosaccharide region. No differences in LPS profiles were seen at various stages of the growth cycle (Davies et al. 1991).

The structure of the 0 antigen of serotype T10 lipopolysaccharide has been elucidated by NMR spectroscopy (Richards and Leitch 1989). Uncommon features of the LPS include the presence in some serotypes of rhamnose in the core oligosaccharides; and, in other serotypes, of sialic acid (Utley et al. 1992). The common antigen of Gram-negative bacteria is present in *P. haemolytica* (Tsai et al. 1988).

LPS can directly induce bovine endothelial cell injury in vitro by an ill-defined mechanism (Brieder et al. 1990) and this toxic effect may be reduced by neutrophils (Brieder et al. 1991). LPS can also stimulate TNF-α release from bovine alveolar macrophages (Bienhoff et al. 1992). Purified LPS exhibits the same activity in standard biological assays for endotoxin, as do similar fractions from other Gram-negative organisms (Rimsay et al. 1981).

Envelope proteins

Examination of envelope preparations by SDS-PAGE shows major differences between strains of *P. haemolytica* and of *P. multocida*, thus making separation of the two species possible on this basis (Knights et al. 1990; Rossmanith et al. 1991). Within *P. haemolytica*, the two biotypes A and T are also distinguishable by these means, but not the individual serotypes. The proteins are most likely located on the surface of the envelope and their expression differs according to whether the organisms are grown in vivo or in vitro (Donachie and Gilmour 1988). Nevertheless, antigens unique to in vivo grown organisms are recognised by protective serum derived from animals vaccinated with killed, in vitro grown organisms, which may suggest that these antigens are either precursors of, or share antigenic determinants with, other *P. haemolytica* proteins (Confer et al. 1992).

The surface composition of *P. haemolytica* is modified by the availability of iron (Deneer and Potter 1989) and iron-regulated OMP may be expressed in vivo (Morck et al. 1991). Given that in vivo conditions are iron depleted, features of the organism such as surface structure, hydrophobicity and virulence factors are probably affected in this situation.

The isolation of outer membranes and inner membranes of *P. haemolytica* A1 allowed for identification of their major proteins (Squire et al. 1984). Two major proteins, 30 kDa and 42 kDa, and several minor OMP, were found in the outer membrane, while the inner membrane contained five proteins in approximately equal amounts. A number of extraction methods used in the preparation of vaccines do not release significant amounts of the two major OMP. Antibodies against the 30 kDa protein are able to recognise 30 kDa and 15 kDa proteins in all serotypes of *P. haemolytica* (Craven et al. 1991). In addition, the 30 kDa protein can also be detected in untypeable strains (Simons et al. 1992).

Exotoxin

During the logarithmic growth phase, *P. haemolytica* produces a heat-labile protein cytotoxin (Baluyut et al. 1981). Supplementation of the cell culture medium with serum or serum proteins increases the speed and concentration of toxin production (Gentry et al. 1986). In addition, antigenic profiles, particularly in the higher kilodalton range, are affected by the presence of serum protein in culture media, as well as by the age of the culture (Confer and Durham 1992). This exotoxin acts as a pore-forming cytolysin (Clinkenbeard et al. 1989b) that causes damage to susceptible cells by forming transmembrane pores in plasma membranes. These pores have functional diameters of around 1 nm and they allow dissipation of K^+, Na^+ and Ca^{2+} transmembrane gradients (Clinkenbeard et al. 1989a). Following pore formation the cytolysin induces a Ca^{2+} dependent lysis of susceptible cells.

Specifically the toxin is lytic only for leucocytes, and in particular ruminant cells, which accounts for its terminology as a leukotoxin. Leucocytes affected include bovine macrophages, lymphocytes and cultured lymphoma cells, together with neutrophils from a number of ruminant species. Additionally the leukotoxin is able to lyse platelets, again from ruminant species only (Clinkenbeard and Upton 1991). This species specificity of cells susceptible to the action of leukotoxin is matched by the high susceptibility of ruminants and low susceptibility of non-ruminants to infection by *P. haemolytica* biotype A. Bovine lymphocyte blastogenesis is also inhibited by sublethal concentrations of the

leukotoxin (Majury and Shewen 1991), and the same effect may be observed on lymphocytes from other animal species. Further non-cytocidal effects of leukotoxin include stimulation of respir tory burst and degranulation of neutrophils (Maheswaran et al. 1992) and generation of arachidonic acid metabolites with potent chemotactic activity (Henricks et al. 1992).

The leukotoxin is a protein of about 100 kDa (Lo et al. 1985) that is heat-labile, oxygen stable, pH stable, non-dialysable and water soluble (Chang et al. 1986). The structural gene for the cytotoxin shares significant sequence homology with the α-haemolysin of *E. coli* (Lo et al. 1987), and the two proteins of *P. haemolytica* leukotoxin, designated LKtA and LKtC, are homologous to the *E. coli* HlyA and HlyC products respectively (Strathdee and Lo 1987).

There is also sequence homology with the leukotoxin gene from *Actinobacillus actinomycetemcomitans* (Kolodrubetz et al. 1989), *Morganella morganii*, *Proteus vulgaris* and *Proteus mirabilis* (Koronakis et al. 1987) and immunological cross-reactivity with the haemolysin of *Actinobacillus pleuropneumoniae* (Devenish et al. 1989). A pure DNA sequence encoding *P. haemolytica* leukotoxin has now been patented, as has an *E. coli* transformed by a plasmid vector containing this sequence and used for production of recombinant leukotoxin. Crude leukotoxin administered via the gut induces a pulmonary immune response in calves (Bowersock et al. 1992).

Plasmids

Plasmid-mediated antibiotic resistance has been demonstrated in *P. haemolytica* (Zimmerman and Hirsch 1980; Chang et al. 1987). The presence of plasmids is not universal within the species, but they can be recovered from *P. haemolytica* strains derived from a range of animal species (Haghour et al. 1987). Each strain carries only one plasmid, and plasmids are generally small (<3.4 MDa). A relationship between drug resistance and plasmid isolation has been found in a limited number of serotype 1 field isolates (Rossmanith et al. 1991). Not all plasmids present are associated with antibiotic resistance; and conversely, multiple resistance can be found in the absence of plasmids. Although all strains of *P. haemolytica* serotype A1 are lysogenic, no association has yet been shown with the production of toxin or other product.

Extracellular enzymes

The majority of A serotype, but not T serotype, strains produce neuraminidase (Frank and Tabatabai 1981), which may be cell-associated or in culture supernatants (Otulakowski et al. 1983). A neutral protease is also produced (Otulakowski et al. 1983). Little is known about the haemolysin that gives the species its name, and research of the past 30 years has told us no more than that its production is not plasmid mediated (Chang et al. 1987).

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The Interaction Between *Pasteurella haemolytica* and Ovine Mammary Gland Epithelium: Antigen Recognition, Adherence and Cytokine Production

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Abstract

Pasteurella haemolytica is a common cause of ovine mastitis in the UK. We have used milk whey and serum from immunised sheep and from clinical cases of mastitis to investigate antigen recognition in the ovine mammary gland. Total membrane protein or outer membrane protein (OMP) preparations of bacteria grown under iron-replete or iron-restricted conditions have been used in isotype-specific immunoblots. This has demonstrated clear differences in the pattern of antigen recognition by serum and mammary secretions. In particular, IgA antibody appears to recognise a number of large molecular weight antigens that are not apparent in IgG_2 or IgM immunoblots.

The interaction of *P. haemolytica* with ovine mammary gland epithelium has been studied in vitro by adding bacterial surface proteins to primary mammary epithelial cell cultures. After incubation at 37 °C for 10 min or 4 °C for 2 hours and repeated washing, epithelial cell preparations were run in immunoblots with antisera against *P. haemolytica*. A 70-kDa, pasteurella-derived, iron-regulated OMP was identified. The results suggest that this protein may be involved in binding *P. haemolytica* to mammary epithelium. Electron microscopy of in vitro preparations of bacteria with mammary epithelial cells has demonstrated *P. haemolytica* on and in the cells.

Further information on this host-pathogen interaction has been obtained by using the polymerase chain reaction to study cytokine production by mammary epithelial cells following interaction with the membrane proteins of *P. haemolytica*. These results indicate that mammary gland epithelial cells produce interleukin 6 and granulocyte and macrophage colony stimulating factor following interaction with membrane proteins of bacteria grown under iron-replete conditions.

PASTEURELLA haemolytica is a normal inhabitant of the upper respiratory tract of healthy sheep. It is also associated with disease processes such as pneumonia, septicaemia and mastitis (Jones 1991a,b). It is suggested that the main source of infection of *P. haemolytica* for the teats of the ewe is the mouth of the lamb (Jones 1991a). The infection usually follows an acute or hyperacute course, but in some cases subacute or subclinical mastitis may occur (Watkins and Jones 1992). The main feature is the rapid establishment of very severe inflammation, which is lethal.

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Several virulence factors are recognised in *P. haemolytica*, such as lipopolysaccharide (LPS), glycocalix, outer membrane proteins (OMP), fimbriae, and a very potent leukotoxin. The virulence mechanisms were recently reviewed by Confer et al. 1990. However, very little is known about the way in which *P. haemolytica* interacts with the ovine mammary gland and whether that interaction can influence the subsequent character of the mucosal immune response.

Materials and Methods

Bacteria

Most of the *P. haemolytica* strains used in this study were clinical isolates from ovine mastitis. Strains S19, S39 and S44 belong to the serotype A1. Strains ES26L and GW3 were kindly provided by Prof. J.E.T. Jones (Royal Veterinary College, London);

ES26L is an A9 isolate that consistently induces clinical mastitis, and GW3 is an isolate responsible for sub-clinical mastitis.

Some other strains were also used, for comparative purposes: an A1, an A2 and a non-typeable isolate from clinical cases of ovine pneumonia, and an A1 isolate from a clinical case of bovine pneumonia.

All the bacteria were maintained in liquid nitrogen and subcultured onto blood agar overnight, followed by culture in brain–heart infusion broth (BHI) at 37 °C, in a 5% CO_2 atmosphere. The iron-restricted growth conditions were achieved by adding 200 μM of the iron-chelator α-α'-dipyridyl to the broth (Deneer and Potter 1989).

Membrane preparations

Bacteria grown to the mid-exponential phase were harvested, washed twice in 10 m*M* HEPES, and sonicated 4 times for 30 sec at 5 A, in a Dawe Instruments sonicator (London). The supernatant was collected after centrifugation at 800 × g for 5 min and was designated total membrane preparation (IRTM). It was aliquoted and kept frozen at −20 °C. The OMP-enriched fraction (IROMP) was obtained by sarcosinate treatment of the IRTM preparation (Squire et al. 1984).

Isotype recognition of protein epitopes

This part of the study was performed using SDS–PAGE (Laemmli 1970) and immunoblotting (Towbin et al. 1979). Briefly, the proteins of both membrane preparations were separated in acrylamide electrophoresis gels and blotted onto nitrocellulose (BIO-RAD) or Immobilon (Millipore) sheets. The immunoblots were reacted with serum or milk whey at the appropriate dilutions for 1 hour at room temperature. After extensive washing, they were incubated with the different monoclonal antibodies overnight, at 4 °C. The monoclonal antibodies used were raised against sheep immunoglobulins: anti-IgA (84 2F9), anti-IgG_2 (192 4F10), and anti-IgM (102 JH). The immunoblots were then reacted with anti-mouse immunoglobulin raised in a goat and conjugated with peroxidase and developed with DAB or α-chloronapthol.

Mammary epithelial cell culture

Mammary glands from adult ewes were obtained from a slaughter house. The epithelial cells were collected by gently brushing the mammary cisternae and were transferred immediately into Hank's balanced salt solution (HBSS) containing antibiotics. The cells were washed twice in HBSS and plated to a density of 2×10^5 onto a variety of substrates: plastic, glass, collagen gels, and polyester (Melinex, Agar Scientific) or polystyrene membranes (Costar).

The cells were grown to confluence in a medium containing lactogenic hormones (hydrocortisone 1 μg/mL, prolactin 6.25 IU/mL, and insulin 2.5 μg/mL). The cells were characterised as epithelial by cytokeratin staining with monoclonal antibodies (Cyto-1, Sigma).

Bacterial adherence

For testing adherence to cultured mammary epithelial cells, the different isolates of *P. haemolytica* were grown for 12 hours on blood agar plates, harvested, and washed twice with HEPES 10 m*M*. The bacteria were resuspended in HBSS and the concentration adjusted to 10^6 cells/mL. Epithelial cells were grown to confluency on 24-well tissue culture plates (Nunc). The epithelial cell monolayer was washed twice with HBSS and the bacteria were incubated with the cells for 10 min at 37 °C, followed by 2 hours at 4 °C. The non-adherent bacteria were then thoroughly washed with HBSS. The epithelial cells were processed by thin-sectioning and observed by transmission electron microscopy.

Ligand-binding assay

This assay is a modification of the technique originally described by Oblas et al. (1983) for the study of the interaction of proteins with mammalian cells. Briefly, epithelial cell monolayers were incubated with whole bacteria (10^6/mL), IRTM (200 μg/mL) or IROMP (100 μg/mL), for 10 min at 37 °C and 2 hours at 4 °C. After extensive washing, the cell monolayer was lysed using the SDS–PAGE sample buffer, with SDS and 2-mercaptoethanol. This lysate was run on SDS–PAGE, blotted onto Immobilon and reacted with antiserum against *P. haemolytica*.

Epithelial cell stimulation

Epithelial cells were grown to confluency in 6-well tissue culture plates (Nunc). Confluent monolayers were stimulated with the *P. haemolytica* membrane preparations (IRTM) in culture medium from which hydrocortisone was omitted 24 hours before. The final protein concentrations were 1, 10, 20, 50 and 100 μg/mL and the cells were stimulated for 24 hours.

LPS from *Escherichia coli* serovar O127:B8 (Sigma) was also used as a stimulant, at the same concentrations as the IRTM.

Cytokine gene expression

The expression of the genes coding for IL-1α, IL-1β, IL-6, and granulocyte and macrophage colony

stimulating factor (GMCSF) was investigated by the polymerase chain reaction (PCR). Briefly, total RNA was isolated from the epithelial cells with RNAsol (Promega), according to the manufacturer's instructions. The correspondent cDNA was then synthesised and the PCR performed using specific primers.

Results

Isotype specific recognition of surface antigens

The surface antigens of *P. haemolytica*, IRTM and IROMP produced different recognition patterns when reacted with the different immunoglobulin isotypes in serum and milk whey. Immunoblots of total membrane proteins and of the OMP-enriched fraction of *P. haemolytica* S19 were reacted with serum and milk whey from ewes with mastitis and ewes repeatedly immunised with an available commercial vaccine against pasteurellosis. Although there were slight differences in the recognition pattern of all the isotypes, the most striking feature was the recognition pattern of IgA. In serum, from both mastitis and hyperimmunised ewes, proteins from all the range of sizes were detected. When reacted with mastitis milk whey, there was a clear preferential recognition of proteins with a molecular weight of 100 kDa and 120 kDa. Other strains of *P. haemolytica* isolated from clinical mastitis revealed a similar antigenic profile. In contrast, IgA in colostral whey from hyperimmunised ewes showed strong recognition of two proteins of 70 kDa and 90 kDa.

Adherence of *P. haemolytica* to cultured mammary epithelial cells

When log-phase bacterial cultures were incubated with mammary epithelial cells and then examined by transmission electron microscopy, it was possible to see some bacteria in close contact with the epithelial cells. These bacteria were not removed by extensive washing of the cell monolayer. In addition to adherence, it was also evident that bacteria were present inside the epithelial cells. They were internalised after 2 hours incubation at 4 °C.

Identification of surface proteins that bind to epithelial cells in vitro

When total membrane preparations of *P. haemolytica* (200 μg/mL) were used in the ligand-binding assay, we could detect proteins with molecular weights of 94 kDa and 110–120 kDa bound to epithelial monolayers. When IROMP preparations (100 g/mL) were used, the major OMP could also be detected, in addition to those mentioned above.

Cytokine gene expression

Cultured mammary epithelial cells were able to express the genes coding for some cytokines, under different stimulation conditions. Il-1α was present in all the cells tested, with or without stimulation. Il-1β was induced by low doses of *E. coli* LPS (1 and 10 μg/mL), but not by higher doses of LPS nor by *P. haemolytica* IRTM preparations. Il-6 was detected in epithelial cells exposed to as little as 1 μg/mL IRTM preparation and throughout the whole range of concentrations tested, but not in LPS-stimulated cells. GMCSF could be induced by 10–100 μg/mL of *P. haemolytica* IRTM, but not by any LPS concentration tested.

Discussion

Crude total membrane preparations (IRTM) containing the iron-regulated proteins, and OMP-enriched fractions (IROMP), when separated by electrophoresis and reacted with serum or milk whey, showed marked differences in the way they were recognised by the different antibody isotypes. No predominant protein was recognised by total IgG, or by the IgG_2 or IgM isotypes, although there were some minor differences. In contrast, IgA in mastitis whey seemed to preferentially recognise proteins with a very specific range of molecular weights of 110 kDa and 120 kDa.

The identity of these antigens is unknown, but it is interesting that an iron-regulated membrane protein of similar size, 100 kDa, has been identified as a specific bovine transferrin receptor (Ogunnariwo and Schryvers 1990; Yu et al. 1992). It is known that, of all secretory organs, the ruminant mammary gland has the least well developed IgA system and that there is a predominance of IgG_1 relative to IgA (Craven and Williams 1985). However, IgA synthesis can be stimulated by local infusion of antigen during mammary gland involution (Sheldrake and Husband 1985). The main effect of IgA in the gland may be to block bacterial adhesion to mammary gland epithelium (Craven and Williams 1985).

In order to gain additional information about the mechanisms by which *P. haemolytica* exerts its pathogenic effect within the mammary gland, we looked at the interaction between clinical isolates and cells derived from normal mammary epithelium. Transmission electron microscopy showed bacteria associated with the surface of the epithelial cells as well as within the cytoplasm of the same cells. The mechanism by which the internalisation process occurs is not known. Surface proteins that trigger binding and uptake into target cells have been identified in several microorganisms (Melo and Pechere 1990). The major OMP and the 94–120 kDa *P.*

haemolytica surface antigens were able to associate with the epithelial cells and may thereby play a role in the intracellular entry of the bacteria.

Epithelial cells, by virtue of their location, are likely to be the first mammary cells to interact with pathogens. Other epithelial cells have been shown to modulate the local cellular environment by releasing cytokines and other chemotactic factors for neutrophils and lymphocytes (Elmsie et al. 1992; Hedges et al. 1992).

The present study demonstrates that cultured mammary gland epithelial cells express the genes coding for some pro-inflammatory cytokines. The IL-1α gene seems to be contitutively expressed in this in vitro system independently of any bacterial stimulation. It could be either a feature of ovine mammary epithelial cells, or an artifact due to the culture conditions. IL-1β is induced only at lower doses of *E. coli* LPS stimulation. The two cytokines IL-6 and GMCSF seem to be induced by a specific interaction with *P. haemolytica* surface antigens, as they are not detected in the control unstimulated cells or in the *E. coli* LPS-stimulated ones.

IL-6 plays an important role in regulating antibody production in vitro as well as in vivo. During inflammatory processes, IL-6 mediates the final maturation of proliferating B cells into specialised immunoglobulin-secreting plasma cells and participates with IL-1 and TNF in the induction of acute-phase reaction (Elmsie et al. 1992). Using a mouse model, IL-6 was shown to induce high rate IgA secretion in IgA-committed B cells (Beagley et al. 1989). GMCSF has a broad range of activities in addition to stimulating the growth of granulocyte and macrophage colonies in vitro. GMCSF stimulates the antimicrobial activity of mononuclear phagocytes, as well as being a potent stimulator of eosinophil and neutrophil function (Gregory et al. 1991). So far, studies regarding cytokine production and activity in the ruminant mammary gland have been focused on IL-2 (Sordillo et al. 1991) and IFN-gamma (Sordillo and Babiuc 1991).

The present in vitro study suggests that other cytokines, such as IL-6 and GMCSF, may play an important role in the local response to bacterial invasion of the mammary gland, particularly by *P. haemolytica*. The interaction between surface antigens of *P. haemolytica* and epithelial cells is an early event in the establishment of mastitis. Some of the bacterial surface antigens bind in vitro to the epithelial cells. Ovine mammary epithelial cells respond to in vitro stimulation by *P. haemolytica* surface antigens and express the genes coding for IL-6 and GMCSF. These cytokines are important inflammatory factors and could be involved in the local immune response to *P. haemolytica*.

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Hyaluronic-Acid Mediated Adhesion of *Pasteurella multocida* to HeLa Cells

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Abstract

Adhesion of bacteria to host cells is a crucial step in colonisation and disease pathogenesis. In this connection, further studies were conducted to analyse the attachment mechanisms of *Pasteurella multocida* to HeLa cells. Of 10 strains of *P. multocida*, belonging to serotypes A, B, D and E, all six of serotype A strains adhered strongly to HeLa cells. Exposure of the six strains to several physical, chemical and enzymatic protein-denaturating treatments did not affect adhesion. However, treatment of the bacteria with hyaluronidase clearly reduced their adhesive capacities. Pre-treatment of the HeLa cells with hyaluronic acid, or with extracts of the adherent pasteurellae containing hyaluronic acid, also reduced adhesion. Thus, it could be suggested that adhesion of the six *P. multocida* A strains was mediated by capsular hyaluronic acid.

IN the process of infection, bacterial adhesion to host cells is significant in the pathogenesis of disease. This research, on the adhesive capacity of *Pasteurella multocida*, was stimulated by a report of Glorioso et al. (1982) on adhesion of *P. multocida* serotype A (Carter) to HeLa cells.

Materials and Methods

Pasteurella multocida strains

The 10 strains of *P. multocida* used in this study (Table 1) were cultured overnight at 37 °C in brain-heart infusion (BHI) (Gibco, Eggenstein, FRG). Then the bacteria were centrifuged at 11 000 × g and the pellets were re-suspended in HANKS balanced salt solution (HBSS) (Sigma, Deisenhofen, FRG) and adjusted to an optical density of 1.0 at 620 nm. The suspensions were stained with fluoresceine isothiocyanate at pH 7.4 (Valentin-Weigand et al. 1987).

Cell cultures

HeLa S_3 cells, provided by H. Muller, Institute of Pharmacology, University of Giessen, were cultured on glass slides in minimal essential medium (MEM) (Sigma) containing 10% foetal calf serum (Gibco) for 24 hours at 37 °C under 5% CO_2 and then transferred to Petri dishes containing 4 mL of HBSS. Prepared in this manner, the HeLa cells served in subsequent adhesion assays.

Adhesion assays

Of the fluoresceine-stained bacteria, 100 μg were added to each glass slide of HeLa cells, incubated for 1 hour at room temperature, and then washed three times in HBSS containing 0.05% Tween 20 (Sigma). The numbers of bacteria adhering to 5–20 randomly chosen HeLa cells per slide were counted by fluorescence microscopy.

For further analysis of their adhesive activities, the unstained bacterial preparations were subjected to the following (bacterial surface-altering) treatments prior to the adhesion assays:

- 1 hour at 95 °C in HBSS at pH 5 (Tojo et al. 1988)
- 6 hours at 37 °C in saline containing 0.5*M* KSCN (Ringler et al. 1985)
- 1 hour at 25 °C in 6*M* guanidinium chloride, pH 2 (Chhatwal et al. 1984)
- exposure to either 250 units/mL trypsin (Gibco), 100 mg/mL pronase E (Merck, Darmstadt, FRG) or 50 units/mL testicular hyaluronidase (Sigma) respectively in modified HBSS (mHBSS) (without calcium and magnesium) (Sigma) for 1 hour at 25 °C.

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After each of these treatments the bacteria were stained with fluoresceine and tested for adhesion.

Determinations of binding sites for adhesions on the HeLa cells were attempted by 'blocking' experiments. Sets of HeLa cells were exposed for 1 hour at 25 °C to one of the following substances contained in HBSS:

- 10 mg/mL N-acetylglucosamin (Sigma)
- 10 mg/mL D-glucuronic acid (Sigma)
- a mixture of 10 mg/mL N-acetylglucosamin and 10 mg/mL D-glucuronic acid
- 0.5, 1, 2, 4 or 10 mg/mL of hyaluronic acid (Sigma)
- crude dialysed KSCN extract from the pasteurellae (Ringler et al. 1985).
- crude hyaluronidase extract from the bacteria (Carter 1972).

Uronic acid content of the extracts was estimated according to Dische (1968), with D-glucuronic acid and hyaluronic acid as standards. After each treatment, 100 L of fluoresceine-stained bacteria were added to the HeLa cells.

Results

All six *P. multocida* strains of serotype A adhered strongly, with 50 or more bacteria per HeLa cell. The four strains of the other serotypes (B, D and E) adhered to a significantly lower degree, with fewer than five bacteria per HeLa cell (Table 1). Adhesions first became evident after 5 min and reached highest levels after 30 min of incubation at room temperature.

Adhesive activities of the pasteurellae were neither reduced nor enhanced after pre-treatments of bacteria with heat at pH 5, KSCN, guanidinium chloride, trypsin or pronase. However, adhesion diminished after exposure of the adherent pasteurellae to hyaluronidase (Table 2).

Pre-incubation of the HeLa cells with N-acetylglucasamin, D-glucuronic acid or a mixture of both did not affect adhesion. On the other hand, pre-incubation of the cells with at least 1 mg hyaluronic acid per mL HBSS markedly reduced the extent of adhesion. The KSCN and hyaluronidase extracts from the adherent strains containing more than 50 μg of hyaluronic acid per mL also inhibited adhesion (Table 3 and Fig. 1).

Table 1. Adhesive capacities of *Pasteurella multocida* strains.

Strain	Serotype (Carter)	Adhesive capacity*
1013-6[1]; (NCTC 10322)	A	61.2 (14.6)
815-6[1]; (Carter T4967)	A	61.4 (13.9)
A4N[2]	A	59.8 (11.3)
B575N[2]	A	60.7 (9.3)
165/92[3]	A	65.7 (13.3)
755[4]	A	64.0 (10.0)
844-5[1]; (NCTC 10325)	D	4.7 (1.8)
844-6[1]; (NCTC 10323)	B	0[5]
815-7[1]; (Carter 1309)	B	3.0 (1.1)
844-7[1]; (NCTC 10326)	E	0[5]

NCTC, National Collection of Type Cultures, Central Public Health Laboratory, London, United Kingdom
* Expressed by number of adhering bacteria per HeLa cell (standard deviation)
[1] Kindly supplied by Zentrum fur Hygiene und Medizinische Mikrobiologie, Marsburg, FRG
[2] Strains isolated from infections of rabbits
[3] Cultured from rabbits by Zentral Tierversuchsanlage, Medizinische Hochschule, Hannover, FRG
[4] Isolated from human bronchitis by Institut fur Medizinische Mikrobiologie, Giessen, FRG
[5] Adhesions to the cells were not higher than the background adhesion

Discussion and Conclusion

Bacterial adhesion to host cells could facilitate the establishment of infection for *P. multocida*. Haemagglutinating substances as well as fimbriae have been described as adhesions (Pestana de Castro et al. 1980; Fortin and Jacques 1987; Trigo and Pijoan 1988). Previous authors have studied the interaction of pasteurellae with respiratory epithelial cells (Rivera et al. 1986; Jacques 1987; Nakai et al. 1988; Trigo and Pijoan 1988; Chung et al. 1990). Glorioso et al. (1982) reported the adherence of *P. multocida* serotype A (Carter) to HeLa cells. Their findings indicated that a fimbrial protein was the substance used for adhesion.

Our results confirm the adhesion of *P. multocida* serotype A to HeLa cells. While the extent of adhesion could not be markedly reduced by protein-denaturating procedures, adhesion decreased significantly after treatment of the bacteria with hyaluronidase. The degree of adhesion also diminished after treatment of the HeLa cells with hyaluronic acid or bacterial extracts containing hyaluronic acid. Thus, we concluded that hyaluronic acid in the capsule of *P. multocida* serotype A strains (Rosner et al. 1992) could play a role in the bacterial adhesion to HeLa cells. The observation that the monomers of hyaluronic acid, N-acetylglucosamin and D-glucuronic acid, did not affect adhesion was in agreement with Turley (1991), who discussed the necessity of hexamers or decamers of hyaluronic acid for adhesion to specific receptors or extracellular proteins in host tissues.

Table 2. Adhesive capacities of adherent *P. multocida* strains after physical, chemical or enzymatic treatments.

	Adhesive activities of *Pasteurella multocida* strains*						
Strains	Untreated bacteria	Heat (pH 5)	KSCN	Guanidinium chloride	Pronase	Trypsin	Hyaluronidase
1013-6	62.9 (33.5)	53.1 (24.2)	56.0 (14.0)	69.0 (19.1)	58.0 (28.8)	53.4 (16.3)	8.2 (6.8)
815-6	65.9 (16.8)	76.6 (28.2)	59.2 (19.3)	97.2 (17.8)	64.9 (17.9)	43.6 (14.2)	14.4 (9.6)
A4N	64.2 (12.0)	60.0 (7.5)	60.5 (11.2)	59.4 (5.0)	50.2 (11.9)	52.4 (11.3)	4.3 (2.5)
B575N	64.0 (14.0)	64.2 (8.3)	61.0 (9.3)	59.4 (9.7)	60.6 (7.2)	50.8 (9.8)	4.6 (1.8)
165/92	59.4 (9.1)	63.6 (6.9)	59.9 (9.1)	58.6 (11.1)	50.8 (5.4)	55.6 (6.9)	26.1 (8.4)
755	62.5 (12.4)	61.2 (7.6)	57.2 (7.4)	55.8 (5.8)	51.4 (7.0)	52.2 (14.2)	14.3 (6.9)

* Numbers of adherent pasteurellae per HeLa cell; standard deviation in parentheses

Table 3. Adhesion of pasteurellae after pre-treatments of the HeLa cells.

	Adhesive activities of *Pasteurella multocida* strains*				
Strain	Untreated control	N-Acetylglucosamin	D-Glucuronic acid	N-Acetulglucosamin + D-Glucuronic acid	Hyaluronic acid
1013-6	62.9 (18.5)	60.3 (21.8)	48.6 (10.7)	50.8 (7.2)	8.2 (4.8)
815-6	66.9 (16.8)	55.7 (19.8)	56.7 (17.4)	61.4 (14.3)	5.0 (3.4)
A4N	63.6 (12.0)	60.8 (11.0)	62.0 (12.4)	62.6 (9.4)	2.6 (1.1)
B575N	69.6 (16.0)	63.4 (10.5)	67.6 (10.4)	61.0 (6.9)	2.8 (1.4)
165/92	64.4 (11.4)	64.6 (14.8)	62.2 (6.1)	60.8 (8.8)	3.0 (1.3)
755	63.0 (7.3)	66.0 (11.2)	60.2 (11.6)	66.6 (9.4)	2.9 (1.4)

* Numbers of adherent pasteurellae per HeLa cell; standard deviations in parentheses

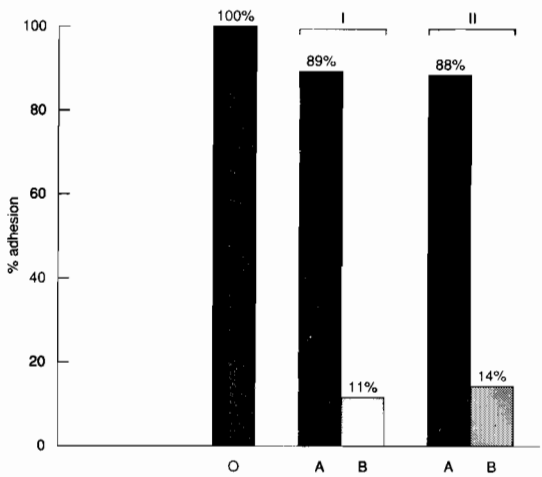

Fig. 1. Average inhibition determinations of bacterial adhesion after pre-treatments of HeLa cells with KSCN (I) or hyaluronidase (II) extracts from the non-adherent (A) and the adherent (B) *P. multocida* strains.
I, KSCN treatment conditions
II, hyaluronidase treatment conditions
O, average adhesion values of adherent pasteurellae to untreated HeLa cells (= 100%) (Tables 1-3).
A, containing $<200\ \mu g/mL$ hyaluronic acid
B, containing $>500\ \mu g/mL$ hyaluronic acid

Several attempts have been made to develop a protective vaccine against pasteurellosis in rabbits, mainly caused by *P. multocida* serotype A (Manning et al. 1989). These vaccines contained KSCN extracts from the bacteria (originally described by Mukkur 1979), purified bacterial outer membranes (Lu et al. 1991), or living pasteurellae (DiGiacomo et al. 1987). The vaccines protected animals against sickness induced by homologous *P. multocida* strains, but did not prevent colonisation of nasal cavities (Lu and Pakes 1981; Ringler et al. 1985; Lu et al. 1987; DiGiacomo et al. 1987; Lu et al. 1991). This may be explained by the fact that hyaluronic acid, which acts as mediator of the adhesion, is a poor immunogen.

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Serology and Virulence of Haemorrhagic Septicaemia *Pasteurella multocida* Isolated from Domestic and Feral Ruminants

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Abstract

Two serotypes (B:2 and E:2) of *Pasteurella multocida* cause haemorrhagic septicaemia (HS) of cattle and buffalo. Recent findings show that serotypes B:1, B:3,4 and B:4, as well as serotype B:2, cause HS in feral ruminants (antelope, deer, elk and bison). HS strains from domestic and feral ruminants were examined for hyaluronidase production, virulence for young turkeys, and toxin production. All of 54 serotype B:2 strains produced hyaluronidase, whereas other serotypes in group B and serotype E:2 did not. Of three serotype B:3,4 strains, all were virulent for young turkeys. None of three serotype B:2, three serotype E:2 and one serotype B:4 strains was virulent. Toxin production was tested in mice. Toxin was not detected in sonicates of any of ten serotype B:2, seven serotype E:2 and two serotype B:3,4 strains.

HAEMORRHAGIC septicaemia (HS) is an acute septicaemic disease principally affecting cattle and buffalo. In these domestic species, HS is a primary pasteurellosis caused by two specific serotypes of *Pasteurella multocida*, serotypes B:2 and E:2 (De Alwis 1992). Occasionally, a disease that is clinically similar to HS of cattle and buffalo is reported in feral ruminants (Gochenour 1924; Nordkvist and Karlsson 1962; Jones and Hussaini 1982; Franson and Smith 1988). Serologic examination of *P. multocida* isolated from HS-like disease in feral ruminants has shown some strains to be of the B:2 serotype, whereas other strains have the group B capsule antigen but different somatic antigen (Rhoades and Rimler 1992).

Although the B:2 serotype is not known to cause disease in avian species, other *P. multocida* strains with the B capsule antigen have been isolated from birds occasionally. Sometimes these avian strains have the same antigenic formula as those isolated from feral ruminants.

Rabbit antibodies directed against the specific capsule antigen of serotype B:2 passively protect mice against other group B strains, regardless of somatic serotype or animal origin. Although passive immune crossprotection can be demonstrated in mice, differences in pathogenic properties among the serogroup B strains have not been adequately demonstrated.

In this study, strains of *P. multocida* that cause HS in cattle, buffalo and swine, and those that cause HS-like disease in feral ruminants, were compared for potential pathogenic properties; i.e. hyaluronidase/chondroitinase activity, virulence for avian species, and toxin production.

Materials and Methods

Bacteria

Strains of *P. multocida* isolated from different animal species in different countries were from the National Animal Disease Center culture collection.

Hyaluronidase/chondroitinase

Bacteria were tested for hyaluronidase and chondroitinase activity after 24 hours growth on heart-infusion agar containing substrate (Smith and Willett 1968).

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Virulence

Capsulated bacteria grown on dextrose starch agar for 18 hours at 37 °C were used. Cultures were suspended in tryptose broth and adjusted tubidometrically to contain 109 colony-forming units (CFU) per mL. Serial 10-fold dilutions were made, and groups of five 9-day-old turkeys were inoculated intraperitoneally or intramuscularly with 10^3, 10^4, 10^5 or 10^6 CFU. Turkeys were examined daily for 9 days, at which time they were killed and necropsied.

Toxin production

CD1 mice (22 g; Harlan-Sprague-Dawley) were inoculated with filter-sterilised sonicates of bacteria, as previously described (Rimler and Brogden 1986).

Results and Discussion

Hyaluronidases are enzymes that have been associated with invasive mechanisms of bacteria, helminths, and snake venoms. Most bacterial hyaluronidases have activity for hyaluronic acid, but not for chondroitin. All of 54 serogroup B:2 strains produced an enzyme with both hyaluronidase and chondroitinase activity (Table 1). Other serotypes did not possess activity for either substrate.

The B and E serogroups differed regarding virulence for young turkeys (Table 2). Strains of the B:3,4 serotype were virulent, with at least 1000 CFU causing death of all birds usually within 48 hours, regardless of route of inoculation. The B:2, B:4 and E:2 serotypes did not produce clinical disease at any level of bacteria tested, and no lesions were seen at necropsy. Deaths in groups inoculated with the two serotype B:1 strains were sporadic. Four turkeys died among the various groups with one B:1 strain, whereas 10 turkeys died among the various groups with the other strain. Gross lesions were not seen in the remaining turkeys at necropsy.

Table 1. Hyaluronidase/chondroitinase production by *P. multocida*.

Number positive/ total	Serotype	Source
0/4	B:1	antelope, swans
54/54	B:2	bison, deer water buffalo sheep, swine, yak
0/8	B:3,4	cattle, deer, elk
0/5	B:4	bison, turkeys
0/6	A:2	bighorn sheep, turkeys
0/13	E:2	cattle, swine

Table 2. Virulence of HS *P. multocida* for 9-day-old turkeys.

Strain	Serotype	Source	Origin	Virulence
P-1495	B:1	antelope	USA	−/+[a]
P-1511	B:1	antelope	USA	−/+
M-1404	B:2	bison	USA	−[b]
P-4983	B:2	buffalo	Sri Lanka	−
P-4996	B:2	swine	India	−
P-1458	B:3,4	bison	USA	+[c]
P-4675	B:3,4	deer	UK	+
P-5227	B:3,4	elk	USA	+
P-5325	B:4	bison	Canada	−
P-4099	E:2	cattle	Zambia	−
P-4109	E:2	cattle	Sudan	−
P-4367	E:2	cattle	Nigeria	−

[a]sporadic death occurred among different groups (see text)
[b]no deaths occurred at $< 10^6$ CFU
[c]5/5 turkeys died at $< 10^3$ CFU

The B:1, B:3,4, and B:4 serotypes have been isolated from disease in avian species (Rhoades and Rimler 1992; Rhoades et al. 1992). Because these serotypes can be isolated from mammalian sources and are sometimes virulent for avian species, transmission of certain strains between birds and mammals may be possible under natural conditions.

Although toxin has been identified in serogroup A and D strains of *P. multocida*, a similar toxin was not found in 19 strains of the B or E serogroups (Table 3).

Table 3. HS *P. multocida* found negative for toxin production.

No. strains tested	Serotype	Source	Origin
10	B:2	bison, deer, buffalo, swine, yak	China, India, Malaysia, Philippines, USA
2	B:3,4	elk	USA
7	E:2	cattle	Nigeria, Mali, Zimbabwe

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Virulence, Purification, Structure, and Protective Potential of the Putative Capsular Polysaccharide of *Pasteurella multocida* Type 6:B

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Abstract

Initial experiments carried out with protein-free, enriched capsular polysaccharide (CPS), purified lipopolysaccharide (LPS), and potassium thiocyanate (KSCN) extract revealed that, while all were antiphagocytic in an in vitro phagocytic uptake assay, CPS was the most potent. As a prelude to evaluating its vaccine potential against haemorrhagic septicaemia, a procedure was developed to isolate the putative CPS in a highly purified state. The monosaccharide composition and the linkage structure of the CPS thus purified revealed that it contained mannose, galactose and arabinose, and was a branched polymer. The molecular weight of CPS under non-denaturing conditions was 900 000 Da, suggesting it to be a decamer. Purified CPS in its native state was found to be non-toxic and non-protective.

CAPSULAR polysaccharides (CPS) are important surface determinants present on some pathogenic Gram-negative and Gram-positive bacteria, e.g. pneumococci, meningococci, *Haemophilus* spp. and clostridia. They have been considered to constitute an important virulence attribute because of their antiphagocytic effect typified by pneumococci (White 1979). As such, considerable efforts have been expended on elucidating the structure of CPS of human pathogens and on evaluation of their potential as vaccines (Cruse and Lewis 1989). The genus *Pasteurella* has several species that are encapsulated and are involved in the etiology of economically significant diseases of food-producing livestock; e.g. *P. haemolytica* (shipping fever pneumonia), *P. multocida* type A (shipping fever pneumonia, swine enzootic pneumonia, fowl cholera, snuffles in rabbits), *P. multocida* type D (atrophic rhinitis in swine) and *P. multocida* types B and E (haemorrhagic septicaemia in cattle and buffalo).

Consistent with the majority of bacterial polysaccharides, various CPS types of *P. multocida* (and *P. haemolytica*) mentioned above are based on distinct immunological specificity. While the structure of the oligosaccharide repeating unit of the CPS of *P. multocida* type A and some serotypes of *P. haemolytica* has been elucidated (Adlam et al. 1984, 1985a,b) no such information is available on the structure of types D, B or E. However, procedures for isolation of the CPS of *P. multocida* types B and E, and evaluation of their protective potential in mice and cattle, have been reported (Penn and Nagy 1976; Nagy and Penn 1976). Further, although the antiphagocytic nature of the *P. haemolytica* A1 CPS (for the phagocytosis of these organisms by alveolar macrophages in vitro) was recently reported (Czuprynski et al. 1991) no information on the modulatory role of CPS of *P. multocida* serotypes is available. It was the aim of this investigation to: (a) determine the antiphagocytic potential of crude or purified CPS of *P. multocida* type B; (b) evaluate and/or develop a procedure for its purification; and (c) determine its monosaccharide composition and linkage structure.

Materials and Methods

Bacterial strain and growth conditions

P. multocida type 6:B strain C82, obtained through the courtesy of Dr Chandrasekaran of the Veterinary

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Research Institute, Ipoh, Malaysia, was used throughout this investigation. The culture was stored frozen in brain–heart infusion broth (BHI) containing 20% glycerol in a biofreezer at −70 °C.

The organisms were grown for 12–14 hours in BHI in 10 L capacity fermenters. Concentration and washing were accomplished using the Amicon hollow-fibre concentrator (model DC2).

Animals

Specific pathogen free BALB/c mice, 6–8 weeks of age, were obtained from the University of New South Wales Animal Facility. They were housed in cages (10 per cage) and supplied with food and water ad lib.

Determination of minimum moribund dose

Mice were injected subcutaneously with 0.1 mL of different dilutions (10^{-7}–10^{-9}) of a 12-hour-old BHI culture of *P. multocida* type 6:B grown from a single colony on a sheep blood agar (SBA) plate. The medium used for preparation of dilutions was Hanks medium 199, pH 7.2. The inoculated mice were monitored four times per day over a period of 96 hours for the onset of the moribund state and mortalities. In challenge experiments, mice were inoculated subcutaneously with 0.1 mL 10^{-7} dilution in Hanks medium 199 and monitored for 96 hours for the onset of the moribund state and mortalities. The minimum moribund dose (MMD) of *P. multocida* types 6:B strain C82 was found to be 2 CFU.

Analytical methods

Total carbohydrate concentration was determined by the method of Dubois et al. (1956) using glucose as standard. Protein content was measured by a modified Lowry procedure (Markwell et al. 1978) and 2-keto-2 deoxyoctulosonic acid (KDO) measured according to Karkhanis et al. (1978). Nucleic acid and protein peaks in chromatographic runs were monitored by absorption at 260 and 280 nm respectively.

Preparation of LPS and KSCN extract

LPS was prepared according to Westphal and Jann (1965), whereas the KSCN extract was prepared according to Mukkur (1979).

Purification of CPS

The initial method used for purification of CPS (CPSI) was that of Penn and Nagy (1976), whereas the second method used was developed in this investigation (CPSII) and is described in the text under Results.

Determination of component monosaccharides of CPS, and linkage structure

The absolute molar content of monosaccharides present in CPS of *P. multocida* type 6:B and linkage structure were determined through the courtesy of Oxford Glycosystems Ltd, UK. The monosaccharide composition analysis involved the use of anhydrous methanolic HCl to liberate monosaccharides as the 1-O-methyl derivatives, subsequent N-acetylation of any available primary amino groups, and the conversion of individual monosaccharides in per-O-trimethylsilyl (TMS) methyl glycosides.

An aliqout of the TMS methyl glycoside was separated on a GLCMS system by capillary GLC using a CP-SIL8 column. Identification of individual methyl glycosides was based on retention time and mass spectrum by comparison with standard reference TMS methyl glycosides. Quantitation of individual TMS methyl glycosides was achieved using the signal from a flame ionisation detector (FID).

A known amount of a monosaccharide standard (scyllo-inositol) was added (prior to addition of methanolic HCl) to both the mixture of standard monosaccharides and to the sample. This served as an internal standard so that the relative molar response factor of the individual TMS methyl glycosides could be calculated for each monosaccharide standard. These response factors were then used to determine the absolute molar monosaccharide content of the carbohydrate polymer. For linkage analysis, the protocol outlined below was followed.

A weighed portion (500 μg) of the carbohydrate polymer was cryogenically dried over activated charcoal at 198 °C ($< 10^{-6}$ bar) for 3 days. The polymer was then methylated by dissolving in anhydrous dimethyl sulphoxide (500 μL) and treated with methylsuphinyl methyl sodium in dimethyl sulphoxide (400 μL, 2*M*) and anhydrous methyl iodide (300 μL) over 6 hours, with constant stirring. The methylated carbohydrate polymer was purified by partitioning the reaction mixture in chloroform (0.8 mL) and washing with (a) sodium thiosulphate solution (2 mL, 0.1% w/v) and (b) 10 × 2 mL water, the aqueous layer being discarded in each case. After evaporation of chloroform the methylated carbohydrate polymer was hydrolysed by formic acid: trifluoroacetic acid:water (10:1:2) at 100 °C for 16 hours.

The hydrolysing reagent was removed by evaporation under reduced pressure and the methylated monosaccharides produced were reduced by incubation in aqueous sodium borohydride (5 mg/mL, pH 11.0) at 30 °C for 4 hours. Excess borohydride was destroyed by the addition of acetic acid and the

reaction mixture was dried under reduced pressure. The partially methylated alditols were purified by repeated (×10) flash evaporation of the mixture with methanol.

Finally the partially methylated alditols were 0-acetylated by treatment with pyridine (5 mg/mL; 150 μL) at ambient temperature for 4 hours. The acetylating reagent was removed by evaporation under reduced pressure, and the partially methylated alditol O-acetates were taken up in dichloromethane (0.3 mL) and washed (×3) with 1 mL water. The dichloromethane layer was dried under reduced pressure and the residue taken up in 20 μL dichloromethane. An aliquot (1 μL) of this mixture was injected onto a CPSil8 capillary G-C column (30 m) and permethylated alditol acetates (PMAA) were identified by their retention times to known standards and by mass spectrometry.

Molecular weight determination

The apparent molecular weight of the purified CPS was determined by gel filtration on a Sepharose 6B column (2.0 × 100 cm) equilibrated with PBS, pH 7.2, at a flow rate of 15 mL/hour. The void volume (Vo) and total bed volume (Vi) were determined using Blue Dextran 2000 (Pharmacia) and 2,4 dinitrobenzoic acid-Na salt respectively. Calibration of the column was performed with dextran polymers of varying molecular weights — 39, 73.5, 298 and 500 kDa (Sigma Chemical Co., St Louis, USA). Fractions were monitored for total carbohydrate.

To determine whether the CPS was aggregated, a purified sample was chromatographed on Sepharose 6:B (2.6 × 100 cm), equilibrated with 0.1*M* citrate buffer (pH 2.3) containing 0.1% zwittergent as detergent.

Toxicity and protective potential of purified CPS (CPSII)

To investigate the protective potential of the purified CPS, mice were injected with graded doses of 5, 10, 20, 50, 100 g and 300 g of the CPS, with or without Freund's incomplete adjuvant. Mice were observed for any signs of depression or toxicity for 1 week and were bled from the retro-orbital plexus on day 26. They were then challenged on day 28 with 100–200 CFU of live organisms administered subcutaneously. The mice were observed for 4 days and mortalities recorded. Animals found moribund were euthanased immediately and counted as mortalities.

Measurements of phagocytic uptake in vitro

The phagocytic uptake was measured using ^{3}H-labelled *P. multocida* type B and ovine mammary neutrophils as described previously (Mukkur and Inman 1989). The ^{3}H-organisms were de-encapsulated by suspension in phosphate-buffered saline containing 0.08*M* KSCN, pH 7.2, by incubation at 37 °C for 30 min in a gently shaking water-bath. This was followed by centrifugation (6000 × g) for 20 min and the cell pellet resuspended in Hank's balanced salt solution (HBSS) to a density of 5 × 10^8 CFU/mL. After removing the cytophilic IgG_2 from neutrophils, the latter were suspended in 0.1% gelatin HBSS to a final concentration of 5 × 10^7 cells/mL. To 1.4 mL of warm HBSS containing 0.2 mL of neutrophils were added various quantities (10–100 g carbohydrate) of CPSI, LPS or KSCN extract followed by 0.2 mL of encapsulated or de-encapsulated labelled *P. multocida* type 6:B. The reaction mixture was incubated at 37 °C for 15 min and subjected to gradient centrifugation in gelatin accordingly to van Dissel et al. (1986).

For determination of radioactivity, sedimented neutrophils were first solublised in 5 mL of NCS tissue solubliser and then transferred to vials containing 5 mL OCS (organic counting scintillant) scintillation solution (Amersham, Australia) for determination of radioactivity in a liquid scintillation counter.

Statistical analysis

Student's *t* test was used to determine the significance of differences between the $\log_{10}$ of the mean values for the phagocytic uptake percent.

Results

Phagocytic uptake experiments

In phagocytosis experiments reported in this paper, the CPS of *P. multocida* type 6:B strain 82 purified according to Penn and Nagy (1976) (CPSI) was used. Such preparations were found to be contaminated with up to 20% LPS, as revealed by KDO analysis. The KSCN extract, on the other hand, is known to contain CPS, LPS, nucleic acids and proteins. The phagocytic uptake of encapsulated versus de-encapsulated *P. multocida* type B revealed that the percentage of de-encapsulated organisms phagocytosed was significantly higher than that of the encapsulated organisms.

When purified CPSI was added to the phagocytosis reaction mixture in increasing amounts, there was a significant inhibition of phagocytic uptake of de-encapsulated *P. multocida* by the ovine mammary neutrophils (Table 1). There was also a substantial, although statistically not significant, reduction in the phagocytic uptake of de-encapsulated organisms on addition of Westphal LPS or KSCN extract in

increasing amounts to the phagocytosis reaction mixture.

Table 1. Inhibition of phagocytic uptake of *P. multocida* type 6:B by ovine mammary neutrophils with specific CPS.

Organism	Phagocytic uptake percent ± SEM
Untreated *P. multocida*	6.5 ± 1.8
Treated *P. multocida**	23[a] ± 4.5
Treated *P. multocida** purified capsular polysaccharide	
(i) 10 μg	17 ± 2.3
(ii) 50 μg	13 ± 2.7
(iii) 100 μg	7.8[b] ± 2.1
Treated *P. multocida** Westphal lipopolysaccharide	
(i) 10 μg	21 ± 2.1
(ii) 50 μg	18 ± 2.4
(iii) 100 μg	16.5[c] ± 1.7
Treated *P. multocida** KSCN extract	
(i) 10 μg	20.7 ± 1.8
(ii) 50 μg	17.1 ± 3.1
(iii) 100 μg	14.3[d] ± 1.0
Treated *P. multocida** Pneumoccocal capsular Polysaccharide (100 μg)	24.3 ± 2.9

Significant differences: (b) vs (a) ($p < 0.01$), (b) vs (c) & (d) ($p < 0.05$)

* Refers to organisms that have been decapsulated, as indicated in Materials and Methods

Development of method for purification of CPSII

Because of relatively high level of contamination of CPS prepared according to Penn and Nagy (1976) (CPSI), a new procedure for purification of the CPS of *P. multocida* type 6:B was developed (Table 2). This method involved extraction of the CPS with a solvent comprising 0.1% zwittergent and 0.1*M* citrate buffers, pH 2.3, at 41 °C for 30 min, followed by precipitation with 1% cetavalon to remove nucleic acid contamination.

Polysaccharides in the cetavalon supernatant were precipitated sequentially with ethanol and acetone. The precipitate was dissolved in water and treated sequentially with 0.1*M* $CaCl_2$ and sodium acetate to precipitate LPS, protein and nucleic acid contaminants. The supernatant obtained following acetate precipitation was concentrated by pervaporation and subjected to anion exchange chromatography on DEAE-Sephacel (Pharmacia). After elution of the fall-through peak in 0.05*M* Tris-HCl, pH 8.0, further elution was carried out using a linear sodium chloride gradient (0–0.2*M* NaCl) (Figure 1). After elution of the major gradient peak, the remaining bound material comprising the majority of LPS and nucleic acid contaminants was eluted off the column with 0.5*M* NaCl.

The major gradient peak was concentrated by pervaporation and subjected to molecular sieve chromatography on Sepharose 6B equilibrated with citrate-zwittergent buffer (Figure 2) when the leading peak, representing the putative capsule with substantially reduced LPS, protein and nucleic acid contamination, was obtained (CPSII). This purified CPS was used throughout the rest of this investigation. The final yield of CPSII was 3.5%, with only little contamination with protein and nucleic acid and no detectable contamination with LPS (Table 2). Confirmation of the absence of any major contaminants was also obtained by nuclear magnetic resonance spectrometry (data not shown).

Table 2. Purification of CPS of *P. multocida* type 6:B.

Purification step	Total carbohydrate (mg)	Nucleic acid	Contamination (%) with		Percent yield
			LPS	Protein	
Detergent extraction	537.2	55.8	71.1	ND	100
Cetavalon supernatant	385.7	19.3	36	21.1	71.8
Ethanol precipitate	368.8	11.2	41	22.3	68.7
Acetone precipitate	277.0	13.2	28.5	17.8	51.6
$CaCl_2$ supernatant	261.4	1.9	10.9	3.8	48.7
Acetate supernatant	253.5	1.5	19.4	3.5	47.2
Anion exchange chromatography	22.2	1.7	6.7	ND	4.1
Molecular sieve chromatography	18.6	1.7	Not detectable	0.56	3.5

LPS, lipopolysaccharide; ND, not done

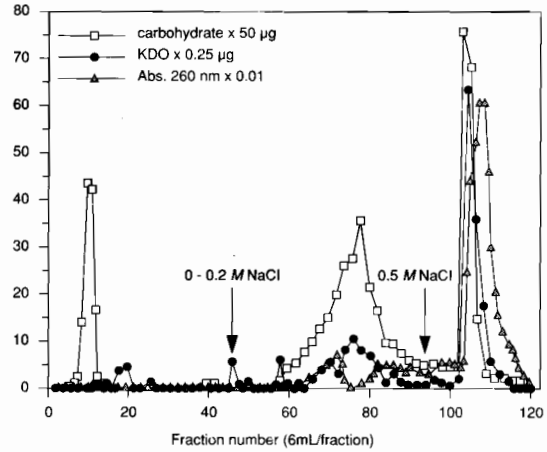

Figure 1. 10 mg of solvent-extracted CPS was loaded on a DEAE-Sephacel column (2.0 × 50 cm) and equilibrated with 0.05*M* Tris buffer, pH 8. Arrows indicate the start of the NaCl gradients. Fractions were monitored for absorbance at 260 nm, total carbohydrate and KDO.

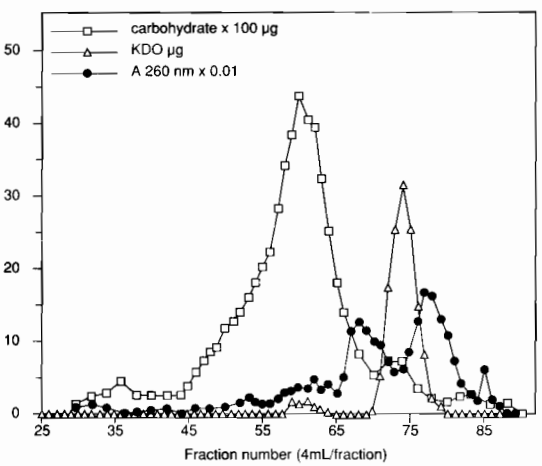

Figure 2. 10 mg of the CPS-rich peak from the ion-exchange column was loaded on a Sepharose 6B column (2.6 × 100 cm) and equilibrated with 0.1*M* citrate buffer, pH 2.3, containing 0.1% zwittergent. Fractions were monitored for absorbance at 260 nm, total carbohydrate, and KDO.

Molecular weight of purified capsular polysaccharide (CPSII)

The molecular mass of purified CPSII determined by gel filtration in zwittergent buffer was 90 000 Da, whereas it was 900 000 Da when the solvent used was PBS (data not shown).

Monosaccharide composition and linkage analysis

The CPSII was composed of arabinose, mannose and galactose in the ratio of 0.5: 2.0: 0.8 (Table 3). No other monosaccharides were detected in the carbohydrate polymer. The total ion current profile (not shown) of the carbohydrate polymer revealed the following PMAA derivatives:

(1) terminal linked arbinofuranosyl
(2) 4 substituted xylopyranosyl
(3) terminal mannopyranosyl
(4) terminal galactopyranosyl
(5) 2 substituted mannopyranosyl
(6) 4 substituted glucopyranosyl
(7) 3 substituted mannopyranosyl
(8) 3 substituted galactopyranosyl
(9) 6 substituted hexopyranosyl
(10) 6 substituted hexopyranosyl
(11) 2,6 di-substituted mannopyransoyl
(12) 3,6 di-substituted galactopyranosyl
(13) 3,4,6 di-substituted hexopyranosyl
(14) 2,4,6 tri-substituted hexopyranosyl
(15) 2,4,6, tri-substitued hexopyranosyl

Table 3. Monosaccharide composition of purified CPS of *P. multocida* type 6:B.

Monosaccharide*	Absolute molar content (nmol/mg)	Ratio
Arabinose	156	0.5
Mannose	613	2.0
Galactose	215	0.8

* Total monosaccharide content = 1.02 µ mol

One PMAA derived from L-arabinose (component 1) — namely 1,4 di-O-acetyl 2,3,5 tri O-methyl arbinitol — was detected, thereby indicating the presence of terminal non-reducing L-arabino-furanosyl residues within the polymer. This clearly indicated that the carbohydrate polymer had some degree of branching.

Four PMAAs derived from D-mannose (components 3, 5, 7 and 11) — namely 1,5 di-O-acetyl 2,3,4,6 tetra-O-methylmannitol, 1,2,5 tri-O-acetyl 3,4,6 tri-O-methyl mannitol, 1,3,5 tri-O-methyl 2,4,6 tri-O-methyl mannitol, and 1,2,5,6 tetra-O-acetyl 3,4 di-O-methyl mannitol — were detected, indicating the presence of terminal non-reducing D-mannopyranosyl, 2 substituted D-mannopyranosyl, 3 substituted D-mannopyranosyl and 2,6 di-substituted D-mannopyranosyl residue. The presence of 1,5 di-O-acetyl 2,3,4,6 tetra-O-methyl mannitol and 1,2,5,6 tetra-O-acetyl 3,4 di-O-methyl mannitol confirmed that the polymer was branched and not linear. The

high solubility of the polymer in both water and dimethylsulphoxide suggested that 1-6 linkages were within the polymer backbone and that the branching position was at C2.

Three PMAAs derived from D-galactose (components 4, 8 and 12) — namely 1,5 di-O-acetyl 2,3,4,6 tetra-O-methyl galactitol, 1,3,5 tri-O-acetyl 2,4,6 tri-O-methyl galactitol, and 1,3,5,6 tetra-O-acetyl 2,4 di-O-methyl galactitol — were detected, indicating the presence of terminal non-reducing D-galactopyranosyl, 3 substituted D-galactopyranosyl and 3,6 di-substituted O-galactopyranosyl residues within the carbohydrate polymer. The concentration of 1,5 di-O-acetyl 2,3,4,6 tetra-O-methyl galactitol was low, and probably represented the end terminus of the polymer chains. However, the presence of 1,3,5,6 tetra-O-acetyl 2,4 di-O-methyl galactitol clearly indicated that the carbohydrate polymer was branched at D-galactopyranosyl residues at positions 3 or 6. The high solubility of the polymer in both water and dimethyl sulphoxide indicated that 1-6 linkages were present within the backbone of the polymer, thereby suggesting that the branch points occurred at position C3 of D-galactopyranosyl residues.

Components 2 and 6 — namely 1,4,5 tri-O-acetyl 2,3 di-O-methyl xylitol and 1,4,5 tri-O-acetyl 2,3,6 tri-O-methyl glucitol — were almost certainly environmental contaminants arising from cellulose/hemicellulose, and can be ignored. Components 13, 14 and 15 represent unidentified PMAAs derived from 3,4,6 and two 2,4,6 tri-substituted hexopyranoses. It was difficult to assign the significance of these; they may represent contaminants or multiple branching points within the polymer, bearing in mind that microheterogeneity is commonly found in polysaccharides. The fact that series of PMAAs were detected from both D-mannose and D-galactose indicated either that the carbohydrate polymer contained a complex repeating unit (6 units or more) or that several different polymer species were present. Based on compositional analysis, the ratio of D-mannose:D-galactose:l-arabinose was 2.0:0.8:0.5, and, assuming that the repeating unit is based on this ratio, several speculative structures can be proposed, again assuming that 1-6 linkages are present within the polymer.

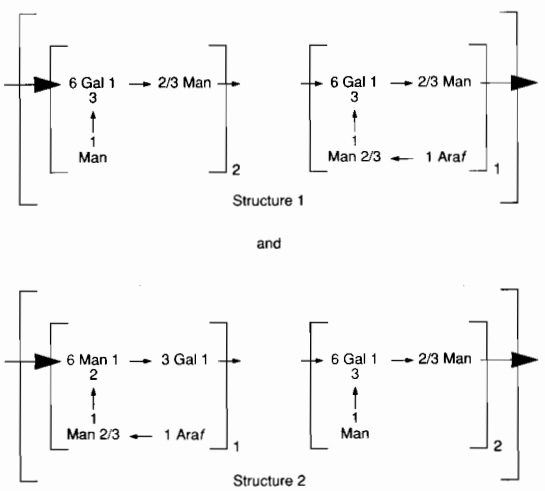

Figure 3. Speculative linkage structures.

The above two structures are speculative, since others can be drawn. They should not be taken to represent final linkage structures.

Toxicity and protective capacity of purified CPS (CPSII) of *P. multocida* type 6:B

Purified CPS was found to be non-toxic to mice, up to a dose of 300 μg, as tested so far. However, immunised mice were not protected against experimental challenge with *P. multocida* type 6:B (Table 4). This was so regardless of whether mice were immunised with a single dose (with or without adjuvant) or with multiple doses.

Table 4. Protective potential of purified CPS of *P. multocida* type 6:B.

Total vaccine dose (μg)	Immunisation regime: μg CPS administered at week			Number of mice surviving/total number challenged
	0	1	2	
Administered in water				
50	50	—	—	0/4
50	50	15	25	0/4
100	10	—	—	0/4
100	15	25	60	0/4
300	300	—	—	0/4
300	50	100	150	0/4
Administered in FIA*				
50	50	—	—	0/4
100	100	—	—	0/4
300	300	—	—	0/4
Controls	—	—	—	0/4

* FIA, Freund's incomplete adjuvant

Discussion

It was clear that purified CPS was significantly more antiphagocytic than purified LPS or KSCN extract, thus constituting an important virulence factor of *P. multocida* type 6:B and an important vaccine candidate. Purified CPS occurs in its native state as a

decamer, as evidenced by the fact that its molecular weight in PBS was 900 000 Da, whereas in detergent it was 90 000 Da. The high molecular weight characteristic of CPS was also suggested by Penn and Nagy (1976), although its size was not estimated.

The CPS was non-toxic and also non-protective in mice (as reported this paper) or rabbits (Chandrasekaran et al., these Proceedings, Session 4). Similar results were reported by Penn and Nagy (1976), who also found their purified CPS preparation to be non-protective in rabbits. Penn and Nagy (1976) also reported that cattle stimulated with purified CPS developed mouse protective antibodies. We did not adopt this approach because of our recent demonstration of an apparent lack of relationship between the passive mouse protection test and indirect capsular haemagglutination with active protection in buffalo (Chandrasekaran et al., these Proceedings, Session 4). Despite repeated immunisation of mice or rabbits with purified CPS with or without adjuvants (aluminium hydroxide or Freund's incomplete adjuvant), we were unable to generate precipitating antibodies. This finding was in contrast to that reported by Penn and Nagy (1976).

Although we are currently developing a CPS-specific ELISA for measuring antibody responses to CPS of *P. multocida* type 6:B and studying their role in protection, two objectives need to be addressed. The first is the availability of a quality-controlled reproducible method for coating purified CPS on the ELISA plates. The second involves ascertaining the specificity of the reaction: one of the monosaccharides present in CPS (galactose) is related to a monosaccharide (D or L glycero-D-mannoheptose) present in the LPS of *P. multocida* type 6:B (Rimler et al. 1984).

In contrast to the findings of Penn and Nagy (1976), the procedure reported in this investigation ensured a very low level of contamination with extraneous components, including proteins, nucleic acids or LPS. Analysis of the monosaccharide composition revealed the presence of arabinose in a furanose configuration, and the linkage analysis revealed the CPS to be a branched polymer. This was in contrast to that reported for CPS of *P. haemolytica* (Adlam et al. 1984, 1985a,b). However, it cannot be categorically stated that the arabinose, mannose and galactose existed in the polymer as the native monosaccharides, since any N- or O-substituents that are acid labile would have been hydrolysed. It is certain that no acid resistant substituents are present on any of the monosaccharides to a detectable extent. Further, any O-glycosidic linkages in the polymer that are not acid liable (an extremely rare phenomenon) would not be cleaved, and the 1-O-methyl derivatives of the relevant monosaccharides would not be detected.

Finally, since permethylation analysis gives only a partial sequence and no information on anomeric linkages, it is necessary to produce defined oligosaccharides using degradative techniques, such as the use of endo-glycosidases or, as a last resort, non-specific chemical degradative techniques, and obtain their sequence.

Research aimed at enhancing the immunogenicity of purified CPS with a view to evaluating its protective potential, in the first instance in mice, is currently in progress.

Acknowledgments

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Serotype- and Genus-Specific Protein Antigens of *Pasteurella multocida* and their Role in Immunity to Infection

Ramdani[1] and B. Adler[1]

Abstract

Three monoclonal antibodies (MAbs) — designated DM-1 and DM-2 and DM-4 — were produced against protein antigens of *Pasteurella multocida* strain M1404. Immunoblots indicated that DM-1 and DM-2 reacted strongly with a 33 kDa protein, and to a lesser extent with 29 kDa and 42 kDa proteins. DM-4 reacted with a 36 kDa protein. All reactive antigens, except for the 42 kDa protein, appeared to be located in the cytoplasm and/or periplasm. The MAbs could not agglutinate *P. multocida* unless the organisms were pretreated with 1*N* HCl. None of the three MAbs were able to protect recipient mice against *P. multocida* infection, nor were they able to opsonise these organisms for phagocytosis by murine macrophages. Active immunisation with the reactive proteins conferred only partial protection (25–60%) against challenge infection. The results imply that these proteins play only a minor role in protection from *P. multocida* infection.

LIPOPOLYSACCHARIDE (LPS) and proteins are major components of the outer membrane of Gram-negative bacteria (Hancock and Nikaido 1978). Most studies on immunity have focused on LPS (Mutharia and Hancock 1983). Protein extracts from *P. multocida*, as well as from other Gram-negative organisms, frequently contain LPS (Gregory 1986). Conversely, Bain and Knox (1961) found that LPS preparations of *P. multocida* contained protein antigens. It has therefore been difficult to determine the role of each antigen type in immunity to infection with *P. multocida*.

Bain (1955) described a protective protein antigen in some strains of *P. multocida* that was immunogenic in mice. However, this preparation contained significant amounts of LPS and the protein was not characterised further. Dhanda (1960) also reported isolating a predominantly protein antigen from some strains of *P. multocida* and he found a similar protective response to that shown earlier by Bain (1955).

Monoclonal antibodies (MAbs) have been used by many investigators to isolate and to purify protein antigens, and also to identify the role of proteins in immunity to infection. For example, Lu et al. (1987) identified a 37.5 kDa outer membrane protein (OMP) of *P. multocida* as the reactive antigen for a protective MAb. MAbs may also facilitate the identification of bacterial serotype. Zollinger et al. (1984) serotyped Neisseria meningitis based on MAbs against OMP. Lida et al. (1990) used MAbs to classify *Actinobacillus pleuropneumonia.*

The aims of this study were to produce MAbs against protein antigens of *P. multocida* and to determine whether such antibodies would confer protection against this pathogen. The results would provide information on the role of protein antigens in immunity to *P. multocida* infection.

Materials and Methods

P. multocida strain M1404 was maintained as described by Ramdani et al. (1990). Sub-cellular fractions were prepared from sonicated organisms using sucrose density gradients (Ito et al. 1977). The enzyme immunoassay (EIA), electrophoretic (sodium dodecyl sulphate-polyacrylamide gel electrophoresis, SDS-PAGE) and immunoblotting techniques used have been described previously (Dawkins et al. 1991; Ramdani and Adler 1991).

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Table 1. Protective ability against lethal infection with *Pasteurella multocida* of individual MAbs, immune mouse serum (IMS) and normal mouse serum (NMS) in groups of three mice.

MAbs or controls	Mouse survival (day)							Mean time to death	Percent protection
	1	2	3	4	5	6	7		
DM-1	3	1	1	1	0	0	0	2.3	0
DM-2	3	0	0	0	0	0	0	2.0	0
DM-4	3	0	0	0	0	0	0	2.0	0
IMS	3	3	3	3	3	3	3	—	100
NMS	3	0	0	0	0	0	0	2.0	0

The bacterial proteins used for raising MAbs were eluted from SDS–PAGE gels according to the method described by Hager and Burgess (1980), as initial attempts to remove LPS completely from *Pasteurella* extracts by affinity chromatography with polymyxin B–acrylamide gel had been unsuccessful. Spleen cells obtained from a mouse immunised with the eluted proteins were fused with NS-1 cells to produce MAbs (Ramdani and Adler 1991).

Ammonium-sulphate precipitated immunoglobulin fractions (1 mL) of the three individual MAbs (equivalent to 1 mL of ascitic fluid) were injected intraperitoneally into groups of three mice. Two additional groups of three mice were injected with immune mouse serum (IMS) and normal mouse serum (NMS), which served as positive and negative controls respectively. All mice were challenged with 100 viable organisms 24 hours after the antibody injection.

The proteins used to immunise mice in the challenge experiments were prepared according to the method described by Judd (1988). Briefly, a sonicated extract of *P. multocida* was separated on 12.5% SDS–PAGE gel and transblotted onto nitrocellulose. Based on the immunoblot reaction of the sonicate and DM-1, the protein antigens were designated P-42, P-33 and P-29. The region of nitrocellulose containing the appropriate antigen was excised and then dissolved and particularised. Each of the protein antigens P-42, P-33 and P-29 was prepared from 20 lanes of transblotted sonicate antigens. Each particularised protein antigen was mixed with incomplete Freund's adjuvant (IFA), and injected intramuscularly into groups of five mice at a dose of 0.2 mL per mouse. Five mice were also injected with nitrocellulose alone in IFA, as a control group. Each group was immunised four times, at weekly intervals. Eight weeks after the final immunisation the mice were bled and their sera were tested by EIA against sonicate and LPS antigens and also by immunoblotting against homologous sonicate.

Results

Antibody classes of MAbs

The immunoglobulin isotypes of DM-1, DM-2, and DM-4 were determined by gel immunodiffusion (Adler and Faine 1983) and found to be IgG_1, IgG_1 and IgG_3 respectively.

Serological reactions of the three MAbs

Antibody activity of the three MAbs in ascitic fluid was detected with EIA using homologous sonicated whole bacterial cells. Each exudate, when diluted 1:100, reacted with an absorbance of between 0.55 and 0.88. Immunoblotting the MAbs against sonicate showed that DM-1 and DM-2 both reacted strongly with a band at 33 kDa and to a lesser extent with bands at 29 kDa and 42 kDa (Fig. 1). The main band recognised by DM-4 was a 36 kDa antigen with slight reaction with a band of 33 kDa (Fig. 1). Reactivity with the three MAbs was completely abolished by Proteinase-K treatment of the nitrocellulose blots (data not shown).

Hybridoma supernatants of the three MAbs agglutinated whole bacteria treated with 1N HCl, with titres of 1024 (data not shown). Immune mouse serum had a similar agglutination titre, while normal mouse serum was negative. However, none of the MAbs or immune serum could agglutinate untreated organisms (data not shown).

Reaction of MAbs with cellular fractions

The reactions of the three MAbs with membrane, cytoplasmic and periplasmic fractions were analysed by immunoblotting, in order to determine the cellular location of the reactive antigens. The results (Fig. 2) showed that DM-1 and DM-2 reacted with 29 kDa and 33 kDa bands present in both cytoplasmic and periplasmic fractions, as well as with a protein band of 42 kDa that was present in the membrane fraction. DM-4 reacted with a band of 36 kDa present in both cytoplasmic and periplasmic fractions.

Table 2. Protection of mice following immunisation with nitrocellulose-bound proteins P-42, P-33 or P-29, nitrocellulose alone (N/C) or sonicate (Son).

Immunisation	Challenge (CFU) (Strain)	Mouse survival (day) 1	2	3	4	5	6	7	Mean time to death	Percent protection
P-42*	10^2 (M1404)	9	6	6	6	6	6	6	2.0	66.8
P-42	10^4 (M1404)	4	4	1	0	0	0	0	2.5	0
P-42	10^4 (PBA100)	5	4	4	2	1	1	1	2.5	20
P-33	10^2 (M1404)	4	1	1	1	1	1	1	2.0	25
P-29	10^2 (M1404)	5	3	3	3	3	3	3	2.0	60
N/C	10^2 (M1404)	5	5	0	0	0	0	0	2.0	0
N	10^2 (M1404)	5	0	0	0	0	0	0	1.0	0
N	10^4 (M1404)	5	0	0	0	0	0	0	1.0	0
Son	10^2 (M1404)	5	5	5	5	5	5	5	—	100
Son	10^4 (M1404)	4	4	4	4	4	4	4	—	100

*Combined results from two vaccinated groups.
CFU, colony-forming units; N, normal (control) mice.

Protective capacity of the three MAbs

The ability of the three MAbs to protect mice against infection with *P. multocida* was determined. The results (Table 1) showed that none of the MAbs conferred protection to recipient mice. The positive and negative control groups gave the expected results.

To confirm that antibodies to the reactive antigens (P-29, P-33, P-42) were not protective, mice were immunised with these proteins and then challenged with a lethal dose (100 viable organisms). Percent protection of each group was recorded (Table 2). Vaccination with P-29 conferred 60% protection, vaccination with P-33 gave 25% protection, and vaccination with P-42 gave 67% protection.

Discussion

Three MAbs were produced against protein antigens of *P. multocida* strain M1404. Two MAbs (DM-1 and DM-2) reacted with three protein bands at 29 kDa, 33 kDa and 42 kDa, while one MAb (DM-4) reacted with a protein band of 36 kDa, based on immunoblotting. Turner (1983) suggested that staining of multiple bands by MAbs is frequently seen when antibodies are produced against complex antigens, such as whole bacterial cells. Suggestions for possible causes for this reaction were discussed by Lane and Koprowski (1982). The sensitivity of the MAb-reactive antigens (nitrocellulose blots) to Proteinase-K treatment and the failure of the MAbs to react with LPS in an EIA (data not shown) confirmed the protein nature of the reactive epitopes. Although characterisation of the MAbs is continuing, preliminary evidence suggests that DM-1 and DM-2 may be specific for *P. multocida* strains of Heddleston type 2 (including the homologous strain M1404), while DM-4 reacts broadly with all 16 Heddleston serotypes and as well as with other *Pasteurella* spp.

The reaction of DM-1 and DM-2 with sub-cellular fractions indicated that the major 33 kDa antigen, as well as the 29 kDa antigen, were found in the cytoplasmic and periplasmic fractions. The 42 kDa antigen, which also reacted with the same MAbs, was found in the membrane fraction. The 36 kDa protein antigen reacting with DM-4 was also found in the cytoplasmic and periplasmic fractions. The detection of antigens in both cytoplasmic and periplasmic fractions indicates that either the cellular fractions were inadequately separated or the reactive proteins are in more than one cellular location. The inability of the MAbs to agglutinate untreated *P. multocida*, as well as the observation that antibody activity in

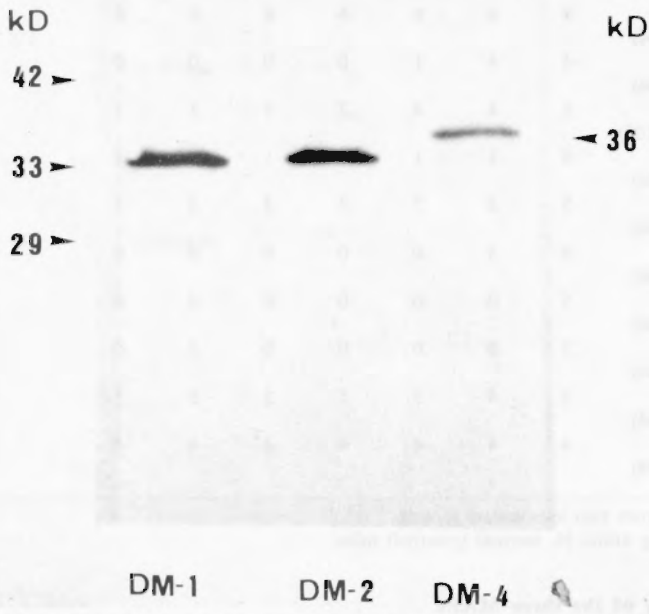

Fig. 1. The immunoblot profiles of DM-1, DM-2 and DM-4 against homologous sonicate. DM-1 and DM-2 showed identical reactions with antigens of 29 kDa, 33 kDa and 42 kDa, indicated by the arrows on the left. DM-4 reacted mainly with a 36 kDa antigen, shown on the right.

the culture supernatants was not removed by extensive absorption with intact bacteria (data not shown), supported the conclusion that the MAbs did not react with surface antigens. Modification of the bacterial surface with 1N HCl was apparently required to expose the reactive epitopes for cells to be agglutinated. When *P. multocida* organisms were treated with Triton X-114, all three MAbs reacted with soluble material located in the aqueous phase (data not shown).

In this study, none of the MAbs protected against a lethal dose of 100 organisms of homologous *P. multocida* (Table 2). In contrast, immune mouse serum gave 100% protection in this experiment and could also protect against 10^4 organisms. The findings suggest that antibodies to these protein antigens are not protective. The three anti-protein MAbs did not opsonise *P. multocida* for phagocytosis by mouse macrophages (data not shown).

Active immunisation with P-29, P-33 and P-42 conferred only partial protection (25–67%), confirming the limited role of these particular proteins in immunity to infection. The protein-vaccinated mice produced moderate levels of the antibody as measured by EIA against sonicated antigens (data not shown). It is not surprising that immunisation of the mice with the protein antigens gave only partial protection against challenge, given the fact that the 33 kDa and 29 kDa antigens were found to be subsurface proteins. There was some evidence that 42 kDa protein may be partially membrane associated, and mice immunised with this protein were partially protected. In contrast, other workers have found that OMP in combination with LPS antigens can be excellent immunogens or protective antigens (Lugtenberg et al. 1984). It is clear that further work is required to confirm the role of these proteins in immunity.

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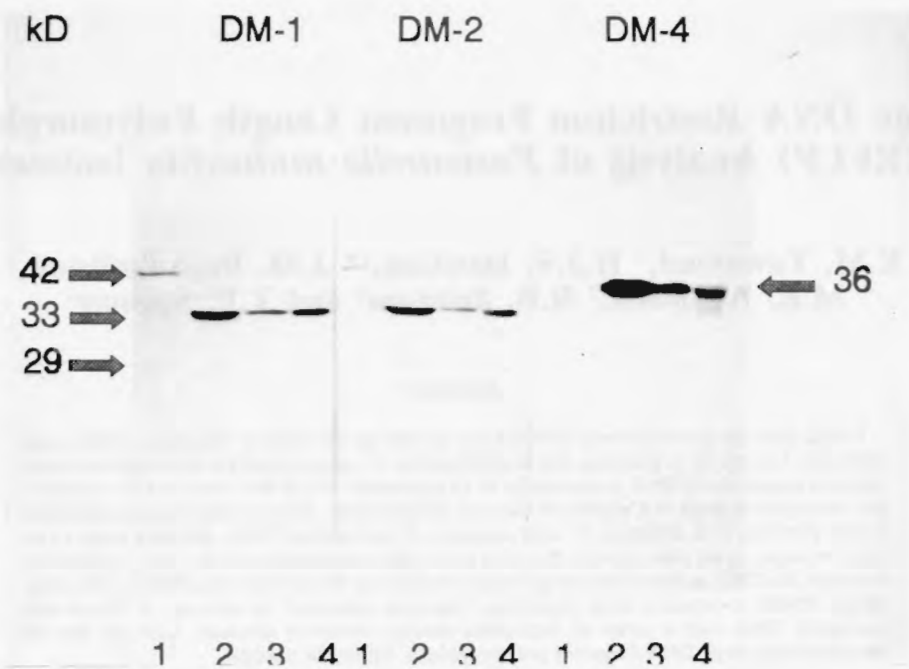

Fig. 2. Immunoblotting reactions of three MAbs, DM-1, DM-2 and DM-4, with cell fractions obtained by sucrose density gradient centrifugation. The reactive bands are arrowed. A, total membrane fraction; B, cytoplasmic fraction; C, periplasmic fraction; D, sonicate

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Large DNA Restriction Fragment Length Polymorphism (RFLP) Analysis of *Pasteurella multocida* Isolates

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Abstract

Pulsed field gel electrophoresis (PFGE) has opened up the field of microbial genetics and molecular biology by overcoming the size limitations of conventional gel electrophoresis and allowing separation of DNA fragments up to 10 megabases. PFGE has permitted the construction of restriction maps of a number of bacterial chromosomes. Karyotyping of various parasites is now possible, as is mapping of large segments of mammalian DNA, allowing work to be done on large, intact gene clusters. By using restriction endonucleases with a long recognition sequence, the DNA is cleaved into large fragments that can be resolved using PFGE. This study utilises PFGE to separate large restriction fragments generated by cleavage of *Pasteurella multocida* DNA with a series of infrequent-cutting restriction enzymes, with the aim of demonstrating large DNA-fragment polymorphisms within the species.

THE development of pulsed field gel electrophoresis (PFGE), first described by Schwartz et al. (1983), initiated renewed interest in microbial genetics and the study of genomic organisation. Conventional agarose and polyacrylamide gels have been the main methods of DNA separation in molecular biology. These techniques are limited in their effectiveness, as they are only capable of resolving DNA fragments up to 40 kb in length (Dawkins 1989). Resolution of DNA molecules greater than this threshold limit is poor, with the DNA exhibiting size-independent mobilities and co-migration within an agarose gel matrix.

PFGE has overcome the size limitations of conventional electrophoresis methods by forcing the DNA molecules to reorient periodically between two electric field directions (Lai 1991). The original apparatus consists of a complex electrode array in conjunction with an electrical switching unit (Dawkins 1989). Non-uniform fields in both directions were found to provide the best resolution, but in order to achieve narrow bands, one uniform and one non-uniform field was used. The contour-clamped homogeneous electric field apparatus (CHEF) alternately pulses at 120°, using voltages applied along opposing sides of a hexagonal array of electrodes clamped to a predetermined electric potential (Chu et al. 1986). Each time the field direction is altered, the DNA fragments must reorientate along the new field before again migrating forwards. Smaller molecules have a capacity to turn and reorientate more quickly than larger ones, and DNA mobility becomes a function of size and molecular mass. The programmable switching unit of the CHEF-DR II system (Bio-Rad) provides precise control over the electrical field. It allows a continuously adjustable range of switch times and will enhance resolution within a 0.1–10-megabase DNA size range. The increased size separation now possible is critical in the construction of restriction maps of bacterial genomes.

Structural analysis by PFGE can determine size and organisation of the genome, with chromosomal studies showing that most bacteria contain a circular genome (Bergström et al. 1991). PFGE is also thought to be the most definitive technique with regard to estimation of genome size. Other techniques have resulted in size estimations varying between 10 and 20% from those obtained using PFGE (Krawiec and Riley 1990).

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Genetic studies of eukaryotes using PFGE have allowed electrophoretic karyotyping to be made of these organisms. Intact chromosomes can be resolved with PFGE thus enabling a full chromosomal analysis to be carried out. The combination of PFGE and restriction endonuclease digestion of genomic DNA can provide important information regarding genetic variability between bacterial strains. The mean genomic content of guanine and cytosine in eubacterial DNA varies from 25 to 75% (Muto and Osawa 1987). The occurrence of particular restriction endonuclease sites can be determined by the genomic G + C content, with the size of the recognition sequence being an additional factor. Restriction enzymes such as Not I, Sfi I and Sma I recognise sequences mainly composed of G + C. Therefore, in a bacterium with a low G + C content, such as *Pasteurella multocida*, these enzymes will not cut the DNA as frequently as they would with a G + C-rich bacterial genome. Such enzymes will alter their cutting frequency depending on the frequency of the particular recognition sequence in the genome. Restriction enzymes with an 8 or 10 base pair recognition sequence are particularly infrequent cutters (e.g. not I and Sfi I), and are commonly used in mammalian chromosomal studies. Thus, different 'rare-cutting' endonucleases can be specifically chosen for various bacterial genera, with the aim of yielding informative electrophoretic profiles (Corich et al. 1991).

The aim of this study was to examine fourteen *P. multocida* isolates through the use of PFGE and infrequent-cutting restriction enzymes. *P. multocida* is the causative agent in one of the most economically important livestock diseases of Southeast Asia — haemorrhagic septicaemia (HS). There have been many typing systems developed to serologically classify the *P. multocida* species, and a definitive method to confirm HS-pathogenicity is still to be found. PFGE has been shown to be a reliable indicator of bacterial strain differentiation and may offer greater discrimination than conventional typing methods such as ribotyping (Grothues and Tümmler 1987; Rood and Cole 1991; Prevost et al. 1992).

Materials and Methods

Bacteria

The 14 *P. multocida* isolates used in this study are listed in Table 1. They were kindly provided by the Regional Veterinary Laboratory, Benalla, Victoria. The strains listed here are a broad representation of the species with regards to HS pathogenicity and geographical location. All isolates were grown on blood agar (BA) and incubated overnight at 37 °C in 5% CO_2.

DNA Preparation for PFGE

Bacteria were grown overnight in heart infusion broth (HIB) at 37 °C with agitation. Cells were harvested by centrifugation for 10 min at 3000 rpm, washed once, and resuspended with an equal volume of 0.05*M* EDTA, pH 8.0. Then 0.3 mL of cell suspension was mixed with 0.9 mL of 1.0% low-melt preparative grade agarose (Bio-Rad, Richmond, CA) at 40–50 °C, then pipetted into a plug mould (Bio-Rad) and allowed to solidify. Cell lysis was performed by incubating one to four plugs in Chromosomal Proteinase K Buffer (0.5*M* EDTA, pH 8.0; 0.01*M* Tris, pH 8.0; 1% Sarkosyl) at 45 °C for 1–2 days. Plugs were washed with 0.05*M* EDTA, pH 8.0, at room temperature for 30 min each and stored at 4 °C until further use.

Table 1. Details of the 14 *P. multocida* isolates used in the study.

Reference number	Identity/origin	Clinical history	Carter type	HS-ELISA
0020 (964)	Malaysia	HS, ox	ND	+
0043 (ATS5)	Lyn, Ireland		ND	+
0113 (945)	Tantabin	HS (Roberts 1)	ND	−
0119 (948)	Bai, Rangoon	HS	ND	+
0121 (952)	Dedye	HS (Roberts 1)	ND	+
0131 (973)	Izatnager 25	HS avirulent, buffalo	ND	+
0140 (989-A)	Queensland (11B)	wound, cattle	B	−
0141 (990)	Baghdad	HS (Roberts 1)	ND	+
0148 (2415)	Bunia 11, Africa	HS, cattle	E	+
0150 (2417)	SI, Africa	HS, cattle	E	+
0332 (M1404)	NADC, Ames	Hedd 2, HS, bison	B	+
0348 (P932)	NADC, Ames	Carter B, HS buffalo	B	−
0349 (P934)	NADC, Ames	Carter D, pigs	D	−
0350 (P1234)	NADC, Ames	Carter E, HS, cattle	E	+

Restriction endonuclease digestion

Prior to digestion, the agarose plugs were washed twice in double distilled water at room temperature, twice with 50 × volume TE (10m*M* Tris, pH 8.0, 1m*M* EDTA, pH 8.0) at room temperature, once in double distilled water on ice, again in TE, pH 8.0, on ice, and finally once in 100 μL of 1 × restriction buffer on ice. All washes were performed for 30 min. Then 40 μL of restriction enzyme (Promega, Madison, WI) and 2 μL of acetylated bovine serum albumin (BSA, 1 mg/mL) were added to the digestion buffer and allowed to stand for 30 min on ice before incubating at the appropriate temperature overnight, as recommended by the enzyme manufacturer. Two restriction enzymes, Not I and Sma I, were used in the study. Incubation overnight was done at 37 °C and 22 °C respectively. 12 μL of 0.5*M* EDTA, pH 8.0, was added to terminate the enzyme reaction.

PFGE analysis

Electrophoresis was performed using the CHEF-DR II apparatus, with pulse times as determined by the desired fragment size range. The Not I-digested samples were run with a ramped pulse time of 10–150 s for 24 hours at 180 V. Sma-I-digested samples were separated using a 5–35 s switch ramp at 180 V for 20 hours. Lambda concatemers (Promega) and *S. cerevisiae* (Bio-Rad) chromosomes were used as molecular weight size standards. After electrophoresis, the gels were stained with ethidium bromide followed by destaining in distilled water and photographed with UV illumination (Fig. 1).

Results and Discussion

This study used a PFGE technique to examine chromosomal DNA from 14 *P. multocida* isolates. The chosen isolates included strains isolated from animals with HS, reference strains, and non-HS isolates covering a broad representation of the species. The origin of the strains varied widely geographically, and included Asia, Africa and North America.

Chromosomal DNA was isolated from each strain and examined after digestion of the DNA with infrequent-cutting restriction enzymes. Since the introduction of PFGE, there has been a vast expansion in knowledge regarding this technique and its applications. Consequently, there is a greater range of restriction endonucleases designed to be specifically applicable with the technique. Here, analysis of the restriction fragments generated by digestion with Sma I and Not I showed diverse electrophoretic profiles. Some of the profiles demonstrated correla-

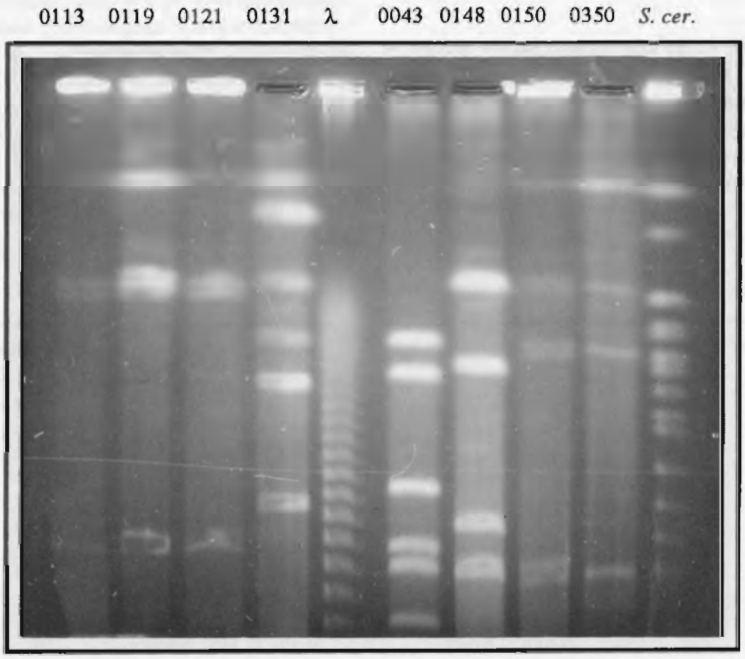

Fig. 1. Large DNA separation of fragments produced after cleavage of chromosomal DNA from *P. multocida* isolates with Not I, an infrequent-cutting restriction enzyme. Separation was done using the CHEF-DR II apparatus (Bio-Rad), with a 10–150 s pulse time for 24 hours at 180 V.

tion with previous serotypic designation. The two Carter E isolates, 0150 and 0350, showed identical profiles, which were quite distinct from the other HS isolates. The fact that PFGE examines the entire genome instead of a highly specific region of DNA, as in ribotyping studies, supports the hypothesis that PFGE has a greater correlation with serotypic properties than does rDNA analysis.

Interestingly, 0140 (Carter B, HS -ve) is the only isolate examined thus far that does not appear to have a recognition site for Not I under the conditions used in this study. All other strains generated three or four restriction fragments after cleavage with Not I, exhibiting the relative infrequent number of cutting sites. Sma I digests of the *P. multocida* isolates showed greater homogeneity among the Asian strains than those with Not I. Isolate 0131 was the only Asian isolate exhibiting slight variation (i.e. lacking the 150-kb fragment present in the other strains).

There has been no evidence to show the presence of any plasmids in these *P. multocida* isolates. Plasmids have been previously reported as being associated with virulence genes for some organisms. PFGE would have explicitly demonstrated the presence of a plasmid. Any plasmid present with virulent properties would be shown as a single band in all HS isolates, due to its inability to be cut by these restriction enzymes.

Present uses of the PFGE technique include electrophoretic karyotyping, long-range genomic mapping, large DNA cloning, the study of pathogenic chromosomal alterations, and the structural analysis of chromosomes. PFGE has also been applied to classification of bacteria, and may offer greater discrimination than conventional typing methods. Future directions include the use of different infrequent-cutting enzymes, in the hope of demonstrating some correlation between chromosomal analysis and HS pathogenicity. Modification of the running conditions may also prove useful, with increased resolution over varying size ranges.

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Ribosomal DNA (rDNA) Analysis of *Pasteurella multocida* Isolates

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Abstract

Fourteen isolates of *Pasteurella multocida*, the majority obtained from animals with haemorrhagic septicaemia (HS), were characterised by ribotyping. This technique uses a combination of restriction fragment length polymorphism (RFLP) analysis and labelled DNA (rDNA) specific for portions of rRNA to detect genotypic variation. It was determined that HS strains could be classified by the presence or absence of a simplified rDNA gene restriction pattern after digestion of genomic DNA with Pst I and probed with 23S rDNA from *Mycoplasma capricolum*. This system has a 93% sensitivity and a 71% specificity for HS. Cleavage of DNA with Eco RI demonstrated relative homogeneity and isolates could be grouped on the basis of their country of origin. There have been many typing systems developed to classify the *P. multocida* species serologically, and to date a method to definitively confirm HS pathogenicity is still lacking. This study demonstrates that ribotyping may be useful in understanding the pathogenicity and epidemiology of *P. multocida*.

HAEMORRHAGIC septicaemia (HS) is a peracute disease of cattle and buffalo, caused by specific serotypes of the bacterial species *Pasteurella multocida* (Dawkins et al. 1990). Unlike most organisms, *P. multocida* can cause disease with variable clinical signs and pathology in a wide range of mammalian and avian hosts. This characteristic of *P. multocida* has led to continuing attempts to group or classify the various types of the species and to correlate types with particular disease syndromes (Brogden and Packer 1979).

Five main typing systems have been developed to classify the *P. multocida* serotypes. Isolates are typed on the basis of their immunologic and serologic characteristics, primarily the antigenic properties of the capsular and cell-wall components (Johnson et al. 1988). Each system is based on a separate strain collection, and uses a variety of assay procedures. This results in inconsistencies between the typing systems, and difficulties occur when comparing results from different research groups (Brogden and Packer 1979). In addition, reports indicate that a significant proportion of isolates are 'untypeable' (Namioka and Bruner 1963; Manning 1982).

Nevertheless, HS isolates have been typed as Roberts type 1, Little and Lyon type 2, Heddleston type 2, Carter types B and E, and Namioka types 6:B and 6:E (Johnson et al. 1991). Little is known of the properties determining serotype specificity, virulence or pathogenicity, although the somatic (O-group) antigen could be one explanation (Namioka and Murata 1961). Studies utilising polyacrylamide gel electrophoresis (PAGE) have successfully classified various bacterial species. Studies by Johnson et al. (1991) of protein and lipopolysaccharide (LPS) antigens of *P. multocida* isolates have indicated a correlation between electrophoretic patterns and serotypic properties, although no protein(s) unique to HS were identified.

In recent years, molecular techniques have been applied increasingly to bacterial taxonomy and species differentiation. The two most widely used techniques have been restriction endonuclease analysis (REA) and ribosomal RNA (rRNA) typing, also known as ribotyping. REA involves restriction endonuclease cleavage of genomic DNA and agarose

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gel electrophoresis to separate the DNA fragments. Thus REA provides specific differentiation between strains without relying on phenotypic expression (Stull et al. 1988). The banding patterns produced by this technique are often very complex, making interpretation of results difficult. Alternatively, a minor genetic change in the DNA restriction sequence could produce a marked alteration in the banding pattern.

Ribotyping, like REA, utilises restriction enzyme digestion and agarose gel electrophoresis. The additional use of Southern blotting and rRNA probes reduces the complexity of the restriction patterns and highlights restriction fragment length polymorphisms (RFLPs) within the genome. RFLP studies can be used to discriminate between strains by detecting minor chromosomal changes that do not always correspond to a variation in phenotypic expression (Fox et al. 1991). The use of probes derived from rRNA sequences can provide a broad spectrum of hybridisation with bacterial chromosomal DNA, as rRNA has been more highly conserved (Stull et al. 1988). Therefore, a rRNA probe can be used to study the molecular epidemiology of a variety of bacteria.

The aims of this study were to carry out a molecular analysis of *P. multocida* Carter types B and E isolated from animals with HS by REA and ribotyping, and compare these characterisations with those of a number of non-HS serotypes.

Materials and Methods

Bacteria

The 14 strains of *P. multocida* included in the study have been previously documented by Dawkins et al. (1990) and Johnson et al. (1991). They are described in Table 1. All isolates were routinely assessed for purity on sheep blood agar (7% sheep blood, 3.9% Colombin agar), incubated overnight at 37 °C with 5% CO_2, and stored in glycerol-based broths (−80 °C).

DNA preparation

Bacterial DNA was extracted essentially as described by Sambrook et al. (1989). Briefly, isolates were cultured on blood agar, harvested in saline and pelleted by centrifugation. Bacterial cells were resuspended in Lysis Buffer (10m*M* Tris pH 7.6, 10m*M* EDTA, 50m*M* NaCl, 0.2% sodium dodecyl sulphate (SDS)) containing Proteinase K (2 mg/mL, Promega, Madison, WI, USA) and incubated at 45 °C for 4 hours. The DNA was sequentially extracted three times with equal volumes of equilibrated phenol (BDH), chloroform:isoamyl alcohol (24:1 v/v) and ether. The DNA was precipitated with ethanol, washed in 70% ethanol, dried, and resuspended in 10m*M* Tris-HCl, pH 8.0, 1m*M* EDTA, pH 8.0 (TE). The absorbance of an aliquot of each preparation was read at 260 nm and 280 nm to determine the DNA concentration.

Restriction endonuclease digestion of DNA

Bacterial DNA (5–7 μg per lane) was cleaved with 30 units of Eco RI or Pst I per sample, according to the supplier's instructions (Promega). The resulting fragments were separated by electrophoresis on a 0.9% agarose gel (Bio-Rad, Richmond, CA, USA) in Tris-acetate buffer (40m*M* Tris-acetate, 2m*M* EDTA, pH 8.0). Following electrophoresis, the DNA fragments were stained by ethidium bromide (1 mg/mL), examined and photographed by UV transillumination. Bacteriophage λDNA (Promega), digested with Hind III restriction endonuclease, was used as a molecular size marker.

Table 1. Details of the 14 *P. multocida* isolates used in the study.

Reference number	Identity/origin	Clinical history	Carter type	HS-ELISA	Ribotype
0020 (964)	Malaysia	HS, ox	ND	+	+
0043 (ATS5)	Lyn Ireland		ND	+	+
0113 (945)	Tantabin	HS (Roberts 1)	ND	−	+
0119 (948)	Bai, Rangoon	HS	ND	+	+
0121 (952)	Dedye	HS (Roberts 1)	ND	+	+
0131 (973)	Izatnager 25	HS avirulent, buffalo	ND	+	+
0140 (989-A)	Queensland (11B)	wound, cattle	B	−	−
0141 (990)	Baghdad	HS (Roberts 1)	ND	+	+
0148 (2415)	Bunia 11, Africa	HS, cattle	E	+	+
0150 (2417)	SI, Africa	HS, cattle	E	+	+
0332 (M1404)	NADC, Ames	Hedd 2, HS, Bison	B	+	+
0348 (P932)	NADC, Ames	Carter B, HS buffalo	B	−	+
0349 (P934)	NADC, Ames	Carter D, pigs	D	−	−
0350 (P1234)	NADC, Ames	Carter E, HS, cattle	E	+	+

Probes

The probe used in this study was a fragment eluted from an agarose gel of the plasmid pMC5, double-digested with Eco RI and Pst I. pMC5 was obtained from Dr C.J. Morrow, Veterinary Research Institute, Attwood, Victoria. The 2.8-kb fragment from digested pMC5 plasmid contains some of the 23S ribosomal RNA (rRNA) operon from *Mycoplasma capricolum*. The probe was labelled using the Promega Prime-a-Gene labelling system according to the manufacturer's directions, and [α $-^{32}$P] dCTP (Amersham International plc, UK). Incorporated DNA was eluted from the mixture by spun column chromatography (Sambrook et al. 1989), then used directly in a hybridisation reaction.

Southern transfer of DNA and hybridisation

The electrophoresed gel was soaked in 0.25*M* HCl for 15 min, then in 0.4*M* NaOH for 30 min with shaking. The DNA fragments were then transferred to a nylon membrane (Hybond-N+, Amersham) as described by Sambrook et al. (1989), with the aid of a Bio-Rad Vacuum Blotter (1–3 psi for 30 min). Following transfer, the membrane was immersed in 0.2*M* Tris-HCl, pH 7.0, 2 × salt sodium citrate (SSC) for 10 min, then air-dried and subsequently used for hybridisation or stored at 4 °C. Prehybridisation of the membrane was carried out for 1–4 hours at 65 °C in hybridisation buffer containing 10% polyethylene glycol 8000 (10% PEG 8000, Sigma), 1.5 × SSPE (for 2 L, 18.6 g NaCl, 4.1 g $NaH_2PO4.H_2O$, 1.1 g EDTA, pH 7.4), 7% SDS and 0.125 mg/mL denatured salmon-sperm DNA. Denatured, labelled probe DNA was added to the buffer and allowed to hybridise overnight at 65 °C. The membrane was washed twice at 65 °C in 0.1 × SSC/0.1% SDS for 10 min each, blotted dry, and analysed by autoradiography. For multiple hybridisations, radiolabelled probe was removed from the membrane by incubation in 0.5% SDS at 100 °C for 30 min.

Results

Experimental digestion with a spectrum of restriction endonucleases showed that Eco RI and Pst I cleavage with subsequent hybridisation gave the most informative restriction patterns. REA revealed complex banding patterns, with the isolates appearing homogeneous. Any variability was hard to distinguish due to the band complexity (Fig. 1). Southern blots of Eco RI and Pst I digestions of the 14 *P. multocida* isolates using the *Mycoplasma* rRNA probe generally demonstrated the presence of common band(s) for each ribotype. Representative results of the ribotyping experiments are shown in Figures 2 and 3.

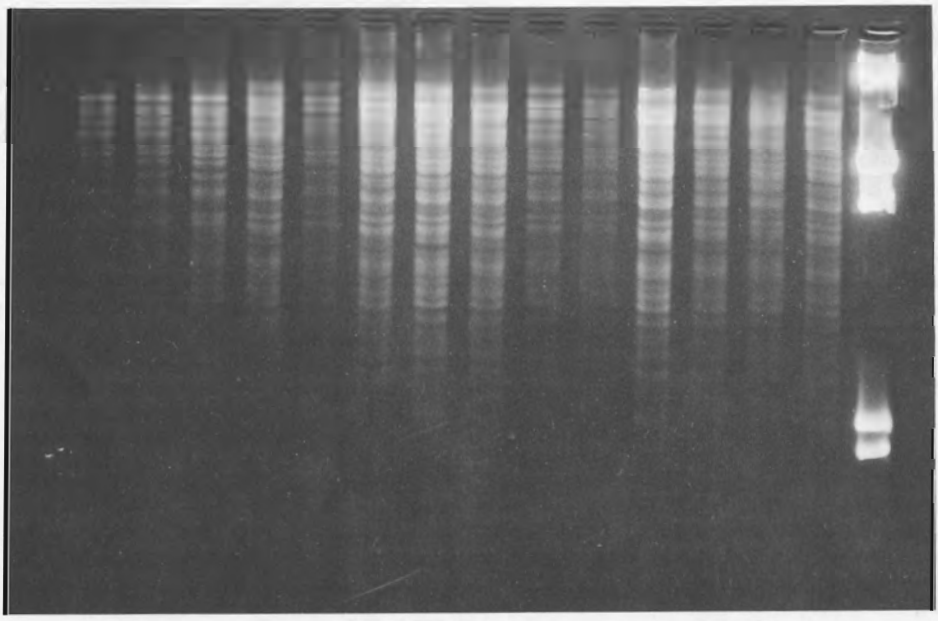

Fig. 1. REA analysis of DNA from *P. multocida* isolates with Eco RI, showing the very complex REA patterns and the difficulty in interpretation and analysis.

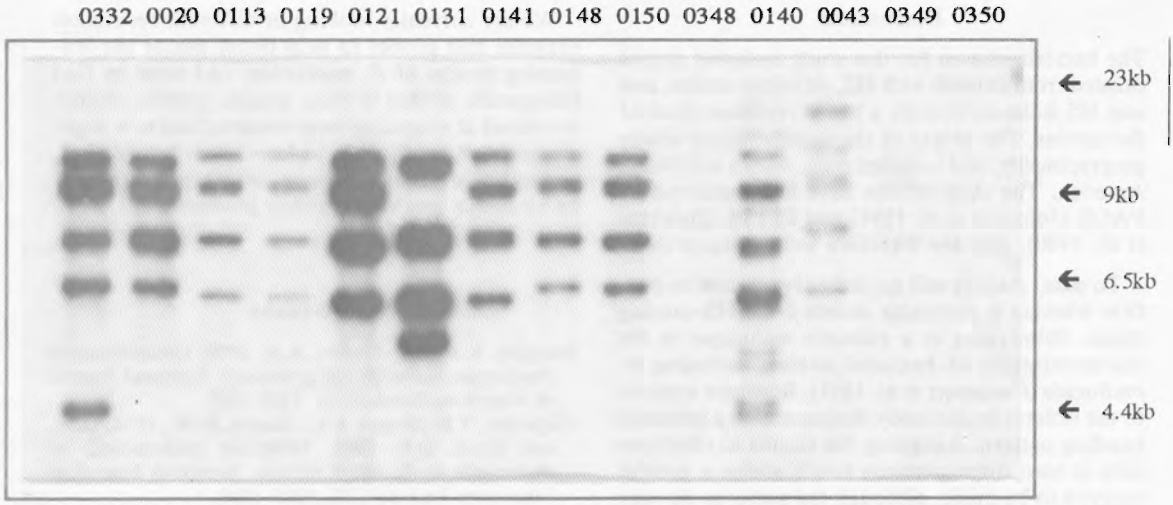

Fig. 2. Eco RI digest of genomic DNA from *P. multocida* isolates. Ribotyping was done using the 23S DNA coding region from *Mycoplasma capricolum* in the plasmid pMC5. The ribotype shows the less-complex pattern of RFLP and the ease of analysis and interpretation in comparison to REA.

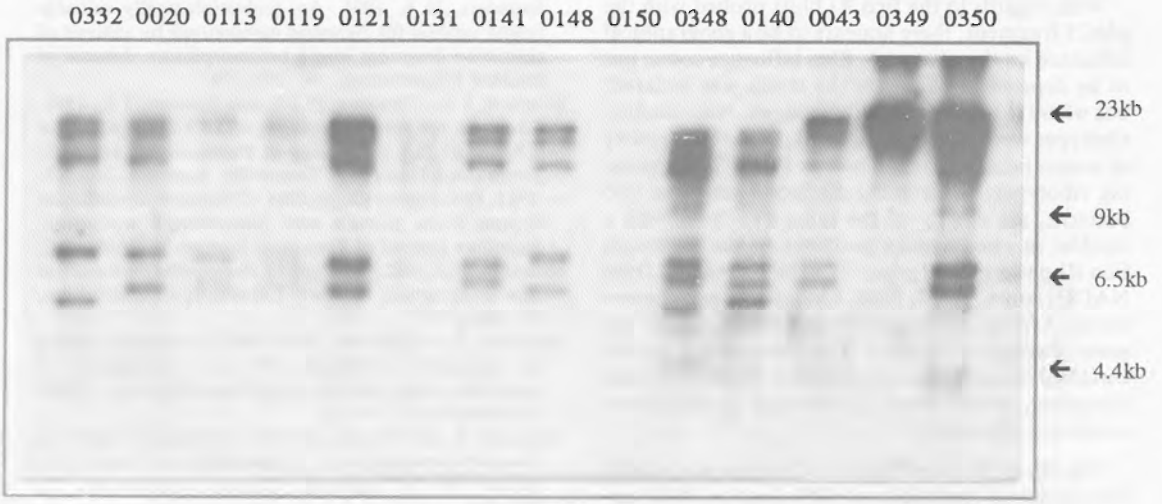

Fig. 3. Ribotyping using the 23S from pMC5 following Pst I digestion of *P. multocida* DNA, showing less-complex patterns and ease of interpretation in comparison to REA.

After probing, similar ribotypes were shown when the isolates were digested with Eco RI, although minor variation was seen with the smaller fragment (5.5–5.9 kb). Similarly, with the Pst I digest, there were common bands, enabling a distinct pattern to be determined. RFLP patterns were assigned arbitrarily as Pattern 1 (Eco RI blot) and Pattern 2 (Pst I blot). Pattern 1 consisted of four bands: 10.7 kb, 9.0 kb, 7.0 kb, and either 5.9 or 5.5 kb; whereas fragment sizes in Pattern 2 were 19.5 kb, 16 kb, 12 kb, and either 7.0 or 6.6 kb. The presence of the Pattern 2 was highly specific for HS isolates. All isolates that tested positive in the HS-ELISA were shown to exhibit Pattern 2 when probed with pMC5. The two strains designated as false negatives by HS-ELISA were shown to be positive by the HS pattern of the Pst I-digest.

Discussion

The bacteria chosen for this study included strains isolated from animals with HS, reference strains, and non-HS isolates covering a broad representation of the species. The origin of the strains varied widely geographically, and included Asia, Africa and North America. The same strains have been analysed by PAGE (Johnson et al. 1991) and ELISA (Dawkins et al. 1990), and are therefore well characterised.

To date, there is still no definitive system to confirm whether a particular isolate is an HS-causing strain. Ribotyping is a valuable technique in the characterisation of bacterial strains, including *P. multocida* (Carpenter et al. 1991). Ribotype analysis of the isolates in this study demonstrated a common banding pattern. Assigning the strains to ribotypes with at least three common bands allows a general analysis to be made, although the patterns do vary slightly within each group. In an attempt to categorise these strains with respect to HS pathogenicity, Pst I ribotypes were correlated with respect to the presence of the arbitrarily assigned Pattern 2.

With regards to the Eco RI blots probed with the pMC5 fragment, there appears to be a geographical influence on the ribotypes. This influence seems not to be dependent on where the strain was isolated, but where it was stored and passaged. Nine distinct ribotypes were seen using Eco RI, with the majority of strains being of either ribotype 1 or 2. The remaining ribotypes, while being distinct from these two patterns, are mostly of the same structure, with a doublet at one or more positions of the dominant Eco RI pattern. The group of strains obtained from NADC, Ames, (0332, 0348, 0349, 0350) and the two South African strains (0148 and 0150), are of the same ribotype, ribotype 1. The Asian strains can be classified as another group (ribotype 2) with only one exception, 0020, which is identical to the group exhibiting ribotype 1.

The HS-ELISA developed by Dawkins et al. (1990) has an overall specificity of 99%, and an 86% sensitivity, which includes five false negatives and only one false positive. This study using rDNA typing has confirmed the HS type strains and the false negative rate in the ELISA. It appears that a Southern blot of Eco RI-digested *P. multocida* isolates can be useful from an epidemiological perspective, while Pst I blots may have some significance with respect to HS pathogenesis.

We are currently looking at different restriction enzymes and probes to help better define the HS-causing strains of *P. multocida*, and hope to find HS-specific probes to these strains. Further studies are aimed at extending these observations to a larger group of *P. multocida* isolates. It is hoped that, through this characterisation, an understanding of its virulence mechanisms may be elucidated.

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Molecular Epidemiology of *Pasteurella multocida* Using Ribotyping

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Abstract

Ribotyping is a molecular typing method that highlights restriction site heterogeneity within ribosomal RNA (rRNA) genes and their adjacent regions. Previous studies have demonstrated the potential use of the method in taxonomic and epidemiological studies to trace and define disease trends. We have applied the technique to study epidemiological trends in *Pasteurella multocida* infections of large ruminants. Eco RI digestion fragments of genomic DNA of *P. multocida* isolates of Carter serogroups B and E were transferred by Southern blotting to nylon membranes. Two sets of rRNA gene restriction patterns were obtained by hybridising P^{32}-labelled 16S fragments from: (1) a phylogenetically remote Gram-negative organism and (2) a haemorrhagic septicaemia-causing *P. multocida* isolate. Distinct ribotypes of *P. multocida* could be distinguished following autoradiography. The specificity of rRNA gene restriction patterns varied with the probe employed, the geographical origin of the isolate and, to some extent, with the host species. The results demonstrate that ribotyping may provide a widely applicable system to investigate the molecular epidemiology of *P. multocida* infections.

VARIOUS typing systems have been developed in attempts to differentiate *Pasteurella multocida* isolates. The systems most commonly used involve serotyping according to capsular (Carter 1955; Rimler and Rhoades 1987) and somatic (Heddleston et al. 1972) antigens. Problems encountered have included lack of correlation between the different typing systems (Brogden and Packer 1979), and difficulties in typing many isolates (Manning 1982).

Despite the importance of *P. multocida* in veterinary diseases, there has been only limited application of molecular genetics to the study of this organism. Classifications based on genetic relatedness can avoid potential pitfalls associated with variable expression of phenotypes. The most recent advances in DNA technology have provided new typing methods based on characterisation of the genotype, such as restriction endonuclease analysis (REA), and the use of DNA and RNA probes.

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REA (Sambrook et al. 1989) provides an alternative means of characterising and differentiating between closely related strains of bacteria. Restriction enzymes cut the DNA at specific nucleotide sequences and produce a set of DNA fragments that, when separated by electrophoresis, provide a characteristic band or fingerprint of the respective genome. This technique has been used successfully in studies on the pathogenesis of *P. multocida* infection in pigs (Butteschon and Rosendal 1990); the differentiation of *P. multocida* isolates from reference vaccine strains in turkeys (Kim and Nagaraja 1990); and the characterisation and classification of *P. multocida* Carter serogroup B and E isolates (Wilson et al. 1992). A disadvantage of REA is that, depending on the restriction enzyme used, it may be difficult to interpret results when a multitude of fragments are produced and subtle differences may be missed. The use of labelled nucleic acid probes to highlight restriction site heterogeneity can overcome these problems.

Snipes et al. (1989) used REA and rRNA probes to characterise *P. multocida* isolates genotypically to differentiate strains for epidemiological studies. This procedure, which compares highly conserved

rRNA genes and their adjacent sequences, has been named 'ribotyping'.

The purpose of this study was to attempt to ribotype *P. multocida* isolates from large ruminants in order to identify small genotypic differences that may relate to geographical and host species differences.

Materials and Methods

Bacterial isolates

P. multocida isolates were obtained from the pasteurella reference collection held at the Regional Veterinary Laboratory, Benalla (Johnson et al. 1991). Reference strains 0332/M1404 and 0348/P932 were isolated in North America from bison with haemorrhagic septicaemia (HS). Strains 0140/989-A (Queensland 11B) and 0131/973 (Izatnager 25) are classic *P. multocida* strains. Although there is a paucity of information on the history of the HS isolates used, all are believed to have originated from clinically affected animals. All, except 0106 and 0348/P932, reacted positively in the HS-ELISA (Dawkins et al. 1990).

Media and growth conditions

All isolates were grown routinely on sheep blood agar (7% sheep blood, 3.9% Columbia agar) and stored in glycerol broths (−70 °C) and as lyophilised cultures. For use, bacteria were harvested from lawn cultures grown overnight at 37 °C (5% CO_2) on sheep blood agar.

Purifying genomic DNA

A modification of a simplified procedure (Troyer et al. 1990) was used to extract genomic DNA from *P. multocida* isolates. Bacterial cells were harvested by centrifugation at 12 000 rpm for 5 min at room temperature and the resulting pellet was stored at −80 °C until used. The pellet was resuspended in 500 μL of buffer (20m*M* Tris, 100m*M* EDTA, pH 8.0) in a microfuge tube. Lysozyme was added (4 mg/mL) and the tube was placed on ice for 5 min. SDS (1%), NaCl (0.5*M*) and Proteinase K (2 mg/mL) were added and the tube was incubated at 55 °C for 2 hours. Following the addition of 500μL of a 25:24:1 mixture of phenol:chloroform: isoamylalcohol, the tube was inverted vigorously until a white emulsion formed, held at −20 °C for 30 min and then centrifuged at 12 000 rpm for 15 min at 4 °C. The aqueous phase was transferred to another microfuge tube and the phenol:chloroform: isoamylalcohol extraction was repeated twice, followed by a wash with 500 μL chloroform. DNA was precipitated from the aqueous phase by adding sodium acetate to a final concentration of 0.3*M* and 2 volumes of ice cold ethanol, followed by centrifugation at 12 000 rpm for 15 min at 4 °C. The nucleic acid pellet was washed once with 70% ethanol, dried and redissolved in TE buffer (10m*M* Tris, 1m*M* EDTA, pH 8.0, 50 μL/mL RNase), incubated for 10 min at 37 °C, centrifuged for 5 s in a microfuge, and stored at −20 °C.

Probes

The heterologous probe (Probe 1) was prepared using the 4.8-kb fragment isolated from an Eco RI digest of pMC5 (Glaser et al. 1984). This fragment contains a 5S rRNA gene (1.8 kb), a 23S rRNA gene (2.8 kb) and part of a 16S rRNA gene (0.2 kb) of *Mycoplasma capricolum*. The homologous probe (Probe 2) was prepared using the 1.5-kb fragment isolated from a Bam H1 digest of the plasmid vector pUC19. This fragment contained a virtually complete 16S rRNA sequence (minus about 30 nucleotides from the 3′ terminus) of *P. multocida* reference strain M1404/0332. The fragment was obtained from a genomic DNA preparation of M1404/0332 by polymerase chain reaction (PCR) amplification (Saiki et al. 1988) using universal primers (Lane 1991). The locations of the primers in the 16S molecule are indicated in Figure 1.

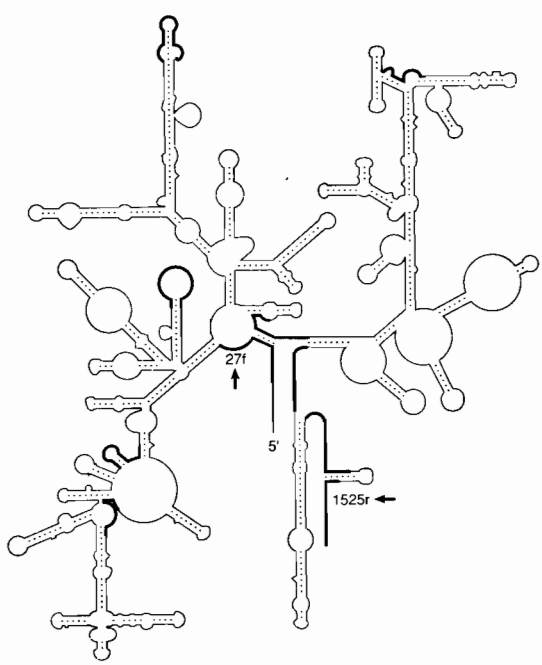

Fig. 1. A tracing of *Escherichia coli* 16S rRNA showing the annealing positions of the universal primers used in the PCR amplification of the 16S rRNA gene of *P. multocida* isolate 0332/M1404. The resulting 1.5-kb fragment was used as the homologous probe (Probe 2).

Restriction endonuclease digestion of genomic DNA

Purified bacterial DNA was digested with Eco RI in a reaction mixture containing the appropriate buffer as recommended by the manufacturer (Promega). Following overnight incubation at 37 °C, the digested samples were mixed with loading buffer and electrophoresed in 1% agarose gels at 80 mA for 4–5 hours in TAE buffer (0.04*M* Tris-acetate, 100m*M* EDTA). The gel was stained with ethidium bromide and photographed under short-wave UV transillumination through a Wratten 23A gelatin filter.

Southern blot analysis

Prior to transfer, agarose gels were depurinated twice in 0.25*M* HCl for 8 min. Restriction fragments contained in agarose gels were blotted onto nylon membranes (Hybond N+, Amersham) by capillary transfer under alkaline conditions for 16–18 hours at room temperature, essentially as described by Southern (1975). Membranes were washed briefly in 5 × SSC (1 × SSC is 0.15*M* NaCl, 0.015*M* sodium citrate, pH 7), wrapped in plastic film and stored at 4 °C.

Hybridisation with heterologous and homologous probes

Purified insert DNA was labelled with ^{32}P-dATP (Bresatec) using either the GIGAprime DNA Labelling Kit (Bresatec) or the Nick Translation Kit (Promega) to a specific activity of 2 × 10^9 cpm/ μg. Unincorporated label was removed using Geneclean (Bresatec). Prehybrisation and hybridisation was performed essentially as described by Sambrook et al. (1989). Nylon membranes were prehybridised for 2 hours at 42 °C in prehybrisation solution (6 × SSC, 5 × Denhardt's reagent, 0.5% SDS, 100 μg/mL denatured salmon sperm DNA, 50% formamide). The prehybridisation solution was replaced with hybridisation solution (6 × SSC, 0.5% SDS, 100 μg/mL denatured salmon sperm DNA, 50% formamide) prior to the addition of radiolabelled probe. Hybridisation was for 16–18 hours at 42 °C. The washing sequence consisted of 2 × SSC and 0.5% SDS for 5 min at room temperature, 2 × SSC and 0.1% SDS for 15 min at room temperature, 2 × SSC and 0.5% SDS for 30 min at 37 °C, and 2 × SSC and 0.5% SDS for 30 min at 68 °C. The membranes were then washed briefly in 2 × SSC, wrapped in cling film, and autoradiographed with Kodak X-OMAT AR film with intensifying screens.

Results

Restriction endonuclease digestion

Digestion of genomic DNA with Eco RI yielded a homogenous distribution of DNA fragment lengths for all *P. multocida* isolates tested (data not shown).

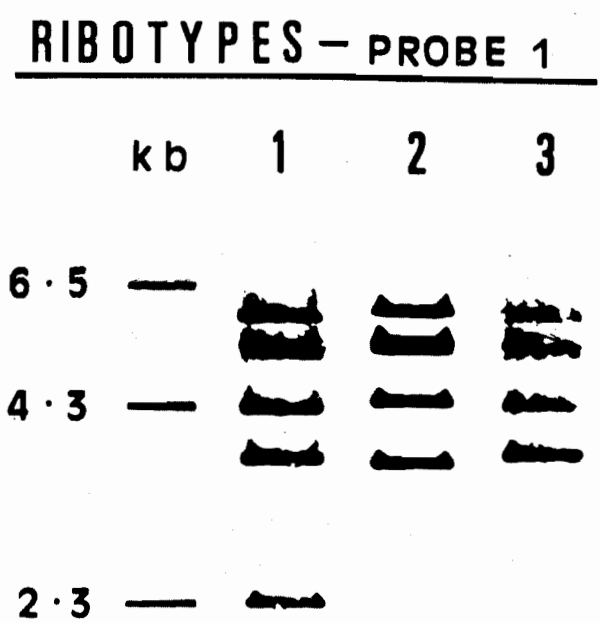

Fig. 2. Autoradiograph of Southern blot of Eco R1-digested genomic *P. multocida* DNA probed with the 4.8-kb insert from pMC5 (Probe 1). Ribotype patterns have been designated as 1, 2 and 3.

Hybridisation using heterologous rDNA probe

Following Southern blotting, the Eco RI digests of the *P. multocida* isolates were hybridised with Probe 1. Hybridisation band patterns for all isolates fell into three clearly distinguishable patterns (Fig. 2). Three hybridisation bands between 4.3 kb and 6.5 kb were common to all isolates. Ribotypes were characterised by additional hybridisation bands as follows: ribotype 1, bands at 3.5 kb and 2.3 kb; ribotype 2, a band at 3.3 kb; and ribotype 3, a band at 3.5 kb. Ribotype 1 was observed in *P. multocida* isolated in North America from bison (reference strains 0332 and 0348; 0089, 0104, 0105, 0106 and 0107). Ribotype 2 was seen in *P. multocida* strains isolated in Southeast Asia from cattle and buffalo (0127, 0131, 0019, 0365, 0417 and 0243) and in one cattle isolate from Africa (0149). Ribotype 3 was observed in *P. multocida* strains isolated in Africa from cattle (0350, 0148, 0152 and 0150) and in the Australian 11B isolate.

Hybridisation using homologous rDNA probe

The nylon membrane was stripped and hybridised with Probe 2. Nine to ten bands were detected with each isolate, producing five clearly distinguishable band patterns (Fig. 3), denoted I, II, III, IV and V. (This numbering system is for convenience only and should not be confused with currently used serotyping systems.) Although Probe 2 produced more hybridisation bands than Probe 1, the banding patterns between the two probes were closely related. All the bands observed in ribotype 1 (Probe 1) were present in ribotypes I and II; ribotype 2 (Probe 1) correlated with ribotypes III and IV; and ribotype 3 (Probe 1) correlated with ribotype V. The graphical representation of the dominant bands in the ribotype

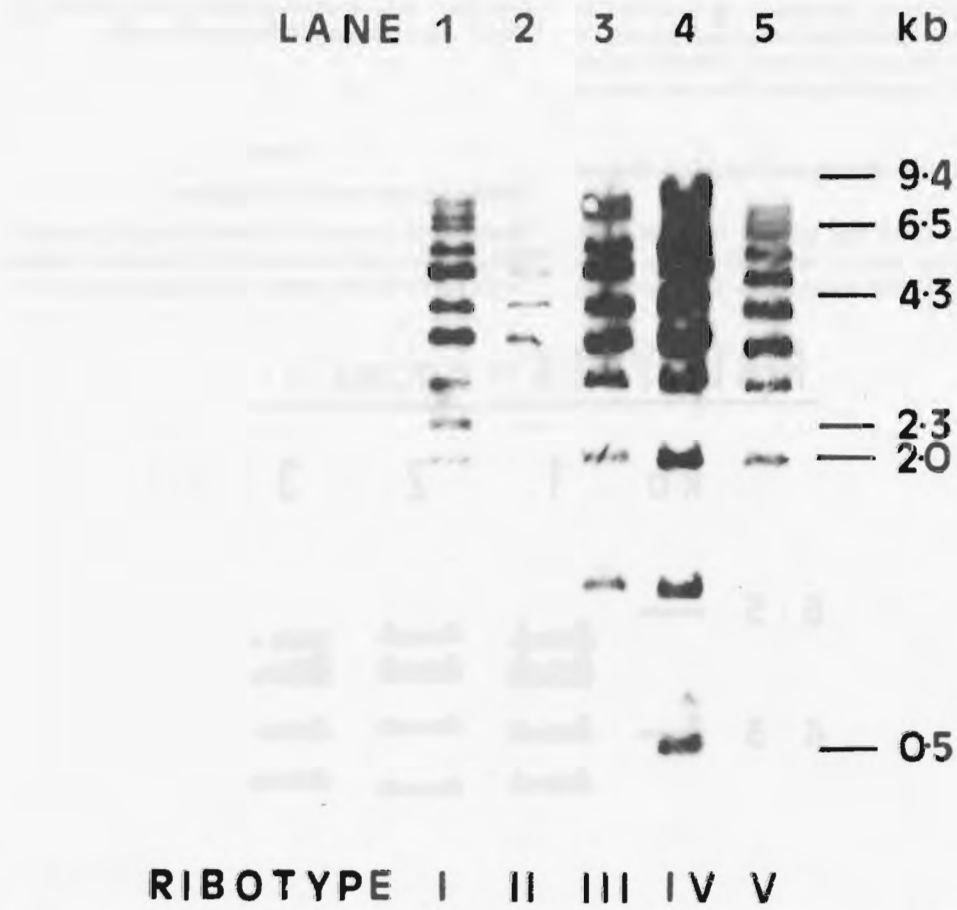

Fig. 3. Autoradiograph of Southern blot of Eco R1-digested genomic *P. multocida* DNA probed with the 1.5-kb fragment representing the 16S rRNA gene of *P. multocida* isolate 0332/M1404. Ribotype patterns have been designated as I, II, III, IV and V.

patterns (Fig. 4) shows similarities in the banding patterns with six bands common to all isolates. In addition to the band positions already described with Probe 1, two bands between 9.4 kb and 6.5 kb and a band at 2.0 kb were common to all isolates. Ribotypes II and V differ from ribotype I by only 1 band at 3 kb and 2.3 kb respectively. Ribotype IV differs from III only by the presence of a band at 0.5 kb.

Ribotype I was seen with six *P. multocida* strains: reference strain 0332; 0089, 0106, 0104, 0105, and 0107. Ribotype II was only observed with the reference strain 0348. Ribotype III was seen in five Asian *P. multocida* strains: 0127, 0019, 0365, 0417, and 0243; and in one African isolate: 0149. Ribotype IV was only observed with the reference strain 0131, and ribotype V was associated with four African isolates (0350, 0148, 0152 and 0150) and the Australian 11B isolate.

Discussion

P. multocida isolates of the Carter B and E serotypes were characterised according to their rRNA gene restriction patterns. Two sets of rRNA gene restriction patterns were obtained by hybridising Southern-blotted *P. multocida* genomic DNA fragments from Eco RI digests with (1) pMC5, a clone containing part of the 16S, and the entire 23S and 5S, rRNA genes from *M. capricolum*; and (2) a specific 16S rRNA probe from *P. multocida*. Each probe generated ribotypes (Figs 2 and 3) that were identified by characteristic banding patterns. The different ribotype patterns indicate sequence variation in the highly conserved rRNA genes.

In the present study, some hybridisation bands were common to all isolates, regardless of serotype. Probes that target more conserved regions of the genome tend to emphasise the similarities between

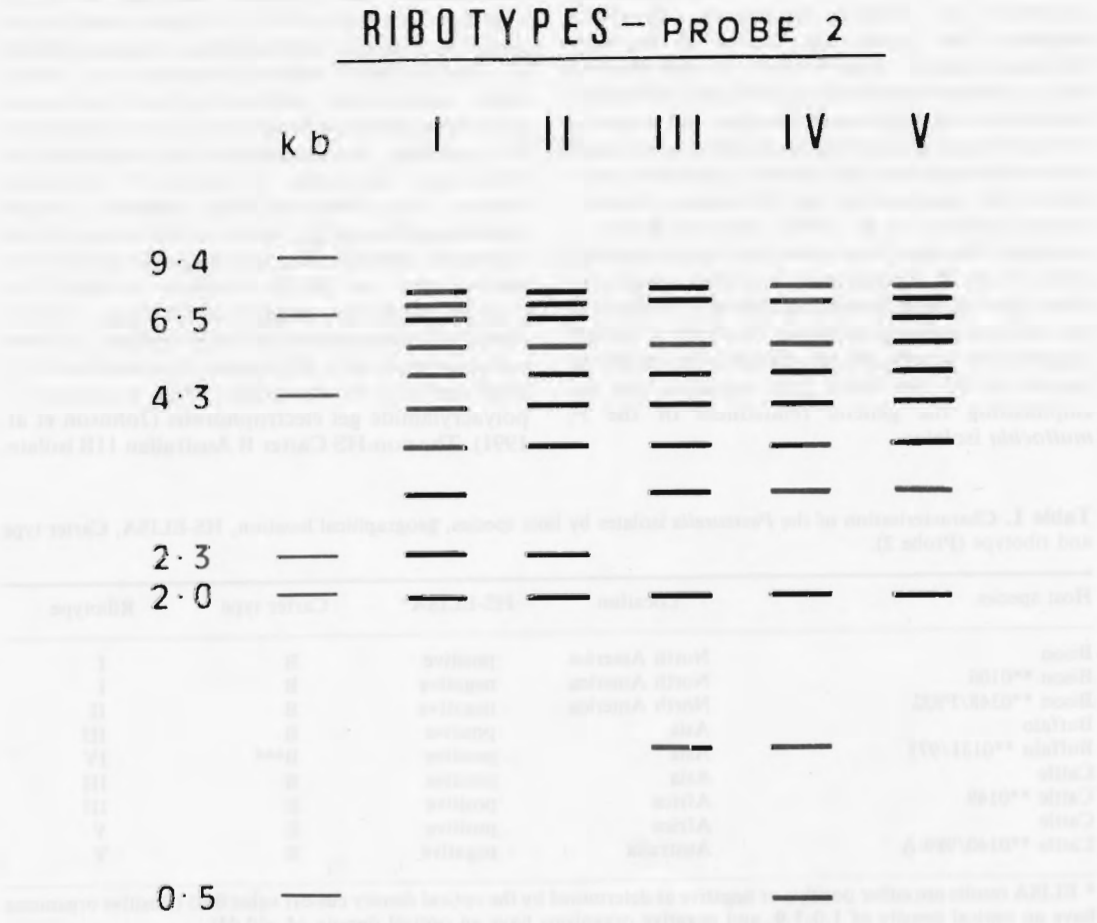

Fig. 4. Graphical representation of the ribotypes shown in Figure 3.

strains, producing fewer bands and identifying related strains (Morrow et al. 1990). As Probe 1 was cloned from *M. capricolum,* a phylogenetically remote organism with respect to *P. multocida*, the ribotype patterns observed are most likely to represent similarities between the isolates that are related to the conserved regions of the rRNA gene. These results show that particular ribotypes tend to be associated with the geographic origin and, to a lesser extent, with the host species of the *P. multocida* strains. These findings are consistent with the observation that protein profiles of HS isolates could be placed into two groups on the basis of country of origin, with isolates of North American and Asian origin (Carter B) forming one group and those of African origin (Carter E) the other (Johnson et al. 1991).

The PCR-generated Probe 2 is a nearly complete DNA copy of the double-stranded 16S rRNA gene sequence of a *P. multocida* isolate, containing both conserved and variable regions of 16S rRNA sequence. This probe has proven to be more sub-species specific than Probe 1, in that we were able to demonstrate distinct genotypic differences between strains of the same serotype, and strains of different serotypes. Sub-species probes tend to target the variable regions of the genome, producing more bands and emphasising the differences between strains (Morrow et al. 1990). As with Probe 1, particular ribotypes were associated with geographic origin of the *P. multocida* strains. The presence of all the hybridisation bands recognised by Probe 1 in the ribotype patterns produced by Probe 2 further suggests that these bands are related to the conserved regions of the 16S rRNA gene sequence, and are emphasising the genetic relatedness of the *P. multocida* isolates.

Despite distinct differences in the ribotype banding patterns of individual isolates, we were unable to identify patterns or bands unique to HS-causing isolates. However, HS isolates did follow general trends in the ribotype patterns observed using the homologous probe (Probe 2) (Table 1). North American HS isolates were ribotype I, Asian HS isolates (with the exception of 0131) were ribotype III, and African HS isolates (with the exception of 0149) were ribotype V. Ribotype IV was unique to isolate 0131, which was obtained from the collection of Professor R.V.S. Bain (University of Sydney), and is described as being avirulent. The African Carter E HS isolate 0149 had an identical ribotype to the Asian Carter B HS strains.

Ribotype II was seen only with isolate 0348. The history of this isolate is vague, although a recent publication (Wilson et al. 1992) suggests that it is derived from the Buffalo B reference strain 0332/M1404. The anomalous reactions of isolates 0348 and 0106 in the HS-ELISA have raised doubts about the virulence of these isolates. Immunoelectron microscopy (IEM) studies (Dawkins et al. 1991), using monoclonal antibodies that recognised lipopolysaccharide epitopes on the outer surface of the organism, were employed to investigate the HS-antigen expression of several *P. multocida* isolates. Both 0348 and 0106 showed a varied immunogold staining pattern, with less than 10% of organisms labelled. The percentage of bacteria that reacted were as heavily labelled as HS-ELISA positive organisms, suggesting dichotomy in the phenotypic expression within these isolates. This was not associated with differences in protein or LPS band patterns or intensities when examined by polyacrylamide gel electrophoresis (Johnson et al. 1991). The non-HS Carter B Australian 11B isolate,

Table 1. Characterisation of the *Pasteurella* isolates by host species, geographical location, HS-ELISA, Carter type and ribotype (Probe 2).

Host species	Location	HS-ELISA*	Carter type	Ribotype
Bison	North America	positive	B	I
Bison **0106	North America	negative	B	I
Bison **0348/P932	North America	negative	B	II
Buffalo	Asia	positive	B	III
Buffalo **0131/973	Asia	positive	B***	IV
Cattle	Asia	positive	B	III
Cattle **0149	Africa	positive	E	III
Cattle	Africa	positive	E	V
Cattle **0140/989-A	Australia	negative	B	V

* ELISA results are either positive or negative as determined by the optical density cut-off value 0.05 (Positive organisms have an optical density of 1.0–1.9, and negative organisms have an optical density of <0.05)
** These isolates were unique in their characterisation
*** avirulent — unknown context

which had the same ribotype as most of the African HS Carter E strains in the present study, was negative by HS-ELISA and IEM in the same study. Although some cattle breeds of African origin are found in Australia, we do not have sufficient clinical and epidemiological information on Australian *P. multocida* strains to comment further.

The use of rRNA, particularly 16S RNA, for comparative molecular studies is now well established (Lane et al. 1985; Olsen et al. 1986; Chuba et al. 1988) and applications of ribotyping to epidemiological investigations have been published (Stull et al. 1988; Snipes et al. 1989). This study is a preliminary investigation of the use of ribotyping for classification of *P. multocida* isolates on the basis of genotype. These results may not necessarily be representative of all *P. multocida* isolates from large ruminants, and should be considered valid only for the *P. multocida* strains used here. Future investigations may provide more insight into the genetic properties of the strains, and may form the basis for a stable and reliable typing system based on genetic relatedness.

Acknowledgments

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SESSION 2: THE DISEASE — PATHOGENESIS, PATHOLOGY, EPIDEMIOLOGY

Pasteurellosis: The Disease

N.J.L. Gilmour[1]

Abstract

The epidemiology and pathogenesis of pneumonic pasteurellosis in cattle and sheep caused by *Pasteurella haemolytica* biotype A, systemic pasteurellosis in sheep caused by *P. haemolytica* biotype T, and haemorrhagic septicaemia in cattle caused by *P. multocida* are compared with the aim of highlighting features of each that may be relevant in future research.

PNEUMONIC and systemic pasteurellosis and haemorrhagic septicaemia (HS) have been described comprehensively (Gilmour and Gilmour 1989; Frank 1989; Carter and De Alwis 1989), so it is not necessary to elaborate here on the descriptions of these diseases; rather similarities and differences will be discussed in an attempt to define gaps in our knowledge and indicate lines of future research.

Pneumonic Pasteurellosis

Epidemiology

Pasteurella pneumonia is caused by *P. haemolytica* biotype A serotypes and occurs in most of the sheep-rearing countries of the world. *P. haemolytica* is present in the upper respiratory tract of sheep of all ages. A crucial question is: What converts these healthy carriers into cases of clinical disease? Predisposing factors fall into two main categories: first, management and environment, in which proof is circumstantial; and second, infectious agents that have been incriminated epidemiologically in association with outbreaks of pneumonic pasteurellosis. Experimental production of the disease with a predisposing virus (e.g. parainfluenza virus type 3) followed by aerosols of *P. haemolytica* of biotype A has demonstrated the link between virus infections and pasteurellosis.

Pneumonic pasteurellosis occurs in sheep of all ages. In lambs less than 3 weeks old the disease is hyperacute, with a generalised infection; between 3 and 12 weeks the disease is an acute infection lasting 2 or 3 days, characterised by pleurisy and pericarditis. The signs of acute pneumonia — hyperpnoea and dyspnoea — are present in older sheep. In flock outbreaks, the first manifestations are sudden deaths and very ill sheep that die quickly. Within a few days acute cases disappear, to be replaced by cases with the more obvious signs of pneumonia. Mortality is typically about 5%, and morbidity up to 10%. Other flock signs are oculo-nasal discharges and coughing. Untreated, subacute cases that survive develop chronic pneumonia with the lesions in the apical parts of the lungs, and pleural adhesions.

The prevalence of pneumonic pasteurellosis within flocks varies from year to year, as does the prevalence in districts or countries. This may be due to rising and falling immunity to the predisposing viral infection and to the pasteurellae themselves. Although it has been shown that lambs that have recovered from acute pneumonic pasteurellosis are immune to reinfection, nothing is known of specific immunity in the carriers. Immunity from vaccination appears to be serotype specific. As the spectrum of serotypes in nasal carriers is wide and constantly changing, the investigation of this aspect has been little researched, as has the phenomenon of innate resistance.

Pneumonic pasteurellosis in calves has both similarities to and differences from pneumonic pasteurellosis in sheep. It is caused by *P. haemolytica*, almost always of serotype A1, in contrast to the disease in sheep, which is predominantly caused by serotype A2, although serotypes A1, 6, 7 and 9 are also involved, with other A serotypes more rarely.

In cattle the carrier state differs from that in sheep. *P. haemolytica* is isolated in culture from nasal swabs

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of fewer healthy cattle than of sheep. This may be because in cattle the organism forms only a small proportion of the nasal bacterial flora, and is therefore less readily detected. The prevalence and numbers of *P. haemolytica* in the nasopharynx increase during stress conditions that predispose to pneumonic pasteurellosis.

The disease in calves most often occurs in newly weaned animals that have been through markets and mixed together. As in sheep, the causes are multifactorial — a mixture of stress and predisposing infection, with the final, acute illness caused by *P. haemolytica*. A combination of environmental stress and non-lethal viral infections compromise the defence mechanisms. In North America, the syndrome, called shipping fever because of its association with relocation as described above, is a prime cause of economic loss to the beef industry.

Pneumonia in adult cattle is also caused by *P. multocida* serotypes A and D. It tends to be a more sporadic disease, affecting individual animals.

Pathogenesis and pathology

In the normal, healthy, unstressed animal the cellular defence mechanisms of the lungs and the mucociliary ladder serve to clear pasteurellae, which are deposited there in aerosols from the nasopharynx. When the defence mechanisms are compromised, the organisms in the alveoli multiply. It has been postulated that initial multiplication occurs in the nasopharynx, and that the lungs are subsequently deluged with large numbers of bacteria they cannot cope with. Once multiplication in the alveoli has begun, the virulence determinants exert their influence to produce alveolar oedema, inflammatory cell exudate and interalveolar haemorrhage.

Spindle-shaped macrophages or 'oat-cells', which form whorls and streams between adjacent alveoli, are pathognomonic in both cattle and sheep.

Experimental production

Research into pneumonia caused by *P. haemolytica* was impeded for many years by the absence of reproducible and standardisable methods for experimental infection. For studies into pathogenesis and immunity, it is desirable to produce a disease similar to the natural one via the natural route, in this case via the respiratory tract, with a similar number of organisms to that which may realistically be expected to occur in nature. Some compromises have to be made. It is advantageous to produce disease in a higher proportion of experimental animals than occurs under natural conditions, so that group sizes in vaccine and therapy trials can be minimised. Production of pneumonia proved almost impossible in conventional sheep, unless very high numbers of organisms were given intratracheally. Specific pathogen free (SPF) lambs infected with parainfluenza virus type 3 and 7 days later exposed to aerosols of *P. haemolytica* have proved to be a generally reliable model of the natural disease. Takes of 80–90% are possible.

Systemic Pasteurellosis in Sheep Caused by *P. haemolytica* Biotype T Strains

Systemic pasteurellosis is an important disease of sheep in Britain. However, although it has been reported in other countries (e.g. the USA and Hungary), it appears to be less common worldwide than pneumonic pasteurellosis. In Britain, it causes sudden death in weaned lambs between September and December. Its occurrence in flocks is sporadic, and mortality is typically 3–5%. Asymptomatic carriage of biotype T strains occurs in the tonsils of many sheep, rather than in the nasopharynx, as with biotype A strains. Stressful environmental and management factors have been implicated as predisposing causes, but clinical disease does not invariably follow exposure to these.

Pathogenesis and pathology

A model for the pathogenesis of the disease was derived from in-depth, quantitative bacteriology and from histopathology of natural cases of the disease. Usually the shepherd is presented with one or two dead sheep. Any sheep seen alive are recumbent and comatose, with copious, frothy, sometimes blood-stained, nasal and oral discharges. The duration of illness does not exceed 3–4 hours in most cases.

At necropsy there is extensive subcutaneous haemorrhage over the neck and thorax, acute congestion of the pleurae, pericardium and lungs and straw-coloured pleural and pericardial effusions. The lungs are oedematous and often uniformly plum-coloured. Petechiae are present in heart muscle, liver and spleen. The tonsils are inflamed, with ulceration of the epiglottis and the pharynx in the region of the tonsillar crypts. Ulceration is common in the oesophagus, and may be found also in the abomasal mucosa. *P. haemolytica* can be cultured from these sites, but not in large numbers. Histologically the characteristic lung lesions, apart from capillary congestion and oedema, are the result of bacterial emboli. These occur with or without, depending on the age of the lesions, a halo of inflammatory cells. Large numbers of organisms (up to 10^{10}/g) are cultured from affected lungs. Counts from liver, spleen and kidneys are always lower. *P. haemolytica* is always isolated from the blood of clinical cases,

but there is no evidence that the disease is a true septicaemia (i.e. with organisms multiplying preferentially in the blood).

It is possible that the bacteria enter the body by way of the pharyngeal, tonsillar, oesophageal or abomasal ulcers, and then move via lymphatic or venous drainage to the terminal capillaries of the lungs. It is not known if significant bacterial multiplication takes place at these portals of entry. However, massive multiplication takes place in the lungs, with subsequent spillover into other organs and the blood. The bacteria multiplying in the lungs produce the toxins that elicit the lung changes and lead to anoxia and death.

Experimental production

While it is possible to kill sheep with large numbers of *P. haemolytica* of biotype T, the disease produced is not the same pathologically as the natural one, and administration of organisms in aerosols to SPF lambs has failed to cause disease. There has been only one report of production of the natural disease by manipulation of the diet (Suarez-Guemes et al. 1985).

As SPF lambs could not be infected by the respiratory route, our aim was to administer the organisms at an equivalent stage to that which occurs between tonsil and lung in the natural situation. Graded doses in 1/2 $\log_{10}$ steps were given to 9-week-old SPF lambs subcutaneously in the prescapular region. The outcome was highly dose dependent: fewer than 5×10^8 organisms killed only the occasional lamb, but 10^9 organisms killed 100% of the lambs within 5 hours. In the latter lambs, the disease was not bacteriologically or pathologically typical of the natural one. However, in lambs given the smaller dose, typical systemic pasteurellosis was produced consistently in about 75%. Clinically apparent disease occurred 9–12 hours after infection, and seldom as late as 24 hours after infection. The results confirm the original hypothesis for the pathogenesis of the disease, namely a non-respiratory-tract portal of entry with the disease following from multiplication of the organisms in embolic lesions in the lungs. The precise trigger mechanisms for the initial alimentary tract ulceration is not known, and neither is the question of whether or not this ulceration occurs only in those animals that subsequently develop systemic T disease.

Haemorrhagic Septicaemia

HS is a specific form of pasteurellosis in buffalo and cattle and occasionally in other species and is caused by *P. multocida* serotype 6:B in Asia and serotype 6:E in Africa. HS was the subject of a recent review (De Alwis 1992), and it is intended here only to highlight aspects of the disease that emphasise how it is similar to or differs from the other forms of pasteurellosis described above.

Epidemiology

As with *P. haemolytica* in pneumonic pasteurellosis, HS-causing *P. multocida* occurs in the nasopharynx of apparently healthy animals, especially in-contacts in recent outbreaks. This carriage tends to be transient, and does not explain adequately the epidemiology of HS. The recent discovery of a more-prolonged tonsillar carriage (latent carriage), with intermittent flare-ups of subclinical infection with nasopharyngeal carriage (active carriage), did much to provide a reasonable hypothesis for the pathogenesis of the disease.

It is thought that HS occurs when an active carrier (i.e. excretor) comes in contact with a susceptible animal (i.e. one with neither innate resistance nor active immunity acquired through a previous latent carrier – active carrier cycle). The introduction of an active carrier into an area where the disease is not endemic and the population is therefore highly susceptible leads to an explosive outbreak, with spread of infection from clinical cases to in-contact animals. In areas where the disease is endemic and seasonal, the low prevalence can be explained by the fact that there is more active immunity. Clinical disease will occur only when immunity wanes, or when an unexposed host is encountered.

It is difficult to explain how an unexposed host can be encountered in endemic areas. Even if it has been born after a previous outbreak, it must have been in contact with survivors of that outbreak. These survivors must include latent and occasionally active carriers, from which infection is possible. It is more likely that the susceptible host is one in which other factors have compromised immunity. At the moment these factors can only be defined as 'stress'.

Pathogenesis and pathology

As with *P. haemolytica* T disease in sheep, there is no evidence that HS is in fact a true septicaemia. Since the earliest clinical sign noticed is oedematous submandibular swelling, it is tempting to suggest that initial bacterial multiplication occurs in the nasopharyngeal region, with subsequent spread to the lungs and ultimately to other organs and the blood, as in T disease in sheep.

Immunity

Vaccination is the preferred method of control for all three forms of pasteurellosis described. However, there are few, if any, highly efficient vaccines. A brief consideration of some aspects of immunity is worthwhile.

First, innate immunity. Not all naive animals exposed to pasteurellae and to the same stresses as their companions develop the disease. The immunologists should seek to determine what factors in the immune system differ in resistant and susceptible animals. Second, active immunity. Again, little is known about active immunity. It is clear that all carriers are not immune in pneumonic and systemic pasteurellosis in sheep, since carriers become clinical cases under the influence of environmental stresses or other infectious agents. True active immunity may occur following subclinical infection, and it has been shown that sheep that recover from acute pneumonic pasteurellosis as a result of antibiotic therapy are solidly immune to further disease from experimental challenge. Further, it was shown that antigens produced in vivo were important in this immunity.

Populations of calves with higher titres of leukotoxin-neutralising antibodies have a lower susceptibility to shipping fever than do those with lower titres. It is not known whether these antibodies result solely from the carrier state, or from previous subclinical infections. In HS, De Alwis has postulated that subclinical nasopharyngeal recrudescences of tonsillar infections maintain immunity. It is not known which antigenic determinants stimulate this immunity. An understanding of the nature of this immunity is probably crucial to the development of improved vaccines for HS. The similarities and differences in the epidemiology, pathogenesis, pathology and immunity of pneumonic pasteurellosis, systemic pasteurellosis and haemorrhagic septicaemia are worthy of study in looking for leads for future research into the prevention of all three diseases.

Acknowledgments

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Bovine Tonsils as Reservoirs for *Pasteurella haemolytica*: Colonisation, Immune Response, and Infection of the Nasopharynx

G.H. Frank,[1] R.E. Briggs[1] and B.M. DeBey[1]

Abstract

Studies were conducted to determine whether *Pasteurella haemolytica*-colonised tonsils could serve as a reservoir for *P. haemolytica* infection of the nasopharynx. Calves' tonsils were inoculated with *P. haemolytica* by instillation into the palatine tonsillar sinuses. Pasteurellae were counted in tonsil washes and aspirated nasal secretions. All calves shed from the tonsils for at least several weeks, whereas only 14 of 28 calves shed *P. haemolytica* in the nasal mucus, usually for less than 1 week. Colonised calves responded serologically to *P. haemolytica* and to leukotoxin. Most calves did not exhibit clinical disease. However, some younger calves (3–5 months) had severe clinical disease ranging from acute submandibular oedema with pneumonia to invasion and destruction of the medial retropharyngeal lymph nodes. Some older calves (7–15 months) exhibited more chronic systemic invasion with joint involvement. After nasal shedding had ceased for at least 1 week, calves were infected with infectious bovine rhinotracheitis virus intranasally. Numbers of *P. haemolytica* in tonsils did not increase, but 7 of 21 calves began shedding *P. haemolytica* in their nasal secretions. Nasal shedding usually lasted for more than 3 days.

These studies show that bovine tonsils can harbour *P. haemolytica* for long periods and can be reservoirs for *P. haemolytica* infection of the nasopharynx. Knowledge of the ecological and physiopathologic relationship of the tonsil with *P. haemolytica* could improve understanding of the pathogenesis of pneumonic pasteurellosis and lead to new means of protection.

FIELD and laboratory studies on bacterial colonisation of the nasopharynx suggest that *P. haemolytica* serotype 1 can survive for long periods in healthy calves in sites other than the main nasal passages (Frank and Smith 1983; Frank 1986, 1988; Frank et al. 1986, 1989). These sites would serve as reservoirs of *P. haemolytica* in healthy calves, and colonise the nasopharynx during times of stress or respiratory viral infection. Heavy colonisation of the nasopharynx often occurs prior to the development of pneumonic pasteurellosis. Since *P. haemolytica* has been isolated from tonsils of sheep and cattle at necropsy, they appeared to be a likely reservoir.

We conducted three experiments to follow *P. haemolytica* colonisation in calves' tonsils after inoculation, and to determine the nasal shedding pattern in tonsil-colonised calves. We had previously observed calves whose nasal mucus was culture-negative for *P. haemolytica*, later begin shedding during an infectious bovine rhinotracheitis (IBR) virus infection. Therefore, our second objective was to determine whether IBR virus infection would elicit an increase in the *P. haemolytica* population in the tonsils and shedding of *P. haemolytica* in nasal mucus.

Materials and Methods

Experimental design

Calves were infected with *P. haemolytica* serotype 1 by instilling a culture (approximately 10^9 colony-forming units, CPU) into their palatine tonsillar sinuses with a modified pipette (Fig. 1) inserted through a speculum (Fig. 2). All calves were culture-negative for *P. haemolytica* in pre-exposure tonsil wash and nasal mucus specimens. Serial tonsil washes and nasal mucus and serums were collected. The numbers of *P. haemolytica* in tonsil washes and nasal

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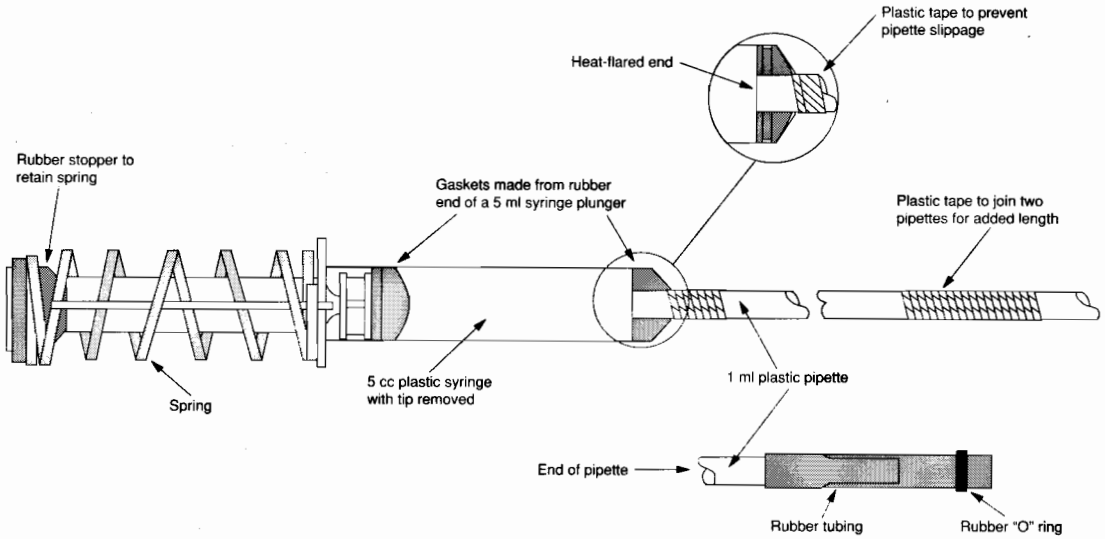

Fig. 1. Tonsillar sampling device.

mucus were determined. Right and left tonsil washes of each calf were assayed separately, while nasal mucus aspirates from each nostril were pooled.

Antibody titres were determined by an indirect haemagglutination procedure and by leukotoxin neutralisation (Frank and Briggs 1992). One week to 1 month after *P. haemolytica* infection, calves were inoculated intranasally with IBR virus using an aerosol apparatus (Frank et al. 1986).

Calves were observed for signs of respiratory tract disease, and rectal temperatures were recorded daily.

Collection of specimens

Tonsillar washings were collected by instilling 3 mL of Dulbecco's buffered saline *P. haemolytica* 7.5 into each palatine tonsillar sinus, and immediately aspirating the fluid with the same type of modified pipette used for bacterial instillation. Nasal mucus was aspirated by suction from each nostril through a modified pipette into a trapping tube (Fig. 3).

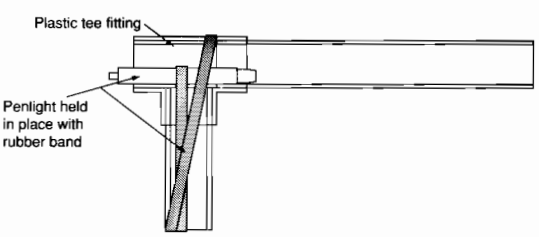

Fig. 2. Speculum made from plastic plumbing pipe, for tonsil sampling.

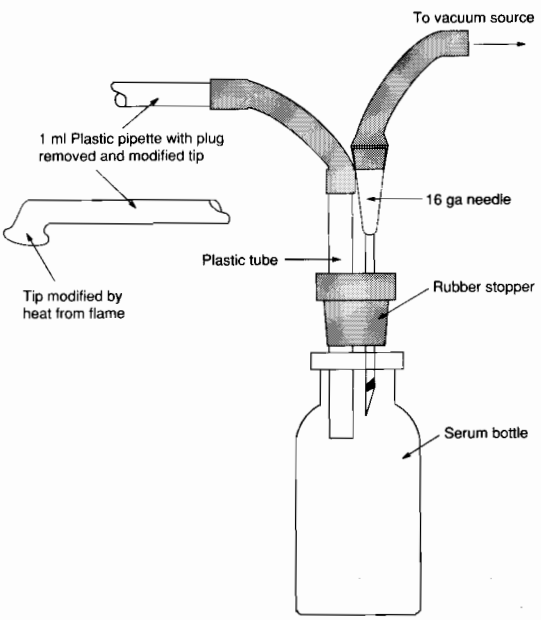

Fig. 3. Nasal mucus aspiration apparatus.

Quantitation of *P. haemolytica* from specimens

The tonsil wash and nasal mucus samples were sonified to disperse *P. haemolytica* throughout the specimens (Frank et al. 1986). Sonified samples were serially diluted and spread onto blood agar plates. After overnight incubation at 37 °C, the *P.*

haemolytica colonies were identified (Frank and Wessman 1978; Frank 1982) and counted.

Results

Extreme variations were often found in the numbers of *P. haemolytica* in washes from the two tonsils of individual calves. In some cases, one tonsil was culture-negative while the other contained large numbers of *P. haemolytica*.

Calves had varying levels of pre-exposure serum indirect haemagglutination titres to *P. haemolytica*, and leukotoxin-neutralisation titres. All calves tested responded to tonsillar colonisation with increases in both types of antibody.

Experiment 1

Ten steers weighing 250–300 kg were inoculated with *P. haemolytica* — two with serotype 2, and eight with serotype 1. Calves were exposed to IBR virus on day 25. Tonsil wash and nasal mucus specimens were collected twice weekly, beginning at day 0.

Clinical observations

After *P. haemolytica* inoculation, calves did not have fevers or clinical signs of disease. The IBR virus exposure elicited signs of mild respiratory tract disease. All calves had febrile responses (rectal temperature >39.4 °C) following IBR virus exposure, beginning 2–3 days after exposure and lasting 4–6 days.

P. haemolytica *isolation*

After *P. haemolytica* instillation, the organism was recovered from tonsil wash specimens for at least 3 weeks. The numbers of *P. haemolytica* serotype 1 were constant for approximately 2 weeks, then began decreasing. The numbers of serotype 2 isolate began decreasing immediately (Fig. 4). The nasal mucus of one calf inoculated with serotype 1 contained *P. haemolytica* on days 4 and 7, and that of another calf contained *P. haemolytica* on day 4. All other nasal mucus samples were culture-negative for the 25-day period before IBR virus inoculation.

The *P. haemolytica* population in tonsil wash specimens was not increased after IBR virus exposure. No isolations were made from the nasal mucus of calves that had been inoculated with serotype 1. Two of the serotype 1 calves were re-exposed by intranasal instillation on day 32 to determine whether their nasal passages could be colonised with *P. haemolytica*. They were susceptible to colonisation, as they shed *P. haemolytica* in their nasal mucus for at least 7 days. One *P. haemolytica* serotype 2-inoculated calf shed in the nasal mucus from days 29 to 35. The other shed from day 29 to day 32.

Experiment 2

Twelve Holstein and Jersey steers, aged 3–15 months and weighing 75–260 kg, were used. Eight calves were exposed to *P. haemolytica* serotype 1 on day 0, and 4 were exposed on day 7. All calves were exposed to IBR virus on day 14. Tonsil washings and nasal mucus were collected twice weekly.

Clinical observations

Infection of the tonsils with *P. haemolytica* caused clinical illness in some calves. Response to infection was varied and included acute submandibular oedema with pneumonia; invasion and destruction of the draining medial retropharyngeal lymph nodes; a more chronic systemic invasion with joint involvement; and the absence of clinical disease. Of the 8 calves exposed on day 0, 4 had acute infections. One calf died and another was euthanased on day 1. Two calves were euthanased on day 2 because of acute respiratory distress. One calf with a more chronic infection was euthanased on day 9.

The four calves with acute infections were febrile by day 1. They had visible, submandibular swelling and severe dyspnoea with forced, audible expiration. There was massive oedema in the soft tissues around the larynx and trachea. The lungs of two calves were pneumonic, while those of two others were grossly normal.

The more chronically infected calf was febrile, and its appetite suppressed. Its tonsils were swollen and there was submandibular swelling and nasal discharge When euthanased on day 9, the submandibular swelling was reduced, but the carpal and hock joints were swollen. There were small, multifocal lung abscesses and pleural adhesions. The retropharyngeal lymph nodes were necrotic. The tonsils contained fibrin casts. Joint fluids were cloudy and contained extensive fibrin deposits.

Three of the seven remaining calves had clinical signs of infection. These included fever, suppressed appetite, swollen tonsils, and occasionally swollen joints.

After IBR virus exposure, five of the seven calves had fevers for at least 4 days. Three calves necropsied on days 21 and 23 had swollen, abscessed tonsils and/or retropharyngeal lymph nodes.

P. haemolytica *isolation*

After *P. haemolytica* inoculation, *P. haemolytica* was recovered from tonsil washes of all calves. The numbers of *P. haemolytica* began decreasing

immediately, and two calves were culture-negative by day 14 (Fig. 4). Only two of eight calves shed *P. haemolytica* in the nasal mucus after *P. haemolytica* exposure and before IBR virus exposure (Fig. 5).

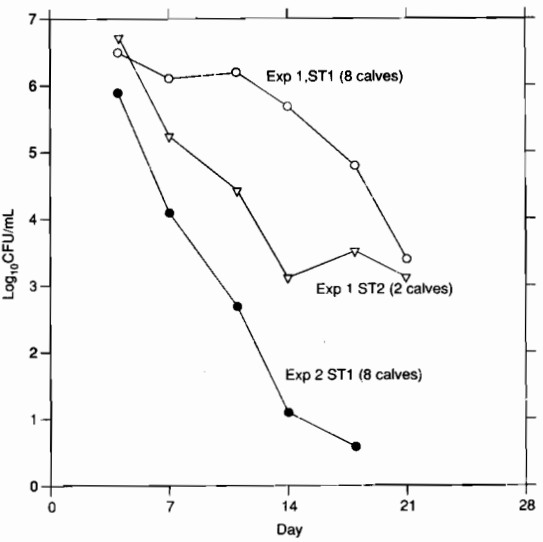

Fig. 4. Mean recovery of *P. haemolytica* from tonsillar wash specimens from calves. CFU, colony forming units.

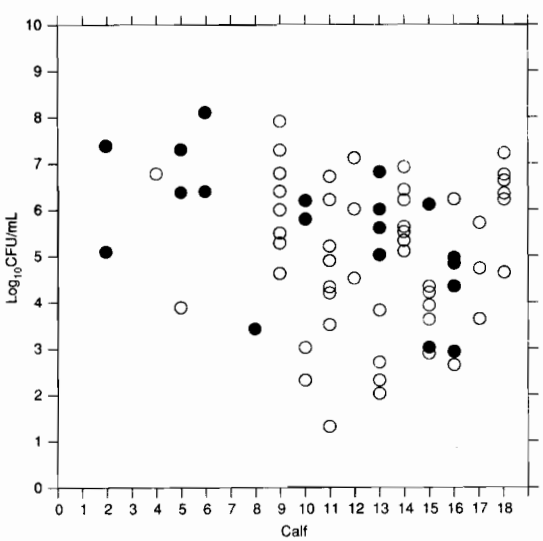

Fig. 5. *P. haemolytica* isolations from the nasal mucus of calves in experiment 2 (calves 1–8) and experiment 3 (calves 9–18). Isolations before IBR virus infection, open circles; after IBR virus infection, closed circles. CFU, colony forming units.

After IBR virus infection there was no measurable increase in the *P. haemolytica* population of the tonsils. Four of seven calves, all of which had ceased shedding *P. haemolytica* prior to IBR virus exposure, began to shed *P. haemolytica* in the nasal mucus (Fig. 5).

Experiment 3

Ten calves, 10–11 months old and weighing 180–230 kg, were used. Four calves were infected with *P. haemolytica* serotype 1 by tonsillar instillation (conventional exposure). Four others were exposed by inserting cotton soaked in a *P. haemolytica* culture into their palatine tonsillar crypts (implant-exposed), and two were exposed conventionally with *P. haemolytica* serotype 2. Calves were tranquillised during exposure, and tonsillar washes were not collected in order to minimise the risk of tonsillar trauma. Conventionally exposed calves were infected with IBR virus at 11 days and implant-exposed calves were infected at 9 days.

Clinical observations

After *P. haemolytica* instillation, serotype 2-inoculated calves had no clinical signs of disease. Of the four conventionally exposed serotype 1 calves, two were febrile and one of those had submandibular swelling and dyspnoea. Necropsy of that calf on day 3 revealed enlarged tonsils and necrotic areas in the retropharyngeal lymph nodes and oedema in the soft tissue around the larynx and trachea. The lungs and trachea were grossly normal. One clinically normal calf euthanased on day 9 exhibited no gross lesions.

Of the four implant-exposed calves, three were febrile, and two of those had submandibular swelling. One became dyspnoeic and was euthanased on day 8. The calf had severe bilateral pneumonia with fibrinous adhesions and the trachea was hyperaemic. The tonsils were grossly normal.

After IBR virus infection all seven of the remaining calves were febrile for at least 4 days and developed white IBR virus-induced plaques on the nasal mucosa. Three calves were later euthanased. One of them had chronic lung lesions and haemorrhagic retropharyngeal lymph nodes.

P. haemolytica *isolation*

Tonsil washes were not collected during this experiment, but after *P. haemolytica* instillation all calves shed *P. haemolytica* in the nasal mucus for an extended time (Fig. 5). The seven IBR virus-exposed calves ceased nasal shedding of *P. haemolytica* prior to exposure. Four of the calves began shedding *P. haemolytica* in the nasal mucus after IBR virus exposure (Fig. 5).

Discussion and Conclusion

Recovery of *P. haemolytica* from tonsil wash specimens for several weeks indicates that the tonsil is a site in which *P. haemolytica* can be carried for long periods by healthy calves. Nasal mucus specimens collected from calves with colonised tonsils were usually culture-negative for *P. haemolytica*, but sporadic isolations were made from some calves. This indicates that small numbers of *P. haemolytica* reaching the nasopharynx were not able to establish colonisation. Our past studies indicate that *P. haemolytica* reaching the nasal passages of healthy calves are cleared rapidly (Frank et al. 1986, 1989).

However, cattle with culture-negative nasal mucus can become culture-positive for *P. haemolytica* during IBR virus-induced respiratory tract disease (Frank et al. 1986) and during transport-induced stress (Frank and Smith 1983; Frank 1985; Frank et al. 1989). This indicates that the nasopharynx is susceptible to colonisation at these times. Because of their proximity to the upper respiratory tract, tonsils are an ideal reservoir for *P. haemolytica* in the healthy calf for colonisation of the nasopharynx during stress or viral respiratory tract disease.

In experiment 1, the nasal passages did not become colonised as a result of an IBR virus-induced respiratory tract disease. Even so, these same calves were susceptible to colonisation of the nasopharynx by *P. haemolytica*, as evidenced by colonisation of the nasal mucus after being re-exposed to *P. haemolytica* by intranasal instillation on day 32.

In experiment 2, however, four of the seven calves that survived to be exposed to IBR virus shed *P. haemolytica* in the nasal mucus. All of the calves had high serum indirect haemagglutination and leukotoxin-neutralisation titres at the time of nasal shedding. Therefore, the *P. haemolytica*-colonised tonsil can be a source of inoculation for the nasopharynx, even in seropositive cattle. Immune status of the calves may be influenced by prior experience with *P. haemolytica* serotype 1, but this has relatively minor effect on the level of nasal colonisation (Frank 1988).

Considering experiment 1 in which *P. haemolytica* did not cause evident clinical disease after tonsillar instillation (Frank and Briggs 1992), the systemic disease encountered in experiments 2 and 3 was unexpected. The clinical effects appear to be related to age, since calves that became acutely infected were among the youngest, while some of the older calves became chronically infected. The smaller calves used in experiment 2 may have been more susceptible than the larger calves used in experiment 1.

The calves that were clinically affected had *P. haemolytica* in the tonsillar tissue, *P. haemolytica* invasion to the draining medial retropharyngeal lymph nodes, and oedema of the surrounding tissues. Whether the invasion of the tonsil and surrounding tissues occurred as a result of trauma during instillation of *P. haemolytica* into the tonsillar sinuses or because of the ability of *P. haemolytica* to invade the tonsil is unknown.

The invasion and soft tissue response likely, led to lung involvement in two of the acutely infected calves in experiment 2. There was *P. haemolytica*-containing fluid present in the pharynx and swelling of the tissues anterior to the larynx caused by oedema of the surrounding tissues. The swelling caused forced inspiration and expiration, which appeared likely to have caused aspiration of *P. haemolytica*-containing fluids into the lung. Alternatively, *P. haemolytica* invasion of the tonsils could have led to pneumonia via the vascular route.

Calves with necrotic retropharyngeal lymph nodes and lung and hock-joint involvement indicated that *P. haemolytica* can become systemic after entry through the tonsils. Lung involvement after *P. haemolytica* invasion has been shown to occur experimentally in calves after intravenous inoculation of *P. haemolytica* (Thomas et al. 1989).

In the bovine tonsil the epithelium of the tonsillar sinus is infiltrated with lymphocytes and degenerated epithelial areas are normally present (Pelagalli et al. 1983). It is possible that *P. haemolytica* could invade such an area, since it produces a leukotoxin that will kill lymphocytes (Benson et al. 1978). Also, there is evidence that *P. haemolytica* could damage the epithelial cells, because *P. haemolytica* has been shown to produce a soluble factor (probably lipopolysaccharide) that is toxic to bovine pulmonary endothelial cells in vitro (Breider et al. 1990). Tonsillar trauma, however, is a natural possibility when rough forages are consumed (Frank and Briggs 1992). Therefore, several possible mechanisms exist for *P. haemolytica* invasion of tonsillar tissue. In some cases after invasion it is possible that the *P. haemolytica* could proceed to cause pneumonic pasteurellosis.

The anatomic structure of bovine tonsils (Pelagalli et al. 1983) and techniques for collecting a biopsy specimen of the tonsils in live cattle have been described (Sweat et al. 1963). Others have isolated *P. haemolytica* from bovine and ovine tonsils. Tonsils from adult sheep collected at an abattoir carried mostly T biotypes of *P. haemolytica*, whereas mostly A biotypes were carried in the nasopharynx (Gilmour et al. 1974). In a study in which sequential tonsil swab specimens were collected from ewes and their lambs,

it was concluded that tonsils of lambs could be colonised shortly after birth by *P. haemolytica* acquired from their dams (Al-Sultan and Aitken 1985). *P. haemolytica* serotype 2 or serotype 1 has been isolated previously from the tonsils and/or retropharyngeal lymph nodes of calves from which the nasal conchae, trachea and lung tissues were culture-negative (Shoo and Wiseman 1990).

P. haemolytica has been isolated from the tonsils of naturally infected calves (Shoo and Wiseman 1990). We have shown that *P. haemolytica* can be carried for long periods in the tonsils of healthy, unstressed calves without causing disease (Frank and Briggs 1992). Experiments 2 and 3 demonstrate that tonsillar infections with *P. haemolytica* can develop into systemic infections. We have also shown that IBR virus infection does not cause an increase in the *P. haemolytica* population in the tonsil, but can result in shedding of *P. haemolytica* in the nasal mucus. Since IBR virus can elicit nasal shedding of *P. haemolytica* in calves with colonised tonsils, the tonsil can serve as a reservoir for *P. haemolytica* infection of the nasopharynx.

Acknowledgments

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Haemorrhagic Septicaemia Carriers Among Cattle and Buffalo in Malaysia

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Abstract

Pasteurella multocida Carter type B, the haemorrhagic septicaemia (HS) serotype, can be passively carried in the lymph nodes of the upper respiratory tract of cattle and buffalo. A survey was conducted in Malaysia on cattle and buffalo in abattoirs to isolate *P. multocida* from the nasopharynx and lymph nodes and to establish the serotypes present in endemic and nonendemic areas. Types A, B and D, and some untypeable organisms, were isolated from carrier animals. Three percent of the animals from HS-endemic areas were positive for *P. multocida* Carter type B. Except for one isolate from the nasopharynx, all isolates were from lymph nodes. The movement of carriers from endemic to nonendemic areas may be important in the spread of haemorrhagic septicaemia, and other diseases caused by *P. multocida*.

PASTEURELLA multocida Carter type B, the haemorrhagic septicaemia (HS) serotype, has been shown to be passively carried in lymph nodes of the upper respiratory tract of cattle and buffalo (Singh 1948; Omar et al. 1962; Wijewantha and Karunaratne 1968; Mohan et al. 1968; Mustafa et al. 1978; Hiramune and De Alwis 1982; Wijewardana et al. 1986). These passive or dormant carriers do not show clinical signs or excrete the organisms through their nasal passages, and are thus not infective. The state of dormancy can only be confirmed by culture of lymph nodes obtained at slaughter. However, dormant carriers can become active carriers when they are stressed. At this stage the animals are infective, and pasteurellae are shed in the nasal passages and can be isolated from nasopharyngeal swabs.

Singh (1948) found 3.5% carriers in a group of live animals swabbed through the external nares and 7% carriers from lymph nodes of slaughtered animals. Wijewantha and Karunaratne (1968) found 15% carriers among slaughtered animals. Mustafa et al. (1978) found carrier rates of 0–5% in nonendemic areas and 44.4% in endemic areas. Hiramune and De Alwis (1982) found no carriers in nonendemic areas and 2.7% in endemic areas. Wijewardana et al. (1986) obtained results similar to Hiramune and De Alwis (1982). It is likely that the number of carrier animals in cattle and buffalo populations influences the frequency of HS outbreaks in endemic areas.

The HS carrier in Malaysia was demonstrated by Omar et al. (1962) by the isolation of *P. multocida* Robert type 1 from the tonsil of a clinically healthy buffalo. Subsequently, pasteurellae of Carter types A, B and D were isolated from cattle and buffalo slaughtered in an abattoir. Since further information on HS carriers in endemic and nonendemic areas is important to develop appropriate prevention and control programs, a survey was conducted on cattle and buffalo in abattoirs and in the field to isolate and establish the serotypes of *P. multocida* from the nasopharynx and lymph nodes of cattle and buffalo from endemic and nonendemic areas.

Materials and Methods

The abattoir survey was conducted in an endemic area (Kelantan), 2 weeks after an outbreak had occurred; and in four nonendemic areas (Ipoh, Shah Alam, Seremban and Melaka). A total of 325 cattle, comprising Kedah-Kelantan, local Indian Dairy and crossbreds, and 46 buffalo (*Bubalus bubalis*, swamp type) were examined. The animals were mostly adult males of unknown vaccination history.

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Pre- and post-slaughter nasopharyngeal and pharyngeal swab samples were taken to screen for *P. multocida*. Each sample was washed in 5 mL tryptose broth in bijour bottles. Retropharyngeal lymph nodes and tonsils were collected in clean plastic bags. All samples were transported to the laboratory immediately, in an icebox.

In addition, a field survey was conducted on 205 cattle and 15 buffalo in Kota Baru and Trengganu, representing endemic areas, and Ipoh and Shah Alam, representing nonendemic areas. Nasopharyngeal swabs were taken in a manner similar to that used in the abattoir survey.

Screening for *P. multocida*

Pieces of tonsil and retropharyngeal lymph nodes were removed aseptically and crushed in about 3 mL of tryptose broth to allow the organism to elute into the broth. A 0.5-mL aliquot of well-shaken broth containing lymph nodes and tonsil and a 0.5 mL aliquot of broth containing swab samples, were inoculated intraperitoneally into a mouse. Broth that killed mice within 72 hours was suspected of containing pasteurellae.

Isolation and identification of *P. multocida*

Heart blood from dead mice was cultured in tryptose blood agar and MacConkey agar and the plates were incubated at 37 °C for 12–24 hours. Smears of heart blood were made and the organisms identified by their staining reaction to Gram's and methylene blue stains. Bacteria that grew on tryptose blood agar, but not on MacConkey Agar, were Gram-negative coccobacilli characterised by a bipolar appearance with methylene blue stain, and were oxidase- and catalase-positive. They were considered to be *P. multocida* (Carter 1967; Namioka 1978).

The slide agglutination test (Namioka and Murata 1961) was used for the capsular typing of isolates. All *P. multocida* type B isolates identified by the simplified capsular typing method were confirmed by IHA (Carter 1955).

Mucoid types of *P. multocida* were grown in the presence of hyaluronidase to remove capsular hyduronic acid (Carter and Rundell 1975). Treated organisms were then subjected to IHA or simple agglutination tests (simplified capsular typing) for serotyping. Known hyaluronidase-positive *P. multocida* types were used as sources of hyaluronidase.

P. multocida organisms not of type B or A were typed by the acriflavine flocculation test (Carter and Subronto 1973).

Results

A total of 49 (13%) of *P. multocida* isolates were obtained from 371 cattle and buffalo samples. Only one (0.3%) of the 44 isolates from cattle was isolated from the nasopharynx, while the rest were from lymph nodes. All five buffalo isolates were obtained from lymph nodes. No isolates were obtained from pharyngeal swabs of slaughtered cattle or buffalo, or from the nasopharynx of live animals in the field.

Of the isolates typed, 44 (11.8%) were from cattle, of which 6 (2.1%) were of type A, 11 (3%) type B, and 5 (2%) type D. Of the five (11%) isolates from buffalo, 1 (2%) was of type B and 1 (2%) of type D. Twenty-one (6%) of the isolates from cattle and 3 (7%) from buffalo could not be typed. No type E isolates were made from either cattle or buffalo.

The 12 (3%) type B isolates from cattle and buffalo were from an endemic area and were collected in Kelantan 2 weeks after an outbreak of haemorrhagic septicaemia. No isolates were made from the nonendemic areas.

Discussion

Abattoirs are convenient and relatively inexpensive sources of samples for epidemiological studies. The selection of abattoirs in this study was based on the availability of laboratory facilities for processing samples. The survey confirmed that HS carriers are present in cattle and buffalo in Malaysia. The *P. multocida* isolates from carrier animals were of types A, B and D. Some of the *P. multocida* organisms isolated could not be typed into any of the known serotypes.

From the 371 abattoir samples studied, 49 (13%) yielded *P. multocida*. No isolates reacted to type E antiserum, while 12 (3%) reacted to type B antiserum. It is known that *P. multocida* type E organisms exist only in the African continent (Bain et al. 1982). The isolation of *P. multocida* types A and D, along with nontypeable *P. multocida*, suggested that cattle and buffalo could succumb to *P. multocida* infections other than haemorrhagic septicaemia.

The findings that all type B isolates were from a district in Kelantan, where an outbreak of HS occurred 2 weeks before the survey was conducted, and that no isolates were obtained from the nonendemic areas of Ipoh, Shah Alam, Melaka and Seremban, confirm the belief that carrier animals are found close to outbreak areas (Mustafa et al. 1978; Hiramune and De Alwis 1982; Wijewardana 1986).

The tonsil and retropharyngeal lymph nodes were sampled because they were the most consistent sites for the isolation of *P. multocida* in carrier animals (Wijewardana 1986). Animals that carried *P. multocida* organisms in their lymph nodes were shown to be passive carriers (Wijewardana et al. 1986; Carter and De Alwis 1989). These animals may discharge the organisms to the environment when stressed, causing the next outbreak of disease. However, the current methods of detecting carriers are based only on nasal swabs in the case of active carriers and culture of lymph nodes from slaughtered animals in the case of passive carriers. The inability to detect carriers in the field poses a problem when moving animals from endemic to nonendemic areas.

Active carriers are those animals from which *P. multocida* organisms could be isolated from the nasopharynx (Wijewardana et al. 1986; Carter and De Alwis 1989). In this study, only one active carrier of type B was detected in cattle from endemic areas (Kelantan) and none from nonendemic areas, confirming previous studies (Hiramune and De Alwis 1982; Wijewardana et al. 1986).

The results of the abattoir survey demonstrated that cattle and buffalo in HS-endemic areas in Malaysia harbour *P. multocida* type B in the lymph nodes of the upper respiratory tract. The movement of these carriers from endemic to nonendemic areas may be important in the transmission of the disease.

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Epidemiology of Pasteurella Pneumonia in Pigs

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Abstract

Enzootic pneumonia is an important problem affecting the pig industry worldwide. Generally it is regarded as being caused by *Mycoplasma hyopneumoniae*. While *Pasteurella multocida* is also commonly recovered from lesions, little is known of the role this organism plays in the disease process.

Two field surveys were conducted in New South Wales (NSW), Australia, during 1989–90. *P. multocida* was recovered from 49% and 61% of lungs respectively. The organism was present in at least some lungs from all 31 herds examined. There was a strong association between lesion severity and frequency of recovery of the organism. Positive cultures were made from approximately 10% of normal lungs, 35% of lungs with low-grade lesions, 60% of lungs with medium-grade lesions, and 80% of lungs with high-grade lesions. There were similar recovery rates from active lesions and chronic lesions; and the recovery rate from younger (14–18-week-old) pigs was similar to that from older (22–26-week-old) pigs. Sequential nasal swabbing of pigs from weaning to slaughter showed that upper respiratory tract infection with *P. multocida* was very common and that most nasal isolates were capsule type D. However, the appearance of the capsule type A strain in nasal-swab cultures coincided with the onset of clinical signs of pneumonia. Most pulmonary isolates were capsule type A. Growth-rate depression was evident only when there was a combination of high lung pneumonia scores and pulmonary infection with *P. multocida*.

PORCINE enzootic pneumonia is one of the most economically significant diseases of the pig industry throughout the world. In Australia, it has been estimated to cost $A55–70 per sow per year, or $A20 million annually, due to depression of growth rate, medication costs, and some contribution to mortality in pigs with concurrent disease (Cutler and Gardner 1988). Affected pigs develop lesions of consolidation in the antero-ventral portions of the lungs, sometimes associated with slight to moderate pleurisy. Coughing is the predominant clinical sign, and pigs with enzootic pneumonia often show illthrift (Ross 1981). It is a disease of high morbidity, with abattoir surveys in a number of countries establishing a prevalence of 35–80% in slaughter-age pigs (Gois et al. 1980; Osborne et al. 1981; Pointon and Sloane 1984; Cutler 1987).

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Mycoplasma hyopneumoniae is generally considered to be the primary cause of enzootic pneumonia. However, *P. multocida* is also commonly recovered from lesions (Osborne et al. 1981; Pijoan et al. 1984; Morrison et al. 1985). Despite the significance of this organism in a number of important diseases of livestock, little is known of its role in porcine enzootic pneumonia. Two field surveys were carried out in New South Wales (NSW), Australia, during 1989–90, in order to determine more clearly the significance of *P. multocida* infection in this disease complex.

Materials and Methods

Design of surveys

The first survey consisted of a case series study of 1283 pigs from 31 herds with enzootic pneumonia. From each herd, a single batch of pigs consigned to slaughter was monitored through one of eight abattoirs involved in the study. Farms selected were convenience sampled from commercial herds with greater than 50 sows in the central-western and mid-coastal areas of NSW. Herd size varied from 50 to

2500 sows. From information supplied by the abattoirs, pigs were classified as either pork or bacon animals. In Australia, pork pigs are generally younger, lighter animals (14–18 weeks old), while bacon pigs tend to be older, heavier animals (20–25 weeks old). Farms sampled specialised in the production of either pork or bacon pigs, and no cull batches of pork pigs were included in the survey.

Most abattoirs dehaired pigs using the traditional immersion in a hot water tank. However, one abattoir used a hot steam method of scalding, in which pigs were suspended head down and sprayed. This second method avoided microbial contamination of lungs with faecal and skin organisms during immersion.

The second survey consisted of a cohort study involving 110 pigs from a single commercial piggery. Nasal swabs and blood samples were taken at 2-weekly intervals from weaning at 3 weeks of age to slaughter at 25 weeks. Individual growth rate data was collected for each animal.

Lung scoring

Lungs were collected at slaughter and pneumonic lesions scored (0–55), according to the method of Goodwin (1971) and Pointon et al. (1987). Lungs were also scored for pleurisy (0–3), and pneumonic lesions were classified as either active or chronic according to recognised pathological criteria (Pointon and Sloane 1984). Briefly, active lesions were defined as oedematous in nature, greyish in colour, and of soft consistency. Chronic lesions were firm and dry, plum-red in colour, and often had shrunken and depressed borders to neighbouring non-pneumonic lung tissue.

Lung and nasal culture

In the first survey, a representative sample of lungs from each consignment of pigs was submitted for bacteriological culture. A total of 653 lungs were cultured. From these, the lobe with the largest lesion (or in normal lungs the left cardiac lobe) was selected and culture performed by direct inoculation of lung fluid on 5% sheep blood agar and MacConkey agar, followed by incubation at 37 °C for 18 hours. Plates were then examined for growth, and colonies identified as *P. multocida* on the basis of colony morphology, colour, odour, lack of growth on MacConkey agar, and judicious use of Gram staining. Other colonies were classified as *Streptococcus* spp., *Staphylococcus* spp., coliforms or miscellaneous, using the above criteria. The identity of *P. multocida* isolates was further confirmed by capsular typing using the methods of Carter and Subronto (1973) and Carter and Rundell (1975). Strains untypeable by these methods were confirmed as *P. multocida* using standard biochemical tests (Carter 1981).

In the second survey, every pneumonic lobe of each lung (and one normal lobe, where available) was cultured on 7% horse-blood agar. Nasal swabs were taken from 60 pigs at 2-weekly intervals from weaning to slaughter using thin-tipped aluminium-shafted swabs inserted 6–8 cm into the left nares, after being premoistened in Hanks 199 medium. Nasal swabs were directly inoculated onto selective 7% horse-blood agar containing neomycin sulphate (2 μg/mL) and bacitracin (3.5 μg/mL). From 60 lungs, 174 lobes were also cultured for the presence of *Haemophilus* spp., using chocolate agar with a *Staphylococcus* nurse colony under microaerophilic/high carbon dioxide conditions in candle jars.

Results

The prevalence of pneumonia in the two surveys was 46% and 71% respectively. This was within the range observed by most authors. The prevalence of high-grade lesions — i.e. those of lung score 10 or greater — was 22% in the first survey and 35% in the second. The prevalence of pleurisy in the first survey was 6%, however lungs from three herds accounted for most of these lesions, and the rest of the consignment batches examined were relatively pleurisy free. In the second survey, 22% of lungs showed some pleurisy, although most had only low-grade lesions.

P. multocida was recovered from 49% and 61% of all lungs in the first and second surveys respectively. These results refer to all lungs cultured, regardless of extent or quality of lesion, and include results from normal lungs. In the second survey, but not the first, all lobes with pneumonic lesions were cultured. This resulted in a 20% increase in the isolation rate of the organism. There was no apparent association between the recovery of *P. multocida* and the presence of pleurisy.

P. multocida was recovered from at least some lungs from all 31 herds included in the surveys; i.e. there was a herd prevalence of 100%.

There was a strong association between the recovery of *P. multocida* and lesion score (Table 1). The organism was much more likely to be recovered from high-grade lesions than from lungs without lesions or with low-grade lesions, and there was a step-wise increase in isolation rate as lesion score increased, from approximately 10% of normal lungs to approximately 80% of high-grade lesions.

The recovery of *P. multocida* was similar from younger pork pigs (48%) and older bacon pigs

Table 1. Association between recovery of *P. multocida* and severity of enzootic pneumonia lesion.

Lesion score	% lobes positive for *P. multocida*	
	Survey 1	Survey 2
Normal lobes	12.8	10.7
Low-grade lesions (score 1–3)	31.0	36.4
Medium-grade lesions (score 4–7)	58.7	58.3
High-grade lesions (score 8–10)	79.7	77.8

(44%). Active lesions of pneumonia were as likely to be *P. multocida* positive on culture as more chronic lesions in both surveys (Table 2). There was no apparent association between the recovery of *P. multocida* from lungs and the sex of the pig or the season of year in which the lungs were cultured.

Table 2. Association between recovery of *P. multocida* and stage of lesion.

Stage of lesion	% of lobes positive for *P. multocida*	
	Survey 1	Survey 2
Active	53.9	62.1
Chronic	48.6	42.0

A comparison of the recovery of different organisms from lungs scalded according to the traditional hot water tank method and the steam scalding method (Table 3) showed that, while the isolation of other organisms declined markedly when carcases were steam scalded, *P. multocida* remained as the predominant microorganism. *Streptococcus* spp. were the next most commonly recovered organism from pneumonic lesions. *Haemophilus* spp. were not detected in any of the lungs specifically cultured for these organisms.

Serial nasal-swab cultures showed that nearly all pigs became infected with *P. multocida* between weaning at 3 weeks and slaughter at 25 weeks (Table 4), and some pigs were infected as early as 3 weeks of age. Most nasal isolates belonged to capsule type D. Capsule type A organisms did not appear in nasal-swab cultures until the pigs were 15 weeks old, and coincided with the onset of coughing in the herd.

The growth performance of pigs was assessed in relation to the presence of pneumonic lesions and the recovery of *P. multocida* from those lesions (Table 5). Only pigs with high-grade lesions of pneumonia from which *P. multocida* could be isolated showed a marked decrease in growth performance. Pigs with lungs from which *P. multocida* was not recovered had a growth rate similar to that of pigs with normal lungs, regardless of the severity of those lesions.

Table 4. Cumulative percentage of pigs with at least one nasal swab positive for *P. multocida*, according to age at sampling.

Age of pigs (weeks) at nasal swabbing	Cumulative % of pigs' nasal-swab positive for *P. multocida*
3	4.8
5	16.1
7	33.8
9	41.9
11	54.8
13	67.7
15	72.6
17	74.2
20	77.4
23	88.7

Of the 372 pulmonary isolates, 92% of strains were capsule type A, 1% capsule type D, and 7% were untypeable. Of the 138 nasal isolates, 73% were type D, 11% were type A, and 16% were untypeable.

Table 3. Association between recovery of *P. multocida* and other organisms and scalding system used during slaughter.

Scalding system	% of lobes positive for each organism				
	Pasteurella	*Strep.*	*Staph.*	Coliforms	Other
Hot water tank	45.1	21.8	13.8	22.4	7.4
Steam	55.8	8.4	2.1	2.1	1.1

Table 5. Association between growth rate of pigs, recovery of *P. multocida*, and severity of enzootic pneumonia lesions.

Lung score and lung culture	Mean dressed weight (kg)	Mean growth rate (g/day)
All pigs with normal lungs	64.5	371
All pigs with low-grade lung score (< 10)	64.3	370
All pigs with high-grade lung score (> 10)	62.2	357
Pigs with high-grade lung score and lung culture negative for *P. multocida*	65.0	374
Pigs with high-grade lung score and lung culture positive for *P. multocida*	60.4	347

Discussion

In both surveys the prevalence of pneumonia was typical of the industry, and *P. multocida* was shown to be associated with pneumonic lesions frequently. The true recovery rate of the organism may be somewhat higher — pigs in which *P. multocida* pneumonia contributed to mortality or to early culling would not have been included in the surveys. Additionally, only a small amount of lung fluid was used to inoculate plates; i.e. the cultural technique used was a simple one, suitable for screening a large number of samples.

Other workers have used a variety of approaches to increase the recovery rate of *P. multocida*. Pijoan et al. (1984) used a combination of broth and mouse inoculation to increase the recovery rate from pig pneumonic lesions by 17%. The recovery rate was higher in the second survey, although this was influenced by the different system used to select lobes for culture; i.e. several lobes per lung were cultured in the second survey, compared to only one per lung in the first survey. Culturing the lobe with the largest lesion only was shown to give 80% of the recovery rate of culturing all pneumonic lobes.

P. multocida was shown to be widespread, rather than being a problem of particular farms, in that at least some lungs from each herd were culture-positive. Previous microbiological surveys of pig pneumonia (Osborne et al. 1981; Pijoan et al. 1984; Morrison et al. 1985) did not identify the herd of origin of lungs examined, and did not demonstrate clearly the widespread involvement of *P. multocida* in enzootic pneumonia across herds.

The strength of the association between lesion size and *P. multocida* recovery rate suggests that the organism significantly exacerbates the condition by extending the size and duration of lesions, thereby contributing to the pathology, clinical signs and economic loss of enzootic pneumonia.

The recovery of *P. multocida* from approximately 10% of normal lungs was somewhat higher than expected. It is probable that normal lungs are constantly clearing inhaled or aspirated *P. multocida*, and this would account for some normal lungs being *P. multocida*-positive on culture. It is also possible that grossly normal lungs may contain areas of microconsolidation, visible only on histopathological examination, and these areas may harbour the organism. Such areas have been noted in experimentally induced *P. multocida* pneumonia in pigs (White et al., unpublished).

There is some evidence that *P. multocida* becomes involved in the development of enzootic pneumonia lesions at an earlier, rather than later, stage. The organism was recovered with a similar frequency from younger pork pigs and older bacon animals, and from earlier active lesions and older, more chronic lesions. Early involvement would allow the organism to make a greater contribution to the development of the disease. Further studies on the timing of *P. multocida* involvement in enzootic pneumonia may require serial slaughtering experiments, to determine the recovery rate of the organism at different stages of lesion development. Serological studies are currently in progress.

Many organisms besides *P. multocida* and *M. hyopneumoniae* have been recovered from porcine enzootic pneumonia (Gois et al. 1975, 1980) and this has made interpretation of the relative importance of *P. multocida* difficult. However, when the contamination of lungs with hot water from the scalding tank was avoided by the use of a steam scalding system, the recovery rate of other organisms markedly declined, whereas that of *P. multocida* rose moderately. *P. multocida* was the predominant organism isolated from lungs from steam-scalded pigs, and the moderate increase in isolation rate may have been due to the lack of overgrowth of pasteurellae by coliforms etc., which sometimes occurred in the lung cultures from tank-scalded pigs. The role of *Streptococcus* spp. in the enzootic pneumonia complex requires further elucidation.

Most animals appeared to acquire a *P. multocida* infection of the upper respiratory tract. There was little correlation between successive swabs from the same animal, and this probably reflects the relative insensitivity of nasal swabbing as a diagnostic test for pasteurella infection. ELISA studies also suggest

that most pigs in the study became infected with *P. multocida* by the time of slaughter (White et al., unpublished). The organism has also been recovered more frequently from the tonsillar crypts than from the anterior nares of pigs (van Leengoed et al. 1986). The pigs that were nasal-swab positive at weaning probably acquired infection from their dams. This was followed by a gradual increase in the proportion of pigs with at least one positive nasal-swab culture during the period of survey (Table 4), presumably due to horizontal transmission among cohorts. The appearance of capsule type A strains in nasal-swab cultures occurred at the same time as the onset of clinical signs of pneumonia. Whether this increase in the population of type A strains in the upper respiratory tract preceded or followed the initial development of pneumonia lesions is unclear.

Two factors appeared to be required in order for pigs to suffer a marked decline in growth performance: first, the presence of larger lesions of lung score 10 or more; and second, the presence of *P. multocida* in those lesions. It is likely that *P. multocida* is responsible for much of the economic loss associated with porcine enzootic pneumonia, and that *M. hyopneumoniae* is the most common initiator of the lung damage that allows *P. multocida* to become established. It is interesting to speculate that, under adverse environmental conditions (e.g. high ammonia levels), *P. multocida* may be able to colonise the lungs without the assistance of other microorganisms. It has been well demonstrated, experimentally at least, that a variety of insults other than *M. hyopneumoniae* can be used to induce pneumonic pasteurellosis in pigs, including an adenovirus (Smith et al. 1973), a live attenuated swine fever vaccine (Pijoan and Ochoa 1978), the pseudorabies virus (Fuentes and Pijoan 1987), embryonated *Ascaris suis* eggs (Raynaud et al. 1977), and *P. multocida* in conjunction with large doses of saline (Hall et al. 1988).

Further work in this project is concentrating on characterisation of the Australian pig pneumonia isolates of *P. multocida* with respect to somatic serotypes, toxigenicity, outer membrane protein profiles and biochemical profiles, and serological and protection studies.

Acknowledgments

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The Epidemiology of Haemorrhagic Septicaemia in Sri Lanka

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Abstract

Clinical haemorrhagic septicaemia (HS) has been reported in Sri Lanka since the beginning of the present century. Serological confirmation was made only in 1955. The disease is endemic in buffalo and cattle that are mainly free-roaming on the dry plains covering about two-thirds of the land area. It is seldom seen in intensively reared exotic animals in the hill country.

More losses occur in buffalo than in cattle. Few reports are available of the disease in pigs and wild elephants. Goats are highly resistant to HS serotypes. Morbidity in cattle and buffalo is highly variable, and the pattern varies in endemic and nonendemic areas. Case fatality is near 100%. In endemic areas, mostly young animals are affected. The spread of outbreaks is related to rainfall.

Varying proportions of animals in endemic areas are immune carriers, a state that results from non-lethal natural exposure. The persistence of the organism in the tonsils has been demonstrated both experimentally and under natural conditions. A prolonged latent carrier state with intermittent shedding has also been demonstrated. Currently, the changing epidemiological patterns following intensive vaccination are being studied.

Sri Lanka is a tropical island of 65 000 km^2, which displays a range of climatic conditions based on altitude and rainfall. Broadly, hill country (> 1000 m above mean sea level), mid country (300 m–1000 m) and low country (< 300 m) are recognised; as well as wet (> 2000 mm), intermediate (1250–2000 mm) and dry (< 1250 mm) zones. Agricultural patterns in the country are governed by these climatic conditions, thus giving rise to a number of 'agro-climatic' zones. The incidence and distribution of animal diseases are related to the types and numbers of livestock and their husbandry practices, which are in turn related to the agro-climatic zones.

History

A disease clinically resembling haemorrhagic septicaemia (HS) has been recorded in Sri Lanka since the beginning of the 20th century. The first occasion the disease broke out in epidemic proportions was in 1955–56, when nearly 10 000 deaths were recorded. It was during this epidemic that the organism was first identified by R.V.S. Bain as Roberts Type I (Dassanayake 1957; Perumalpillai and Thambiayiah 1957), and vaccination was introduced.

In the early years, vaccination was carried out in an ad hoc manner, with no strategically planned program supported by definite epidemiological knowledge. Vaccination failures were therefore common. This situation gave rise to considerable speculation that different types and strains may exist. In the early 1970s a collection of 50 isolates from different parts of the country were serotyped and conclusively identified as Carter's type B and Namioka's type 6; i.e. serotype 6:B (De Alwis and Panangala 1974). More recently, the Sri Lankan HS isolates were identified as Heddleston's type 2 (Wijewardana, unpublished). No variant cultures have been associated with outbreaks of HS.

Since the major epidemic of the mid-1950s, a definite pattern of incidence and distribution of HS has been established. In the dry plains in the north-western, north-central, south-eastern and eastern regions of the country, which cover approximately two-thirds of the land area, the disease has become endemic, and regular annual outbreaks occur. In these areas, moderate to large herds of indigenous

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animals roam freely. In a restricted land area in the hill country, where exotic animals or their crosses are reared intensively, the occurrence of HS is exceptional. In the mid and low country, wet and intermediate regions of the south-western part of the island, sporadic outbreaks occur from time to time, often resulting from spread of infection from endemic areas, by transport of animals (Fig. 1).

Species Affected

The main species affected are buffalo and cattle. Epidemiological studies have shown that buffalo are more susceptible than cattle. A study in selected endemic areas showed that there was no significant difference between the herd infection rates over a 3-year period in the 870 herds of buffalo and 803 herds of cattle investigated. The mean mortality, however, was higher in buffalo than in cattle, and this difference was highly significant (De Alwis and Vipulasiri 1980). Another study of outbreaks in both endemic and nonendemic areas showed that mortality in buffalo was three times higher than in cattle (De Alwis 1981).

Disease caused by HS serotypes have been reported in Sri Lanka in other species of animals. There have been rare, sporadic outbreaks in pigs (De Alwis, unpublished), and two reports of the disease in wild elephants (De Alwis and Thambithurai 1965; De Alwis 1982a; Wickremasuriya and Kendaragama 1982).

Since goats are found roaming freely in HS endemic areas, they were for long suspected to be reservoir hosts. In an abattoir study (Wijewardana et al. 1986a), no isolations were made from the naso-pharynx or associated lymph nodes of goats. None of these goats showed any antibody against HS. Attempts to transmit the disease naturally to goats by tying them in close contact with clinically diseased buffalo failed, and no antibody response was detected in any of the goats. Direct experimental infection of goats by the sub-cutaneous or intranasal routes using doses of up to 10^4–10^6 cattle lethal doses proved futile, except in a very few animals (Wijewardana et al. 1986b). The overall conclusion was that goats were highly resistant to infection with HS-causing pasteurellae.

Fig. 1. (a) Climatic zones in Sri Lanka; (b) Incidence of haemorrhagic septicaemia in Sri Lanka.

Morbidity, Mortality and Case Fatality

In general HS occurs in situations where early disease detection is difficult. In such situations, once clinical disease is established, death is almost certain, with case fatality being near 100%. Morbidity however, is highly variable and will range from less than 5% to nearly 90%. Many factors appeared to influence morbidity; e.g. the species affected (cattle or buffalo), age, endemicity of the area, and herd size. The higher susceptibility of the buffalo has already been dealt with.

Age

Studies in HS-endemic areas have shown that, in cattle and buffalo, while morbidity among under 2-year-olds was 30–32%, the corresponding figure for over 2-year-olds was 3–5% for cattle and 8–9% for buffalo (De Alwis and Vipulasiri 1980). An analysis of the mortality patterns in two outbreaks showed that 91% and 84% respectively of all HS deaths occurred in the 6–18-month age group (De Alwis et al. 1976).

Endemicity

Studies showed that, in endemic areas, outbreaks occurred frequently, but the morbidity in each outbreak was low and only young animals were affected. When occasional sporadic outbreaks occurred in nonendemic areas, however, all age groups were affected and morbidity was high (De Alwis and Vipulasiri 1980).

Herd size

In Sri Lanka, the herd size in cattle and buffalo is related to the system of management. Large herds usually roam freely, grazing on common grasslands in the village and drinking in common village tanks. The smaller herds are mostly confined. It has been found that in small herds of fewer than 10 animals, incidence is low, but when the disease does occur, morbidity is high. The larger herds, on the other hand, experience more frequent outbreaks, but in each outbreak morbidity is low. One study showed that, in endemic areas, the percentage of herds of more than 50 animals infected over 3 years was four to five times higher than the figure for small herds of less than 10 animals (De Alwis and Vipulasiri 1980).

Seasonal Incidence

Outbreaks of HS have generally been associated with the rainy season. An epidemiological study that reviewed HS status in the 1970s, when immunisation programs were weak, found that outbreaks occurred throughout the year. The distribution of outbreaks was fairly constant from January to July, but a steep rise in the number of outbreaks began in August and reached a peak in October. This trend roughly corresponded to levels of the organism and presumably denotes its degree of dissemination (De Alwis and Vipulasiri 1980). Since 1984 the immunisation programs have been strengthened and rationalised and mass vaccination is carried out during the period June to August, with the resultant abolishing of the peak (Hettiarachchi 1991) (Fig. 2).

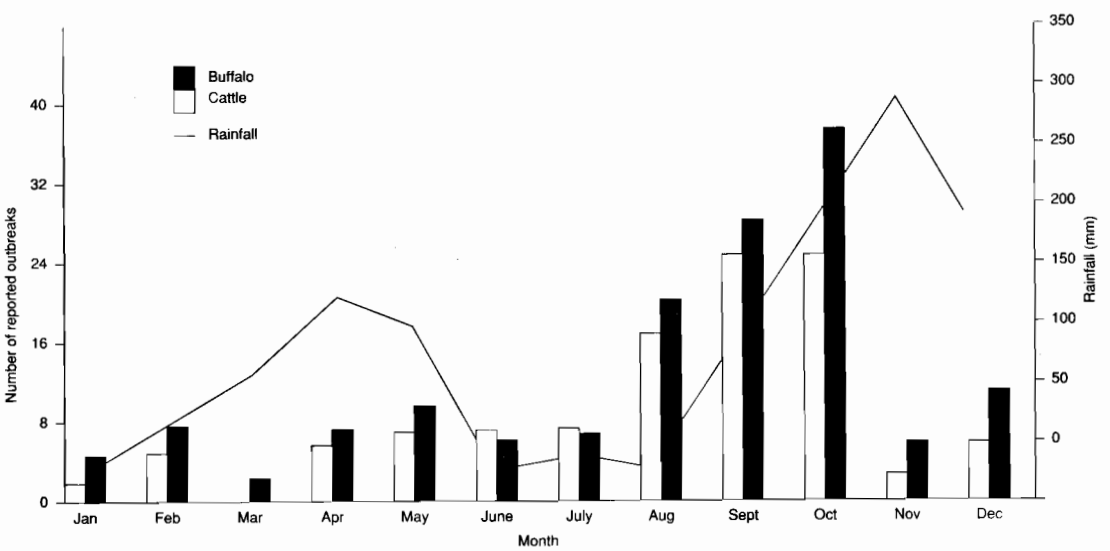

Fig. 2. Distribution of haemorrhagic septicaemia outbreaks in buffalo and cattle as related to the annual rainfall pattern.

Naturally Acquired Immunity

Studies on the antibody status of unvaccinated cattle and buffalo in Sri Lanka in high-, moderate- and low-incidence areas showed that 36%, 7% and 0.47% of animals respectively had antibodies to the indirect haemagglutination assay (IHA) (De Alwis and Sumanadasa 1982). In another study, where 26 unvaccinated buffalo calves were kept exposed to an outbreak of HS, it was found that those that were unaffected developed antibodies detectable by IHA in 2–3 weeks and the antibodies were detectable for at least 8 months (De Alwis 1982b). A similar study in three field outbreaks in unvaccinated herds showed that 80–100% of animals that survived developed high antibody levels (De Alwis et al. 1986).

It is evident that different morbidity patterns in endemic and nonendemic areas are due to the different proportions of naturally immune animals. The morbidity pattern is thus governed by the phenomenon of naturally acquired immunity, which in turn is dependent on recent exposure to disease. During the rainy season, outbreaks spread. At the end of this season, therefore, most surviving animals have acquired natural immunity. By the next year's annual rainy season, a substantial number of new animals would have reached the susceptible age, when the next outbreak occurs. The cycle thus continues.

The Carrier Status

The earliest study on HS carriers in Sri Lanka was by Wijewantha and Karunaratne (1968). In nasal and pharyngeal swabs of cattle originating from HS-endemic areas, typical *P. multocida* 'Asian type I' was isolated from 14% and the 'Australian type I' from 0.6% of animals. The latter non-HS serotype is designated 11:B (Namioka:Carter) or B:3,4 (Carter:Heddleston). In a more recent survey in a group of cattle at the same abattoir, Wijewardana et al. (1986a) isolated typical HS-causing pasteurellae from 0.6% of swabs from the external nares; from 1.4% of swabs from the nasopharynx; and from 2.2% of cultured retropharyngeal lymph nodes.

Hiramune and De Alwis (1982) found no carriers among 250 animals examined in a HS nonendemic area, and nearly 3% carriers among 589 animals examined in endemic areas. They further found that, in the latter group, the carrier rate varied considerably from one location to another, and was in general related positively to recent outbreaks of HS. In small groups recently exposed to HS, up to 23% of animals were carriers.

More recently, De Alwis et al. (1986) swabbed the nasopharynx of animals that had survived recent outbreaks repeatedly, at regular intervals. They found that the carrier status, as evidenced by the presence of the pasteurellae in the nasopharynx, was a transient one. Further, different animals showed up as carriers on different days. In order to further elucidate the nature of the carrier status, carriers were produced experimentally by controlled exposure and observed for up to 1 year. Using a marked organism, the intermittent appearance of the organism in the nasopharynx was confirmed. By slaughter of these carrier animals, pasteurellae were isolated, not only from the nasopharynx, but also from the lymph nodes associated with the respiratory tract, the most consistent site being the tonsils, from which site they were recovered even 8 months after a single exposure (De Alwis et al. 1990).

A current survey of the presence of HS-causing pasteurellae in the tonsils of abattoir animals has confirmed the tonsils as a site of persistence under natural conditions (Wijewardana et al., unpublished). Thus the presence of 'latent carriers' that harbour pasteurellae in their tonsils only, and 'active carriers' where the organism is also present in the nasopharynx, has been established. It also became evident that, in most earlier studies, the carriers detected had only been those in the 'active' state, and not the 'latent' ones. Attempts to clear carriers by antibiotic therapy failed, although the organism was sensitive to the antibiotics used in vitro. This phenomenon was explained by Hordagoda and Belak (1990). These workers, using a peroxidase-antiperoxidase technique, established that the pasteurellae in the tonsils were lodged in the crypts, and not in the tonsillar tissue, and hence not exposed to parenterally administered antibiotics.

Conclusions

The epidemiological studies on HS in Sri Lanka carried out over the past two to three decades have:

- established the identity of the single serotype involved;
- recognised the presence of endemic and nonendemic areas;
- established that buffalo have higher susceptibility than cattle;
- recognised the seasonal incidence pattern and its possible dependence on climatic and immunological interactions;
- recognised morbidity patterns in endemic and nonendemic areas, and noted a possible relationship to the phenomenon of naturally acquired immunity;
- recognised species-specific and age-specific morbidity patterns;
- recognised the presence of latent and active carriers, and identified the site of persistence in latent carriers;

- postulated that the initiation of an outbreak results from a latent carrier becoming active, shedding pasteurellae through nasal secretions, and infecting in-contact susceptible animals. (It is unlikely that a carrier itself will break down into a clinical case as was earlier believed, on account of its immune status.)

A few grey areas remain to be investigated; e.g. the factors that cause a latent carrier to become active are as yet unknown. In addition, to reproduce HS experimentally by infection through the natural routes of infection, large numbers of organisms are required. How an active carrier could transmit such large numbers of organisms is still uncertain. It is not known whether intrinsic or extrinsic factors cause variation in the infective dose under natural conditions. Current and future epidemiological studies on HS in Sri Lanka will be directed towards these areas.

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The Effect of the *Pasteurella multocida* Toxin in Calves

W. Erler[1] and D. Schimmel[1]

Abstract

Twenty-seven *Pasteurella multocida* strains were isolated from affected lungs of calves. Of six strains belonging to serotype D, three strains produced toxin. This suggested that toxigenic *P. multocida* strains participate in the formation of pneumonic lesions in calves. The intratracheal application of different toxin preparations to calves subsequently showed that it is possible to induce lung lesions with purified toxin. The lesions are lobular to lobularly confluent in distribution and catarrhal in character. The intramuscular application of a crude toxin preparation to five calves resulted in atrophy of the nasal conchae in four animals. On the basis of these results, it would appear that toxigenic *P. multocida* plays an important role in both pneumonia and atrophic rhinitis of calves.

In recent years a number of papers have been published on the importance of the dermonecrotic toxin of *Pasteurella multocida* in the pathogenesis of progressive atrophic rhinitis of pigs. The triggering of this disease by the toxin is now undisputed (Rutter and Mackenzie 1984).

In this paper, we report on isolating toxigenic *P. multocida* (as well as non-toxic *P. multocida* and *P. haemolytica*) from pneumonic lungs of calves; and on the importance of the toxin in producing lung lesions and atrophic rhinitis in calves.

Materials and Methods

Calves aged 3–8 weeks from different herds, with clinical signs of enzootic pneumonia, were transported to the Institute and euthanased. Parts of macroscopically altered lungs were taken for microbiological examination and the isolation of *P. multocida*. The *P. multocida* strains isolated were differentiated serologically by the method of Carter, and toxin production was determined using the guinea pig skin test, tissue culture with embryonic bovine lung cells, and an ELISA with monoclonal antibodies.

A crude toxin was prepared from a toxigenic strain by ultrasonic extraction. From this extract, the toxin was purified by size-exclusion chromatography on Sephadex G 200 and ion-exchange chromatography with DEAE-Sephacel. The purity of the toxin was checked by PAGE. The amount of toxin was determined by an ELISA.

For the animal experiments we used calves of 4–6 weeks of age. The animals were challenged intratracheally with different toxin preparations. One week later they were euthanased and the extent of lung lesions was assessed on the scale: 0 = without a change, to 3 = very marked changes.

In addition, a crude toxin preparation ($3 \times 50\ \mu g$) was administered intramuscularly to five calves.

Results and Conclusion

Results of isolation, typing and toxin production of *P. multocida* strains from affected calf lungs are given in Table 1. From the 27 strains isolated, 1 belonged to type A, 7 to type D or A/D, and 12 strains were not typeable (nt). Fifty percent of the D strains examined produced toxin, but none of the nt strains. These findings demonstrate that toxigenic *P. multocida* strains participate in the formation of pneumonic lesions in calves.

The results of intratracheal application of purified toxin preparations to calves are shown in Table 2. From these, it is evident that it is possible to induce lung lesions with the purified toxin. The lesions are lobular to lobularly confluent, and catarrhal in character.

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Table 1. Isolation, serotyping and toxin production of *P. multocida* strains from affected lungs of calves.

		Serotype			
		A	D	A/D	nt
Strains isolated	27	1	7	7	12
Toxin					
examined	12		6	1	5
produced	4		3	1	0
Percent	33.3		50	100	0

Table 2. Results of intratracheal administration of *P. multocida* toxin to calves.

Application	Number of animals	Severity of pneumonia				
		0	1	2	3	mean
Purified toxin (25 μg)	10	0	1	3	6	2.5
Buffer control	10	7	3	0	0	0.3

The crude toxin preparation (3 × 50 μg) given intramuscularly to five calves resulted in swelling of the liver and spleen. In addition, four of the calves developed atrophic changes in the nasal conchae.

On the basis of the results published by Frymus et al. (1991) and Chrisp and Foged (1991) on the effects of the toxin of *P. multocida* in rabbits, and our own results (Schimmel and Erler 1991), it can be concluded that toxigenic *P. multocida* plays an important role in both atrophic rhinitis and pneumonia of pigs, calves and rabbits.

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The Pathology of Experimental Haemorrhagic Septicaemia in Cattle and Buffalo

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Abstract

Two buffalo and two cattle that had not been vaccinated against haemorrhagic septicaemia (HS) were challenged with 8 × 10^7 colony-forming units of *Pasteurella multocida* type B:2 (Carter:Heddleston) by subcutaneous injection. In addition, one bovine that had been vaccinated with a commercial oil adjuvant HS vaccine (PusVetma, Surabaya, Indonesia) and eight cattle that had been vaccinated with four other strains of *P. multocida* isolated from cattle were challenged with the same organism. The non-vaccinated animals died 24–60 hours post challenge, with clinical and pathological changes similar to those described in natural cases of acute HS. The other nine cattle remained clinically normal. They had no significant lesions at necropsy and no organisms were cultured from their organs or blood. It was concluded that each of the strains used as a vaccine was able to protect against a subsequent challenge with the B:2 strain of *P. multocida*.

THE published literature on haemorrhagic septicaemia (HS) contains relatively little on the pathology of the disease, despite its importance in many countries around the world (Losos 1986). HS is regarded as one of the most economically important diseases of cattle and buffalo in Indonesia, yet little material from HS outbreaks is received by the veterinary diagnostic laboratories.

The experiments described here were designed to reproduce HS in un-vaccinated animals and to determine the cross-protection between *P. multocida* strains that normally affect cattle. The paper also outlines the pathological features of the experimental disease in cattle and buffalo in Indonesia.

Materials and Methods

Two cattle and two buffalo that had not been vaccinated against HS, and one bovine that had been vaccinated with a commercial oil adjuvant HS vaccine containing *P. multocida* Katha strain (PusVetma, Surabaya, Indonesia), were challenged with 8 × 10^7 colony-forming units (CFU) of *P. multocida* type B:2 (Carter:Heddleston) by subcutaneous injection in the left side of the neck. An identical challenge was given to eight cattle that had been vaccinated by multiple injections of a formalin-killed bacterin of one of four strains of *P. multocida* — type 14 (Heddleston), type 7:A (Namioka:Carter), type 11:B (Namioka:Carter), or type B:2 (Heddleston:Carter) — previously isolated from cattle.

Following challenge, the animals were observed at regular intervals for the duration of the experiment. At each observation period, venous blood was collected from the jugular vein and the clinical condition and rectal temperature of the animals was recorded. Animals were necropsied at the time of death or when the animal was moribund. The vaccinated cattle were euthanased at the end of the experiment by the intravenous injection of sodium pentobarbitone 325 mg/mL (Lethabarb, Arnolds of Reading, Melbourne, Australia).

Results

Clinical findings

The clinical findings in the non-vaccinated cattle consisted of extensive swelling at the injection site and

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a rise in body temperature. The swellings were first observed 4 hours after the injection, and the febrile response was seen after 12–18 hours. A temperature rise was noted in one buffalo, but the other, which died after 24 hours, had neither of these clinical signs. Apart from these specific signs, the clinical syndrome was one of lethargy, recumbency and finally death.

The time to death of the non-vaccinated animals ranged from 60 hours in the cattle to 24 and 31 hours respectively in the buffalo.

Gross pathology

The gross pathological changes were similar in all of the non-vaccinated animals examined in this study. However, the lesions were most severe in the buffalo. There was extensive, subcutaneous oedema in the tissues surrounding the injection site. The epidermis and dermis were thickened, and the underlying musculature was pale and oedematous. The oedema extended from the brisket to the neck, and to the sub-mandibular region and larynx in the buffalo that died after 24 hours, but was more localised in the other animals. The left precapsular lymph nodes, which drained the injection sites, were swollen and oedematous, with thickened capsules. In cross section the lymph nodes were diffusely reddened and contained numerous 3–5-mm-diameter pale, necrotic foci. The other lymph nodes examined were of normal appearance.

There were no other gross lesions in the cattle, but both buffalo had congested lungs, especially in the cranial lobes, stable foam in the larger airways, thread-like pleural adhesions, and numerous ecchymotic haemorrhages on the epicardial and endocardial surfaces. In the buffalo that survived for 31 hours, the larynx was diffusely hyperaemic.

Histopathology

The microscopic lesions at the injection site consisted of severe oedema and fibrin deposition in the subcutaneous tissues and between and surrounding the muscle fibre bundles. This was accompanied by necrosis and degeneration of muscle fibres and vasculitis and thrombosis of blood and lymphatic vessels. The associated inflammatory cell response was made up of neutrophils and macrophages, with occasional bacterial colonies and micro-abscesses scattered throughout.

The lymph nodes draining the injection site had fibrin and oedema in the perinodal tissues. This was accompanied by thrombosis and vasculitis of capsular blood vessels. The nodes were very oedematous, with fibrin deposition throughout, and the subcapsular and medullary sinuses were packed with neutrophils. There were numerous foci of necrosis scattered throughout both cortex and medulla. There were no histopathological changes in the other lymph nodes examined.

Large numbers of bacteria were a feature of tissue taken from the site of the injection and from the lymph nodes of the cattle, but not the buffalo. However, bacterial emboli in the adrenal glands and spleen were a feature in the buffalo.

The lungs showed relatively mild purulent interstitial pneumonia, congestion and haemorrhage, and a variable amount of oedema. Lymphatics were dilated, and sometimes contained neutrophils.

There were no significant gross or microscopic changes in any of the vaccinated bovines.

Bacteriology

P. multocida was re-isolated from all non-vaccinated animals from a variety of samples including blood, tonsils, parotid and sub-maxillary lymph nodes, lung, spleen and oedema fluid. *P. multocida* was not isolated from blood or any other tissues from the vaccinated animals.

Discussion

The clinical and pathological findings in these animals were similar to those reported in reviews by Losos (1986) and De Alwis (1992). As expected, the buffalo were more severely affected than the cattle and had a shorter survival time. Pyrexia, which occurred after 10–12 hours, was a feature in the cattle and in the longest surviving buffalo, but not in the severely affected buffalo that died after 24 hours. This animal's survival time was the same as that given by Bain (1963), but shorter than the survival times given by other authors (Losos 1986; De Alwis 1992). A surprising find was the lack of observable swelling at injection sites in the buffalo, even though the oedema was very severe at necropsy. This was in contrast to the cattle, which had a detectable swelling from 4 hours onward. Our findings are the opposite of those reported by Losos (1986), where subcutaneous oedematous swellings were more common in buffalo than cattle.

The lesions observed in the lymph nodes in the animals in this study were more severe than those recorded by other authors, who reported swelling, congestion and hyperaemia, but not frank necrosis (Siew et al. 1970; Bastionello and Jonker 1981; Losos 1986). Experimental cases described by Rhoades et al. (1967) had little or no lymph node enlargement.

In addition, there was more necrosis and haemorrhage at the injection site in our animals than in the experimental infection following subcutaneous injection reported by these authors. In this respect our cases resemble the naturally occurring cases recorded by Bastianello and Jonker (1981), which had similar lesions in the vicinity of the joints and lungs. These differences may represent strain variations or be due to method of infection. The lung lesions found in our cases were mild, but similar to those reported elsewhere. Gross and microscopic changes were not observed in the gastrointestinal tract. All animals in this study developed septicaemia, as determined by the presence of bacteria in a variety of tissues and re-isolation of the challenge organism from several locations.

The failure to produce significant pathological changes or bacterial colonisation in the vaccinated animals suggests that each of the strains of HS used as a vaccine provided protection against subsequent challenge with virulent B:2 organisms.

Finally we would suggest that more effort be placed on attempting to confirm the clinical field diagnosis of HS by bacterial isolation. We believe that the disease is easily confused with other acute fulminating diseases of cattle and buffalo, and that laboratory confirmation of diagnosis will assist in establishing the true incidence of the disease.

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Pasteurella multocida and *Pasteurella haemolytica* Infections in Ruminants and Pigs in Southern New South Wales

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Abstract

Pasteurella spp. were isolated from 219 disease incidents in southern New South Wales over a 5-year period. *Pasteurella haemolytica* was recovered on 108 occasions (sheep 65, beef cattle 33, dairy cattle 5 and other animal species 5) and *Pasteurella multocida* also on 108 occasions (pigs 65, sheep 19, beef cattle 12, dairy cattle 4 and other species 8). Other *Pasteurella* spp. (including *P. gallinarum* 4 isolations, and *P. ureae* 1) were recovered on 11 occasions. Eight submissions yielded two strains of *Pasteurella* spp.

Disease incidents were classified on the basis of the pathological findings. *P. haemolytica* and *P. multocida* infections in sheep were associated with pneumonia as the principal pathological condition (26 and 11 submissions respectively), pneumonia as an incidental pathological finding (15 and 2), mastitis (16 and 1), perinatal septicaemia/stillbirth (8 and 2), and abnormal semen (1 and 2).

In beef cattle, the two species were associated mainly with pneumonia as the principal diagnosis (24 and 7). In dairy cattle they were associated with pneumonia (2 and 3) and mastitis (2 and 1).

In pigs *P. multocida* was associated with pneumonia as the principal pathological condition (31 submissions), incidental pneumonia (6), and atrophic rhinitis (24). Toxigenic strains of *P. multocida* were isolated from 5 submissions (4 type D and 1 type A) and non-toxigenic strains from 7 submissions derived from piggeries with clinical atrophic rhinitis while non-toxigenic strains were isolated from 12 submissions derived from piggeries free of the disease. Pleurisy (12) and pericarditis (6) were commonly seen in association with pneumonia.

The antibiotic sensitivity patterns of the isolates are presented.

PASTEURELLA infection occurs commonly in many animal species (particularly sheep, pigs and cattle) in Australia. There are a range of *Pasteurella* spp. involved, particularly *P. multocida*, and *P. haemolytica* types A and T. They are associated with infection of the respiratory tract (pneumonia, pleurisy and rhinitis) and the reproductive system (mastitis and metritis). There are, however, few reports of pasteurellosis in Australia in the literature.

There are reports on *P. multocida* and atrophic rhinitis in pigs (Love et al. 1985, Mercy et al. 1986, Eamens et al. 1988, Gardner et al. 1989); *P. haemolytica* infection in neonatal foals (Peet et al. 1977); *P. multocida* septicaemia in fallow deer (Carrigan et al. 1991); *P. haemolytica* mastitis in ewes (Kabay and Ellis 1989; Philbey 1990); *P. haemolytica* type A mastitis in a goat (Ryan and Greenwood 1990); *P. multocida* (Walker et al. 1979; Reece and Coloe 1985; Ireland et al. 1989) and *P. anatipestifer* (Munday et al. 1970; Grimes and Rosenfeld 1972; Rosenfeld 1973; Reece and Coloe 1985) in birds. A comprehensive review of pasteurellosis has been recently published (Eamens 1990).

This study reports the isolation of pasteurella from pathological material submitted to the Regional Veterinary Laboratory (RVL), Wagga Wagga, New South Wales, Australia.

Materials and Methods

Over the period July 1987 - June 1992, pathological material from a wide range of animal species submitted to RVL, Wagga Wagga, from the south-western region of New South Wales was routinely

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cultured for *Pasteurella* spp. on 7% sheep blood agar (Oxoid, Basingstoke, Hants., UK, Blood Agar Base No 2) and incubated at 37 °C in air with 10% CO_2. Between January 1988 and April 1989, in the course of a survey on atrophic rhinitis, a number of pig herds were sampled for *P. multocida*, using nasal swabs cultured onto Pedersen's NB medium and tested for toxigenicity (Eamens et al. 1988). From November 1991 to June 1992, sick or dead animals from selected pens in two large beef feedlots were sampled for *Pasteurella* spp. using nasal swabs, or by culturing other tissues. Isolates were confirmed as pasteurella by standard methods (Cowan 1974). Isolation of *P. multocida* from a number of pigs in a piggery was regarded as one isolation for the purposes of this report.

Tissues for histopathology were fixed in neutral buffered formalin, sectioned, and stained with haematoxylin and eosin by standard methods.

Antibiotic sensitivities were performed by the Oxoid Multodisk diffusion technique according to the method of Simmons and Craven (1980). Antibiotics tested were neomycin 30 μg, compound sulphonamide 300 μg, tetracycline 30 μg, sulphamethoxazole/trimethoprim 25 μg, ampicillin 10 μg, furazolidone 100 μg, lincospectin 150 μg and streptomycin 10 μg.

Results

Pasteurella spp. were recovered from 219 submissions, which yielded a total of 227 isolations. Two distinct strains were isolated from 4 sheep, 2 beef cattle, 1 dairy cattle and 1 pig submission. The distribution of these isolates among the animal species is shown in Table 1.

Isolates included under *P. haemolytica* included 95 identified as type A (57 from sheep, 31 from beef cattle, 3 from dairy cattle, 3 from birds and 1 from a laboratory rabbit) and 8 identified as type T (4 from sheep, 1 from beef cattle, 2 from dairy cattle and 1 from an alpaca). Five isolates were not typed beyond *P. haemolytica*.

Table 1. Number of isolates of *Pasteurella* spp. recovered from various animal species (227 isolates).

Isolates	Animal species*					
Pasteurella spp.	1	2	3	4	5	Total
P. multocida	19	12	4	65	8	108
P. haemolytica	65	33	5	0	5	108
Other species	2	0	1	1	7	11
Total	86	45	10	66	20	227

* 1 sheep, 2 cattle (beef), 3 cattle (dairy), 4 pigs, 5 other species

The other animal species from which *P. multocida* was isolated included poultry and dogs (3 isolates each) and a laboratory rabbit and a cat (1 isolate each).

Three isolates included under other *Pasteurella* spp. were confirmed as *P. gallinarum* (from birds) and *one* as *P. ureae* (from mastitis in a goat). Six isolates could not be identified at the species level.

For sheep, beef cattle, dairy cattle and pigs, the clinical disease categories from which the *Pasteurella* spp. were derived are shown in Table 2.

Perinatal mortality in lambs due to pasteurellosis was confirmed on 10 occasions (9 from one property, which was intensively monitored during a research project) yielding *P. haemolytica* type A on 6 occasions, *P. haemolytica* type not determined on 2 occasions and *P. multocida* on 2 occasions.

The principal pathological conditions diagnosed in the various species are shown in Table 3.

Considering only the principal pathological conditions, in sheep with pneumonia *P. haemolytica* type

Table 2. Clinical syndrome or other reason reported for the various animal species yielding *Pasteurella* spp. (227 isolations).

Species	Clinical syndrome/other reason*							
	1	2	3	4	5	6	7	Total
Sheep	31	12	16	10	6	5	6	86
Beef cattle	13	17	1	1	2	1	10	45
Dairy cattle	2	5	3	0	0	0	0	10
Pigs	13	23	1	1	4	18	6	66
Other species	7	9	1	0	0	1	2	20
Total	66	66	22	12	12	25	24	227

* 1 death, 2 respiratory signs, 3 mastitis, 4 perinatal mortality, 5 illthrift, 6 monitoring, 7 other

A was involved on 20 occasions, type T on 2 occasions, and *P. multocida* on 11. Sheep with mastitis yielded *P. haemolytica* type A on 16 occasions, while *P. multocida* was recovered on only 1 occasion.

Table 3. Principal pathological abnormality observed in the various animal species yielding *Pasteurella* spp. (227 isolations).

Species	Pathological condition*						
	1	2	3	4	5	6	Total
Sheep	39	18	2	9	3	15	86
Beef cattle	32	0	0	2	0	11	45
Dairy cattle	6	3	0	1	0	0	10
Pigs	32	1	11	1	3	18	66
Other species	5	1	4	2	0	8	20
Total	114	23	17	15	6	52	227

* 1 pneumonia, 2 mastitis, 3 upper-respiratory-tract conditions, 4 septicaemia with hepatitis, 5 gastroenteritis, 6 other pathological conditions

In beef cattle with pneumonia, *P. haemolytica* type A was isolated 23 times, while *P. multocida* was recovered 7 times. Pyrexia in feedlot cattle was associated with both *P. haemolytica* type A (4 isolates) and *P. multocida* (2).

In dairy cattle, pneumonia was associated with *P. multocida* (3 cases) and *P. haemolytica* types A and T (1 case each). Mastitis in dairy cattle was associated with *P. haemolytica* type A (2) and *P. multocida* (1).

P. multocida was virtually the only strain isolated from pigs. Toxigenic strains of *P. multocida* were isolated from 5 submissions (4 type D and 1 type A) and non-toxigenic strains from 7 submissions derived from piggeries with clinical atrophic rhinitis, while non-toxigenic strains were isolated from 12 submissions derived from piggeries free of the disease.

Histopathological examination was performed on lungs from 48 ovine cases. *P. haemolytica* type A and type T, and *P. multocida*, were associated with fibrinous pneumonia (13, 0 and 1 case respectively out of a total of 16) and with bronchopneumonia (15, 1 and 5 out of 22 cases). There were 3 cases of verminous pneumonia, 2 cases of suspected mycoplasmal pneumonia, 2 cases of inhalation pneumonia and 1 case of interstitial pneumonia.

Examination of 26 beef cattle lungs revealed *P. haemolytica* type A and *P. multocida* associated respectively with fibrinous pneumonia (15 and 6 out of 21) and bronchopneumonia (2 and 1 out of 4). Only 4 dairy cattle lungs were examined, 2 with fibrinous pneumonia and 2 with bronchopneumonia.

A total of 34 pig lungs were examined. *P. multocida* was associated with 15 cases of bronchopneumonia, 9 cases of enzootic pneumonia, 9 cases of fibrinous pneumonia, and 1 case of interstitial pneumonia.

Stress of shipment is considered a factor in precipitating pneumonia in cattle — so-called 'shipping fever'. There were 7 cases with a history of recent transport: 1 dairy (with fibrinous pneumonia due to *P. multocida*) and 6 beef (2 with bronchopneumonia with either *P. haemolytica* type A or *P. multocida*; and 4 with fibrinous pneumonia, 3 caused by *P. haemolytica* type A and 1 by *P. multocida*).

The results of antibiotic sensitivity testing of *P. haemolytica*, *P. multocida* and other *Pasteurella* spp. are reported in Table 4.

Table 4. The in vitro sensitivity of 154 *Pasteurella* spp. isolates to a range of antibiotics.

Antibiotic	*Pasteurella haemolytica*	*Pasteurella multocida*	*Pasteurella* other
Ampicillin	100*	98	80
Tetracycline	100	97	90
Sulpha methoxazole/ Trimethoprim	99	88	90
Lincospectin	98	88	90
Furazolidone	92	45	90
Neomycin	71	66	100
Sulphonamide	68	57	80
Streptomycin	43	34	80
No. isolates	79	65	10

* % of isolates sensitive to the antibiotic

Discussion

Submissions received at RVL, Wagga Wagga, average approximately 4000 per year, with 50% from sheep, 34% from cattle, and 5% from pigs. Because of the bias in selection of pathological material submitted to the laboratory (some specimens being derived from research projects and surveys), the results of this study are not a true indication of the prevalence of the various *Pasteurella* spp. or the clinical or pathological conditions associated with infection. Nonetheless, pasteurellae were found to be a relatively common cause of disease in sheep, cattle and pigs.

Mortality, respiratory disease and mastitis were by far the most common clinical entities detected, while

pneumonia, mastitis, hepatitis and atrophic rhinitis were the most common pathological abnormalities. This is consistent with the expected pattern of disease associated with this group of bacteria in other countries (Eamens 1990).

P. multocida was essentially the only strain isolated from pigs. *P. haemolytica* type A was most common in beef cattle and sheep, with type T being relatively rare, and *P. multocida* represented about 30% of isolates in these two animal species.

Mastitis in sheep is a more common problem than is generally recognised, with the vast majority of cases being attributable to *P. haemolytica* type A. Perinatal mortalities in lambs due to pasteurella septicaemia were also quite common, although most were derived from one property. The incidence of this infection is probably underestimated, because of the infrequency with which newborn lambs are examined and cultured.

With the stress of transport and mixing of animals from a large number of sources considered a major factor in the initiation of respiratory disease, the major expansion in the beef feed-lotting industry in recent years is likely to greatly increase the incidence of pasteurellosis. Although relatively rare in New South Wales to date, 7 cases of pasteurellosis following transport were identified in the current study.

No attempt has been made in this report to analyse the age of the animals involved or the economic impact of pasteurellosis.

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Haemorrhagic Septicaemia of Buffalo in Sabah, Malaysia

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Abstract

Buffalo provide approximately 35–40% of the beef requirement of Sabah, Malaysia. The animals also provide draught power in the agricultural sector and serve as important 'living assets' in the farming community. Haemorrhagic septicaemia (HS), caused by *Pasteurella multocida*, is the major killer disease of buffalo in Sabah. Since the first outbreak in 1973 in the Interior and West Coast regions, the disease has continued to kill 100–150 buffalo annually. A total of 23 outbreaks were recorded for the period 1983–91. In contrast, no outbreaks have been recorded in the cattle population. The occurrence of HS is influenced by several factors. Cases are more frequently reported immediately after heavy rain. Low vaccination coverage makes the stock more susceptible, while the often extensive nature of buffalo keeping exposes healthy animals, particularly young stock, to 'carriers'. Regular vaccination is still the best solution. The percentage of animals vaccinated needs to be increased; and precautions need to be intensified when animals are introduced to the Tawau and Sandakan HS-free regions for draught power.

BUFFALO (*Bubalus bubalis*) provide 35–40% of the beef requirement in Sabah, Malaysia. The buffalo population, as estimated in 1991, is about 65 000 head (Mokhtar and Yeo 1991). The majority of animals are found in the West Coast and Interior regions of the state, and 95% are contained in single-owner herds of less than 10 animals (Yeo, unpublished).

Besides being a major contributor of beef, buffalo also play an important role in providing draught power in the agricultural sector. They are used for padi (rice) cultivation in the Interior and West Coast regions, while in the Tawau and Sandakan regions they are used to haul and transport oil palm fruit due to the rugged terrain. For the farming community, buffalo are 'living assets', which are sold for cash in times of need.

The first outbreak of haemorrhagic septicaemia (HS) in Sabah was recorded in 1973 in the Interior and West Coast regions. About 1200 buffalo died (Bacon 1986). Since then, HS has become the most single important infectious disease, causing between 100 and 150 deaths annually.

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The epidemiology of the disease, which is caused by *Pasteurella multocida* serotype 6:B, is not well documented. This paper discusses the disease in Sabah using the limited data collected from 1983 to 1991.

Materials and Methods

A retrospective analysis was carried out on all reports confirmed positive for *P. multocida* by the Animal Research Centre in Kepayan. 'Spatial' distribution by districts, the estimated period of occurrence and animal mortality were derived from the reports, which were rarely detailed in nature. Observations made during visits by the authors to HS-affected areas in the state over the past 5 years are also presented.

Results

Table 1 shows the occurrence of HS in buffalo in Sabah from 1983 to 1991, as obtained from laboratory reports.

Clinical signs

Fever and dullness were frequently detected. This would be followed by respiratory distress with profuse salivation. Respiration was often rapid and

Table 1. The occurrence of haemorrhagic septicaemia in buffalo in Sabah from 1983 to 1991.

Year	District	Period of occurrence	Estimated mortality
1983	Kota Belud	February–April	88
1983	Kota Belud	June–August	41
1983	Tambunan	October–December	33
1984	Tambunan	March–June	69
1984	Tambunan	August–September	42
1985	Kota Kinabalu	January–March	14
1985	Kota Kinabalu	July–August	5
1985	Penampang	July–August	6
1985	Tambunan	September–November	56
1986	Keningau	January–March	54
1986	Kota Marudu	July–September	49
1987	Kota Kinabalu	January–March	7
1987	Membakut	January–March	51
1987	Membakut	May–June	32
1987	Keningau	October–December	35
1988	Papar	May–September	78
1989	Papar	March–April	40
1989	Papar	June–July	29
1989	Papar	September–November	54
1990	Papar	January–March	51
1990	Papar	November–December	47
1991	Penampang	January–February	14
1991	Kota Marudu	April–May	37

'noisy', and severe dyspnoea could occur if animals were handled. Occasionally mucous membranes were cyanotic. Oedema of the submandibular region was more frequent than oedema of the brisket or anterior limbs.

The fatality rate was almost 100%. In most acute cases, animals died 2–3 days after showing signs. Buffalo that were not tended usually chose to continue wallowing and finally died in pools of water.

No cases of HS were recorded in cattle herds even though abundant cattle could be found in several districts — such as Kota Belud, Keningau and Kota Marudu — where HS of buffalo has been reported. Cattle and buffalo herds are traditionally not kept in close proximity with each other by small or large holders.

Necropsy

There were general toxic changes associated with septicaemia, but no pathognomonic lesions. Oedema of the lungs and subcutis was usually pronounced. Variable lesions included haemorrhages in the heart and petechiation of mucosal surfaces of the upper respiratory tract.

Transmission

In most cases, affected animals had a history of contact with infected animals within or between herds. This contact could have occurred as a result of animal movement to open markets or tamus, between districts, or within a district for draught and other purposes. Common grazing or wallowing grounds including streams and rivers were also implicated as reservoirs for susceptible buffalo.

Deaths due to HS were more common in younger animals. More than 90% of cases involved animals less than 2 years old or animals that remained unvaccinated due to the extensive nature of management.

Most outbreaks occurred during the wetter months (Table 1). Fourteen out of 23 outbreaks occurred between April and December, when the south-west monsoon usually prevails. Of these, 79% occurred during the onset and middle of the monsoon and only three during the last 3 months of the year. Nine outbreaks began during the first 3 months of the year when the north-east monsoon, which is drier, prevails.

Area vaccination coverage

The average percentage of buffalo vaccinated annually against HS in some designated districts is tabulated in Table 2.

Table 2. Estimated average percentage of vaccination against haemorrhagic septicaemia.

District	Estimated average (%)
Kota Belud	12
Tambunan	29
Kota Kinabalu	19
Penampang	24
Keningau	15
Kota Marudu	15
Membakut	38
Papar	33
Kudat	27
Sandakan	65
Tenom	33
Tuaran	12
Beaufort	29

Discussion

According to this analysis, HS of buffalo occurs at all times of the year. This is similar to observations made by De Alwis (1992). The majority of outbreaks reported here occurred at the onset and middle of the south-west monsoon period, which lasts from April to November.

The north-east monsoon, which can bring as much as 600 mm of rain in the wettest month (Thomas et al. 1986), does not seem to influence the occurrence of HS in the Kudat district. This could be due to the fact that buffalo are present in small numbers and are sparsely dispersed in the region, and the animals are rarely worked during the rains as padi is not an important cultivation in the region. Hence animals are not subjected to undue stress, although conditions may be wet and humid.

Our records do not show any major HS outbreak in cattle in the state. This is different from the situation in Peninsular Malaysia (Joseph 1979; Sharee and Salim 1991). Traditionally buffalo and cattle are rarely kept in close contact in Sabah, even in smallholder farms. Cattle husbandry is generally more 'intensive'. Buffalo are usually, though not always, kept extensively or left to roam. Whether the difference in management of these two species, coupled with a smaller cattle population of almost half the buffalo population, could explain the non-existence of HS in cattle is still unknown.

Vaccination coverage in 13 districts (Table 2) ranged between 12 and 65%. In the eight districts where outbreaks of HS had been reported, the average coverage appears to be lower than in those without outbreaks, although a few exceptions like Membakut and Papar have comparatively high vaccination coverage. It is estimated that vaccination coverage in the whole state is between 20 and 25% (Yeo, unpublished).

From most cases observed, the clinical signs were those indicative of acute syndromes of the disease. Farmers usually do not inform the veterinary authorities until after infected animals develop oedematous swelling and respiratory distress. Therefore, the animals commonly do not respond to antibiotic treatment.

In peracute cases, buffalo die without premonitory signs in common grazing grounds or wallowing pools and are left to rot or are not properly disposed of. This situation, which was also observed by Sharee and Salim (1991) in Peninsular Malaysia, creates sources of infection for other buffalo.

In open markets or tamus, where buffalo gathered from various districts are for sale, infected or carrier animals could be inadvertently introduced to become sources of infection. This is particularly true in the Kota Belud district, where animals from as far afield as Kota Marudu, Tambunan and Keningu have been known to transmit or harbour not only HS bacteria but also parasites such as *Fasciola* spp.

Buffalo, particularly in padi growing areas, are subjected to greater stress during the wet seasons, as a result of extra workloads of land tilling and ploughing before padi planting. Feed sources are usually not specifically provided then, thus depriving the working animals of necessary energy requirements. As a consequence, buffalo in padi growing areas are more prone to HS than are those in the Tawau and Sandakan districts, where animals do not face feed shortage and the work output expected from them is constant or routine. Furthermore, buffalo in plantations are kept under better management conditions.

Conclusions

HS outbreaks in Sabah are influenced by a number of factors including the monsoons and the immune status of buffalo herds, and to a degree by husbandry systems. Efforts towards reducing this killer disease in buffalo should include higher and effective vaccination coverage and educating farmers on better husbandry and proper movement controls. Factors inducing stress of buffalo should be alleviated where possible.

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Haemorrhagic Septicaemia in Vietnam and its Control

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Abstract

Vietnam is situated in the middle of Southeast Asia and has a tropical monsoonal climate. The dry season usually occurs from November to February, and the wet season from April to September. Every year, haemorrhagic septicaemia (HS) causes heavy losses in cattle and buffalo. There are marked climatic differences between the southern and northern parts of the country, and the aim of this study was to examine and compare the seasonal incidence of HS in the southern Mekong River delta and the northern Red River delta during 1984–86.

Figures for temperature, relative humidity and rainfall for the dry and rainy seasons in the two deltas, together with morbidity and mortality rates caused by HS, are given in Table 1. In both regions, HS was most prevalent in the wet season, during periods of high air temperatures and heavy rainfall. In general the two deltas had similar climatic and disease patterns, the main differences in the Red River delta being lower air temperatures and HS morbidity and mortality rates during the dry season. These results are in agreement those of others (Manniger 1934; Jasin 1952; Vittoz 1952) and indicate that annual vaccination should occur immediately before the beginning of the wet season.

Since 1957, aluminium-hydroxide absorbed HS vaccine has been used to vaccinate cattle and buffalo. The vaccine has low potency and provides only short-term protection, despite the large (20 mL) dose given. Major outbreaks still occur in much of the country. More recently, oil adjuvant vaccines have been evaluated because of their higher potency and persistent immunity.

The oil adjuvant vaccine is prepared from liquid cultures of *P. multocida* grown in a 20 L fermenter to a density of at least 20×10^6 organisms/mL. The organisms are inactivated by the addition of formalin to a final concentration of 0.3–0.5% and then added slowly to a suitable mixture of mineral oil (marcol-52 or paraffin) and emulsifying agent (montanide-888 or melted lanolin) in an emulsifier with a capacity of 100 L. Emulsification is continued for 20–30 min, depending on the volume of the mixture. After overnight storage the mixture is re-emulsified, bottled and stored at room temperature for 2–3 weeks prior to use. Montanide-888, although it is more expensive, is generally used in preference to lanolin, as vaccines made with lanolin are more viscous.

More than 800 000 doses of oil adjuvant vaccine have now been used in more than 10 districts of Vietnam. There have been no problems with storage or distribution under field conditions, as the emulsified vaccine is stable for 12 months at 14–35 °C. The vaccine is also safe, and no shock reactions have been seen in cattle or buffalo. However, the emulsified vaccine does cause some local reaction at the site of injection when administered intramuscularly. (Subcutaneous administration of vaccine should be avoided as it results in the formation of fibrous lumps, 5–10 cm in diameter).

Oil adjuvant vaccine confers immunity for 12 months and offers the potential for eradicating HS. However, despite its high potency, the vaccine has not gained widespread acceptance because of difficulties associated with vaccination and the cost of reagents.

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Table 1. Temperature, relative humidity and rainfall in the dry and rainy seasons in the Mekong and Red River deltas (1984–86), together with figures for morbidity and mortality caused by HS.

Place (season)	Temp. (°C)	Relative humidity	Rainfall (mm)	Morbidity (%)	Mortality (%)
Mekong (dry)	26.5 ± 0.7	74.18 ± 3.77	32.1 ± 7.4	19.6 ± 2.7	3.7 ± 0.5
Mekong (rainy)	27.3 ± 0.1	84.4 ± 0.3	192.9 ± 3.4	33.0 ± 8.5	4.9 ± 0.2
Red (dry)	18.1 ± 0.1	85.9 ± 0.6	35.8 ± 5.9	2.4 ± 0.7	0.4 ± 0
Red (rainy)	27.3 ± 0.2	83.3 ± 0.4	227.1 ± 13.1	16.2 ± 1.7	6.4 ± 0.6

SESSION 3: PREVENTION AND CONTROL OF PASTEURELLOSIS

Prevention and Control of Pasteurellosis

D. Mosier[1]

Abstract

Pasteurellosis of ruminant production animals has a significant economic impact worldwide. In cattle, pasteurellosis mainly takes the form of haemorrhagic septicaemia or pneumonia, whereas pneumonia is most prevalent in sheep and goats. Prevention and control depends on a variety of environmental and management factors, host immunity and therapeutic protocols. Outbreaks are usually associated with stress. During stressful periods there are increased rates of exposure and risk of infection. Although a small percentage of animals have naturally acquired immunity to infection, in most animals vaccination is necessary to stimulate disease resistance. Numerous killed and live bacterial suspensions and bacterial extracts of *Pasteurella haemolytica* and *P. multocida* have been evaluated for their efficacy as vaccines. Considerable variation in effectiveness has been found, based on factors such as the conditions of vaccine preparation and use, the route of administration, the adjuvant, and the presence and concentration of immunologically important antigens. In animals that develop clinical disease, antibiotic therapy can be effective if it is initiated early. Although several types of antibiotics are generally effective, the emergence of antibiotic-resistant strains of pasteurellae can complicate treatment.

PASTEURELLOSIS causes a wide variety of disease syndromes in numerous animal species worldwide. In cattle, the disease mainly takes the form of haemorrhagic septicaemia (HS) and pneumonic pasteurellosis. In sheep and goats the pneumonic form is most prevalent.

HS is an acute highly fatal infection of cattle and buffalo that occurs primarily in tropical Asia and Africa. Disease is caused by *P. multocida* type B:2 in Asia, whereas type E:2 causes disease in Africa. Estimated economic losses due to HS are substantial in both buffalo and cattle, with annual losses of 4000–10 000 reported in some countries (Bain et al. 1982). Reliable estimates of the extent of the losses are often difficult to obtain, however, due to the acute nature of the disease and its common occurrence in remote areas (Carter and De Alwis 1989). Sheep and goats are not highly susceptible to natural or experimental HS and the disease is of minor significance in these species (Carter and De Alwis 1989).

In North America and Europe, pasteurellosis is manifested primarily as pneumonia. Bovine pneumonic pasteurellosis is most frequently caused by *P. haemolytica* type A1 and occasionally by other serotypes of *P. haemolytica* or *P. multocida* type A (Frank 1989). Annual losses due to bovine respiratory disease have been estimated at US$500–800m in the United States and US$3bn worldwide (Drummond et al. 1981; Ratafia 1988). Most ovine cases are caused by *P. haemolytica* type A2 (Gilmour 1980). However, substantial numbers of cases have been associated with other A serotypes, including 1, 6, 7 and 9, and by T serotypes 3, 4 and 10 and *P. multocida* types A and D (Fraser et al. 1982). Pneumonic pasteurellosis is the most commonly diagnosed infectious disease of sheep in England (Gilmour and Gilmour 1989), but its precise economic impact is not well defined.

Pasteurellae are sometimes associated with a variety of other disease syndromes in food production ruminants. *P. haemolytica* biotype T produces systemic disease of young adult sheep (Gilmour 1980). Biotype A occasionally causes septicaemia in lambs, sometimes accompanied by arthritis and meningitis, and mastitis in ewes (Gilmour and Gilmour 1989). Compared to other types of pasteurellosis in ruminants, these diseases are of minimal significance.

Due to the potentially devastating effect of septicaemic and pneumonic pasteurellosis on ruminant animals and their owners, effective prevention and

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control are critical issues. Although many factors influence the development of disease, three of major importance are management, host immunity and treatment.

Management

A common feature of the septicaemic and pneumonic forms of pasteurellosis is the importance of stress and other detrimental factors in predisposing to disease. Whereas some of these factors are not easily controlled, certain management practices can reduce the risk of outbreaks.

HS-causing strains of *P. multocida* are intermittently shed by immune carrier animals that harbour the organism in their tonsils (Carter and De Alwis 1989). Shedding usually occurs following some type of environmental or management-associated stress, resulting in increased exposure of other animals. In well-managed cattle in dry environments, the incidence of HS is low (De Alwis 1981), but in poorly managed cattle in low areas the incidence is higher. Animal density also effects disease incidence (De Alwis and Vipulasiri 1980). In small, herds the incidence is generally low, whereas in large (>50 animals) herds it can be four to five times greater.

Although outbreaks can occur at any time, they are more common and spread more readily during wet weather (Carter and De Alwis 1989). Increased survival and concentration of organisms in wet environments, or increased host susceptibility and increased shedding of organisms by latent carriers due to weather-associated stress, are probable in these conditions. While vaccination can effectively reduce the incidence of disease, the frequency and timing of vaccination are important (Carter and De Alwis 1989). Poor access to animals, inadequate facilities, and the reluctance of owners to vaccinate routinely in the absence of disease all contribute to an increased incidence of disease.

Bovine pneumonic pasteurellosis is also associated with stress factors. *P. haemolytica* A1 is present in small numbers in the nasopharynx of healthy cattle, but numbers increase following stress (Frank and Smith 1983; Hamdy and Trapp 1967). Normal, non-stressed calves are unlikely to develop disease following exposure to *P. haemolytica*. However, extreme environmental conditions, crowding, shipment, viral infection and food and water deprivation increase the chance of infection following exposure (Frank 1989). Current management and marketing practices of mixing, transporting, and crowding of calves are stressors that predispose to pneumonia (Frank 1986). Vaccines are available that are efficacious under controlled conditions. However, with many current management practices, the timing of vaccination is often inappropriate for the stimulation of adequate immunity prior to periods of stress and increased exposure.

The prevalence of ovine pneumonic pasteurellosis varies from year to year, and outbreaks can occur without any apparent stimulus. In most cases, however, environmental or management-associated stress predisposes to disease (Gilmour 1980). One factor commonly associated with outbreaks of pasteurellosis is concurrent infection with parainfluenza-3 virus or other viruses (Davies et al. 1980; Leamaster et al. 1987).

Host Immunity

Haemorrhagic septicaemia

Natural immunity to HS occurs in approximately 10% of buffalo and cattle (Bain 1954). This immunity is due to protective antibodies that develop following non-fatal exposure, and can persist for more than 1 year in some animals (Carter and De Alwis 1989). Animals with high antibody levels, however, act as a source of infection for other animals, since they continue to carry and intermittently shed organisms. Disease in young animals (less than 6 months old) is uncommon, with most fatalities occurring in animals 6–24 months old (Carter and De Alwis 1989). Although natural immunity is stronger and more persistent than vaccine-induced immunity (De Alwis 1982), vaccination is the most practical and reliable method to stimulate host immunity.

Vaccination

Vaccination is a relatively effective method for disease control. However, there is considerable variation in the quality, efficacy, and composition of vaccine preparations (Johnson et al. 1989). Killed, whole organism preparations that have been used include alum-precipitated bacterins, aluminium hydroxide gel bacterins, broth bacterins, and an oil adjuvant bacterin. Live vaccines have also been utilised with variable success (Myint and Carter 1989). Although cross protection and immunologic similarities have been demonstrated between a number of HS-causing strains, freshly isolated or recently passaged local strains are generally used for vaccine production (Carter and De Alwis 1989).

The oil adjuvant bacterin has proven reasonably efficacious (Bain et al. 1982). The vaccine provides 6–9 months of immunity following initial vaccination in young animals, and can protect for approximately 12 months after revaccination (Bain et al. 1982; Myint and Carter 1989). The vaccine is viscous and difficult to administer, undergoes rapid

deterioration at room temperature, has a relatively short storage life, and sometimes causes adverse local reactions (Bain et al. 1982). Attempts to reduce the viscosity of the vaccine usually result in decreased immunity compared to that provided by the conventional oil adjuvant vaccine (Yadev and Ahooja 1983). Recently, however, two oil adjuvant vaccines have been developed that have low viscosity and have stimulated high antibody titres for up to 230 days (Muneer and Afzal 1989).

Antigenic characteristics of oil adjuvant vaccines have been partially examined. Serum from some cattle vaccinated with the Katha strain or local strains of *P. multocida* in oil adjuvant had elevated enzyme-linked immunosorbent assay values to a heat-stable lipopolysaccharide (LPS) antigen (Johnson et al. 1989). However, a substantial number of cattle had little to no antibody, suggesting that the frequency of revaccination was inadequate or that a large proportion of animals were unvaccinated. Serum from animals receiving local strains had fainter staining of LPS bands and relatively more-intense staining of protein bands between 27 and 44 kDa on immunoblots, compared to Katha-vaccinated cattle. Many of the protein bands were shared between Katha- and local strain-vaccinated cattle.

Non-oil adjuvant bacterins are widely used in some endemic regions (Bain et al. 1982). Non-adjuvanted broth bacterins provide only 1.5–2 months immunity and can be associated with shock due to the endotoxin in the bacterin (Carter and De Alwis 1989). In contrast, alum-precipitated and aluminium hydroxide gel bacterins provide protection for up to 4–6 months (Bain et al. 1982). Although vaccine efficacy can vary with production and administration methods, most potency tests demonstrate a protective advantage for use of the vaccines. Efficacy was demonstrated for an alum-precipitated bacterin that provided protection following challenge (Giridhar et al. 1990). In this study complement fixation titres against killed whole-cell bacteria, but not titres against a saline capsular extract, were closely correlated with resistance. Use of an alum-precipitated vaccine in conjunction with levamisole stimulated significantly increased antibody titres in levamisole-treated, compared to untreated, groups (Sharma et al. 1990). The protective capacity and duration of these increased antibody levels were not explored.

A variety of live vaccines for HS have been examined. Streptomycin-dependent mutants of virulent serotypes have been developed and used to immunise cattle and buffalo (Wei and Carter 1978). Although the vaccines were protective, they were not considered to be practical for use in the field. A live isolate of *P. multocida* serotype B:3,4 obtained from a fallow deer (Jones and Huassaini 1982) protected calves against challenge with serotype B:2 9 months after vaccination (Myint et al. 1987).

A lyophilised vaccine derived from the same strain protected three of five subcutaneously vaccinated cattle and three of four intradermally vaccinated cattle against challenge with *P. multocida* serotype B:2 12 months later (Myint and Carter 1989). All vaccinated buffalo survived challenge at 8 or 13 months, whereas all controls died following challenge. The vaccine dose was not pathogenic for buffalo older than 5 months, but three of six 3–5 month old buffalo died following inoculation with a single dose. In a subsequent field trial of the vaccine, 1415 cattle and 303 buffalo were vaccinated subcutaneously (Myint and Carter 1990). No disease outbreaks occurred in the vaccinated animals the following year, but three buffalo died soon after vaccination. Eight months after vaccination, 14 of 19 calves survived experimental challenge with serotype B:2, whereas 2 of 2 unvaccinated controls died.

Since the subcutaneous route of administration in young buffalo resulted in some deaths (Myint and Carter 1989, 1990), the effect of the route of vaccination was evaluated by vaccinating 6745 cattle and buffalo intranasally and 8231 subcutaneously (Carter et al. 1991). Although the intranasal route of vaccination was not protective against standard subcutaneous experimental challenge techniques, substantial reductions in deaths due to HS were reported in the regions where the vaccine was used. Additionally, 15 randomly selected cattle survived experimental challenge 13 months following subcutaneous vaccination, whereas 2 unvaccinated controls died.

Important antigens

Antigens of *P. multocida* responsible for stimulating resistance to HS are not well defined. The capsule is the most likely source of important antigens, and is composed of polysaccharide, LPS, and a variety of proteins (Rimler and Rhoades 1989). Three antigen complexes — alpha, beta and gamma — have been described for *P. multocida* B:2 (Prince and Smith 1966a,b). These complexes corresponded to a polysaccharide–protein complex, a type-specific capsular polysaccharide, and LPS respectively. Since the antigens stimulated antibody responses in naturally and experimentally infected cattle and buffalo (Prince 1969a,b), they were considered to be important immunogens. Subsequently, a variety of studies have attempted to define more adequately the protective roles of *P. multocida* antigens.

Capsular polysaccharides have been extracted by a variety of methods (Mukkur 1977; Tadayon and Lauerman 1981; Giridhar et al. 1990). Some of these

capsular extracts stimulated antibodies associated with resistance to disease. Vaccination with type-specific capsular polysaccharide in oil adjuvant was protective for cattle, but not for rabbits (Penn and Nagy 1974, 1976). The purified capsular antigens of types B and E stimulated antibodies in cattle that were protective against homologous challenge (Nagy and Penn 1974, 1976), but a purified polysaccharide did not protect mice, rabbits, or cattle following challenge (Bain et al. 1982). Some of these inconsistencies in protective ability may be related to the degree of purification of the extracts. The stimulation of antibodies to highly purified capsular material is difficult (Rimler and Rhoades 1989), possibly due to the presence of large amounts of relatively non-immunogenic polysaccharide and the absence of highly immunogenic proteins. In less-purified extracts, proteins or other substances may be present which stimulate antibody production more effectively.

Endotoxin accounts for much of the toxicity associated with HS (Rebers et al. 1967). Due to the important role of LPS in the pathogenesis of disease (Rhoades et al. 1967), inhibition of its activity by antibodies or other substances could play an important protective function. Isolated LPS is not highly protective: non-adjuvanted LPS did not provide significant protection to mice or cattle following homologous challenge (Bain 1963; Schmerr and Rebers 1979; Erler et al. 1983). When combined with carrier substances, antibody production to LPS was enhanced (Rimler et al. 1984; Rimler and Phillips 1986). If immunogens within LPS can be specifically identified, these could possibly provide the basis for an effective vaccine, since serologic similarities exist between the LPS of type E and B strains (Baxi et al. 1970).

In one study, active cross protection occurred between in vivo grown types B:2 and E:2 (Bain et al. 1982). This protection was abolished by heat or proteolytic enzymes, suggesting that the protective substance was a protein antigen. A variety of protein and LPS antigens were recognised on immunoblots using serum from immunised animals (Johnson et al. 1989). Although the serum was protective for mice, the precise antigens responsible for protection were not determined. Additional studies are necessary to characterise more adequately the antigens of LPS that may play a role in immunity to disease.

Protein antigens are generally considered to be important immunogens. Immunisation of buffalo with proteins from a capsular extract of *P. multocida* in oil adjuvant provided protection for 4 months - 2 years (Dhanda 1959; Bain et al. 1982). Similar or identical proteins were later described that stimulated a strong antibody response in mice and buffalo (Bain 1963). Peptidoglycan has been isolated (Feist et al. 1981) and used without success as a vaccine in calves (Erler et al. 1983). Although exotoxins are important virulence factors and immunogens for some types of pasteurella infections, similar toxins have not been associated with HS strains (Rimler and Rhoades 1989).

Due to the greater immunity provided by natural exposure to live organisms compared to commonly used vaccines, an appropriate avirulent live vaccine could prove highly effective in reducing disease. Bacterins or extract vaccines that contain high concentrations of appropriate capsular polysaccharides and proteins may also prove more effective than current vaccines. The identification of common protective antigens between strains and serotypes could provide the opportunity to develop a broadly effective vaccine. Investigation of other routes of administration, such as oral vaccines, could provide a vaccine with greater user acceptance. However, field conditions in which a vaccine will be used must be considered for all future vaccine development studies.

Bovine pneumonic pasteurellosis

Natural infection with *P. haemolytica* results in protection against subsequent infection; however, the duration of the immunity is not known. Cattle naturally exposed to *P. haemolytica* had high antibody titres that were correlated with resistance to challenge (Confer et al. 1984). High levels of leukotoxin-neutralising activity were present in shipped cattle that remained healthy compared to cattle that died of pneumonia (Shewen and Wilkie 1983). Natural infection generally produces immunity of higher quality and longer duration than that acquired by vaccination. This enhanced immunity is most likely due to either the difference in the route of exposure between natural infection and most vaccines, or to the production of certain antigens in vivo that are not produced during in vitro preparation of vaccines.

Vaccination

Vaccine development for bovine pneumonic pasteurellosis has been of considerable interest for many years. Relatively reproducible experimental challenge techniques have aided in the evaluation of vaccines (Jericho et al. 1976; Panciera and Corstvet 1984). Using these and other techniques, viral vaccines, bacterins, live bacterial vaccines and subunit vaccines have been evaluated for their ability to prevent disease.

The efficacy of *P. haemolytica* bacterins varies considerably, but historically they have been considered not to provide consistent protection (Mosier et al. 1989b). In fact, some bacterins seemed to enhance the severity of disease (Friend et al. 1977; Wilkie et al. 1980). A *P. haemolytica* bacterin given by subcutaneous and aerosol routes resulted in more severe lesions than those in controls following challenge (Friend et al. 1977). Similar adverse effects were obtained with a subcutaneous formalin-killed *P. haemolytica* vaccine (Wilkie et al. 1980).

The use of combined *P. haemolytica* and *P. multocida* bacterins has been variably successful. There was substantial reduction in respiratory disease following the use of a bivalent bacterin as well as monovalent *P. haemolytica* or *P. multocida* bacterins (Palotay et al. 1963). In contrast, use of a commercial bivalent bacterin did not reduce respiratory disease significantly compared to that in unvaccinated animals (Martin et al. 1984).

The type of adjuvant has some effect on the efficacy of bacterins. Cattle vaccinated with *P. haemolytica* bacterins in Freund's complete or incomplete adjuvant, but not aluminium hydroxide, were protected against experimental challenge (Confer et al. 1987). In similar studies, *P. haemolytica* and *P. multocida* in aluminium hydroxide and *P. multocida* in oil adjuvant were not effective in reducing respiratory disease (Cardella et al. 1987). However, a positive benefit was obtained with *P. haemolytica* in oil adjuvant. Bivalent and monovalent *P. haemolytica* bacterins in aluminium hydroxide adjuvant did not reduce lesions following challenge (Confer et al. 1985b). Recently, use of a formalin-inactivated, sonicated, adjuvant-free *P. haemolytica* bacterin resulted in significantly less pneumonia following challenge (Jericho et al. 1990). Based on these results, it was suggested that additional studies on bacterins may be warranted.

Subcutaneous or aerosol vaccination with live *P. haemolytica* or *P. multocida* caused increased resistance to experimental disease (Corstvet et al. 1978). Studies with live *P. haemolytica* or *P. multocida* given subcutaneously, intradermally, or by aerosol demonstrated reduced lung lesions and clinical signs compared to controls and bacterin-vaccinated calves (Panciera et al. 1984; Confer et al. 1985b). Field trials of a live lyophilised intradermal vaccine produced variable results (Henry 1984; Smith et al. 1985; Purdy et al. 1986). Vaccination prior to shipment resulted in reductions in the number of sick calves and fatalities compared with unvaccinated controls (Henry 1984). An economic advantage for vaccination was determined, based on a lower incidence of respiratory disease, few relapses, few treatment days, decreased mortality and increased weight gains (Smith et al. 1985). However, another study of the same vaccine demonstrated no significant effect on performance, morbidity and mortality of feeder calves (Purdy et al. 1986).

Live vaccines have been modified in attempts to enhance their protective effects. Vaccination with chemically altered strains of *P. haemolytica* and *P. multocida* passaged in media containing acriflavin hydrochloride yielded favourable results (Kucera et al. 1981, 1983). Cattle vaccinated with streptomycin-dependent mutants of *P. haemolytica* and *P. multocida* had significantly less-severe lesions following challenge than did the controls (Catt et al. 1985; Blanchard-Channell et al. 1987; Chengappa et al. 1989). In field trials, the streptomycin-dependent vaccine provided better protection than a commercial *P. haemolytica* - *P. multocida* bacterin (Kadel et al. 1985). Lyophilised *P. haemolytica* significantly enhanced resistance to challenge (Confer et al. 1986). Vaccination caused a significant increase in antibody titres to somatic antigens, as well as to *P. haemolytica*-associated leukotoxin and a capsular carbohydrate-protein antigen extracted from *P. haemolytica* (Lessley et al. 1985).

Important antigens

P. haemolytica possesses a variety of virulence components that are potentially important immunogens (Confer et al. 1990). These include leukotoxin (LKT) (Markham and Wilkie 1980; Shewen and Wilkie 1982, 1985), LPS (Rimsay et al. 1981), capsular polysaccharides (CP) (Adlam et al. 1984), outer membrane proteins (OMP) (Squire et al. 1984) and fimbrae (Morck et al. 1987; Potter et al. 1988). Specific antibodies to these factors may protect the lung from damage by inhibiting bacterial attachment, by enhancing phagocytosis and pulmonary clearance of bacteria, or by neutralising toxic bacterial products (Confer et al. 1988).

Antibodies to CP or OMP antigens may be important for resistance to pneumonic pasteurellosis. Immunisation with crude capsular extracts provided some protection against experimental disease (Confer et al. 1987, 1988). A saline-extracted *P. haemolytica* antigen provided some protection when injected subcutaneously, but not when given by aerosol (Matsumoto et al. 1984). A saline extract-derived carbohydrate-protein antigen enhanced resistance to challenge when used as a vaccine (Confer et al. 1989). Serum from resistant, but not susceptible, animals had high antibody levels to a variety of specific antigens identified within this extract (Mosier et al. 1989a). Potassium thiocyanate extracts of *P. haemolytica* and *P. multocida* have been used as antigens in several trials (Mukkur 1978; Yates et al. 1983). A potassium thiocyanate extract of *P.*

haemolytica partially reduced mortality and pulmonary lesions compared to controls (Yates et al. 1983). In contrast, 7 of 10 cattle vaccinated with another potassium thiocyanate extract died following challenge, with evidence of severe pneumonia (Cho et al. 1984).

Calves vaccinated with a sodium salicylate extract of *P. haemolytica* in oil adjuvant were protected against challenge (Gilmour et al. 1982). However, in a series of experiments using a similar vaccine, vaccination failed to provide protection consistently (Gilmour et al. 1987). In fact, it was suggested that the sodium salicylate vaccine may have enhanced the severity of experimental disease in some calves.

High antibody levels to partially purified CP or OMP in some of the extracts have been associated with reduction in disease severity following challenge (Yates et al. 1983; Confer et al. 1987, 1989). In some animals this protection was attributed to antibodies to CP. However, antibodies to protein components were also considered to be important for enhanced resistance (Confer et al. 1989). This protection may have been independent of the concurrent presence of antibodies to LKT, since no leukotoxic activity was present in the capsular preparation (Confer et al. 1985). Subsequently, a 30-kDa *P. haemolytica* OMP has been described that stimulates antibodies highly correlated with disease resistance (Craven et al. 1991). In contrast, OMP of *P. multocida* type A did not correlate with protection in mice (Abdullahi et al. 1990).

Antibodies to LKT neutralise toxicity to bovine leukocytes in vitro (Cho et al. 1984; Gentry et al. 1985). Additionally, serum antibodies to LKT have been associated with enhanced resistance to pneumonic pasteurellosis. Cattle exposed to live *P. haemolytica* in vaccines or naturally often develop high serum antibody levels to LKT and have reduced disease severity following challenge (Cho et al. 1984; Gentry et al. 1985; Mosier et al. 1986). In contrast, cattle dying of pneumonic pasteurellosis often have low antibody levels to LKT (Shewen and Wilkie 1983). A variety of protective antigens derived from LKT preparations have been described (Mosier et al. 1989b). However, there is some question about the precise role of antibodies to LKT, since animals with high LKT titres also often have antibodies to other *P. haemolytica* antigens, most notably those of capsular or OMP origin (Yates et al. 1983; Confer et al. 1989).

Vaccination with cell-free, LKT-containing supernatants of *P. haemolytica,* but not recombinant LKT produced in *Escherichia coli*, has been associated with reduced clinical disease and lesion severity following challenge (Shewen et al. 1988; Conlon et al. 1991). In other experiments, supernatants of *P. haemolytica* serotype A1 provided some protection compared to controls and cattle receiving bacterins (Shewen and Wilkie 1988). Subsequent vaccination with supernatants of non-pathogenic *P. haemolytica* A11 resulted in LKT-neutralising titres similar to those produced by vaccination with serotype A1. However, serotype A1 challenge produced more severe lesions in the serotype A11-vaccinated calves than in serotype A1-vaccinated calves. These results suggested that serotype-specific antibody to antigens other than LKT may also be necessary for adequate protection.

Few studies have addressed the effect that antibodies to LPS have on disease resistance. In a retrospective study, no relationship between antibodies to *P. haemolytica* A1 LPS and resistance was demonstrated (Confer et al. 1986a). Other virulence factors that could possibly stimulate protective antibodies include neuraminidase (Frank and Tabatabai 1981), haemolysin (Fraser 1962), and a protease (Otulakowski et al. 1983). Functional properties of these factors have been examined, but their immunogenic properties have not been investigated adequately.

Collectively, these studies of *P. haemolytica* antigens demonstrate that a variety of antigens stimulate antibodies associated with resistance to bovine pneumonic pasteurellosis. Effective vaccines will most likely include combinations of large concentrations of some of these antigens, particularly CP, OMP, and possibly LKT, antigens. The presence of antigens produced in vivo, but not in vitro, has prompted efforts to characterise more adequately the protective capability of in vivo-derived antigens. Although whole bacterins and live vaccines continue to stimulate interest, most future efforts appear to be directed towards development of subunit or recombinant vaccines.

Ovine pneumonic pasteurellosis

Many sheep carry *P. haemolytica* in their nasopharynx, but are not clinically affected with pneumonic pasteurellosis. Colostral immunity in lambs appears to be fairly transient, lasting for approximately 4–5 weeks (Gilmour and Gilmour 1989). However, the duration of immunity following natural infection has not been investigated adequately. Acquired immunity to parainfluenza-3 virus may also enhance resistance, since the use of parainfluenza-3 vaccine has resulted in fewer deaths due to pasteurellosis (Davies et al. 1983; Salsbury 1984; Rodger 1989).

Vaccination

The multiple serotypes of *P. haemolytica* capable of causing pneumonia in sheep complicate the task of developing an effective vaccine. Vaccine development has also been hindered by difficulties with consistent experimental reproduction of the disease (Gilmour 1980; Chandrasekaran et al. 1991). The use of stress, immunosuppression, or intratracheal parainfluenza-3 virus followed by aerosolisation with *P. haemolytica* in specific-pathogen-free (SPF) or conventional lambs are the methods most consistently used to produce experimental infection (Sharp et al. 1978; Chandrasekaran et al. 1991). Such techniques have enhanced opportunities to evaluate experimental vaccines.

Killed vaccines are widely used for disease prevention. Locally isolated *P. haemolytica* A7 and *P. multocida* types A and D in oil adjuvant significantly reduced the lung lesions in five vaccinates compared to four controls following experimental challenge with *P. haemolytica* A7 (Chandrasekaran et al. 1991). However, there was no significant difference between lung lesions in vaccinates and controls following challenge with *P. multocida,* or combinations of *P. multocida* and *P. haemolytica* A7. Furthermore, lung lesions in eight sheep that received a commercial broth vaccine were not significantly different from those in eight unvaccinated controls, following challenge with either *P. haemolytica* A7 alone or in combination with *P. multocida.* The vaccine was also ineffective in preventing natural disease caused by either *P. haemolytica* or *P. multocida* (Zamri Saad et al. 1989). Oil adjuvant vaccines caused local lesions at the site of injection, prompting the use of less-irritating aluminium hydroxide adjuvant vaccines (Gilmour and Gilmour 1989). However, some studies have suggested that non-oil adjuvants do not stimulate as strong an antibody response as do oil adjuvants (Wells et al. 1979a).

Vaccination of conventional lambs with a preparation containing *P. haemolytica* serotypes A1, 2, 6, 7 and 9, and T3, 4 and 10, provided protection against challenge with serotypes A1, 2, and 6 (Wells et al. 1984). Due to inconsistencies in disease reproduction, the experiment was repeated in SPF lambs. Lambs receiving the multivalent vaccine or a monovalent serotype A6 vaccine in Freund's incomplete adjuvant were protected against challenge with *P. haemolytica* A6. In fact, the protection provided by the multivalent vaccine was slightly better than that provided by the monovalent challenge strain.

These studies indicate that variable protection occurs depending on the serotype of bacteria used for vaccination compared to the challenge serotype. It is important to monitor the prevalence of different serotypes in areas of disease outbreaks so that the appropriate serotypes can be incorporated into vaccines (Fraser et al. 1982).

Important antigens

Ovine strains of *P. haemolytica* contain similar antigenic material to the bovine strains. Capsular polysaccharides of *P. haemolytica* stimulate serotype specificity and have been the major focus of the search for important immunogens. While a variety of extraction procedures have been utilised for vaccine studies, the precise antigens responsible for observed effects have not been determined.

Certain substances within sodium salicylate extracts (SSE) of *P. haemolytica* have stimulated resistance to disease. Passive administration of serum from lambs that had recovered from previous experimental infection, or that had been vaccinated with either serotype A2 SSE or serotype A2 SSE combined with heat-killed organisms, protected 94–100% of treated, compared to control, lambs (Jones et al. 1989). In protected lambs, antibodies to SSE correlated best with protection when compared to anti-LPS antibodies, bactericidal antibodies, LKT-neutralisation antibodies, and antibodies reactive in indirect haemagglutination assays. In SPF lambs protected with antiserum from naturally or experimentally exposed lambs, pre-challenge titres to SSE also correlated well with protection.

SSE of several different *P. haemolytica* serotypes have been used as experimental vaccines. Serum from lambs vaccinated against serotype A1 did not provide passive protection to SPF lambs following challenge (Wells et al. 1979b). In contrast, extracts of serotypes A1 and A6 in complete or incomplete Freund's adjuvant protected against homologous challenge, but not heterologous challenge (Gilmour et al. 1979). Similarly, serotype A1 in aluminium hydroxide gel protected against a homologous challenge (Gilmour et al. 1982). However, serotype A2 SSE provided less protection than did heat-killed serotype A2 to homologous challenge, and the combination of serotype A2 SSE with heat-killed bacteria provided only 40–50% protection (Gilmour et al. 1982).

Additional combinations of heat-killed serotype A2 and SSE of serotypes A1 and A6 in Freund's or aluminium hydroxide adjuvant protected against a combined serotypes A1–A2 challenge or challenge by serotypes A1 and A6 (Gilmour et al. 1979). However, the lesions induced in vaccinated animals by challenge with serotype A2 were not significantly different from those in unvaccinated controls. The

poor immunogenicity due to the absence of prominent antigens in serotype A2 most likely accounted for the less-effective protective response to serotype A2 SSE (Gilmour et al. 1979; Gilmour et al. 1983; Adlam 1989). This reduced immunogenicity may partially explain the greater incidence of disease due to serotype A2 compared to other serotypes.

Solid immunity to serotype A2 occurs in lambs that have recovered from previous experimental or natural infection or that have received antiserum from recovered animals (Donachie et al. 1986a,b). Lambs exposed to aerosols of serotype A2 had higher IgG and IgA titres and higher levels of *P. haemolytica*-specific opsonising antibody in lung washings compared to control lambs (Donachie et al. 1986a). Additionally, higher levels of bactericidal antibodies, which correlated with resistance, were also present (Sutherland 1988). These bactericidal antibodies were directed against components of LPS, suggesting that certain antigens within LPS, but not serotype A2 SSE, may be responsible for stimulating this protective response (Donachie et al. 1986a; Sutherland 1988).

Cross reactivity between some *P. haemolytica* serotypes has been described (Burrells et al. 1983; Donachie et al. 1983). The serum of mice and rabbits injected with SSE of several individual serotypes had considerable cross reactivity (Burrells et al. 1983). In SPF lambs infected with serotype A1 SSE there was cross reaction with SSE of serotype A11 (Donachie et al. 1983). Similarly, serum from sheep injected with SSE of *P. haemolytica* serotypes A1, A2, and A6 also cross reacted with serotype A11 (Donachie et al. 1983). Despite this cross reactivity, the collective studies on capsular extracts indicate that antigens from all of the common serotypes should be combined in order to develop a broadly effective vaccine.

Ovine isolates of *P. haemolytica* A1 produce a LKT similar to that described for bovine isolates (Sutherland et al. 1983; Sutherland and Redmond 1986). Leukotoxin has inhibitory and lethal effects on ovine leukocytes and is considered a potentially important factor in the pathogenesis of ovine pneumonic pasteurellosis (Sutherland et al. 1983). Vaccination of sheep with either crude LKT or LKT plus SSE stimulated strong protection against challenge (98% and 86% protection respectively) (Sutherland et al. 1989). However, vaccination with SSE alone provided only 47% protection. These results suggested that something in the crude LKT was primarily responsible for protection. Furthermore, protection was correlated with LKT-neutralising titres and LPS-induced bactericidal activity. It was concluded that LKT-neutralising, bactericidal, or opsonising antibodies were required for protection (Donachie et al. 1986a; Sutherland et al. 1989).

Although LPS probably plays an important role in the development of pneumonic pasteurellosis, potentially important LPS antigens have not been evaluated extensively. Indirect evidence for an important role in resistance was demonstrated by reduction in bactericidal antibodies in serum following absorption with LPS (Sutherland 1988).

A universally effective vaccine could possibly be developed if common protective antigens of important serotypes could be identified and combined in sufficient amounts to stimulate protective antibodies. Capsular polysaccharides and proteins appear to be the most promising source of these protective antigens. Further characterisation of antigens derived from in vivo bacterial growth may also provide opportunities to develop more effective vaccines. Additionally, methods to increase the immune response to components of serotype A2 would enhance the resistance to this important cause of disease.

Treatment

Treatment of pasteurellosis is accomplished mainly by antibiotic therapy and supportive care, and rarely by passive immunotherapy. Successful treatment of pasteurellosis depends on early detection and antibiotic therapy. Delay in the onset of treatment will result in substantially higher mortality rates and economic loss. The selection of antibiotics for use in production animals is affected by a variety of factors, such as their kinetics and spectrum of activity, cost, approval for use in production animals, and residue concerns. In most cases, antibiotics are used when illness is confirmed or suspected. However, prophylactic use in feed or water is practiced in some situations.

In many cases of HS, animals are found dead, reinforcing the need for close monitoring and treatment with antibiotics if fever occurs. Close monitoring is especially important, since treatment is generally effective only in those animals that receive therapy very early in the course of disease (Carter and De Alwis 1989). Successful treatment with intravenous sulphadimidine and intramuscular tetracycline, penicillin–streptomycin or chloramphenicol have been described (Carter and De Alwis 1989). Antibiotic resistant strains appear to be uncommon (Carter and De Alwis 1989), possibly due to high mortality rates in affected animals and low rates of over-use or inappropriate use of antibiotics.

Prophylactic use of antibiotics in feed or water has been attempted in recently transported cattle for the

prevention of pneumonic pasteurellosis. Some of these studies report decreased incidence of disease due to treatment (Schipper and Kelling 1974; Lofgreen 1983). In contrast, prophylactic tetracycline or ampicillin failed to clear *P. haemolytica* from the nasal cavity (Shoo 1989a), and increased mortality has been associated with prophylactic use of antibiotics in some cases (Martin 1985). These mixed results, and the occurrence of antibiotic resistance that is often associated with prophylactic antibiotic use, suggest that the benefits of this practice are questionable (Chang and Carter 1976).

When treating clinical cases of bovine pneumonic pasteurellosis, it was recommended that, if the animal did not respond within 24 hours of initiation of antibiotic therapy, the antibiotic be changed (Morter et al. 1986). In one study the response rates to either erythromycin or amoxicillin alone were unacceptable, but when these were used in conjunction the response rates were substantially better (Morter et al. 1986). Strains of *P. haemolytica* and *P. multocida* resistant to common antibiotics used in production animals have been reported (Chang and Carter 1976; Martin and Meek 1981; Wray and Morrison 1983). Additionally, the incidence rate of antibiotic resistance to many pasteurellae is increasing (Allan et al. 1985; Haghour et al. 1987). In one study, half of the *P. haemolytica* isolates were resistant to penicillin, streptomycin and tetracycline (Chang and Carter 1976). A relatively high incidence of multiple resistance to penicillin, ampicillin, tetracycline and sulphonamide has also been reported (Shoo 1990). In vitro antibiotic sensitivity testing is generally a beneficial practice; however, in vitro results are not always useful predictors of the success of treatment (Mechor et al. 1988).

Resistance to many antibiotics is plasmid mediated (Willson 1990). Plasmids associated with antibiotic resistance have been described for *P. multocida* isolated from an outbreak of bovine pneumonia (Silver et al. 1979). Three plasmids were described, which collectively resulted in resistance to tetracycline, streptomycin and sulphonamide. Antibiotic-resistant plasmids have also been reported for both type A and type T serotypes of *P. haemolytica* (Zimmerman and Hirsch 1980; Boyce and Morter 1986; Chang et al. 1987). Resistance to streptomycin and tetracycline (Zimmerman and Hirsch 1980), as well as to other antibiotics commonly used for the treatment of bovine pneumonia (Boyce and Morter 1986), were described. Bovine strains of both *P. haemolytica* and *P. multocida* have been found to produce beta-lactamase, an enzyme that inactivates penicillin and similar antibiotics (Philippon et al. 1986; Shoo 1989b). Sulbactam, a beta-lactamase inhibitor, combined with ampicillin results in greater efficacy against ampicillin-resistant strains (Farrington et al. 1987; Girard et al. 1987).

Ovine isolates of *P. haemolytica* biotype A are generally more sensitive to penicillin than are biotype T strains (Gilmour, 1980; Adlam, 1989). While antibiotic resistance occurs in ovine strains, most ovine isolates of *P. haemolytica* are sensitive to penicillin and ampicillin and almost all are sensitive to tetracycline (Gilmour and Gilmour 1989).

New generations of antibiotics are continually being developed and tested for the treatment of pneumonic pasteurellosis (Lekeux and Art 1988; Ose and Tonkinson 1988). While it is important that effective antibiotics are available, proper use of those that are currently approved is equally important. Regulations concerning antibiotic withdrawal times and tissue residue levels will most likely be more strictly enforced in the future. Responsible and proper antibiotic use is also necessary to reduce the occurrence of resistance in many pasteurella strains.

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Protection to Haemorrhagic Septicaemia Induced in Mice by Vaccination with Oil Adjuvant and Broth Bacterin Vaccines

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Abstract

Protection induced in mice by oil adjuvant and broth bacterin vaccines was assessed using live challenge infections. Protective immunity was induced by both procedures. Oil adjuvant vaccination induced a 10^5-fold increase in resistance to lethal challenge over control mice, while secondary vaccination induced a further 10-fold increase. Broth bacterin induced a slightly weaker protective response, with 10^4- and 10^5-fold increases in resistance to lethal challenge following primary and secondary vaccination respectively. There was a significant relationship between IgG antibody levels and resistance to challenge ($P = 0.026$). Protection lasted for at least 20 weeks after a primary oil adjuvant vaccination. There was also a strong and significant relationship between IgG antibody levels and the passive protection afforded by serum transfer in each experiment, and the overall correlation was highly significant ($P = 0.00001$). There appeared to be a relationship between protection and the antibody response to major protein bands with the apparent molecular mass M_r 94 000, 80 000, 67 000, 35 000 and 32 000, as well as to the bands in the region of the lipopolysaccharide components of *P. multocida* (approximately M_r 14–15 000). Whether protection resulted from recognition of specific antigens, or was a result of both antibody levels and antibody specificity, remains to be defined.

HAEMORRHAGIC septicaemia (HS) is caused by specific serotypes within the bacterial species *Pasteurella multocida* (Bain 1955; Carter 1961; Dawkins et al. 1990). Annual vaccination with an oil adjuvant vaccine (OAV) is widely practiced and is generally credited with reducing the prevalence and the incidence of HS. Despite this, significant outbreaks still occur in most endemic regions (Bain et al. 1982). Formalin-fixed, whole-cell bacterins are generally used during an outbreak to protect animals at risk, and have been thought to reduce further disease spread and stock losses. Unfortunately, little is known about the host response to vaccination procedures, and even less about the transmission of the disease, or why outbreaks continue to occur despite the intensive vaccination programs (Bain et al. 1982; Johnson et al. 1989).

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The protective immune response to HS vaccination has long been attributed to humoral responses, as protection can be transferred to naive animals with serum from vaccinated animals (Roberts 1947; Bain 1955). As immune cattle sera will also protect mice against a lethal challenge dose, mouse passive protection assays have been used widely in the assessment of the protective antibody levels of cattle and buffalo to HS (Bain 1955; Heddleston et al. 1967; Bain et al. 1982). However, the cumbersome and expensive nature of this qualitative assay prevents its widespread use as a routine test for serological evaluation of protective antibody levels in livestock.

Furthermore, outside of the passive protective effect of sera, very little is known about the kinetics of the antibody response following administration of OAV and bacterin, or even what constitutes a protective response. Even less is known about the specificity of the antibody response or the functional antibody classes involved in protection. Given the economic importance of this disease and the major vaccination campaigns carried out annually, the lack of basic information on protection and responsiveness to different vaccination regimes

presents a major hurdle in vaccine improvement, disease control and animal management. To address these deficiencies, the latest HS-working parties of the Food and Agriculture Organisation (FAO) of the United Nations and the Animal Production and Health Commission for Asia (Bangkok, Thailand, 1987 and 1990) have recommended that the mouse be used to assess new vaccines and vaccination regimes, and that ELISA technology be used to evaluate the immune responses in mice and cattle with a view to defining the protective response.

In this study, ELISA was used to measure the antibody responses of mice to vaccination with OAV and broth bacterin and the protective responses were determined by challenge, and by the ability of serum to provide passive protection to mice. The antibody responses to specific cellular components were also assessed using immunoblotting techniques.

Materials and Methods

Mice

Female BALB/c mice, 6–10 weeks old (Monash University Resources Centre, Clayton, Victoria), were used.

Bacteria

P. multocida strain M1404, supplied by Dr K.R. Rhoades (National Animal Disease Centre, Ames, Iowa), was maintained and grown as described previously (Ramdani et al. 1990). This HS-causing organism was isolated from bison and has been adopted as the type strain for Carter group B and Heddleston type 2 (Brogden and Packer 1979; K.R. Rhoades, personal communication, 1987). Mice were challenged by intraperitoneal injection with 0.1 mL of appropriately diluted broth from logarithmic growth-phase bacterial cultures, and the number of viable organisms injected was confirmed by plate-counting procedures (Ramdani et al. 1990).

Vaccination protocol

Bacteria were grown to a density of 10^9 organisms/mL in brain–heart infusion broth. The bacteria were washed three times in phosphate buffered saline (PBS) by centrifugation and resuspended in approximately 1/10 the original volume of formyl saline. The fixed bacteria were then emulsified in an equal volume of incomplete Freund's adjuvant (Difco Laboratories) and re-emulsified in an equal volume of a 0.85% sodium chloride solution containing 2% Tween 80. The bacterin consisted of formalin-fixed bacteria that were sonicated prior to injection. Mice were vaccinated by subcutaneous injection with 0.2 mL of either OAV or bacterin. Bacterial numbers in each vaccine dose were approximately 5×10^8. Secondary vaccination was given 4 weeks later, with the exception of the kinetics experiment, in which mice were given a primary vaccination only. Groups of at least five mice were bled from the retro-orbital plexus (Ramdani et al. 1990) at weekly intervals, and the sera were pooled from each time point. The same pooled sera were used in the ELISA, passive mouse protection tests and immunoblotting studies.

Experimental design

Diagrammatic representations of the experimental designs are shown below:

Challenge infection

Mice were challenged by intraperitoneal injection of 10^2 to 10^8 viable bacteria 4 weeks after primary and secondary vaccination. In the kinetics experiment, mice were challenged with 10^3 and 10^6 viable bacteria only, as 10^3 was a significant challenge for waning immunity and 10^6 was a discriminating dose for immune mice (Tables 1 and 2). Naive mice and mice injected with a PBS–oil adjuvant were used, and referred to as normal and control mice respectively.

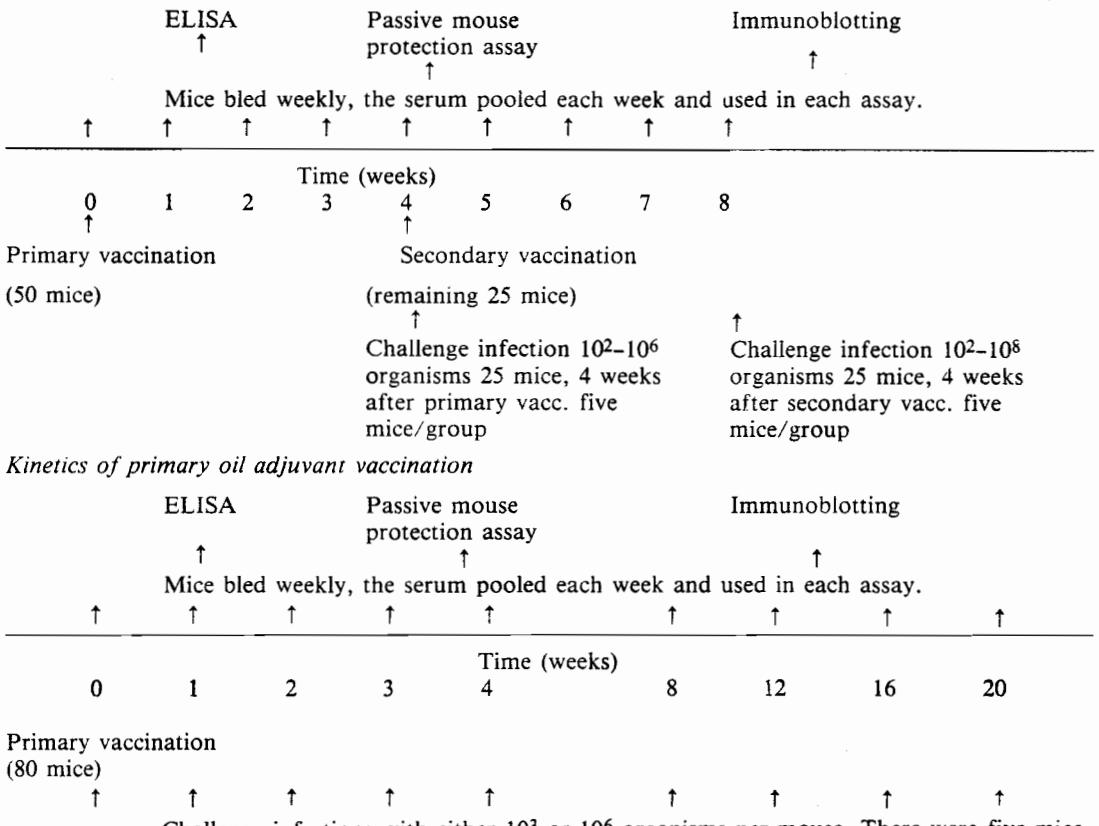
Enzyme-linked immunosorbent assay

The boiled antigen preparation and the ELISA technique have been described by Johnson et al. (1988). The procedures were the same, with the exception that 100 μL of reagents were used in each step, the coating antigen was extracted from *P. multocida* strain M1404, and the horseradish peroxidase-conjugated antibodies used were affinity-purified, goat anti-mouse IgG (whole molecule) and IgM (μ chain specific) from Sigma Chemical company (St Louis, Missouri, USA). Serial serum dilutions were performed on all samples and the results represented in all figures were from a 1/200 dilution.

Immunoblotting

Polyacrylamide gel electrophoresis was performed according to the detailed method of Johnson et al. (1988) and the dissociated bacterial proteins were then transferred to nitrocellulose (Johnson et al. 1989). In this study, the nitrocellulose strips were probed with mouse serum overnight and the conjugate used was sheep anti-mouse immunoglobulin (Silenus Laboratories, Hawthorn, Victoria, Australia) diluted 1/500. Protein bands were identified by Coomassie Blue stain and lipopolysaccharide (LPS) by silver staining (Tsai and Frasch 1982; Johnson et al. 1989, 1991). Molecular weight determinations were made from total protein stains of the nitrocellulose strips.

Oil adjuvant and bacterin vaccination experiments



Kinetics of primary oil adjuvant vaccination

Table 1. Protection induced in mice following primary and secondary oil adjuvant vaccination.

Treatment group	Challenge dose	Percent survival	Mean time to death (days)
Naive control	10^2	0	<1
Primary vaccination			
Adjuvant control	10^2	0	<1
Vaccinated	10^2	80	4*
	10^3	100	—
	10^4	100	—
	10^5	100	—
	10^6	60	2
Secondary vaccination			
Adjuvant control	10^2	0	<1
Vaccinated	10^2	100	—
	10^4	80	8*
	10^6	100	—
	10^7	80	<1
	10^8	0	1

* Individual animals, statistical analysis was not performed

Table 2. Protection induced in mice following primary and secondary bacterin vaccination.

Treatment group	Challenge dose	Percent survival	Mean time to death (days)
Primary vaccination			
Naive control	10^2	0	1
Bacterin	10^2	100	—
	10^3	60	2.5*
	10^4	60	5.0*
	10^5	80	2.0*
	10^6	0	4.6*
Secondary vaccination			
Naive control	10^2	0	<1
Bacterin	10^2	100	—
	10^4	100	—
	10^6	80	1.0
	10^7	40	2.0
	10^8	0	<1

* Small sample sizes required that the times to death be pooled for analysis. Increased survival times were significant ($P<0.05$, Mann-Whitney test)

Passive mouse protection test

Groups of five recipient mice were injected intraperitoneally with 0.2 mL of pooled sera collected from mice at specific time points throughout the vaccination experiments. Twenty-four hours later, these mice were challenged with 100 viable bacteria. The protection afforded by the passively transferred antisera was recorded as the percentage of mice surviving the challenge. The time to death of those that succumbed to challenge was also recorded. Mice were observed for 10 days.

Statistics

Analysis of survival data was performed using either the Mann–Whitney test or the Chi-square test, taking into account the continuity correction. Regression analysis was applied to compare immunoglobulin titre to passive protection and also protection to challenge. Significance estimates for correlations were from Fisher's Exact F-test. The analytical test applied is indicated after each probability value.

Results

Response to oil adjuvant vaccine

Protection against challenge

Mice were challenged with 10^2–10^6 viable organisms after primary vaccination, or with 10^2–10^8 viable organisms after secondary vaccination. The results are shown in Table 1. All normal and control mice died within 1 day. Primary vaccination induced 100% protection against 10^5 organisms, with 60% of mice surviving a challenge of 10^6 bacteria. Mice that succumbed to challenge with 10^6 organisms had a mean time to death of 2 days. Secondary vaccination produced a further increase in protection, with 100% survival following a challenge with 10^6, and 80% survival following a challenge with 10^7, viable organisms. No mice survived a challenge dose of 10^8 organisms.

Antibody response

The antibody responses to primary and secondary OAV vaccination are shown in Figure 1a. One week after vaccination the IgM response was at a peak. It decreased in subsequent weeks to background levels. Pooled normal mouse sera had ELISA optical densities of between 0.125 and 0.15 for both IgG- and IgM-class antibodies. The IgG response rose over the weeks to a peak and plateau at week 6 (2 weeks after secondary vaccination).

Passive mouse protection assay

Pooled sera from the vaccinated mice were injected into eight groups of five mice and the recipient mice were challenged (Figure 1a). The transferred sera taken from OAV-vaccinated mice prior to week 4 did not confer protection and, although the sera resulted in an increased mean time to death, it was not significant when compared with control mice and mice injected with normal mouse serum. However, by week 4 there was a solid 80% protection to challenge. This protection was significant ($P = 0.007$, Chi-square test) when compared with control mice. Sera collected in all subsequent weeks conferred 100% protection. There was a significant positive correlation between the IgG levels and passive mouse protection ($r = 0.93$: $P < 0.001$, F-test). There was no correlation between protection and IgM levels.

Immunoblotting

Figure 1b shows the specificity of the antibody response of OAV-vaccinated mice to the *P. multocida* antigens. Normal mouse sera recognised bacterial cell components of an apparent M_r 37 000, and showed weak recognition of a band at an estimated M_r 14 600, with the latter being in an intense LPS region. One week after vaccination, these bands had increased stain intensity, and antibodies were also recognising additional bands at apparent M_r 94 000, 80 000, 67 000, 50 000, 35 000 and 32 000, and an additional band in the LPS region at an apparent M_r 14 200.

The intensity of staining and the number of bands recognised continued to increase with time after vaccination. A total of at least 17 major antigenic bands in the range M_r 14 200–96 000 were recognised by week 8. Bands that developed strongly in the weeks when serum first conferred protection were at M_r 94 000, 80 000, 67 000, 50 000, 35 000 and 32 000. Another two bands in the LPS region, M_r 14 200–15 000, also became evident at the time protection was conferred by sera.

Response to bacterin

Protection against challenge

Mice were challenged with 10^2–10^6 viable bacteria after primary vaccination, or with 10^2–10^8 bacteria after secondary vaccination. The results are shown in Table 2. Primary vaccination produced 100% protection to challenge with 10^2 organisms, and 60–80% protection to challenge with 10^3–10^5 organisms. The overall protection was significant ($P = 0.011$, Chi-square test). Mice were not protected against a challenge of 10^6 viable bacteria. The mean time to death of the mice that succumbed to challenge was significantly greater than that of control mice ($P < 0.05$, Mann–Whitney test), even among those challenged with 10^6 organisms. Secondary vaccination induced an increased

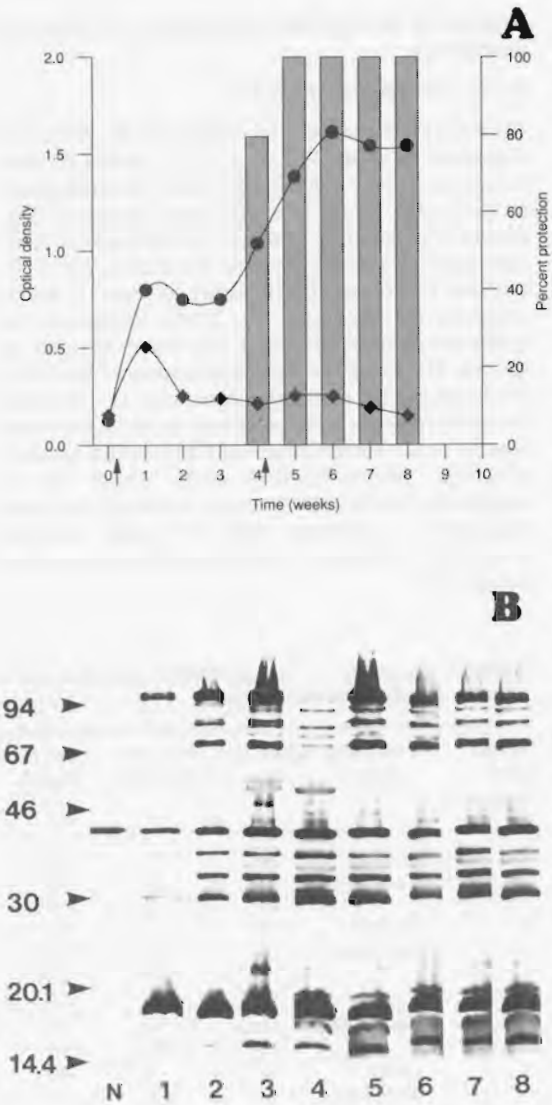


Fig. 1. Results from mice vaccinated with primary and secondary OAV. Panel A shows the IgG (● — ●) and IgM (◆ — ◆) antibody levels to *P. multocida* in mice. Animals were vaccinated at 0 and 4 weeks (arrowed). The bar graph represents the percent passive protection that was afforded to recipient mice using pooled sera from the respective weeks. Panel B shows the immunoblot results from the same batch of pooled sera as those used in the ELISA and the passive protection study. Normal sera (N) and sera from vaccinates at specific weeks (numbered) are shown. The molecular weight markers are shown as $M_r \times 10^{-3}$.

protection, with no mice dying in the low dose range. Mice challenged with 10^6 bacteria had 80% protection, while 40% protection was observed following a challenge with 10^7 bacteria. No mice survived a challenge with 10^8 organisms. Unlike the primary vaccination group, the mice that died did not have longer survival times when compared to control mice.

Antibody response

The IgG and IgM antibody responses to primary and secondary bacterin vaccination are shown in Figure 2a. The IgM response was weak and short-lived with a mild response at weeks 1 and 4. The later was prior to secondary vaccination. There was no evidence of an IgM response to secondary vaccination. The IgG response, on the other hand, rose steadily to week 4 and was increased by secondary vaccination with the peak response being observed at week 5. Thereafter, the IgG levels were seen to plateau.

Passive mouse protection test

Pooled sera from the bacterin-vaccinated mice were injected into eight groups of five mice, and the mice were challenged. The results are shown in Figure 2a. Sera obtained from donor mice 2 weeks after primary bacterin vaccination gave 40% protection against challenge. Sera taken at weeks 3 and 4 afforded recipient mice 80% and 100% protection to challenge. Sera from mice at each time point after secondary vaccination gave 80–100% protection to challenge. There was a significant correlation between passive protection and IgG levels over the weeks 0–4 ($r = 0.99$: $P = 0.0014$, F-test). There was no correlation between protection and IgM levels. The mean times to death of the mice that died in both the primary and secondary vaccination groups were not different from those of control mice.

Immunoblotting

Antibody responses of the bacterin-vaccinated mice to *P. multocida* antigens are shown in Figure 2b. Normal mouse sera again recognised the M_r 37 000 protein and the M_r 14 600 LPS bands. One week after primary vaccination additional protein bands were apparent: M_r 94 000, 36 000 and a band in the LPS region, estimated at M_r 14 200, were stained. By week 2, when the serum conferred 40% protection, antibodies were binding to protein bands at approximately M_r 80 000, 36 000 and 32 000, with increasing stain intensity and bands being evident in the LPS region, estimated M_r 14 200–15 000. As the protection afforded by serum increased to 80 and 100% at weeks 3 and 4, the stain intensity of the these bands, and the number of bands recognised, increased. Secondary vaccination produced an increased stain intensity of all bands, with at least 17 major antigenic components being recognised.

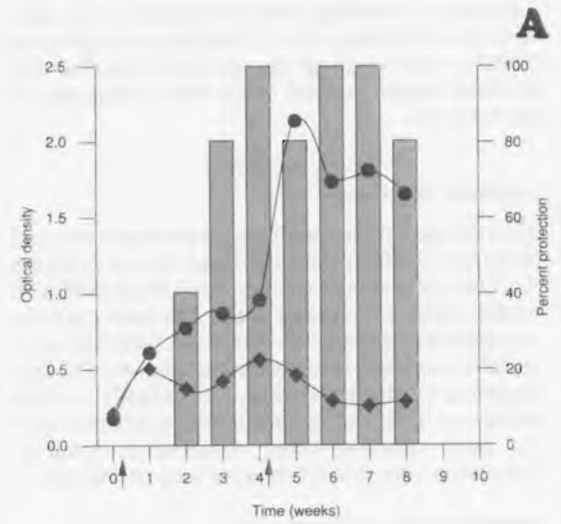


Fig. 2. Results from mice vaccinated with primary and secondary broth bacterin. Panel A shows the IgG (●—●) and IgM (◆—◆) antibody levels to *P. multocida* in mice. Animals were vaccinated at 0 and 4 weeks (arrowed). The bar graph represents the percent passive protection that was afforded to recipient mice using pooled sera from the respective weeks. Panel B shows the immunoblot results from the same batch of pooled sera as those used in the ELISA and the passive protection study. Normal sera (N) and sera from vaccinates at specific weeks (numbered) are shown. The molecular weight markers are shown as $M_r \times 10^{-3}$.

Kinetics of the response to a primary oil adjuvant vaccination

Protection against challenge

Mice were challenged with either 10^3 or 10^6 viable organisms at weeks 1, 2, 3, 4, 8, 12, 16 and 20 after vaccination. Five control mice were also challenged at each time point with 10^2 viable bacteria. The results are shown in Table 3. The protection from vaccination was low in the first few weeks, with 20% survival following 10^3 challenge at week 1, but it progressively increased to 100% protection to challenge doses of 10^3 and 10^6 viable bacteria at week 4. Thereafter, protective responses of 80–100% were seen at each time point up to week 20. The mice that died at weeks 2, 4 and 16 had an increased mean time to death when compared with control animals ($P<0.05$, Mann–Whitney test). There was a significant correlation between antibody titre and resistance to challenge with 10^3 viable bacteria ($r=0.866$: $P=0.026$, F-test) over the course of the experiment.

Table 3. Longevity of the protection afforded by a primary oil adjuvant vaccination.

Weeks after treatment	Treatment groups	Challenge dose	Percent survival	Death (days)
1	Control	10^2	0	<1
	Vaccinates	10^3	20	3.5*
		10^6	0	3.8*
2	Control	10^2	0	1.0
	Vaccinates	10^3	40	3.0*
		10^6	40	2.5[a]
3	Control	10^2	0	1.0
	Vaccinates	10^3	80	1.0
		10^6	100	—
4	Control	10^2	0	<1
	Vaccinates	10^3	100	—
		10^6	100	—
8	Control	10^2	0	<1
	Vaccinates	10^3	100	—
		10^6	80	1.0
12	Control	10^2	0	<1
	Vaccinates	10^3	100	—
		10^6	80	1
16	Control	10^2	0	<1
	Vaccinates	10^3	100	—
		10^6	80	5.0[b]
20	Control	10^2	0	<1
	Vaccinates	10^3	100	—
		10^6	100	—

* Increased survival times were significant ($P<0.05$, Mann–Witney test)

[a] Not significantly different from control animals

[b] Individual animal

Antibody response

The antibody responses for the first 10 weeks after the primary vaccination are shown in Figure 3a. A weak short-lived IgM response was observed 1 week after vaccination. The IgG response rose to a peak and plateau at week 9. Similarly high IgG levels were seen throughout the 20 weeks of this experiment. Consequently, results after week 10 were not shown.

Passive mouse protection test

Sera obtained from mice at specific times after OAV vaccination were injected into 12 groups of five mice and the mice were challenged. The results are shown in Figure 3a. Recipient mice were not protected prior to week 3. By week 3, sera conferred 60% protection with 80% protection being transferred at week 4 ($P = 0.04$, Chi-square test). Thereafter, the protection afforded by the serum was high, with 80–100% protection for the remainder of the 12 weeks. The protection to challenge, while fluctuating between 80–100%, remained significant ($P = 0.002$, Chi-square test). The mean time to death of the vaccinated mice was not significantly different from the control mice, except at weeks 1 and 2 ($P < 0.05$, Mann–Whitney test). There was a significant correlation between IgG levels and protection ($r = 0.807$: $P = 0.005$, F-test), but there was no correlation between IgM levels and protection.

Immunoblotting

The specificity of the antibody responses of the mice to *P. multocida* antigens is shown in Figure 3b. Only weeks 1–4 and weeks 8, 12 and 16 are shown. Pooled normal mouse sera from these mice induced the same bands as seen previously, and primary vaccination also induced bands similar to those seen at the same time in the earlier experiments (Figures 1b and 2b). The bands that appeared at the time serum conferred protection were estimated at M_r 80 000, 67 000, 36 000 and 32 000, and in the LPS region at M_r 14 200–15 000. The number of bands recognised increased in intensity to a peak and plateau at week 5. Thereafter, the staining levels remained the same, with at least 17 major antigenic bands being recognised.

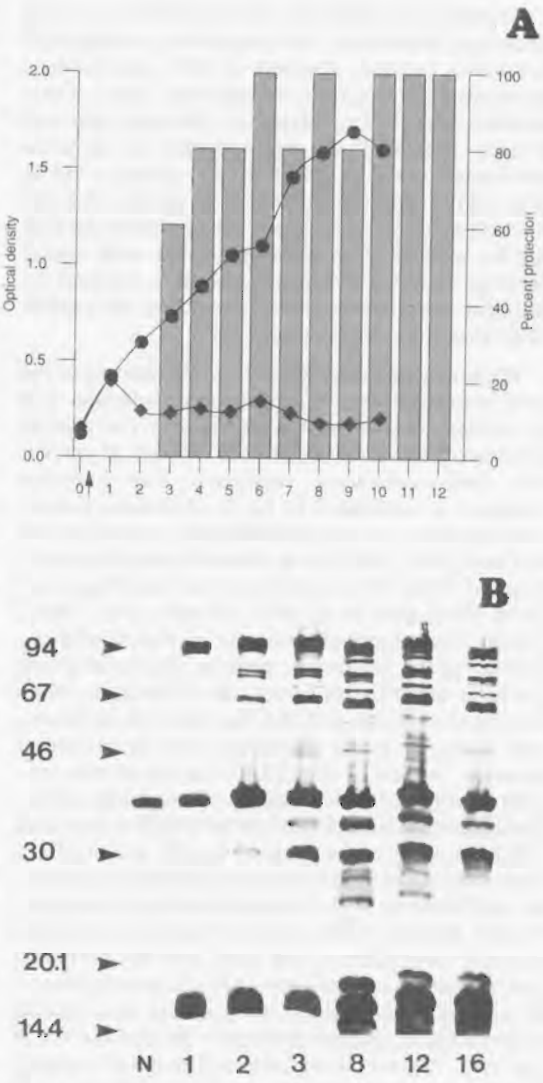


Fig. 3. Results from mice vaccinated with primary OAV. Panel A shows the IgG (●—●) and IgM (◆—◆) antibody levels to *P. multocida* in mice. Animals were vaccinated at week 0 (arrowed). The bar graph represents the percent passive protection that was afforded to recipient mice using pooled sera from the respective weeks. Panel B shows the immunoblot results from the same batch of pooled sera as those used in the ELISA and the passive protection study. Normal sera (N) and sera from vaccinates at specific weeks (numbered) are shown. The molecular weight markers are shown as $M_r \times 10^{-3}$.

Discussion

Protection induced by OAV and broth bacterin, the two commonly used styles of HS vaccines in Southeast Asia and India, were assessed by challenge and passive mouse protection tests, with reference being made to the serum antibody levels and the specificity of the antibodies to *P. multocida* antigens. Primary OAV induced a marked increase in protection to challenge, with 80% of mice being resistant to challenge with 10^6 viable bacteria. This conservatively represents a 10^5-fold increase in resistance, as the estimated lethal dose for mice is less than 10 organisms (Ramdani et al. 1990).

Secondary vaccination induced a further ten-fold increase in protection. By comparison, primary and secondary bacterin resulted in 10^4- and 10^5-fold increases in protection respectively. Thus, OAV induced a level of resistance to challenge that was 10-fold greater than was induced in bacterin-vaccinated mice. In addition, the primary OAV-induced resistance lasted at least 20 weeks, while the protection afforded by bacterin appeared to be falling by week 4. This is in agreement with earlier findings that the protective response induced by bacterin was greatest at week 3 and then fell rapidly after that time (Sinha et al. 1957).

While challenge represents the only real test of the level of protection an animal has to an infection, this is neither economical nor feasible in the routine assessment of vaccines or of the efficacy of current HS field-vaccination regimens. The humoral response is considered to be the dominant protective response, so the passive mouse protection test has been used widely as a relatively reliable assessment of immunity in cattle and buffalo (Nagy and Penn 1976; Bain et al 1982; Sawada et al. 1985). Nevertheless, there are inherent problems with the routine use of this assay: namely, the requirement for large quantities of serum; the difficulty in interpreting the results; and the fact that it is an expensive assay in terms of labour and experimental animals. In view of this, ELISA serology may provide a more convenient means of assessing protective immunity. ELISA systems are sensitive tests, and offer flexibility in the range of specific antigen fractions that can be used to evaluate antibody responses. In conjunction with immunoblotting techniques, ELISA permits the spectrum of bacterial cell components recognised by the host, and the contribution of the major antibody classes in protection, to be defined. Consequently, these assays were used to evaluate the serological response of mice to OAV and bacterin. The sera were then used in passive mouse protection tests to enable correlations to be made between the protective response to vaccination and the antigen components recognised.

The principal response to the OAV and bacterin vaccination was the IgG response, with only mild and transient IgM responses. The kinetics experiment demonstrated that there was a strong and highly significant correlation between the anti-*P. multocida* IgG response and protection against challenge with viable bacteria ($r = 0.807$: $P = 0.005$, F-test). There was also a direct and highly significant relationship between IgG antibody levels to *P. multocida* and the ability of serum to transfer protection (pooled over all experiments, $r = 0.75$: $P = 0.00001$, F-test), thus affirming the major protective role of serum and, in particular, the IgG antibody class.

Serum transfer demonstrated that the protection induced by bacterin was partially protective 2 weeks prior to that seen in mice vaccinated with OAV. Complete protection (100%) was transferred by serum from bacterin vaccinates 1 week prior to a similar level of protection being transferred with sera from OAV-vaccinated animals. The prophylactic use of broth bacterins to induce a rapid immune response in the face of an HS outbreak has been based largely on anecdotal evidence (Bain 1963). The observations in these studies that the broth bacterins induced rapid, but relatively short-lived, protection has confirmed the field observations and earlier mouse studies (Sinha et al. 1957). Despite the rapid development of protective antibodies following bacterin vaccination, the passive protection is not as marked as that transferred by serum from OAV-vaccinated mice. Serum transferred from mice following primary OAV was able to protect recipients for at least 20 weeks.

Immunoblotting demonstrated a relationship between antibody recognition of some bacterial cell components and protection — in particular the protein bands at approximately M_r 94 000, 80 000, 67 000, 36 000 and 32 000. Bands in the LPS region, estimated to be M_r 14 200–15 000, were also observed to stain intensely at the time sera conferred resistance. These observations suggest that antibody to these particular antigens may confer protection. It is interesting to note that the 32 000 band represents a 'type-specific' band for Asian HS isolates (Johnson et al. 1991) and as such may prove to be a candidate antigen for a subunit HS vaccine. Immunisation studies using these antigen components are needed to verify their role in host protection to HS.

The antigen specificity of the murine responses to OAV and bacterin complements the observations of Johnson et al. (1989), in which it was seen that cattle and buffalo sera respond to the same antigens and major protein bands. It remains, however, to more rigorously compare the findings in this study with the responses of cattle and buffalo by conducting a similar experimental vaccination study. It also remains to define which IgG subclasses, and, if any, other immunoglobulin classes, are functional in the protective response. It may prove that one or more of the antigens recognised in the immunoblotting may be used to improve the ELISA systems currently used for HS serology (Johnson et al. 1989, 1991) and may be used as the basis to improve the bacterial identification ELISA (Dawkins et al. 1990).

Quantitation of the protection induced in mice by OAV and bacterin has permitted the critical comparison and analysis of the commonly used styles of HS vaccines. It has also clearly identified the need

for alterations to be made to OAV HS vaccines that will reduce the time it takes the OAV to induce a protective response from the 3–4 weeks seen in this study down to 1–2 weeks. An improvement in the response time would seem feasible, given the rapid, albeit weaker than the OAV, response induced with bacterin. Improved adjuvant procedures and methodology need to be evaluated with the criterion of improving the response time without compromising the level of immunity. There is also a need for extending the longevity of the protective response in order to eliminate the need for annual vaccination, although currently there is no evidence to suggest that long-term immunity can be generated. Mice provide a convenient tool for the analysis of the humoral and the total host response to HS (Ramdani et al. 1990) and may serve a very valuable purpose in improving the HS vaccines and the vaccination regimens. However, great caution should be exercised in extrapolating the responses observed in mice, and even cattle under laboratory conditions, to those of cattle and buffalo under field conditions.

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Comparative Protective Potential of Non-Living Intact Cells and Purified Outer Membrane and Associated Proteins of *Pasteurella multocida* Type 6:B Grown Under Iron-Regulated Conditions

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Abstract

Pasteurella multocida type 6:B grown under iron-restricted conditions was found to be superior in imparting protection against experimental pasteurellosis in mice than *P. multocida* type 6:B grown under iron-replete conditions. Outer membrane and associated proteins (OMAP) prepared from *P. multocida* grown under iron-restricted conditions (DIP-OMAP) were also superior immunogens to those prepared from *P. multocida* grown under iron-replete conditions ($FeCl_3$-OMAP). SDS-PAGE of DIP-OMAP versus $FeCl_3$-OMAP revealed that, in addition to the expected four OMP bands (a, b, c, d), high molecular weight bands (84 kDa and above) were also visible staining more intensely in the DIP-OMAP preparations than the $FeCl_3$-OMAP. Further, at least 10 bands were detected reacting with the convalescent buffalo serum and detectable by immunoblotting.

HAEMORRHAGIC septicaemia (HS) is an economically significant disease, particularly of cattle and buffalo in South and Southeast Asia (Bain et al. 1982). The vaccines in current use include formalin-killed broth bacterin (BB), alum-precipitated vaccine (APV) and an oil adjuvant vaccine (OAV). Both BB and APV are easily injectable, but impart a relatively short duration of immunity (6 weeks — BB; 6 months — APV). On the other hand, the OAV has been shown to impart a longer term immunity; i.e. 12 months (Bain et al. 1982; Chandrasekaran et al., these Proceedings), but is difficult to inject because of its high viscosity. Further, there is no satisfactory method of assessing the potency of different vaccine batches.

This statement was further supported by recent experiments carried out in our laboratory whereby the passive mouse protection test (PMPT) with buffalo sera, previously considered to be satisfactory (Bain et al. 1982), was demonstrated to bear no relationship to active protection in buffalo against experimentally induced HS (Chandrasekaran et al., these Proceedings). Thus it is clear that no satisfactory method is currently available to assess the vaccine potency of different vaccine batches. Coupled with the frequent side reactions encountered in the intramuscular injection of OAV, particularly when injected subcutaneously inadvertently, it is clear that there is a need to identify the most potent immunogens of *P. multocida* type 6:B with a view to developing, not only an in vitro potency assay, but also an improved HS vaccine. The latter would consist of either non-living *P. multocida* type 6:B expressing appropriate amounts of the most potent immunogens, or of purified, cloned and expressed products delivered in either an appropriate easily injectable adjuvant or a live attenuated delivery vehicle such as aromatic dependent *Salmonella* species.

We recently demonstrated that the protective potential of purified LPS was due to its associated proteins, which were of outer membrane origin (Muniandy and Mukkur, these Proceedings, Session 3). Previous experiments designed to evaluate the role of outer membrane proteins in *P. multocida* type A infections in mice have yielded inconclusive evidence (Lu et al. 1988, 1991; Abdullahi et al. 1990). In this

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communication, we report data on the comparative protective potential of intact cells and outer membrane and associated proteins (OMAP) of *P. multocida* type 6:B grown under iron-regulated conditions against experimental pasteurellosis in mice, about which no information is currently available.

Materials and Methods

Bacterial strain

The bacterial strain used in this investigation, *P. multocida* type 6:B strain C82, was obtained through the courtesy of Dr S. Chandrasekaran, Veterinary Research Institute, Ipoh, Malaysia. The culture was kept stored frozen in brain–heart infusion broth (BHI) containing 20% glycerol, in a biofreezer at −70 °C.

Animals

Specific-pathogen-free BALB/c mice, 6–8 weeks old, were obtained from the University of New South Wales Animal Facility. They were housed 10 per cage and provided with food and water ad lib.

Minimum moribund dose

The minimum moribund dose (MMD) of *P. multocida* type 6:B strain C82 was taken to be 2 colony-forming units (CFU) as determined previously (Muniandy and Mukkur, these Proceedings, Session 3).

Bacterial culture conditions

P. multocida type 6:B was grown in BHI as fermenter culture, either in the presence of 0.1*M* ferric chloride ($FeCl_3$) (referred to hereafter as $FeCl_3$-grown) or in 2,2-dipyridyl (20 μg per mL) as an iron chelator (referred to hereafter as DIP-grown) for 18–24 hours. The cultures were washed extensively with cold saline and concentrated using an Amicon hollow-fibre concentrator before use in the extraction of lipopolysaccharide (LPS) or isolation of outer membrane proteins.

Preparation of lipopolysaccharide

The LPS of *P. multocida* type 6:B strain C82 was prepared according to Westphal and Jann (1965) and digested with DNAase, RNAase and Proteinase K using standard procedures. The reaction mixture was then subjected to gel filtration on a Sepharose 6B column (2.6cm × 100 cm) equilibrated with 0.05*M* Tris-HCl, pH 7.2, containing 0.1% zwittergent. Fractions in the leading peak containing KDO was pooled, concentrated by pervaporation and stored frozen (PK-LPS).

Preparation of outer membrane and associated proteins

The outer membrane and associated proteins (OMAP) of *P. multocida* type 6:B strain C82 were prepared according to Snipes et al. (1988). Briefly, pelleted $FeCl_3$ or DIP-*P. multocida* from 10-L culture were suspended in 0.01*M* HEPES buffer, pH 7.4, and disrupted in a French pressure cell at 10 000 psi. After removal of unbroken cells by centrifugation (12 000 × g, 30 min), Sarkosyl (sodium N-lauroyl sarcosinate) was added to give a final concentration of 2.0%. The OMAP were deposited by centrifugation at 40 000 × g for 1 hour, washed three times in 0.05*M* Hepes buffer at pH 7.2, resuspended in 4–5 mL water and stored at −20 °C.

SDS-PAGE and immunoblotting

OMAP were analysed on 12% slab gels with Laemmli buffers (Laemmli 1970) in a Mini-Protean II gel electrophoresis system (BioRad, Australia). The bands in gels were visualised after staining with coomassie blue or following silver staining. Immunoblotting was carried out according to Towbin et al. (1979) using PVDF membranes. The source of antibodies used were mice previously immunised with non-living *P. multocida* type 6:B or OMAP and sera from buffalo convalescing after an outbreak of HS in Malaysia, the detecting antibodies being alkaline phosphatase labelled mouse monoclonal anti-bovine IgG and the substrate system comprising nitroblue tetrazolium (NBT) and bromochloro-indoyl phosphate (BCIP).

Analytical methods

Protein content was measured by a modified Lowry procedure (Markwell et al. 1978). Contamination of OMAP preparations with LPS was assessed by measuring KDO as an indicator (Karkhanis et al. 1978).

Immunisation challenge experiments in mice

Immunisation of mice (four per group) was carried out by the intraperitoneal route, whereas challenge infection was given subcutaneously. For investigating the comparative protective efficacy of $FeCl_3$-grown versus DIP-grown organisms, mice were immunised with 1×10^9 CFU of formalin-killed organisms and challenged with virulent organisms in doses ranging from 1×10^5 to 2.3×10^9 CFU of *P. multocida* grown for 12 hours in BHI. Any dilutions or resuspensions were made in Hank's medium 199. For immunisation with OMAP, mice were immunised with a total of 50 μg or 100 μg administered in a single dose, or in two doses split equally. In the latter case, the second dose was administered 2 weeks after the

first. For challenge infection of mice immunised with OMAP, 0.1 mL of 10^{-7} dilutions of a 12-h BHI culture containing 100–200 organisms were used.

Measurement of antibody levels

Antibodies to LPS and OMAP were determined using an enzyme-linked immunosorbent assay (ELISA) as described previously (Mukkur et al. 1991). The concentrations of LPS or OMAP used for coating the ELISA plates (M129B Dynatech) were 50 ng and 50 μg per mL respectively.

Statistical analysis

Student's *t* test was used to determine the significance of difference between the $\log_{10}$ of the mean values for ELISA titres.

Results

Non-living *P. multocida* type 6:B that had been grown in the presence of 2,2-dipyridyl (i.e. under iron-restricted conditions) imparting better protection to mice against experimental pasteurellosis than *P. multocida* that had been grown under iron-replete conditions (Table 1). This is demonstrated by the fact that mice immunised with $FeCl_3$-grown organisms were only partially protected at a challenge dose of 10^7 CFU per mouse, and were completely unprotected at challenge doses of 10^8 or 10^9 CFU, whereas those immunised with DIP-grown organisms were completely protected at challenge doses of 10^7 CFU, and partially protected when challenged with 10^8 or 10^9 CFU. Antibody titres of immune mice against their respective OMAP preparations were substantial, whereas those against LPS, although still substantial, were significantly lower in magnitude (Table 1).

The level of LPS contamination of the DIP-OMAP or $FeCl_3$-OMAP was 4.5 or 7.5% respectively. The OMAP of DIP-grown *P. multocida* also appeared to impart a superior level of protection to that obtained with the OMAP prepared from $FeCl_3$-grown organisms (Table 2). While vaccination of mice with a single dose of OMAP from DIP-grown organisms imparted complete protection to mice against challenge infection, adoption of a booster vaccination regime was necessary to achieve the same level of protection with the OMAP prepared from $FeCl_3$-grown *P. multocida*.

Coomassie blue staining of the SDS-PAGE gels showed one major OMAP band with a molecular mass of 35 kDa (a) and three other faintly staining OMAP bands (b — 30 kDa; c — 23 kDa; d — 17 kDa). In the OMAP prepared from DIP-grown organisms, additional intensely staining bands, appearing as a doublet with molecular mass of

Table 1. Comparative protection in mice immunised with formalin-killed *P. multocida* type 6:B grown under iron-regulated conditions.

Vaccine	Dose used for challenge (CFU)	No. surviving/ No. challenged	% Protection	Mean ELISA antibody titre (± SEM) against	
				*OMAP	*PK-LPS
DIP-medium					
Experiment 1	1×10^7	4/4	100	54.8 ± 5.5	19.1 ± 3.7
	1×10^6	4/4	100		
	1×10^5	4/4	100		
Experiment 2	2.3×10^9	1/4	25	48.8 ± 5.6	15.7 ± 4.2
	2.3×10^8	2/4	50		
	2.3×10^7	4/4	100		
	2.3×10^6	4/4	100		
$FeCl_3$-medium					
Experiment 1	1×10^7	1/4	25	47.3 ± 7.3	13.7 ± 3.2
	1×10^6	4/4	100		
	1×10^5	4/4	100		
Experiment 2	2.3×10^9	0/4	0	69.3 ± 7.6	24.5 ± 5.0
	2.3×10^8	0/4	0		
	2.3×10^7	3/4	75		
	2.3×10^6	3/4	75		
None (non-immune)	2.0×10^2	0/4	0	2.0 ± 1.3	1.9 ± 1.2

* OMAP and PK-LPS denote outer membrane and associated proteins and Proteinase K-digested lipopolysaccharide respectively

84 kDa and higher, were present. The latter bands could also be seen in the SDS–PAGE gels of OMAP prepared from the $FeCl_3$-grown organisms, although the bands were fainter (figure not shown). A minimum of 14 other faintly staining bands with intermediate molecular weights ranging from 14 kDa to 75 kDa were also visible in both types of OMAP preparations. The intensity of the faint bands was markedly improved on silver staining (Fig. 1).

While there was no significant difference between the antibody titres against OMAP or PK-LPS and mice immunised with formalin-killed organisms grown under iron-regulated conditions (Table 1), all titres were significantly higher than those of non-immune mice. The antibody titres of mice immunised with one dose of OMAP were significantly lower ($P<0.05$) than when the same amount of antigen was administered in two equal doses. However, with the exception of one of the immunisation regimes with DIP-OMAP (100 μg OMAP administered as two doses of 50 μg each), no significant difference between antibody levels against OMAP was evident.

All mice immunised with OMAP also developed an antibody response to the contaminating LPS. Again the anti-LPS titres were significantly lower ($P<0.01$) in mice receiving one dose than in those receiving two doses (Table 2).

On immunoblotting with convalescent buffalo sera, 10 distinct bands could be seen, of which only 3 bands (a, d and those greater than 84 kDa) were of outer membrane protein origin (figure not shown). It should be noted that there were some proteins expressed in the $FeCl_3$-OMAP in higher concentrations than in the DIP-OMAP preparations in the molecular weight range of 35–45 kDa (Fig. 1).

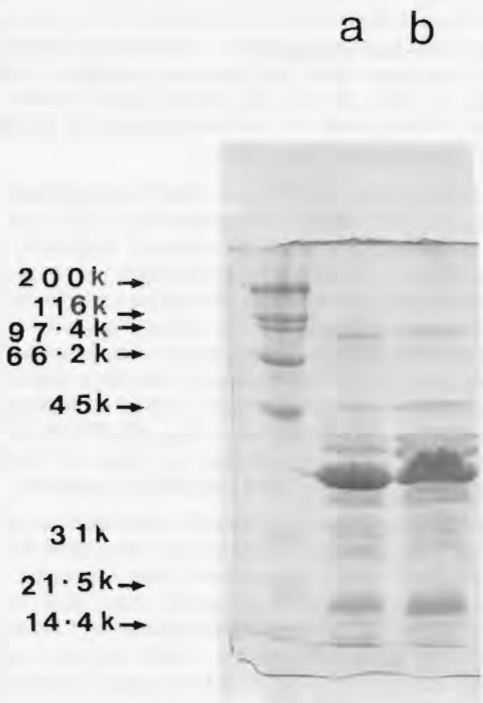

Fig. 1. SDS–PAGE electrophoresis of OMAP prepared from (a) DIP-grown and (b) $FeCl_3$-grown *P. multocida* type 6:B. Estimated molecular weights are shown in kilodaltons.

Table 2. Comparative protection in mice immunised with OMAP of *P. multocida* type 6:B grown under iron-regulated conditions.

OMAP-vaccine dose (μg)	No. surviving/ No. challenged	% protection	Mean ELISA antibody titre (± SEM) against*	
			OMAP	*PK-LPS
$FeCl_3$-medium				
50 (×1)	2/4	50	19.9 ± 4	4.0 ± 2.0
25 (×2)	4/4	100	43.7 ± 3.8	23.8 ± 3.8
100 (×1)	3/4	75	41.1 ± 3.1	12.1 ± 2.3
50 (×2)	4/4	100	44.9 ± 4.9	29.5 ± 2.0
DIP-medium				
50 (×1)	4/4	100	27.0 ± 3.0	2.8 ± 1.4
25 (×2)	4/4	100	57.0 ± 12.5	29.3 ± 4.5
100 (×1)	4/4	100	39.6 ± 9	15.6 ± 4.7
50 (×2)	4/4	100	73.6 ± 1.2	29.3 ± 4.5
None	0/4	0	2.9 ± 1.2	2.7 ± 0.4

* PK-LPS denotes Proteinase K-digested lipopolysaccharide
* ELISA titres for samples marked 'a' were determined at 2 weeks post-immunisation only

Discussion

This experiment demonstrated that formalin-killed, DIP-grown *P. multocida* type 6:B and DIP-OMAP were superior in imparting protection against experimental pasteurellosis to mice than the $FeCl_3$-grown intact cells and $FeCl_3$-OMAP. The level of protection observed with $FeCl_3$-grown organisms was similar to that reported previously using formalin-killed, BHI-grown *P. multocida* type 6:B strain M1404 (Dawkins et al. 1991).

Although the OMAP were clearly contaminated with LPS, as judged by the presence of KDO, and the detection of anti-LPS antibody response in immunised mice and its possible minor role in protection cannot be ruled out, the major contribution to the observed protection was clearly made by the protein components. This is because (a) Proteinase K-digested LPS has been shown to be non-protective in mice (Muniandy and Mukkur, these Proceedings); and (b) mice immunised with 50 μg of DIP-OMAP, with anti-LPS levels similar to those of non-immunised controls, were completely protected.

It has been considered that *P. multocida* grown under iron-restricted conditions in vitro may have an antigenic make up similar to that expressed in vivo. If this assumption is correct, then, given our observation that the protection imparted by LPS was due to its associated protein, which we have suggested as possibly being of OMAP origin (Muniandy and Mukkur, these Proceedings), it appears that superior protection imparted by the DIP-grown organisms and DIP-OMAP could be, at least, partially attributed to the over-expressed, albeit slightly, higher-molecular-mass components (84 kDa and above). Experiments aimed at separating various bands reacting in the immunoblot are currently in progress, with a view to identifying the most potent immunogens of *P. multocida* type 6:B.

Acknowledgments

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Protective Potential of Purified Lipopolysaccharide Versus Conjugated Oligosaccharide of *Pasteurella multocida* Type B in Mice

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Abstract

Purified Westphal lipopolysaccharide (LPS) of *Pasteurella multocida* type B was found to be protective against experimentally induced pasteurellosis in mice. However, the observed protection was abrogated if such LPS was treated with phenol, a protein denaturing agent, or digested with Proteinase K and further purified by gel filtration prior to immunisation. Further, the protein associated with LPS and presumably responsible for the observed protection appeared to be of outer membrane origin. Immunisation of mice with oligosaccharide prepared from LPS and two types of oligosaccharide–protein conjugates failed to confer protection against challenge with virulent organisms.

HAEMORRHAGIC septicaemia (HS) is a killer disease of cattle and buffalo, particularly in South and Southeast Asia, which is socio-economically highly significant (Bain et al. 1982). The vaccines currently in use include formalin-killed broth bacterin (BB) and adjuvanted vaccines; i.e. alum-precipitated (AP) or oil adjuvant (OA) vaccines. The BB and AP vaccines give only short- and medium-term immunity respectively whereas the OA vaccine imparts a longer-term immunity (of 12 months duration) (Bain et al. 1982; Chandrasekaran et al., these Proceedings, Session 3). However, injecting the OA vaccine is difficult due to its high viscosity, and it has to be injected at depth intramuscularly to minimise side effects caused by abscess formation as a result of vaccination. Breakdowns of immunity to HS in areas covered by vaccination have also been reported (Bain et al. 1982) and one possible reason given is the use of vaccine batches lacking adequate potency. Thus it is clear that there is a need to identify the most-potent immunogens of the HS-causing serotype of *Pasteurella multocida*, i.e. type 6:B. In this communication, we present data on the protective potential, in mice, of purified lipopolysaccharide (LPS) before and after treatment with phenol and Proteinase K, oligosaccharide (OPS) derived from LPS, and OPS conjugated to bovine serum albumin (BSA) or diphtheria toxoid.

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Materials And Methods

Bacterial strain

P. multocida type 6:B strain C82 isolated from buffalo with HS, obtained through the courtesy of Dr S. Chandrasekaran, Veterinary Research Institute, Ipoh, Malaysia, was used throughout this investigation. The culture was kept stored frozen in brain-heart infusion (BHI) containing 20% glycerol in a biofreezer at $-70\,^{\circ}C$.

Animals

Specific pathogen free BALB/c mice, 6–8 weeks of age, were obtained from the University of New South Wales Animal Facility. They were housed 10 per cage and supplied with food and water ad lib.

Determination of minimum moribund dose

Mice were injected subcutaneously with 0.1 mL of different dilutions (10^{-7}–10^{-9}) of a 12-h BHI culture of *P. multocida* type 6:B in Hanks medium 199, pH 7.2. Inoculated mice were monitored four times per day over a period of 96 hours for the onset

of moribund state and mortalities. The minimum moribund dose (MMD) of *P. multocida* type 6:B strain C82 was estimated to be two colony-forming units (CFU).

Analytical methods

Total carbohydrate concentration was determined by the method of Dubois et al. (1956) using glucose as standard. Protein content was measured by a modified Lowry procedure (Markwell et al. 1978) and 2-keto-2-deoxyoctulosonic acid (KDO) measured according to Karkhanis et al. (1978). Nucleic acid and protein peaks in chromatographic runs were monitored by absorption at 260 and 280 nm respectively.

Purification of lipopolysaccharide

LPS was prepared according to Westphal and Jann (1965). Briefly, 250 mL of washed, concentrated live bacterial suspension (60 g wet weight) was extracted twice with 90% (w/v) phenol at 68 °C for 30 min and the aqueous phases combined and dialysed exhaustively with running water for 5 days. Nucleic acid was removed by precipitating with cetavalon at 1% (w/v) final concentration. The LPS in the supernatant was precipitated with 10 volumes of ethanol in the presence of 0.5*M* NaCl at 4 °C overnight and centrifuged at 10 400 × g for 30 min. The precipitate was resuspended in about 150 mL distilled water, dialysed against water and centrifuged at 105 000 × g for 4 hours. The gelatinous LPS pellet (LPSI) was resuspended in water and used for toxicity and protection studies. Another lot of LPS was prepared and purified as follows.

The phenol-extracted LPS extract was subjected to cetavalon treatment and ethanol precipitation as stated above, digested with DNAase (100 μg/mL in 0.1*M* Tris buffer pH 7.2 in the presence of 0.02*M* $MgSO_4$) and RNAase (50 μg/mL) at 37 °C for 6 hours. The extract was then digested with Proteinase K (100 μg/mL) for 12 hours at 37 °C, dialysed against water and centrifuged three times at 105 000 × g for 4 hours. The LPS pellet (LPSII) was dialysed against 50m*M* Tris pH 7.2 containing 0.1% zwittergent (3-14, Calbiochem) and subjected to gel filtration through a Sepharose 6B column (2.6 x 100 cm) equilibrated with the above buffer (Fig. 1). The eluted fractions were assayed for total carbohydrate, KDO and absorbance at 260 and 280 nm. The KDO-containing peak, regarded as LPSIII, was pooled and dialysed against water to remove the detergent. Such purified LPS contained 3.3% protein as contaminant.

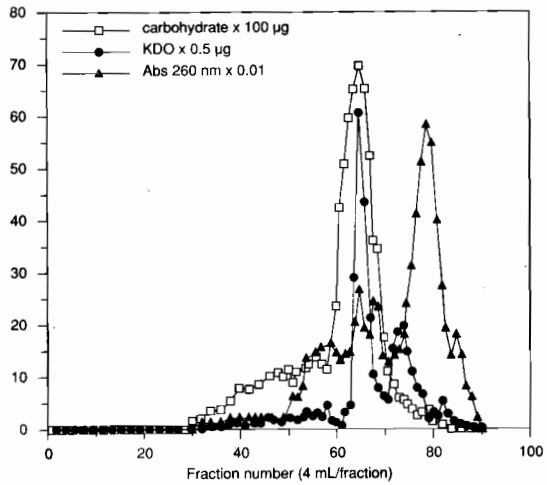

Fig. 1. Gel filtration of LPSII on a Sepharose 6B column

LPSII (50 mg) was loaded on a Sepharose 6B column (2.6 x 100 cm), equilibrated with 0.05*M* Tris buffer pH 7.2 containing 0.1% zwittergent, and 4 mL fractions were collected. Fractions were monitored for total carbohydrate, KDO and absorbance at 260 nm and 280 nm.
carbohydrate × 100 μg
KDO × 0.5 μg
absorbance 260 nm × 0.01
Fraction number (4 mL/fraction)

Preparation of oligosaccharide

Oligopolysaccharide (OPS) was prepared essentially according to Jacques and Dubray (1991). Briefly, 20 mg purified LPSIII was treated with 2% (v/v) acetic acid at 100 °C for 2 hours. After cooling, the precipitated LipidA was removed by centrifugation at 3200 × g for 15 min. The supernatant was dialysed against 50m*M* pyridinium-acetate buffer pH 4.7 and subjected to gel filtration through a Sephadex G-50 column (2 × 100 cm) equilibrated with the same buffer. The column was eluted at a flow rate of 14 mL/hour and 4.0 mL fractions were collected. Fractions were assayed for total carbohydrate, KDO and absorbance at 280 nm. The first major carbohydrate-containing peak was pooled, and concentrated by rotary evaporation.

Synthesis of oligopolysaccharide–protein conjugates

Ten milligrams of the OPS in water (5.0 mL) was oxidised with 0.1*M* sodium periodate ($NaIO_4$) for 10 min in the dark. Oxidation was stopped by the addition of ethylene glycol (0.5 mL), the solution left at 4 °C for 20 min, dialysed against distilled water and then concentrated by rotary evaporation. The

oxidised OPS was conjugated to bovine serum albumin (BSA) and diphtheria toxoid (DT) using indirect conjugation with a spacer according to Cryz et al. (1986). Briefly, 20 mg of 1-ethyl-3-(3-dimethyl aminopropyl) carbodimide and 20 mg of adipic acid dihydrazide (ADH) was added to 10 mg BSA or DT in 0.05*M* sodium phosphate buffer at pH 7.2. The pH was adjusted to 4.8 with 0.1*M* HCl and the mixture kept at this pH for 2 hours at room temperature. The solution was dialysed against 0.05*M* sodium phosphate buffer at pH 8.0.

The oxidised OPS and ADH-BSA or ADH-DT were mixed (1:1) and incubated at room temperature for 6 hours, and 10–15 mg $NaCNBH_3$ was added. The reaction mixture was incubated at room temperature for 6 days after adding 3 drops of toluene and sealing the reaction vessel. The reaction mixture was subjected to gel filtration through an ACA 34 column (2.6 × 100 cm) equilibrated with PBS, and fractions were monitored for carbohydrate and protein. The fractions, appearing immediately after void volume where the carbohydrate and A280 profile coincided, contained the OPS-ADH-BSA or OPS-ADH-DT conjugate. They were pooled and concentrated by membrane filtration using Centricon-10 according to the manufacturer's (Amicon) instructions. Total carbohydrate and protein concentrations of the conjugates were then determined for immunisation.

Preparation of outer membrane and associated proteins

Outer membrane and associated proteins (OMAP) were prepared according to Snipes et al. (1988), the details of which have been described in Kennett et al. (these Proceedings). The OMAP preparation used in this investigation was contaminated with 4.5% LPS.

SDS-PAGE

Various LPS preparations were subjected to discontinuous SDS-PAGE according to Laemmli (1970) using a Mini Protean gel system (BioRad, Australia). Separating gels were formed with 14% acrylamide incorporating 4*M* urea and 0.1% SDS. Stacking gels used were 4.5% without urea. Samples were mixed 3:1 with sample buffer containing 0.1% SDS. Electrophoresis was performed with a constant voltage of 200 V at room temperature for 45 min.

LPS bands were detected by the silver staining method of Tsai and Frasch (1982), with minor modifications, during the developing step. The developer solution, containing 50 mg citric acid in 1L milliQ water, was warmed to 41 °C and 0.5 mL of 37% formaldehyde was added before use. After silver staining and washing, the gel was floated while shaking in the developer solution for 30–45 secs. Development was terminated when the gel reached a desired intensity by adding 1% acetic acid solution. This fast development ensured that only LPS bands and not the protein bands were stained (Brahmbhatt, pers. comm.).

Immunisation challenge experiments in mice

Mice in groups of four or five were immunised intraperitoneally with either 5, 10, 25, 50, 75 or 100 μg of purified LPS or OPS. The OPS-ADH-protein conjugates were injected intraperitoneally at graded doses of 2.5, 5.0, 10, 20 and 40 μg. Blood samples were taken via the retro-orbital plexus 2 days pre-challenge.

Challenge of immunised and non-immune control mice was effected subcutaneously with 100–200 CFU prepared by dilution of a 12-h culture of *P. multocida,* at 4 weeks post-immunisation. Challenged mice were observed for 7 days and mortalities were recorded. Mice euthanased in a moribund state were counted as mortalities.

Antibody levels

Mouse serum antibody levels to LPS were determined using an enzyme-linked immunosorbent assay (ELISA) according to Mukkur et al. (1991) using LPSIII (1.5 μg/mL) as the antigen to coat the ELISA plates (M129B Dynatech). The secondary antibody used was alkaline phosphatase-labelled goat anti-mouse whole IgG molecule (Sigma Chemicals Ltd). Antibody levels against OMAP were also measured by ELISA employing 6.5 μg/mL to coat the ELISA plates.

Results

Toxicity and protective efficacy of purified LPS

The Westphal LPS (i.e. LPSI of *P. multocida* type 6:B strain C82) was found to be toxic to mice at doses greater than 75 μg. However, when immunised mice were challenged with live organisms at 4 weeks post-immunisation, a high level of protection was observed (Table 1).

Effect of phenol and Proteinase K treatment of LPS on its protective efficacy

When the purified LPS (i.e. LPS II obtained after Proteinase K treatment) was used for immunisation of mice at doses of 25–75 μg followed by challenge at 4 weeks post-immunisation, its protective potential was abrogated (Table 2).

Table 1. Toxicity and protective properties of LPSI in mice.

μg LPS inoculated	No. dead/ no. inoculated	No. surviving/ no. challenged	Percent protection	ELISA antibody titre (mean ± SEM)
25	0/5	5/5	100	51.0 ± 8.0
50	0/5	4/5	80	90.0 ± 16.0
75	0/5	4/5	80	75.9 ± 8.0
100	2/5	3/3	100	65.4 ± 5.0
200	4/5	NC	—	NA
300	5/5	NA	—	NA
None	0/4	0/4	0	3.6 ± 1.3

NC, not challenged; NA, not applicable

Table 2. Protective capacity of phenol or Proteinase K-treated LPS (LPSII) in mice.

μg LPS inoculated	Treatment	No. surviving/ no. challenged	Percent protection	ELISA antibody titre (mean ± SEM)
25	Proteinase K	0/4	0	53.4 ± 9.8
50	Proteinase K	0/4	0	54.7 ± 5.3
75	Proteinase K	0/4	0	90.5 ± 18.1
75	phenol	0/5	0	48.5 ± 10.0
Control		0/4	0	4.7 ± 1.3

Protective potential of OPS and OPS-ADH-BSA and OPS-ADH-DT conjugates

The gel filtration profile of OPS following removal of Lipid A revealed the presence of one major peak eluting at the void volume on Sephadex G-50 (Fig. 2). Both the OPS-ADH-BSA and OPS-ADH-DT conjugates eluted at the void volume of the ACA 34 column, indicating the molecular weights of the conjugates to be greater than 350 000 Da (Figs 3 and 4). However, when mice immunised with the OPS and conjugates were challenged with the virulent organisms, no protection was observed (Table 3).

Antibody levels

Regardless of the type of LPS used for vaccination (i.e. LPSI or LPSII), all immunised mice developed high antibody levels against LPS (Tables 1 and 2). However, the titres were comparatively lower in mice immunised with either OPS or OPS-ADH-protein conjugates (Table 3).

The pre-challenge sera of mice immunised with LPSI were also found to have a high level of antibody activity against OMAP prepared from *P. multocida* type 6:B in mice immunised with 75 μg LPSI, the titre being 44.1 ± 4.0.

The SDS-PAGE of purified LPSI, LPSII or a crude extract prepared according to Hitchcock and Brown (1983) revealed that LPS of *Pasteurella*

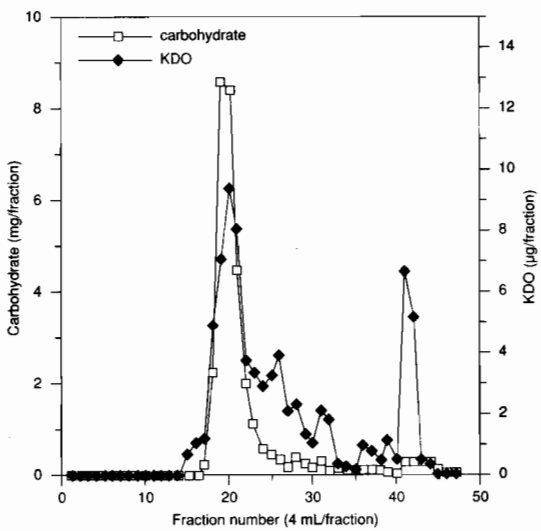

Fig. 2. Isolation of OPS on a Sephadex G-50 column

The concentrated supernatant of LPSIII (treated with 2% acetic acid) containing about 20 mg LPS was loaded on a Sephadex G-50 column (2.0 × 100 cm) and equilibrated with 0.05*M* pyridinium-acetate buffer, pH 4.7. Total carbohydrate and KDO concentrations were determined on the fractions collected.

carbohydrate
KDO
Carbohydrate (mg/fraction)
KDO (μg/fraction)
Fraction number (4 mL/fraction)

Table 3. Protective properties of OPS and OPS-protein conjugates in mice.

Type and amount of antigen inoculated (μg)	No. surviving/ no. challenged	Percent protection	ELISA antibody titre (mean ± SEM)
OPS			
5	0/4	0	14.5 ± 1.1
10	0/4	0	13.7 ± 1.0
25	0/4	0	12.9 ± 0.2
50	0/4	0	22.4 ± 2.2
75	0/4	0	25.8 ± 3.6
OPS-ADH-BSA			
2.5	0/4	0	13.3 ± 1.8
5	0/4	0	19.0 ± 4.7
10	0/4	0	26.9 ± 4.4
20	0/4	0	28.4 ± 2.8
40	0/4	0	32.4 ± 2.1
OPS-ADH-DT			
2.5	0/4	0	23.7 ± 9.41
5	0/4	0	19.9 ± 3.12
10	0/4	0	22.9 ± 4.23
Non-immune	0/4	0	4.7 ± 1.11

multocida type 6:B lacked the typical ladder-like pattern, unlike that of *Escherichia coli* 0:111 or *Salmonella minnesota*. It thus represented rough or perhaps a semi-rough type of LPS for which the term lipo-oligosaccharide as suggested previously (Rimler 1990) seems quite appropriate. However, freezing and thawing or even concentrating purified LPSI or LPSII showed the emergence of a number of high-molecular-weight forms giving the appearance of repeating units (figures not shown) as reported previously (Rimler 1990).

Discussion

With the data gathered so far, it is apparent that protection observed upon immunisation with purified LPS is not due to OPS. This supports observations reported earlier (Ramdani and Adler 1991) using LPS-specific monoclonal antibody in passive mouse immunisation experiments. In addition, the observed immunity does not appear to be due to the whole LPS, as denaturation or digestion of LPS-associated protein was found to abrogate its protective property. On the other hand, Wijewardana et al. (1990) reported their anti-LPS (*P. multocida* type A) monoclonal antibody to be bactericidal and to protect mice completely against homologous challenge. This protection was considered to be associated with Lipid A.

The LPS of *P. multocida* type A or B prepared by phenol water extraction (Westphal and Jann 1965)

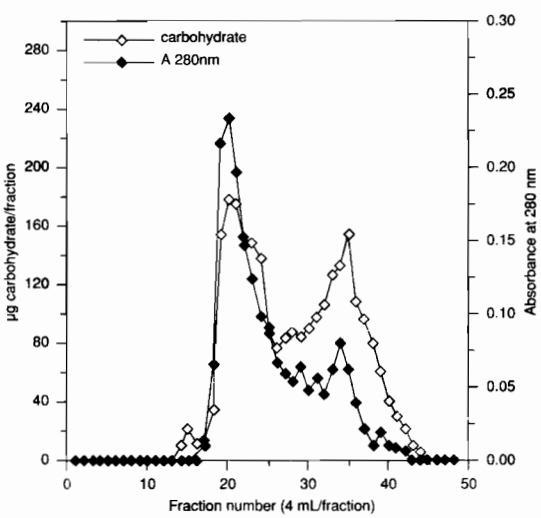

Fig. 3. Gel filtration of OPS-ADH-BSA conjugate on an ACA34 column

Gel filtration of OPS-ADH-BSA conjugate reaction mixture on an ACA34 column (2.0 × 100 cm), equilibrated and eluted with PBS, pH 7.2. Total carbohydrate and absorbance at 280 nm were measured on the collected fractions.
carbohydrate
A 280 nm
Carbohydrate (μg/fraction)
Absorbance at 280 nm
Fraction number (4 mL/fraction)

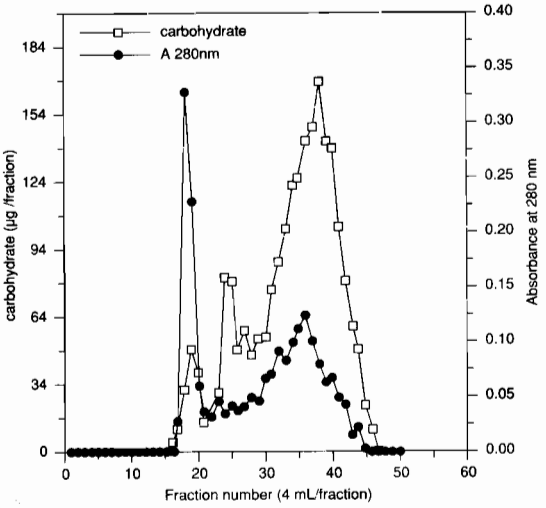

Fig. 4. Gel filtration of OPS-ADH-DT conjugate on an ACA34 column

Gel filtration of OPS-ADH-DT conjugate reaction mixture on an ACA34 column (2.0 × 100 cm), equilibrated and eluted with PBS, pH 7.2. Total carbohydrate and absorbance at 280 nm were determined on the collected fractions.
carbohydrate
absorbance 280 nm
Carbohydrate (µg/fraction)
Absorbance at 280 nm
Fraction number (4 mL/fraction)

has been reported to be a poor immunogen in mice (Bain and Knox 1961; Rebers and Heddleston 1974), but not in chickens, where it was demonstrated to be protective in passive immunisation experiments (Rebers et al. 1980). However, endotoxin of *P. multocida* type A extracted with formal-saline that contained proteins and carbohydrates (free endotoxin) was reported to be protective (Heddleston et al. 1966; Rebers et al. 1967), but the identity and role played by the proteins in protective immunity was not investigated. The fact that pre-challenge sera from mice immunised with untreated LPSI developed substantial antibody levels against OMAP, although contaminated with a low level of LPS (4.5%), suggests that the origin of the immunogenic LPS-associated proteins is the outer membrane of *P. multocida* type 6:B. In this regard it may be pertinent to mention that the protective property of a potassium thiocyanate extract that contained carbohydrates, proteins and nucleic acids was also shown to be associated with the protein component (Mukkur and Pyliotis 1981). This suggestion is also consistent with a recent report (Lu et al. 1991) that antibodies to the outer membrane proteins, but not lipopolysaccharide, inhibit pulmonary proliferation of *P. multocida* type 6:B in mice.

Further work is clearly necessary to identify the nature and origin of the LPS-associated protective proteins, as the latter are likely to constitute one of the significant potent immunogens of *P. multocida* type 6:B.

Acknowledgments

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Immunogenic Effect of *Pasteurella multocida* Extracts in Calves Against Pasteurella Infections

W. Erler[1] and W. Schönherr[1]

Abstract

A sodium chloride extract prepared according to Rebers from the *Pasteurella multocida* strain P383 (type B according to Carter) will provide protection against homologous infection in calves. The immunising effect depends on the strain and the dose used, continues for more than 12 weeks, and can be confirmed by bacteriological, serological and pathological-anatomical tests. Such an extract from a strain of type A (P880) did not produce an immunising effect: in this case, the antibodies induced did not provide protection.

SATISFACTORY protection of calves against *Pasteurella multocida* infections has not been obtained to date. While vaccination against haemorrhagic septicaemia (type B infection) is possible, no effective vaccines against pneumonia in calves caused by *Pasteurella* type A are known. This was illustrated by Mosier et al. (1989) in their review, which recommended that future work concentrate on the immunological value of cell extracts, i.e. defined components of the bacterial cell.

This paper presents the results obtained in immunisation tests in calves using sodium chloride extracts prepared from a *P. multocida* B strain and from a *P. multocida* A strain.

Materials and Methods

Strain P383 (type 6:B) was isolated from a calf that had died from enzootic pneumonia; and strain P880 (type A) was isolated from a piglet. The extracts were prepared in accordance with the instructions given by Rebers et al. (1967). Male and female calves aged 2–4 weeks were used in the experiments. Using different routes, immunisation of the animals was performed with or without addition of Freund's incomplete adjuvant. Three weeks after the last immunisation, the animals were challenged intratracheally with approximately 2×10^9 organisms of strain P383 (once) (Kielstein and Schimmel 1983) or $3–5 \times 10^9$ organisms of strain P880 (three times each) (Schimmel 1987). One week after challenge the surviving animals were euthanased and necropsied. Lung changes were evaluated, and selected organs were examined bacteriologically. Serum antibodies were determined by means of ELISA (Erler et al. 1991).

Results

Immunisation experiments with extracts from strain P383 (type 6:B)

Immunisation was performed subcutaneously at an interval of 2 weeks. A clear dose dependency was observed. The protective dose of immunising antigen was established to be 2×2 mg. Out of 50 animals immunised with 2×2 mg extract, only one calf died after experimental challenge. A total dose of 4 mg of this extract provided 98% protection in calves, whereas challenge infection was lethal for non-immunised animals, which usually died within 2 days from septicaemia.

As a rule, changes in body temperature and disturbances of the general state of health were not observed in the experimental animals after administration of the extract. The general tolerance to it was good in the lower dose range, both with and without the addition of an adjuvant, and the local tolerance was very good when no adjuvant was added. However, shock may occur in individual cases.

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Eight days, on average, after challenge infection, no pasteurellae were isolated from the lungs, pulmonary lymph nodes and trachea, or from the abdominal organs (liver, spleen, kidney) of 70% of the immunised and challenged animals. In animals from which pasteurellae were isolated, usually only single organs were affected. The bacteriological examinations suggested that a septicaemic extension of the organisms was prevented in the immunised animals. The presence of pasteurellae did not correlate with the occurrence of pneumonic changes in the immunised calves.

In contrast to the control animals that died after challenge infection from septicaemia and/or marked pneumonic changes, 47% of the immunised and surviving animals did not show pneumonic changes, despite the high challenge dose administered. Immunisation had a positive effect on the severity and character of pneumonia in the immunised calves after challenge infection: in 73% of the cases, if pneumonia was present it was of low severity; and in 27% of cases it was of moderate severity. There was either no tendency, or at the most a low tendency, towards an extension of pneumonic changes in the immunised animals, in contrast to the situation in control animals that died. Sixty-five percent of lung changes were classed as catarrhal purulent bronchopneumonia. Scarring was taking place in some lesions.

Six of the surviving animals (23%) showed purulent abscess-forming and purulent necrotising bronchopneumonia of a predominantly moderate extension. Fibrinous bronchopneumonia of low severity and with clear-cut margins was observed in three animals (11.5%).

There was a highly significant difference between the immunised experimental animals and the non-immunised control animals in the antibody titres determined in the sera before challenge infection (results not shown). Thirty animals were immunised experimentally subcutaneously with 2 × 2 mg of the extract. After 5, 8, 10 and 12 weeks, two immunised animals each and two non-immunised control animals of equal age were challenged. While all control animals died within 48 hours after challenge, the immunised animals survived. Even 12 weeks after the second immunisation, the animals withstood challenge.

The same infection model was used for the testing of cross immunity. Attempts were made to immunise the animals with the extracts prepared from other *Pasteurella* strains, according to the same procedure. A comparison of the antibody titres obtained with a dose of 2 mg (total dose 4 mg) raised by the strains P383 (6:B), R473 (6:B), PM (7:A) and Bunia II (6:E) shows a decrease in the protective effect. In the order listed, the survival rate with the 2 × 2 mg dose was 90%, 57%, 25% and 17%. The extracts from strains PM and Bunia II did not seem to provide protection against a challenge infection with strain P383. The protective effect of the extract from strain R473, which belongs to the same serotype as strain P383, could be increased considerably by increasing the immunising dose (6 mg and more will result in a 100% protection).

In addition to those contained in the sodium chloride extract, other substances isolated from strain P383 were tested for their immunogenic effect in the calf: lipopolysaccharide isolated with phenol water; mannane isolated from the purification supernatant of this lipopolysaccharide; purified peptidoglycane; and an extract obtained with tricholoroacetic acid. The results showed, however, that neither these extracts nor the compounds isolated had an immunogenic effect. All calves immunised subcutaneously with one of the substances mentioned at a dose rate of 2 × 2 mg, and the controls, did not survive challenge infection, dying within 48 hours of infection.

Immunisation experiments with extract from strain P880 (type A)

In view of the good results obtained with strain P383, we immunised calves at doses of 2 mg. The results of these experiments revealed that, apart from one isolated instance, there was no difference between control and immunised animals in the frequency and severity of pneumonia after experimental challenge. The sodium chloride extract from strain P880 did not provide protection against homologous infection. In six experiments, the antibody levels of the animals had risen significantly after termination of immunisation, as compared to the time before immunisation. The antibody levels in six immunised and six non-immunised animals also differed significantly (results not shown).

These results show that subcutaneous, intramuscular, intradermal, or a combination of subcutaneous and intradermal, administration of the extract, but not intratracheal administration, will result in an immune response. However, a positive immune response will not lead to a parallel decrease in the frequency and severity of pneumonia in the immunised animals.

Although the antibody induced with the sodium chloride extract provided protection against septicaemic *Pasteurella* B infection, it did not provide protection against *Pasteurella* A infection.

Discussion and Conclusion

We were able to show that calves could be protected effectively against homologous infection by means of a sodium chloride extract from a *P. multocida* strain (strain P383, type 6:B) that is highly virulent in calves. The protection produced is dose dependent. Mortality in the infected animals was reduced to almost zero, and the protective effect was confirmed by additional bacteriological, pathological-anatomical and immunological examinations of the calves that survived challenge infection.

We were unable to demonstrate that, by adding Freund's incomplete adjuvant, the dose necessary for protection can be reduced. General tolerance of the extracted vaccine was good. When the adjuvant was omitted, the local tolerance of the extracted vaccine even improved.

Serum antibody levels, as determined by ELISA, were maintained at a high level in vaccinated animals for more than 12 weeks. Eight days after challenge infection, *Pasteurella* organisms were detected in single organs in a low number (30%) of the vaccinated animals. This finding did not correlate with the occurrence of pneumonic changes. There were no pneumonic changes in 47% of all surviving animals; and changes were of low severity in 73% of the animals suffering from pneumonia.

The cross-over experiments (i.e. the preparation of a sodium chloride extract from other strains and challenge with strain P383) suggested that immunisation does not provide sufficient protection in a heterologous system.

The results obtained in infection experiments with *P. multocida* type A did not correlate with the type B findings. Although the sodium chloride extract was immunogenic in calves, antibodies produced were not protective.

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The Antibody Response of Rabbits and Cattle to *Pasteurella multocida*: Assessment of Cross-Reactivity Between Serotypes

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Abstract

The aim of this study was to examine the antibody responses of rabbits and cattle to vaccination with various *Pasteurella multocida* strains using ELISA, immunoblotting and passive mouse protection test (PMPT) procedures. Particular emphasis was placed on assessing the extent of cross-reaction with a reference strain Buffalo B (0332), a haemorrhagic septicaemia (HS) isolate used in the PMPT.

Rabbits were injected with killed whole cell vaccines of the Carter A, B, D and E serogroups and the antibody response measured using an ELISA. Although all sera contained high levels of antibody to the vaccinal strain, there was evidence of cross-reaction between each of the serotypes. Immunoblots also exhibited high levels of cross-reaction. Sera from rabbits vaccinated with the B and E strains protected mice from challenge with Buffalo B (>50% protection, 1:2 dilution). In many cases protection could be observed when the serum was diluted 1:8. In contrast, sera from rabbits vaccinated with strains of the A and D serotypes produced little, if any protection. In an independent study, boiled antigen extracts from the Carter B strains generated high levels of protective antibody.

Sera from cattle vaccinated with the classical HS strains Katha (0132) and Buffalo B were highly protective in the PMPT (93% and 72% respectively, at a dilution of 1:32). In contrast, serum from cattle vaccinated with a deer strain (0431, Carter A) produced only low levels of protection (57%, 1:2 dilution). These results provide insight into the specificity of antibody that is protective against HS.

THE serological characteristics of *Pasteurella multocida* organisms, like other Gram-negative bacteria, are based on the antigenic properties of the capsular and cell wall components (Manning 1982). During the past 50 years, five main serotyping systems have been developed for classifying organisms of this bacterial species. Isolates have been grouped, using a variety of assay procedures, into as few as 3 and as many as 16 serotypes, with little correlation between each of the systems (Brogden and Packer 1978). However, each of the typing systems has, to some degree, underestimated the antigenic complexity of *P. multocida* as a significant proportion of isolates are untypeable (Manning 1982; Jones et al. 1988).

As a subgroup, *P. multocida* isolates from animals with haemorrhagic septicaemia (HS) are relatively homogeneous (Johnson et al. 1991) and 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 (Brogden and Packer 1979; Bain et al. 1982). Despite the many similarities among HS isolates, Bain (1979) noted that some strains were more efficacious than others in protecting mice against several challenge strains.

While the traditional serological tests highlight antigenic differences between *Pasteurella* strains, little is known of the extent of cross-reaction between isolates. Such information is critical when selecting strains for use in vaccines, particularly the multi-component combination vaccines for commercial use. It is also important for the interpretation of diagnostic tests, like the passive mouse protection test (PMPT) and enzyme-linked immunosorbent assay (ELISA), which are routinely used to assess the immune status of animals against HS.

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The main aim of this study was to examine the antibody responses of rabbits and cattle to vaccination with various *P. multocida* strains using ELISA, immunoblotting and passive protection procedures. Particular emphasis was placed on assessing the extent of cross-reaction with a reference strain Buffalo B, a HS isolate used in the PMPT. A secondary objective was to determine whether boiled antigen extracts, used in both agar gel diffusion and ELISA, were able to elicit a protective antibody response.

Materials and Methods

Bacteria

The *P. multocida* strains used in the study were maintained and grown as described previously (Dawkins et al. 1990; Johnson et al. 1991). *P. multocida* strain 0431 was isolated from fallow deer and has been serotyped as Carter A (Carrigan et al. 1991). 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. *P. multocida* strain 0035 is an untyped haemolytic isolate. The origins, clinical histories and serotypes of the other organisms have been described (Dawkins et al. 1990; Johnson et al. 1991). Boiled antigen (BA) was prepared by the method of Heddleston et al. (1972).

Animals

Cross-bred rabbits (IMVS coloured rabbits, Institute of Medical and Veterinary Science, Adelaide, Australia) were used. The animals had no history of *P. multocida* infections and were serologically negative towards this organism. They were injected subcutaneously with 1.0 mL of *Pasteurella* antigen in a double oil adjuvant vaccine (Dawkins et al. 1990). Serum samples, collected after secondary vaccination, were pooled.

Four Hereford × Angus steers were injected subcutaneously with 2.0 mL killed whole cell *P. multocida* organisms in a double oil adjuvant vaccine. Serum samples were obtained both prior to, and 4 months after, secondary vaccination.

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

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 BA extract (Heddleston et al. 1972). Serial dilutions of test serum were added to the wells, 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. The ELISA titre was assigned as the reciprocal of the highest dilution of serum that gave an absorbance of 0.50 at 414 nm.

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 either stained with Coomassie Blue R250 to reveal protein components, or transferred to nitrocellulose for immunoblotting.

Passive mouse protection test

Groups of at least five mice were injected intraperitoneally with 0.2 mL of test serum, diluted in sterile physiological saline. Twenty-four hours later, the mice were challenged with 100 viable Buffalo B organisms, prepared from appropriately diluted log-phase broth cultures. 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 were recorded. In order to quantify the protective capacity of serum, protection was expressed as a protective index (PI), where PI = $\log_2$ serum dilution + proportion of mice surviving.

Results

Response of rabbits to whole cell vaccines

Rabbits were injected with whole-cell vaccines (WCV) using *P. multocida* strains of the Carter A, B, D and E serotypes, and the resultant antibody response was measured using an ELISA (Table 1). All rabbits responded immunologically to the whole-cell *Pasteurella* vaccines, with elevated titres of serum antibodies to the vaccinal strain. Although all antisera also reacted with the antigen extract from Buffalo B, the extent of cross-reaction was most evident in antisera raised against the Carter B and E serotypes. For example, antisera to Insein (0019) and Bunia 10 (0152) showed significant cross-reaction with the challenge antigen. In contrast, antiserum raised against *P. multocida* organisms of the A and D serotypes had relatively low titres against Buffalo B. However, despite these trends, marked differences were noted in the extent of cross-reaction between isolates of the same serotype.

Table 1. Serum antibody titres to *Pasteurella* extracts in rabbits vaccinated with various whole-cell vaccines (WCV). Challenge antigen was extracted from Buffalo B while vaccinal antigen was prepared from the organisms used in each of the *Pasteurella* vaccines. ND denotes serotype not determined, while * indicates sera that were not titrated to end-point values.

Rabbit serum	*Pasteurella* vaccine	Carter type	ELISA titre		Protective index
			Vaccinal antigen	Challenge antigen	
NRS	N/A	N/A	N/A	<200	0
Rb 75	0246 WCV	A	21 000	5 800	0
Rb 76	0347 WCV	A	9 200	3 100	0.2*
Rb 610	0019 WCV	B	16 500	15 000	>4.0*
Rb 615	0130 WCV	B	115 000	15 500	>5.0*
Rb 68	0132 WCV	B	40 000	25 000	>4.0
Rb 73	0243 WCV	B	17 000	17 000	3.7
Rb 77	0348 WCV	B	30 000	19 000	1.7
Rb 72	0288 WCV	D	25 600	1 150	0.1
Rb 74	0349 WCV	D	20 000	4 000	0
Rb 63	0152 WCV	E	155 000	115 000	4.4
Rb 79	0245 WCV	E	6 000	3 000	1.4
Rb 78	0350 WCV	E	20 000	9 000	2.1
Rb 611	0035 WCV	ND	190 000	1 900	0.0

The close relationship between strains of the B and E serotypes was most evident when these rabbit antisera were tested in the PMPT. Antiserum raised against organisms of these serotypes protected mice against challenge with Buffalo B (Table 1). In many cases, protection was still evident when the serum was diluted 1:8. In contrast, serum from rabbits vaccinated against *P. multocida* strains of the Carter A (0246, 0347) and Carter D (0288, 0349) serotypes conferred little, if any, protection against challenge. Normal rabbit serum failed to protect mice.

The specificity of the antisera was analysed using electrophoretically separated fractions of Buffalo B lysate, bound to nitrocellulose strips. Representative immunoblots, shown in Figure 1, indicated that antibodies raised against WCV of each serotype reacted with many of the protein bands in the bacterial lysate. Although some differences were noted between individual sera, no band was identified which correlated with resistance to challenge.

Response of rabbits to crude LPS extract

All six rabbits responded immunologically to BA extracts, with elevated titres of serum antibodies to the homologous antigen (Table 2). Serum from the rabbits injected with BA from the five Carter B strains, Insein (0019), Izatnager (0130, 131 [avirulent]), Katha (0132) and Buffalo B (0332) all protected mice from challenge with Buffalo B (Table 2). However, serum from the rabbit injected with the BA from Bunia 10 (0152, an African HS strain of the Carter E serotype) did not protect mice from challenge, despite an apparently high level of cross-reactive antibodies in the ELISA assay. Antibodies in the serum of a rabbit vaccinated with the non-HS *Pasteurella* (0035) extract did not react with Buffalo B extract and failed to protect mice from challenge (Table 2).

Response of cattle to vaccination

Predictably, all steers responded to vaccination with the appropriate *Pasteurella* vaccines (Table 3). However, serum from cattle vaccinated with the classical HS strains Katha (0132) and Buffalo B had increased titres of antibody to Buffalo B, in comparison with serum from a steer vaccinated against the Carter A strain (0431). Antisera to both HS strains were highly protective in the PMPT (Table 3). In contrast, serum from the steer vaccinated with the Carter A strain was only moderately protective, while normal cattle serum (NCS) was not protective.

Discussion and Conclusion

The results of this study provided evidence of cross-reaction between *P. multocida* strains of the B and E serotype, which confirmed and extended the observations of Bain (1979).

Serum from rabbits vaccinated with B and E strains conferred significant protection against HS challenge; in many cases protection was evident at serum dilutions >1:8. Although, the ELISA results also provided evidence of cross-reaction between isolates of the B and E serotypes, the pattern of reactivity observed was complex. Variations in the extent of cross-reaction between isolates of the same

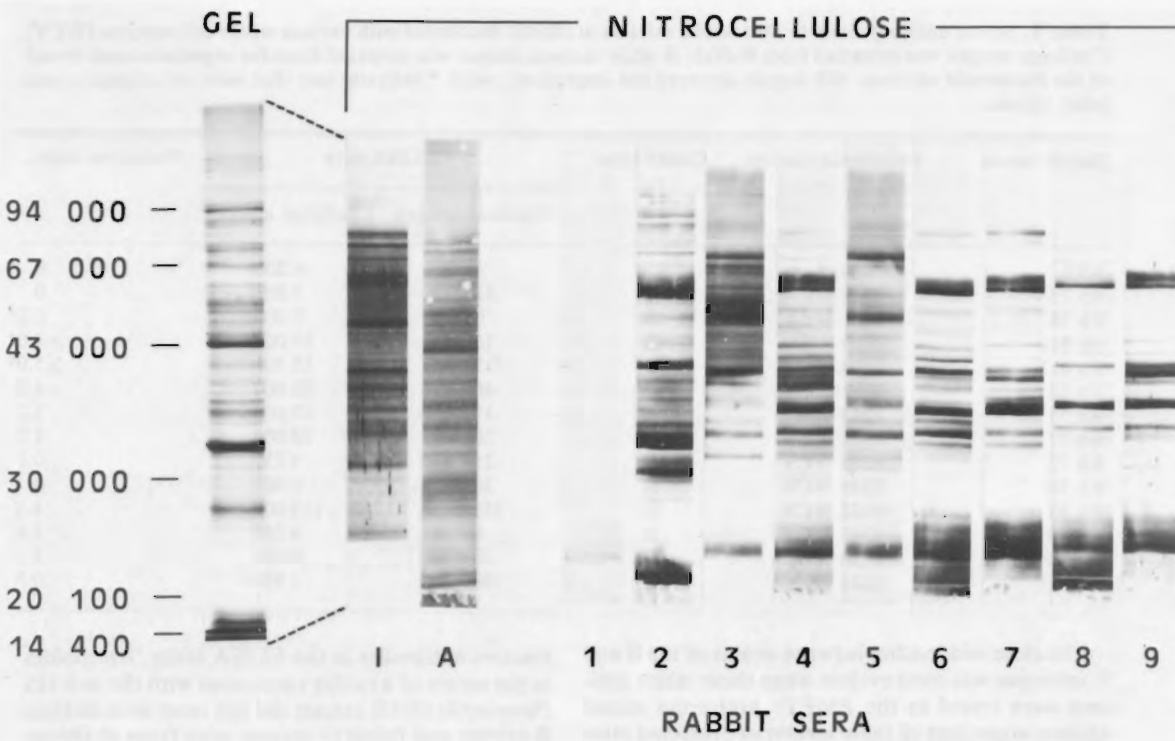

Fig. 1. Comparison of the antibody specificities in rabbit antisera. Lanes: 1, normal rabbit serum; 2, Rb-75; 3, Rb-73; 4, Rb-615; 5, Rb-72; 6, Rb-63; 7, Rb-79; 8, Rb-64; 9, Rb-713. Relevant controls include low molecular weight markers (Pharmacia: lactalbumin [14 400]; trypsin inhibitor [20 100]; carbonic anhydrase [30 000]; ovalbumin [43 000]; albumin [67 000] and phosphorylase b [94 000]), and Buffalo B lysate fractionations on acrylamide gel stained with Coomassie Blue and nitrocellulose strips stained with indian ink (I) and amido black (A).

Table 2. Serum antibody titres to *Pasteurella* extracts in rabbits vaccinated with various boiled antigen vaccines (BAV). Challenge antigen was extracted from Buffalo B while vaccinal antigen was prepared from the strains used in each of the *Pasteurella* vaccines. ND denotes serotype not determined, while * indicates sera which were not titrated to end-point values.

Rabbit serum	*Pasteurella* vaccine	Carter type	ELISA titre		Protective index
			Vaccinal antigen	Challenge antigen	
NRS	N/A	N/A	N/A	<200	0
Rb 64	0019 BAV	B	310 000	270 000	4.1
Rb 612	0130 BAV	B	110 000	86 000	>4.0*
Rb 69	0131 BAV	B	32 000	19 500	>3.0*
Rb 613	0132 BAV	B	35 000	39 000	1.9
Rb 713	0332 BAV	B	17 500	17 500	>3.0*
Rb 614	0152 BAV	E	70 000	39 000	0
Rb 616	0035 BAV	ND	190 000	320	0

Table 3. Serum antibody titres to *Pasteurella* extracts in cattle vaccinated with various whole-cell vaccines (WCV). Challenge antigen was extracted from Buffalo B and vaccinal antigen was prepared from the strains used in each of the *Pasteurella* vaccines.

Bovine serum	*Pasteurella* vaccine	Carter type	ELISA titre		Protective index
			Vaccinal antigen	Challenge antigen	
NCS	N/A	N/A	N/A	400	0
B 1	0132 WCV	B	45 000	34 000	5.9
B 3	0332 WCV	B	18 000	18 000	5.7
B 4	0431 WCV	A	20 000	640	1.6

serotype may be due either to possible antigenic differences between HS isolates or to differences in the composition of the various BA extracts. Boiled antigen is a crude LPS extract that is known to contain protein (e.g. 0332 BA contains approximately 120 μg protein/mL).

Antiserum raised in cattle against *P. multocida* of the A serotype protected mice from challenge with Buffalo B. Although the PI was only 1.57, it was nevertheless significant. *P. multocida* of the A serotype are relatively common pathogens of cattle (Wijewardana et al., these Proceedings), and exposure to these organisms may contribute to natural resistance against HS. High levels of natural resistance to HS have been reported, particularly in endemic areas (Bain et al. 1982).

In this study, the PMPT proved to be a highly specific test for assessing antibody responses to HS organisms. The results obtained emphasise the importance of titration when interpreting PMPT data. Dilution of sera permits discrimination between low levels of natural immunity in un-vaccinated animals and high titre vaccinal responses (e.g. Johnson et al., these Proceedings). Titration also allows vaccinal responses to be assessed quantitatively.

Boiled antigen extracts were highly immunogenic and elicited high titre antibody responses that compared favourably to those observed with WCV. The broad pattern of antibody reactivity seen in rabbits vaccinated with BA (Figure 1) indicates that the proteins associated with these crude extracts elicited significant responses, despite their relatively low concentration. Antibodies to BA extracts from Asian HS strains were protective in the PMPT. It is tempting to suggest that such potent immunogens should be considered for use in HS vaccines.

These studies highlight the complexity involved in analysing immune responses to bacteria. The results demonstrate significant cross-reaction between *P. multocida* isolates of the Carter B and E serotypes. Continuation of this work may provide further insights into the specificity of antibody(s) responsible for protection against HS.

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Characterisation of the Immune Response and Duration of Immunity in Buffalo Vaccinated with Cellular Haemorrhagic Septicaemia Vaccines

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Abstract

The serum antibody response of buffalo immunised with three conventional haemorrhagic septicaemia (HS) vaccines — broth bacterin (BB), alum-precipitated vaccine (APV) and oil adjuvant vaccine (OAV) — and one experimental double emulsion vaccine (DEV) was determined by enzyme linked immunosorbent assay (ELISA), employing lipopolysaccharide (LPS) and boiled or formalin-killed *Pasteurella multocida* type 6:B as antigens. Antibody levels were significantly higher in buffalo immunised with the adjuvanted vaccines (APV, OAV, DEV) than in those immunised with BB alone. Buffalo immunised with BB alone were protected at 6 weeks, but not at 12 weeks, post-immunisation. Buffalo immunised with APV were protected at 24 weeks, but only partially at 52 weeks, post-immunisation. There also appeared to be a relationship between the ELISA titres and protection in buffalo.

All vaccinated buffalo (with the exception of the BB group at 3 months post-immunisation) developed substantial to significant cutaneous delayed-type hypersensitivity reactions.

HAEMORRHAGIC septicaemia (HS) is an enzootic disease of cattle and buffalo in many parts of Asia and Africa. It is caused by *Pasteurella multocida* type 6:B in Asia and type 6:E in Africa. No statistics are available on the annual loss due to the disease in Asia, but it has been estimated at around 100 000 animals (Bain et al. 1982). Although immunisation with the currently available vaccines has reduced HS deaths considerably, there are some major problems encountered in the use of these vaccines. For example, lack of availability of methods for evaluation of the potency of different vaccine batches; occasional breakdowns in immunity in areas covered by vaccination; undesirable side effects, such as post-vaccination shock; and the difficulty of injecting the oil adjuvant vaccine. For these reasons, we have been interested in developing an improved vaccine based on the most potent immunogens.

As a step towards the achievement of this goal, we sought information on the duration of protection and immune response generated as a result of immunisation with vaccines currently in use in the field — broth bacterin (BB), alum-precipitated vaccine (APV) and oil adjuvant vaccine (OAV) — and one experimental double-emulsion vaccine (DEV).

Materials and Methods

Animals

Sixty-seven buffalo, 9–12 months old, were selected on the basis of (a) absence of antibody to *P. multocida* type 6:B measured by indirect haemagglutination (IHA); and (b) a negative passive mouse protection test (PMPT), because of the lack of availability of accepted in vitro indicator tests. All animals were kept at a government animal husbandry station in Malaysia, which had been free of HS outbreaks for the past 10 years.

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Bacterial strain

P. multocida type 6:B strain C82 was used throughout the investigation.

Vaccines, vaccination and experimental challenge

BB, APV and OAV were prepared according to Cheah (1960), Iyer and Rao (1959) and Thomas (1968) respectively. The DEV was prepared according to Herbert (1965). Fifty-one buffalo were vaccinated (10, BB; 13, APV; 16, OAV; 12, DEV) and 16 were used as controls. BB and APV were injected subcutaneously, and OAV and DE intramuscularly, in volumes of 5.0 mL. Vaccinated and control buffalo in groups of two to five animals were challenged at 6 weeks and at 3, 6, 12 and 18 months post-vaccination with 0.5 mL of a 10^{-3} dilution of a 20-h broth culture of *P. multocida* type 6:B strain C82 injected subcutaneously. This dilution contained approximately 3×10^6 colony forming units (CFU), or 300 minimum lethal doses. Challenged animals were observed for 2 weeks, and mortalities were recorded. Moribund buffalo were euthanased and counted as mortalities.

Immunological parameters

Antibody levels and isotype were measured using an enzyme-linked immunosorbent assay (ELISA) as described previously (Mukkur et al. 1991). Antigens used included formalin-killed and boiled intact *P. multocida* type 6:B (1×10^8 CFU per mL), and Westphal liposaccharide (10 μg per mL). Antibody titres were also measured using an indirect haemagglutination (IHA) test. To investigate cutaneous delayed-type hypersensitivity (DTH) of vaccinated and control buffalo, the double skinfold thickness in the caudal fold was measured pre-inoculation, and 48 hours after intradermal inoculation of 0.1 mL of ultrasonicated antigen of *P. multocida* type 6:B strain C82 at a protein concentration of 220 μg per mL. Biopsy specimens of intradermal tissue were taken from representative buffalo showing a significant change in the mean double skinfold thickness to determine the predominant cell type infiltrating the site of inoculation.

Statistical analyses

Student's *t* test was used to determine the significance of differences between the $\log_{10}$ of the mean values for ELISA titres and for the increase in skin thickness measurements. Significance of protection in the immunisation challenge experiments was assessed by Fisher's exact test.

Results

Duration and protection

Results of challenge experiments are shown in Table 1. Buffalo vaccinated with BB were partially protected against experimental challenge at 6 weeks, but not at 12 weeks, post-challenge. On the other hand, buffalo vaccinated with APV were completely protected at 6 weeks and 12 months post-immunisation. Buffalo vaccinated with OAV were completely protected at 6 weeks and at 3, 6 and 12 months post-immunisation against experimental challenge, but were only partially protected at 18 months post-immunisation. Buffalo immunised with DEV were also completely protected at all intervals evaluated, except at 6 weeks post-immunisation, when only partial protection was evident.

All the non-immune control animals succumbed to challenge infection with *P. multocida* type 6:B strain C82 at all intervals evaluated.

Table 1. Protection in buffalo immunised with different types of HS vaccines at various periods post-immunisation.

Challenge interval	Number of buffaloes surviving/ Total number challenged in groups				
	BB	APV	OAV	DEV	Non-immune
6 weeks	3/5	2/3	2/2	1/2	0/4
3 months	0/5	4/4	4/4	4/4	0/4
6 months	ND	4/4	4/4	4/4	0/4
12 months	ND	1/2	3/3	2/2	0/2
18 months	ND	ND	2/3	ND	0/2

BB, broth bacterin
APV, alum-precipitated vaccine
OAV, oil adjuvant vaccine
DEV, double emulsion vaccine
ND, not done

Antibody response

Although there was an increase in the pre-challenge over the pre-vaccination antibody levels in most of the immunised buffalo in all vaccinated groups, statistically significant differences only began to emerge at 3 months post-immunisation (Table 2). The average antibody titres measured in the case of OAV and DEV reached maximum levels at 3–6 months post-immunisation, followed by a drop at 12 months post-immunisation. On the other hand, the average antibody titres in buffalo immunised with APV reached maximum levels at 3 months post-

Table 2. Antibody response of buffalo immunised with various HS vaccines.

Period post immunisation	Vaccine	ELISA antibody titres against antigen					
		FKWC		BWC		LPS	
		Pre-vacc	Pre-chall	Pre-vacc	Pre-chall	Pre-vacc	Pre-chall
3 months	BB	60 ± 14	84 ± 13	81 ± 25	122 ± 22	119 ± 15	225 ± 114
	APV	155 ± 111	369 ± 279	126 ± 79	355 ± 167	121 ± 40	187 ± 66
	DEV	69 ± 30	405 ± 104*	127 ± 42	509 ± 107*	145 ± 54	548 ± 42*
	OAV	124 ± 51	451 ± 99*	171 ± 48	621 ± 67*	580 ± 303	2508 ± 1235*
nonimmunised		—	142 ± 60	—	136 ± 38	—	137 ± 20
6 months	BB	ND		ND		ND	
	APV	62 ± 55	692 ± 286*	192 ± 152	824 ± 90*	110 ± 54	420 ± 200*
	DEV	156 ± 98	965 ± 409*	156 ± 59	578 ± 250*	125 ± 31	438 ± 110*
	OAV	31 ± 18	231 ± 71*	25 ± 11	358 ± 59*	105 ± 73	691 ± 337*
nonimmunised		—	153 ± 70	—	174 ± 65	—	217 ± 92
12 months	BB	ND		ND		ND	
	APV+	123	339	65	288	133	433
	DEV+	187	331	161	190	268	1075
	OAV	139 ± 49	542 ± 311*	74 ± 6	558 ± 238*	109 ± 11	764 ± 403
nonimmunised		—	115	—	110	—	141

BB, broth bacterin
APV, alum-precipitated vaccine
OAV, oil adjuvant vaccine
DEV, double emulsion vaccine
FKWC, formalin-killed whole *P. multocida* type 6:B cells
BWC, boiled *P. multocida* type 6:B cells
LPS, *P. multocida* type 6:B lipopolysaccharide
ND, not done
+ average ELISA titres from two buffalo
* statistically significant at $P<0.05$

immunisation, followed by a drop at 6 months post-immunisation. Preliminary analysis of the antibody isotype in the pre-challenge sera of buffalo at 6 months post-immunisation with OAV revealed the predominant isotypes to be IgG_1 and IgG_2 (Table 3).

Table 3. Antibody isotypes in prechallenge immunoserum obtained from buffalo vaccinated with oil adjuvant vaccine at 6 months post-immunisation.

Serum fraction	Mean percent antibody isotype
IgM	7
IgG_1	48
IgG_2	42
IgA	3

Cutaneous delayed-type hypersensitivity response

With the exception of the BB group at 3 months post-immunisation, vaccinated animals developed substantial to significant cutaneous DTH response (Table 4). Histopathological examination of selected biopsy specimens taken from the site of inoculation showed a predominance of infiltrating lymphocytes, thus confirming the response to be a DTH response (figure not shown).

Discussion

These results provide direct experimental evidence to support previously stated claims that the oil adjuvanted vaccines provided better protection than the APV (Bain et al. 1982). Protection of buffalo vaccinated with BB was partial at 6 weeks post-immunisation and did not last beyond this period.

Table 4. Cutaneous delayed-type hypersensitivity response in immunised and non-immune buffalo.

Type of vaccine used for immunisation	Change in double skinfold thickness (Mean ± SEM) at 48 hours post-intradermal inoculation with soluble ultrasonicated antigen of *P. multocida* type B at weeks (W) or months (M) post-vaccination			
	6W	3M	6M	12M
BB	1.0*	0.6 ±0.3+	ND	ND
APV	3.2	2.6 ± 0.9	2.1 ± 0.4	0.2
OAV	2.6	2.9 ± 0.5	2.1 ± 1.1	6.8 ± 2.6
DEV	4.3	2.6 ± 0.9	1.1 ± 0.6	2.45
Non-immunised	0	0.3 ± 0.01	0.02 ± 0.01	0

BB, broth bacterin
APV, alum-precipitated vaccine
OAV, oil adjuvant vaccine
DEV, double emulsion vaccine
ND, not done
* All figures shown without SEM represent the mean of data from 2 buffaloes only.
+ Statistical significance: All DTH data shown as means ± SEM, with the exception of BB at 3 months post-vaccination, was significantly different from that observed in non-immune control buffaloes ($p<0.05$).

Buffalo immunised with DEV were also protected up to 12 months post-vaccination, although one of the two vaccinated buffalo succumbed to challenge infection at 6 weeks post-immunisation. A similar finding was reported by Neramitmansook et al. (1990). However, because of the partial protection observed, albeit with a small number of buffalo, it is desirable to confirm this observation due to the practical limitations in the use of such a vaccine in the field. Reason for this occurrence is not clear, as the animal in question had a reasonably high antibody titre and DTH reactivity (individual animal data not shown).

An examination of the overall antibody titres in buffalo immunised with various types of HS vaccines revealed that statistically significant differences between immunised versus non-immune animals began to become evident at 3 months post-immunisation. However, comparative analysis of the cumulative antibody titres of pre-challenge sera of vaccinated buffalo that survived or succumbed to experimental challenge revealed a statistically significant difference between the two groups (Table 5). This further confirmed our previous observation that there appeared to be a relationship between ELISA antibody titres and active protection in buffalo (Chandrasekaran et al., these Proceedings).

It could be argued that antibody titres of buffalo immunised in this investigation represented secondary responses. This is because IHA and PMPT used to screen the animals have been shown to be unrelated to active protection in buffalo. In this regard, it is important to note that only 4 of 67 buffalo used in the investigation had high pre-vaccination ELISA titres. Whether this was due to exposure to some other *Pasteurella* species is not clear at the present time — the farm of origin of the buffalo has been free from outbreaks of HS for the past 10 years.

Table 5. Analysis of cumulative serum ELISA antibody titres of vaccinated buffalo that either survived or succumbed following experimental challenge.

Antigen used for coating	ELISA antibody titres in vaccinated buffaloes following experimental challenge			
	Survived		Succumbed	
	Pre-vacc	Pre-chall	Pre-vacc	Pre-chall
FKWC	173 ± 46	551 ± 84*	181 ± 52	274 ± 84
BWC	161 ± 30	488 ± 45*	144 ± 44	224 ± 59
LPS	249 ± 59	752 ± 169*	255 ± 96	437 ± 110

Abbreviations: Pre-vacc and Pre-chall denote pre-vaccination and pre-challenge respectively
* Statistically significant at $p<0.05$

Another interesting find was an apparent relationship between the DTH response and protection in buffalo vaccinated with different HS vaccines, although one of the two buffalo in the DEV group that succumbed to challenge did develop substantial DTH reactivity (4.2 mm). For example, there was no significant difference in the DTH reactivity

between buffalo in the BB group and the non-immunised controls, and they succumbed to challenge infection. Although the comparative role played by IgG_1 versus IgG_2 in the serum of immunised buffalo will need to await the availability of a suitable in vitro assay system, it is clear that any new generation adjuvant may be required to promote the stimulation of not only the appropriate antibody isotype, but DTH reactivity as well.

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Recombinant Vaccines for *Pasteurella haemolytica*

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Abstract

Pasteurella haemolytica vaccines have been used extensively in North American feedlots for a number of years with little impact on the incidence of fibrinous pneumonia, due to a combination of poor management factors and ineffective vaccines. In an effort to develop improved vaccines, we have cloned and expressed the genes coding for leukotoxin and outer membrane proteins in *Escherichia coli*. Vaccination of calves with recombinant leukotoxin, alone and in combination with cellular antigens, significantly reduced morbidity and mortality under experimental and field conditions. Both the full-length molecule and the amino terminal portion effectively induced toxin-neutralising antibodies, and the titre was inversely proportional to the clinical score of challenged animals. Chimeric molecules consisting of leukotoxin plus bovine cytokines or antigenic peptides were also constructed. These fusion proteins were shown to retain the activities of both moieties. These results indicate that recombinant leukotoxin can be used both as an effective immunogen against pneumonic pasteurellosis and as a carrier molecule for the delivery of other polypeptides.

BOVINE respiratory disease (BRD) is an economically important disease complex costing North American beef producers US$0.5bn annually. While the disease is caused by a combination of bacterial and viral infectious agents, stress, and management factors, the principal organism associated with mortality is *Pasteurella haemolytica* serotype 1. Vaccination has been practised for a number of years with little impact on the incidence of disease. One reason for the lack of success of conventional products is that many do not induce leukotoxin (Lkt)-neutralising antibodies, which we feel are necessary for the prevention of pathology associated with *P. haemolytica* infection. The structural gene for leukotoxin (*lktA*) has been cloned and characterised, and therefore could be used for the development of a recombinant subunit vaccine. One vaccine trial using recombinant leukotoxin has been reported, and it was suggested that leukotoxin alone could not induce protective immunity (Conlon et al. 1991).

In this paper, we show that leukotoxin-neutralising antibodies are sufficient for protection against experimental challenge, and that vaccination with leukotoxin plus cellular antigens significantly reduces morbidity and mortality in the field after a single immunisation. Further, the leukotoxin gene can be fused to genes coding for other antigens or biological response modifiers for use in the development of multivalent recombinant vaccines.

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Materials and Methods

Bacterial strains

P. haemolytica serotype 1 strain B122 was isolated from the lung of a calf that died of fibrinous pneumonia (Potter et al. 1988). *Escherichia coli* strains JM105 and W1485 were used as hosts for recombinant DNA work. The media and growth conditions for *P. haemolytica* and *E. coli* strains have been described elsewhere (Potter et al. 1988; Theisen and Potter 1992).

Recombinant DNA techniques

All enzymes and biological reagents were purchased from Pharmacia Canada and were used as recommended by the manufacturer. Other methods have been described previously (Theisen and Potter 1992).

Antigen preparation

The leukotoxin::β-galactosidase fusion protein (Lkt::LacZ) was purified by affinity chromatography using a commercially available column as recommended by the manufacturer (Promega). Full-length recombinant leukotoxin was produced as inclusion bodies. These were extracted with detergent, solubilised and refolded (Harland et al., unpublished). Sodium chloride extracts of *P. haemolytica* and authentic leukotoxin were prepared according to Styrt et al. (1990) and Harland et al. (unpublished).

Immunological techniques

Methods for serological analysis by an enzyme-linked immunosorbent assay (ELISA) will be described elsewhere (Harland et al., unpublished). The anti-leukotoxin titres are presented as the reciprocal of the highest dilution giving an absorbance value greater than the mean background plus two standard deviations. Methods for the assessment of cytokine function included proliferation assays plus precursor frequency analysis (interleukin-2, Hughes et al. 1992) and MHC class II expression plus antiviral assays (interferon-gamma, Hughes et al. 1991).

Vaccine trials

For small-scale vaccine trials, 5–10-month-old calves were purchased from farms in Western Canada. Animals were vaccinated twice by intramuscular injection, 3 weeks apart. Ten days after the second vaccination, they were exposed to an aerosol of bovine herpesvirus-1 (BHV-1) followed by an aerosol of *P. haemolytica* 4 days later. Daily examinations were carried out for 10 days, and each animal was assigned a daily score based on clinical signs of disease. These scores were ranked in order and averaged by group to produce the clinical rank scores presented in Table 1.

A field trial was carried out at a Western Canadian feedlot using recently weaned calves. Animals were randomly assigned to one of four groups at entry. Groups received the *P. haemolytica* vaccine plus a modified live BHV-1 vaccine (MLV), *P. haemolytica* vaccine plus subunit BHV-1 vaccine, BHV-1 MLV or BHV-1 subunit vaccines.

Calves with a temperature greater than 40.5 °C on arrival were excluded from the trial. Calves were checked daily by experienced feedlot personnel for clinical signs of BRD. Blood samples were collected from 5% of the calves at the time of vaccination, and 30 days later, for serological analysis.

Table 1. Clinical and serological responses of cattle to vaccination with (a) authentic and recombinant leukotoxin, and (b) various doses of recombinant leukotoxin.

Group	Clincal rank score	Anti-leukotoxin titre
(a) Placebo	39.2[a]	1 997[a]
Lkt::LacZ	17.3[b]	77 400[b]
Authentic Lkt	20.0[b]	77 000[b]
Extract	15.8[b]	1 152[a]
Lkt + Extract	14.8[b]	93 333[b]
(b) Placebo	21.3[a]	1 305[a]
Lkt::LacZ 100 μg	10.7[b]	42 750[b]
Lkt::LacZ 50 μg	18.7[a]	34 333[b]
Lkt::LacZ 25 μg	22.3[a]	15 117[a,c]
Lkt::LacZ 12.5 μg	26.3[a]	7 833[a,c]

Values with different letters in each column are significantly different ($P < 0.05$).
The adjuvant used in the first vaccine trial (a) was Avridine, while Emulsigen Plus was used in the second trial (b). The clinical rank scores are mean values calculated as described in Materials and Methods.

Results

Cloning and expression of *lktA*

Recombinant clones containing the leukotoxin gene were isolated from a genomic library based on reaction with bovine sera against a *P. haemolytica* culture supernatant. The gene was expressed as a fusion of the amino terminal 376 amino acids to β-galactosidase (pAA101) and as a full-length molecule (pAA352; Fig. 1). Soluble protein was affinity purified from *E. coli* JM105/pAA101 using a monoclonal antibody against the β-galactosidase moiety, while full-length leukotoxin was produced from W1485/pAA352 as inclusion bodies after induction with isopropyl-D-thiogalactopyranoside (IPTG). Inclusion bodies were solubilised with guanidine hydrochloride prior to use.

Protective capacity of a leukotoxin::β-galactosidase fusion protein

In the first vaccine trial, groups of eight calves were vaccinated twice with 100 μg Lkt::LacZ, authentic leukotoxin, an NaCl extract of *P. haemolytica*, an NaCl extract supplemented with authentic leukotoxin, or a placebo, with Avridine as an adjuvant. Following challenge with BHV-1 and *P. haemolytica*, clinical signs of disease were significantly reduced in all vaccinated animals (Table 1) and the only groups exhibiting any mortality were those vaccinated with the placebo (83%) and the NaCl extract (16.6%). Therefore, both authentic and recombinant leukotoxins were equally effective antigens.

A second vaccine trial using recombinant Lkt::LacZ was conducted in which two of the groups (10 calves each) received 100 μg of Lkt::LacZ or a placebo, and the other three groups (6 calves each) received 50, 25, or 12.5 μg fusion protein. In this case, an oil-based adjuvant (Emulsigen Plus) was used. From the results, (b) in Table 1, it can be seen that there was an inverse correlation between the serological response to vaccination and the clinical rank score.

A third trial was conducted using the full length recombinant leukotoxin combined with *P. haemolytica* surface antigens with similar results (data not shown). Therefore, both the Lkt::LacZ fusion protein and the full length recombinant molecules were effective immunogens, and vaccination with these proteins alone or in combination with *P. haemolytica* surface antigens was sufficient for protection against experimental challenge.

Field trial of recombinant leukotoxin

Recombinant leukotoxin (full length) plus a *P. haemolytica* saline extract were mixed with Emulsigen Plus so that each 2 mL dose contained 100 μg of Lkt and 50 μg of extract. Recently weaned calves were vaccinated at entry into a Western Canadian feedlot as described in Materials and Methods. The results (Table 2) show that animals vaccinated with the leukotoxin-based vaccine and a subunit BHV-1 vaccine had significantly lower morbidity and mortality than all other groups. However, when a BHV-1 MLV vaccine was used, less protection was observed, indicating that this vaccine interfered with the response to the *P. haemolytica* vaccine. This is reflected by the lower anti-leukotoxin ELISA titres in this group compared to those in calves that received the BHV-1 subunit preparation (Table 2).

Construction of leukotoxin gene fusions

Gene fusions consisting of the *lktA* gene plus genes coding for bovine interleukin-2 (IL-2), interferon gamma (IFN), somatotropin release inhibitory factor (SRIF), gonadotropin release hormone (GnRH), and a rotavirus VP4 epitope were constructed by cloning the appropriate gene or oligonucleotide downstream of *lktA* in the BamHI site of plasmid pAA352 (Fig. 1). In the case of the cytokines, the fusion proteins retained activity in vitro and in vivo. For example, the responsiveness of peripheral blood mononuclear cells (PBMC) to recombinant bovine IL-2 and the Lkt::IL-2 fusion was 1 in 705 and 1 in 687 cells respectively, as assessed by precursor frequency analysis.

The Lkt::IFN chimera induced MHC class II expression and antiviral activity at levels comparable to bovine IFN. Animals vaccinated with these proteins also developed comparable levels of leukotoxin-neutralising antibodies to animals vaccinated with leukotoxin alone. Therefore, both the Lkt and cytokine portions of these chimeric molecules were active. Likewise, animals vaccinated with the SRIF and VP4 fusions developed antibody against both moieties of these fusion proteins (data not shown). Therefore, leukotoxin may be useful for the development of multivalent vaccines that utilise cytokines for adjuvant activity (IL-2 or IFN) and contain other relevant antigens from bovine pathogens.

Discussion and Conclusions

We have shown that the *P. haemolytica* leukotoxin is protective against experimental challenge with *P. haemolytica* and that protection can be obtained with only the amino terminal 376 amino acids of the molecule. This is in contrast to a recent paper by

Table 2. Clinical and serological responses of cattle to vaccination in the field with a leukotoxin-based vaccine.

Vaccine[1]	No.[2]	Mortality[3]	Morbidity[3]	Anti-leukotoxin titre
PH + Subunit BHV	585	0[a]	119[a]	38 520[a]
PH + MLV BHV	583	5[b]	140[b]	22 993[b]
Subunit BHV	579	6[b]	147[b]	17 050[b,c]
MLV BHV	577	6[b]	154[b]	13 771[c]

[1] PH, Lkt + extract
subunit BHV, subunit BHV-1 vaccine
MLV BHV, modified live BHV1 vaccine
[2] No., number of animals per group
[3] Mortality due to fibrinous pneumonia; morbidity due to BRD only
Values in each column with different letters are significantly different ($P < 0.05$)

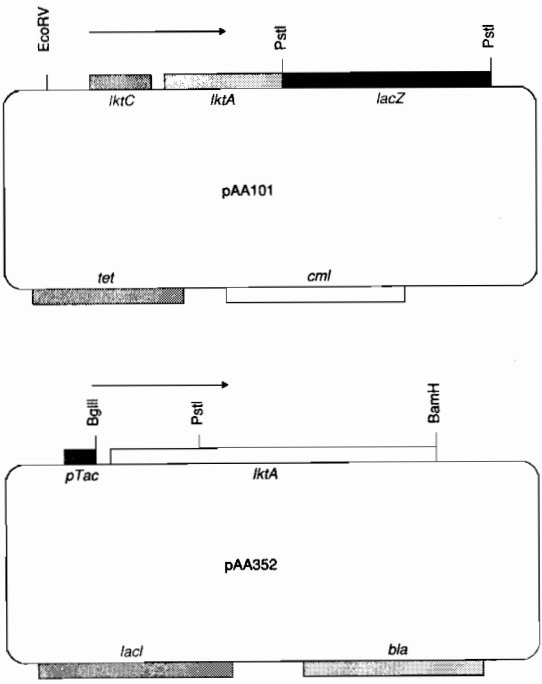

Fig. 1. Structure of recombinant plasmids expressing the leukotoxin gene as (a) a β-galactosidase fusion protein, or (b) a full-length molecule.

lktA,C, P. haemolytica leukotoxin genes
lacZ, β-galactosidase
tet, tetracycline-resistance
cml, chloramphenicolresistance
pTac, tac promoter
bla, β-lactamase

Conlon et al. (1991) in which they reported that recombinant leukotoxin did not protect against experimental challenge unless it was mixed with cellular antigens. However, they did not fully describe the dose of Lkt used or the serological response to vaccination and therefore it is difficult to determine whether there was any correlation between anti-Lkt titres and clinical scores. We found an inverse correlation between the serological response to vaccination and clinical rank score ($P < 0.05$). In the field, vaccination with recombinant leukotoxin combined with *P. haemolytica* surface antigens significantly reduced BRD morbidity and mortality due to fibrinous pneumonia (Table 2). However, when a modified live viral vaccine was administered at the same time, lower seroconversion rates and significantly higher morbidity and mortality were observed. These data indicate that either the MLV vaccine interfered with the response to the *P. haemolytica* vaccine or that the BHV-1 subunit vaccine had immunostimulatory properties. The latter possibility can be ruled out, based on previous studies with the two vaccines (Harland et al., unpublished).

Several chimeric proteins were constructed by fusing *lktA* to genes or oligonucleotides coding for bovine IL-2, IFN, SRIF, GnRH and rotavirus VP4. In the case of the cytokine fusions, both moieties of the molecules were functional. Vaccination of calves with multiple doses of the Lkt::IL-2 chimera induced a higher level of cellular immunity than Lkt alone. This did not result in an increased serological response to Lkt as one might expect (Weinberg and Merigan 1988), probably due to the low quantity of IL-2 used in these experiments (data not shown). However, since the amino terminal portion of Lkt is protective, the molar ratio of IL-2 to Lkt in the fusion protein can be increased by fusing only this part of the leukotoxin gene to the bovine IL-2 gene. Another way of increasing the dose would be to add exogenous IL-2 to the formulation or clone multiple copies of the IL-2 gene into the construct. Whichever method is used, it is clear that the *P. haemolytica* leukotoxin may be useful for the development of multivalent vaccines containing cytokines for adjuvant activity plus heterologous antigens.

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Isolation, Characterisation and Vaccination Efficacy of a Temperature-Sensitive Mutant of *Pasteurella multocida* Type B

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Abstract

It has been possible to protect calves successfully against a lethal challenge infection with a temperature-sensitive mutant of *Pasteurella multocida* type B after having immunised the animals intratracheally four times. After the infection, the immunised animals did not show pneumonic changes.

Up until now, there has been little experience in the characterisation and use of live vaccines against *Pasteurella* infections of domestic animals. Results of vaccine field trials with streptomycin-dependent (Smd) mutants of *Pasteurella multocida* type B were published by De Alwis and Carter (1980) and Wei and Carter (1978). An Smd mutant was also used to control *Pasteurella* infections in the rabbit, with good results (Chengappa and Carter 1980; Lu and Pakes 1981). Schimizu et al. (1987) described the isolation and characterisation of *P. multocida* type D temperature-sensitive (ts) mutants, but did not present results concerning their tolerability and efficacy as vaccines. In poultry, live vaccines have been used successfully against acute *Pasteurella* infections (Bierer and Derrieux 1972; Sulong and Maheswaran 1976).

The research presented here concerns the isolation, characterisation and efficacy of a ts mutant of *P. multocida* type B.

Materials and Methods

The ts mutant was isolated from a *P. multocida* type B field strain after treatment with N-methyl N-nitrosoguanidine and examination of the isolated clones for growth characteristics at different temperatures.

The laboratory animals were ICR mice of 16–20 g bodyweight; and black-spotted, dairy cattle calves aged 2–3 weeks.

The growth characteristics of the organism were tested at different temperatures (33, 37, 39, 41 and 43 °C) over 50 culture-medium passages. Virulence attenuation was investigated after isolation, as well as after the 10th, 30th and 50th passages in ICR mice after intraperitoneal administration of 1×10^8 bacteria.

Testing for tolerability was combined with testing for efficacy. Calves were immunised intratracheally two or four times with $1\text{–}3 \times 10^9$ bacteria, observed clinically, and challenged intratracheally with 3×10^9 bacteria 2 weeks after the last immunisation. Groups of animals to which sterile broth had been administered at the respective immunisation times served as controls.

Results and Discussion

The ts mutant of a *P. multocida* strain serotype B proved to be stable in growth properties and virulence, even after numerous passages. After intratracheal administration, bacterial counts of $1\text{–}3 \times 10^9$ per dose were well tolerated without clinical

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reactions. Intratracheal immunisation of calves with the ts mutant repeated twice protected them from lethal challenge infection, but not against the development of pneumonic changes. Intratracheal immunisation repeated four times also prevented pneumonia after the challenge infection.

According to our knowledge, reports on the application and effect of a live antigen for immunoprophylaxis of the B infection are available by De Alwis and Carter (1980), who used an Smd vaccine. They administered 3×10^{10} bacteria subcutaneously to calves and buffalo and could establish differences in the immune reactions of calves with the aid of the passive mouse protection test, depending on the bacterial concentration and frequency of administration. There was no challenge. Chengappa et al. (1980) reported good results after administration of a Smd vaccine type A in rabbits, even after challenge.

The present study indicated that a temperature-sensitive mutant of *P. multocida* type B could be produced and used in live form to protect against homologous challenge in vaccinated calves. This vaccine may have a role to play in future outbreaks of type B pasteurellosis, both in Europe and in other regions of the world.

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Studies on a Locally Produced Haemorrhagic Septicaemia Vaccine: Duration of Immunity and Safety in Cattle, Buffalo and Goats

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Abstract

In the Philippines, haemorrhagic septicaemia remains an endemic disease causing major economic loss. Its acute nature results in heavy mortalities, primarily in buffalo (carabaos) and cattle. Antibiotics are effective only if given at the early stage of the disease.

The National Animal Disease Situation Report of 1990, by the Bureau of Animal Industry, reported that 17 720 buffalo, 14 331 cattle and 3852 goats/sheep were affected by haemorrhagic septicaemia, with mortalities of 1725, 1057 and 485 respectively.

Locally produced alum-adjuvanted bacterins are being used in the field as prophylactic agents. These bacterins have been prepared using locally isolated strains of *P. multocida* Carter's serotype B.

A study on the duration of immunity and safety of a local fermentor-produced haemorrhagic septicaemia vaccine was conducted using 13 cattle, buffalo and goats. Three cattle, three buffalo and four goats were vaccinated intramuscularly or subcutaneously according to label recommendations. Three buffalo served as unvaccinated controls. Only one batch of vaccine was used.

Safety tests on host and laboratory animals showed no adverse reaction to the vaccine. Results of the passive mouse protection test showed that the duration of immunity in buffalo was at least 4 months, cattle gave inconsistent results, while immunity in goats lasted for 7 months.

It is recommended that the vaccine is used in accordance with the instructions on the label in order to produce maximum protection.

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Evaluation of an Oil Adjuvant Vaccine for Control of Pneumonic Pasteurellosis in Sheep

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Abstract

A trial was conducted to determine the ability of a local vaccine to control experimental pneumonic pasteurellosis in sheep. Sixteen crossbred lambs of about 7 months old were selected and divided into four groups. Lambs in the first two groups were vaccinated intramuscularly with a formalin-killed oil adjuvant vaccine containing a local isolate of *P. haemolytica* A7. Four weeks following the vaccination, lambs in groups one and three were challenged intra-tracheally with *P. haemolytica* A2, and lambs in groups two and four with *P. haemolytica* A7. Surviving lambs were euthanased at day 12 post-infection. Vaccinated lambs had significantly ($P < 0.01$) less extensive pneumonic lung lesions when compared to the unvaccinated animals of the same treatment. In addition, vaccinated animals challenged with *P. haemolytica* A7 had significantly fewer ($P < 0.05$) pneumonic lesions than vaccinated animals challenged with *P. haemolytica* A7.

SINCE the importation of exotic sheep into Malaysia began in 1987 (Hadi 1988) many purebred animals and their crossbred progeny have died of either pneumonic pasteurellosis or haemonchosis (Wan Mohamed et al. 1988). In an effort to control the disease, the Veterinary Research Institute, Ipoh, developed a formalin-killed oil adjuvant vaccine using several local isolates of *P. haemolytica* of unknown serotypes (Chandrasekaran et al. 1987). Subsequently, the vaccine was found to be ineffective in controlling pneumonic pasteurellosis in sheep (Zamri-Saad et al. 1989).

Recently, Bahaman et al. (1991) concluded that the most common serotypes of *P. haemolytica* isolated in Malaysia are A2 and A7. Following this observation, the oil adjuvant vaccine was further modified by incorporating a known *P. haemolytica* serotype A7 (Chandrasekaran et al. 1992). This report describes a trial conducted to evaluate the effectiveness of this modified oil adjuvant vaccine in controlling experimental pneumonic pasteurellosis in lambs.

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Materials and Methods

Animals

Sixteen clinically healthy crossbred lambs of about 7 months old were selected and divided into four groups. Nasal swabs were taken weekly for 5 weeks prior to the experiment in order to detect any pasteurella infection. During the initial 5-week period, the lambs were fed cut grass and supplemented feed at the rate of 0.5 kg/animal/day. Drinking water was available ad lib. At the beginning of the experiment, none of these lambs had *P. haemolytica* or *P. multocida* in its nasal cavity.

Experimental procedure

Table 1 summarises the experimental design. At the start of the trial, lambs in groups one and two were

Table 1. Experimental design of the trial.

Group	No. of animals	Vaccination status	Challenge organism
1	4	+	*P. haemolytica* A2
2	4	+	*P. haemolytica* A7
3	4	—	*P. haemolytica* A2
4	4	—	*P. haemolytica* A7

vaccinated against pneumonic pasteurellosis using the locally prepared formalin-killed oil adjuvant vaccine. The vaccine was given intramuscularly at the rate of 3 mL/animal. Lambs in groups three and four received no vaccine. Vaccination was followed by weekly collection of nasal swabs from all lambs for bacterial isolation prior to experimental challenge with *P. haemolytica*.

Four weeks after vaccination, lambs in groups one and three were challenged intra-tracheally with 1 mL inoculum containing 106 cells of *P. haemolytica* A2 prepared from a 24-h broth culture, while lambs in groups two and four received the same dose of *P. haemolytica* A7. Immediately following the challenge, all lambs were further stressed by intramuscular dexamethasone injections at the rate of 2 mg/kg body weight for 4 days and supplementary feeding was stopped.

The lambs were monitored daily for clinical signs of respiratory disease. All surviving lambs were euthanased 12 days post-challenge, and complete post-mortem examinations were carried out. The extent of the lung lesions was determined according to the method of Gilmour et al. (1983). Lung samples were collected for bacterial isolation to establish a diagnosis of pneumonic pasteurellosis.

Results

Clinical observations

None of the lambs vaccinated with the oil adjuvant vaccine showed any adverse effects to vaccination, but many lambs coughed during the intra-tracheal administration of *P. haemolytica* broth. Intramuscular administration of the oil adjuvant vaccine was difficult, due to its thick consistency.

Two unvaccinated lambs challenged with *P. haemolytica* A2 died of pneumonic pasteurellosis on days 3 and 5 post-infection, following coughing, weakness and rapid respiration. Three other unvaccinated lambs challenged with either *P. haemolytica* A2 or A7 also exhibited rapid respiration, but survived the challenge. None of the vaccinated lambs died or showed obvious clinical signs of respiratory disease.

Macroscopic lung lesions

Many control lambs (75%) had pneumonic lesions of various intensity. The lesions were in the form of red consolidation of the anteroventral portion of the lungs, particularly the apical, cardiac and intermediate lobes. None of the lambs had lesions in the diaphragmatic lobes. Two unvaccinated and one vaccinated lamb challenged with *P. haemolytica* A2 had pleuritis with fibrin deposition adhering the lungs to parietal pleura. The lung lesions were most severe in the control group challenged with *P. haemolytica* A2. All vaccinated lambs that were challenged with either *P. haemolytica* A2 or A7 showed a significant reduction ($P < 0.01$) in the extent of lung lesions when compared to the unvaccinated lambs given the same treatment (Table 2). In addition, the vaccinated lambs challenged with *P. haemolytica* A7 showed a greater reduction in pneumonic lesions than those vaccinated animals challenged with *P. haemolytica* A2 ($P < 0.05$).

Bacteriology

P. haemolytica A2 and A7 were successfully reisolated in pure growths from many lungs with pneumonia.

Discussion

The results of this trial are in general agreement with those of Chandrasekaran et al. (1991). They demonstrate the feasibility of protecting sheep against pneumonic pasteurellosis caused by either *P. haemolytica* A2 or A7 using the locally prepared formalin-killed oil adjuvant vaccine. However, the vaccine appeared to be more efficacious against pneumonia caused by *P. haemolytica* A7 than against pneumonia caused by *P. haemolytica* A2.

Table 2. Average lung lesions in vaccinated and unvaccinated animals infected with *P. haemolytica* A2 or A7.

Group	Vaccination status	Challenge organism	Lung lesion (%)	
			Average	SD
1	+	*P. haemolytica* A2	8.8	6.1
2	+	*P. haemolytica* A7	0.9	1.1
3	—	*P. haemolytica* A2	63.3*	13.6
4	—	*P. haemolytica* A7	22.1*	5.4

* Significant difference ($P < 0.01$) to compared with the vaccinated group of similar treatment

This is probably because *P. haemolytica* A2 antigen was not incorporated in the vaccine (Bahaman et al. 1991; Chandrasekaran et al. 1991) and *P. haemolytica* A7 appeared to be less virulent than *P. haemolytica* A2, based on the extent of lung lesions in the unvaccinated groups (Table 2).

Incorporation of *P. haemolytica* A7 alone in the vaccine will not guarantee full protection against infection by *P. haemolytica* A2. The capsule varies in composition between the different *P. haemolytica* serotypes (Confer et al. 1990). There is also little antigenic similarity between *P. haemolytica* A2 and A7, based on the reactions of monoclonal antibodies with lipopolysaccharides (Durham et al. 1988). However, the cross-protection against infection by *P. haemolytica* A2 observed here was probably due to stimulation by minor antigens distinct from the type-specific antigen, but responsible for low-titre cross reactions (Biberstein 1965; Burrels et al. 1983).

Acknowledgments

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Control of Haemorrhagic Septicaemia in Indonesia — A Short History

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Abstract

The causative agent of haemorrhagic septicaemia (HS) or 'penyakit ngorok' (snoring disease), *Pasteurella multocida*, was first isolated in Indonesia by van Eecke in 1891 (Sumanagara 1959). From 1910 to 1970, attempts were made to control the disease by annual preventive vaccination, based on a killed-broth bacterin. Antiserum obtained from vaccinated animals was also used as a short-term preventive or therapeutic measure (Sumanagara 1959).

In 1962, observations reported at an FAO international meeting on HS indicated that the use of an adjuvant was necessary to produce long-lasting protection in vaccinated animals; and in 1979 an APHCA workshop on haemorrhagic septicaemia recommended the use of aerated broths for improved culture of *P. multocida* (Bain et al. 1982).

Experimental production of an oil adjuvant HS vaccine — funded by the Australian Government and supervised by Professor R. Bain — began in 1970 at the (then) Veterinary Research Institute, Bogor. (Responsibility for the production of HS vaccine was transferred from Bogor to the veterinary biologics facility (PusVetMar) at Surabaya in 1978.) The vaccine consisted of five parts formalin-killed bacterial suspension, four parts liquid paraffin as the dispersing agent, and one part melted lanoline as the emulsifying agent (Syamsudin 1971). A known virulent Carter B-type field strain of *P. multocida* (Katha), obtained from infected blood and stored in a lyophilised state, was used as the seed culture. Alum-adjuvant HS vaccine was also produced.

Aeration and batch culture were necessary to produce sufficient quantities of bacteria economically for either oil adjuvant or alum-precipitated vaccine (Syamsudin 1971; Bain et al. 1982). The medium for batch culture consisted of casein digest supplemented with sucrose, peptone, yeast extract and pancreas suspension. It was contained in 25 L, stainless steel beer barrels. After a defined growth period, the culture was inactivated by the addition of formalin to a final concentration of 0.5%, and left overnight. The vaccine was formulated such that each 3 mL cattle dose contained the equivalent of 2 mg dry weight of bacteria. Each batch was tested for safety by vaccinating two cattle and two buffalo with a double dose.

To assess protection, a group of cattle were vaccinated intramuscularly and challenged 3 weeks later with 1.0 mL diluted broth culture containing 100 million mouse LD_{50} equivalents of a virulent strain of *P. multocida* (Syamsudin 1971).

A passive mouse protection test was used to assess passive protection. Blood samples were taken from three healthy rabbits 3 weeks after they were vaccinated intramuscularly with 2 mL of the vaccine, and the serum was pooled. Four healthy mice were injected subcutaneously with 0.25 mL of the serum and challenged 1 day later with 0.1 mL diluted broth culture containing 100 mouse LD_{50} equivalents of *P. multocida*.

Adjuvanted vaccine produced in Bogor was first used on a large scale in the Natural Preservation and Protection Area of Ujung Kulon (West Java) in 1974, after a number of working buffalo died. The disease outbreak was contained by means of mass barrier vaccination with alum-adjuvant vaccine and the simultaneous use of antisera.

Although vaccination with oil adjuvant HS vaccine is widely practised, and is generally credited with reducing the prevalence and incidence of HS (Bain et al. 1982), outbreaks still occur every year. The main reason for this appears to be incomplete vaccination coverage of susceptible animals in the known endemic areas (Syamsudin 1990). If vaccination coverage is less than 80%, the disease may recur (Bain et al. 1982).

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A pilot HS eradication program, based on mass vaccination over a 3-year period, was undertaken on the Island of Lombok, West Muse Tenggara Province, beginning in 1978. As a result, 89%, 94%, 82%, 93% and 80% of the cattle, buffalo, goat, sheep and pig populations respectively were vaccinated. The program was evaluated in 1981, 1 year after the third annual vaccination. During this time, 53 cases of HS were reported, and culture of 220 pharyngeal mucous membrane samples from animals slaughtered at the abattoir resulted in the identification of five carrier animals. Based on these findings, mass vaccination was continued for another year. In 1985, a follow-up evaluation conducted on 450 abattoir samples obtained from cattle, buffalo, goats and pigs, and on 103 samples from animals suspected of having HS, gave negative findings. The Island was declared free of the disease. However, subsequent evaluations based on culture and serology, together with field reports, indicate that HS is still present on Lombok.

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Clinical Efficacy of a Long-Acting Spiramycin Formulation for the Treatment of Bovine Infectious Enzootic Bronchopneumonia

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Abstract

A large-scale field trial was conducted to assess the comparative efficacy of spiramycin and oxytetracycline in the treatment of the pasteurellosis component of bovine infectious enzootic bronchopneumonia. At the first signs of respiratory disease, a total of 164 cattle were divided into one of four treatment groups. Groups one and three received two injections of spiramycin at a dose rate of 100 000 IU/kg bodyweight. Groups two and four received oxytetracycline at a dose rate of 20 mg/kg bodyweight. Groups one and two received the two injections 48 hours apart, while groups three and four received the two injections 72 hours apart. The clinical condition of the cattle was scored on day 0 (first treatment), on the day of the second injection, and 21 days after the beginning of treatment. The temperature of each animal was recorded daily for 10 days following the start of treatment, and blood was sampled on days 0 and 21 to undertake tests to help establish the aetiological agent — for example, *Mycoplasma bovis*, infectious bovine rhinotracheitis virus, bovine virus diarrhoea virus or respiratory syncitial virus — causing the respiratory condition. The clinical condition, based on illness score assessment, improved markedly, with a 98% recovery rate following spiramycin treatment. The curative efficacy on cattle bronchopneumonia of two injections of spiramycin at a dose rate of 100 000 IU/kg, 48 hours apart, was clearly shown.

THIS report presents the results of a field trial involving various types of cattle and cattle management. The trial was conducted in three different large animal practices in France, where the prevalence of *Pasteurella haemolytica* had been established (Baroux 1991). One-hundred-and-sixty-four cattle were followed clinically over a 21-day period, according to a standard scoring system. The objective was to determine the response of these cattle to two injections, 48 or 72 hours apart, of either spiramycin (Captalin^R, Rhone Merieux) or oxytetracycline (Terramycin long-acting^R, Pfizer) at dose rates recommended by the manufacturer. Efficacy was assessed on the comparative ability of the drugs to improve the clinical status of the animals.

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Material and Methods

Animals

One-hundred-and-sixty-four cattle showing respiratory symptoms were treated. Their weights ranged from 180 to 550 kg, with an average of 340 kg (SD = 70 kg). The cattle were predominantly of the beef type.

Treatment

The cattle were allotted randomly to four different treatment groups. They received two deep intramuscular injections, either 48 hours or 72 hours apart, of either spiramycin at a dose rate of 100 000 IU/kg bodyweight or oxytetracycline at a dose rate of 20 mg/kg bodyweight. Protocols of treatments are described in Table 1.

Table 1. Treatment protocols.

	Antibacterials	
	Spiramycin 100 000 IU/kg	Oxytetracycline 20 mg/kg
2 injections 48 hours apart	Group 1 (n = 41)	Group 2 (n = 40)
2 injections 72 hours apart	Group 3 (n = 45)	Group 4 (n = 38)

Spiramycin: 100 g = 3200 IU (WHO 1962)
100 000 IU/kg = 31.25 mg/kg

Clinical and microbiological survey

Each animal was observed for 21 days. From day 0 (beginning of treatment) to day 10, rectal temperatures were taken daily. At day 0, on the day of the second treatment injection, and at day 21, all the animals were clinically evaluated according to a standard examination method (Raynaud et al. 1986). Respiratory symptoms (respiration rate, nasal discharge, cough) and systemic symptoms (rectal temperature, anorexia, general behaviour) were recorded.

From these data, each animal was assigned a mean comprehensive score (MCS), which indicates clinical status:

MCS < 1.2	= Normal and healthy
1.2 < MCS < 1.8	= Moderately ill
MCS > 1.8	= Severely ill

According to the values of both MCS and rectal temperature, it was possible to make conclusions on the final result of the treatment: a recovery was recorded when the animal returned to normal; a failure was recorded when the animal showed no improvement within 24 hours, either after the first or the second injection.

At day 0 and day 21, blood samples were taken in order to check the antibody titres of each animal for infectious bovine rhinotracheitis (IBR) virus, bovine virus diarrhoea (BVD) virus, respiratory syncitial virus (RSV) and *Mycoplasma bovis*.

Results

Serology

Approximately 10% of cattle showed high titres towards IBR virus, BVD virus and RSV antigens at both day 0 and day 21. However 75% of cattle showed a rising titre (seroconverted extremely well) to RSV between day 0 and day 21. Five percent of cattle seroconverted to *M. bovis*.

Clinical signs

Table 2 gives a general overview of all results. Groups one and three were considered together, as were groups two and four. One animal died in the experiment (mortality on the whole experiment < 0.6%). No death was recorded in the spiramycin-treated group. Recovery in both groups was high, but there was a significant difference between the two. The spiramycin-treated group showed a higher rate of success (98%) than the oxytetracycline-treated group (88%).

All groups of cattle showed an MCS > 1.8 at the beginning of the treatment, indicating that they were affected by severe bronchopneumonia. Following the first injection of antibiotic, the MCS dropped within 2 days (−0.55 for spiromycin- and −0.31 for oxytetracycline-treated batches) and remained low 72 hours after the first injection for groups three and four (Table 3 and Fig. 1).

In parallel with the recovery of the cattle, there was a sharp fall in rectal temperature. Again a decrease was seen within 2 days post-injection (−0.8 °C for spiromycin- and −0.4 °C for oxytetracycline-treated batches). This lasted 72 hours after the first injection for groups three and four (Table 4 and Fig. 2).

For both MCS and rectal temperature parameters, a larger and quicker improvement was registered for the spiramycin-treated groups within 2 days of the first injection.

Table 2. Recoveries, failures and death.

Treatment	No. cattle	Recoveries	Failures	Mortality
Spiramycin 100 000 IU/kg Twice	86	84 (98%)	2 (2%)*	0
Oxytetracycline 20 mg/kg Twice	78	69 (88%)	9 (12%)*	1

* Data significantly different ($P < 0.05$, Yates chi-square test)

Table 3. Clinical response (MCS) at day 0, 48 hours, 72 hours and 21 days after the first injection of antibiotic.

Treatment	MCS			
	Day 0	Day 2–Day 0	Day 3–Day 0	Day 21
Spiramycin 100 000 IU/kg Twice	1.94	−0.55*	0.70	1.01
(no. of animals)	(84)	(41)	(43)	(84)
Oxytetracycline 20 mg/kg Twice	1.79	−0.31*	−0.60	1.01
(no. of animals)	(69)	(35)	(34)	(69)

* Data significantly different ($P < 0.05$)

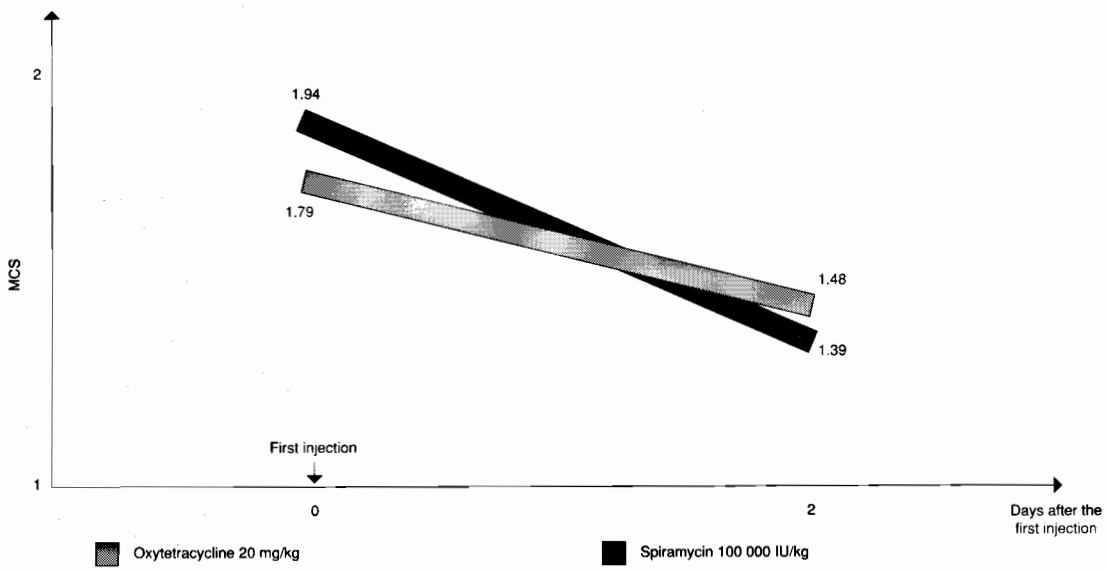

Fig. 1. Evolution of the clinical response (MCS) within 48 hours of the first antibacterial injection.

Table 4. Mean daily rectal temperatures on day 0, and 24, 48 and 72 hours after the first antibiotic injection.

Treatment (Days)	Temperature (°C)				
	0	1–0	2–0	3–0	3
Spiramycin 100 000 IU/kg Twice	40	−0.6	−0.8*	−0.9	39.1
(No. cattle)	(84)	(84)	(84)	(43)	(43)
Oxytetracycline 20 mg/kg Twice	39.8	−0.3	−0.4*	−0.8	39
(No. cattle)	(69)	(69)	(69)	(34)	(34)

* Data significantly different ($P < 0.05$)

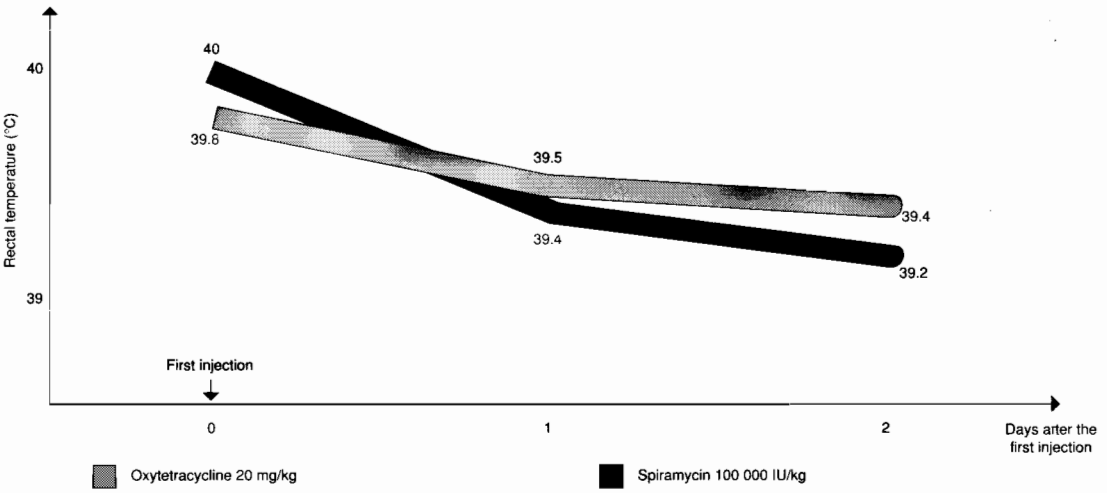

Fig. 2. Evolution of mean daily rectal temperatures within 48 hours of the first antibacterial injection.

Discussion

Serology

In this experiment, seroconversion to RSV occurred in approximately 75% of cattle and there was very little or no involvement of *M. bovis*. Such results are specific to farming areas where the low incidence of *M. bovis* has been clearly demonstrated, as well as the involvement of a mild strain of RSV as an initiator of severe pasteurella complications (Baroux 1991).

Clinical signs

The level of recoveries following spiramycin treatment (98%) was higher than that reported in the current literature (Alzieu et al. 1988). The latter authors performed a trans-tracheal aspiration on 31–100% of cattle as early as the first day of their arrival in feed lot facilities. *M. bovis* was isolated from 68% and *Pasteurella* spp. from 32% of the animals. As mycoplasma infection is difficult to treat, a high mycoplasmal contamination in the Alzieu et al. (1988) study explains the lower level of recovery (75–85%).

To explain the clinical efficacy of spiramycin towards pasteurella, the minimum inhibitory concentration (MIC) of spiramycin towards 97 pasteurella strains was determined and compared with the concentrations of spiramycin reached in the respiratory tract of cattle (Alzieu et al. 1988). Spiramycin shows a very high affinity for lung parenchyma (Raynaud et al. 1987; Espinasse et al. 1990), bronchial secretions (Floc'h et al. 1988), and alveolar macrophages (Harf et al. 1988). It has been demonstrated that even subinhibitory concentrations of spiramycin greatly increase the phagocytosis activity of macrophages and the number of ingested cocci per phagocyte (Desnottes and Diallo 1990).

Data presented in Table 5 and Figure 3 explain the excellent results obtained in this study. In the respiratory tract, the concentration of spiramycin following two injections of 100 000 IU/kg 48 hours apart is higher than the MIC of *P. multocida* and *P. haemolytica*.

Eleven failures occurred following treatments. The nine oxytetracycline failures were treated with spiramycin at a dose rate of 100 000 IU/kg twice, 48 and 72 hours apart. One of these nine cattle did not respond to spiramycin treatment or to any other supporting therapy. Necropsy showed suppurative necrosis involving 66% of the lungs, suggesting that by the time the spiramycin treatment was initiated (24 hours after the first injection of oxytetracycline) lesions were already too extensive to allow recovery.

The two spiramycin failures were treated with colistin at a dose rate of 50 000 IU/kg/day for 3 days with 100% success. This suggests involvement of Gram-negative bacteria sensitive to colistin.

Results in Tables 3 and 4 and Figures 1 and 2 are similar to those published by Alzieu et al. (1988) and Espinasse et al. (1990), who followed a protocol with two injections of spiramycin (100 000 IU/kg) 48 hours apart. Within 2 days of the first injection, they noticed a drop in MCS and rectal temperature similar to the one recorded in this experiment.

Table 5. Comparison between spiramycin concentrations obtained in the respiratory tract and the MIC of *Pasteurella* spp. and *M. bovis* (IU/mL or g).

	Spiramycin 100 000 IU/kg 1st injection			Spiramycin 100 000 IU/g 2nd injection					
Time (hours)	4	24	48	4	24	48	72	96	120
Serum (IU/mL)	3.9	2.3	0.9	5.5	1.1	0.8	0.45	0.3	0.1
P. haemo. MIC	◄				32.5				►
P. multo. MIC (IU/mL)	◄				36.5				►
Bronchial secretions (IU/mL)	30	47	33	47	39	44	62	27	ND
M. bovis MIC (IU/mL)	◄				51.2				►
Lungs (IU/g)	73	96	83	246	96	109	94	38	52
Macrophages (IU/mL)	307	928	1325	/	1780	2646	596	574	814

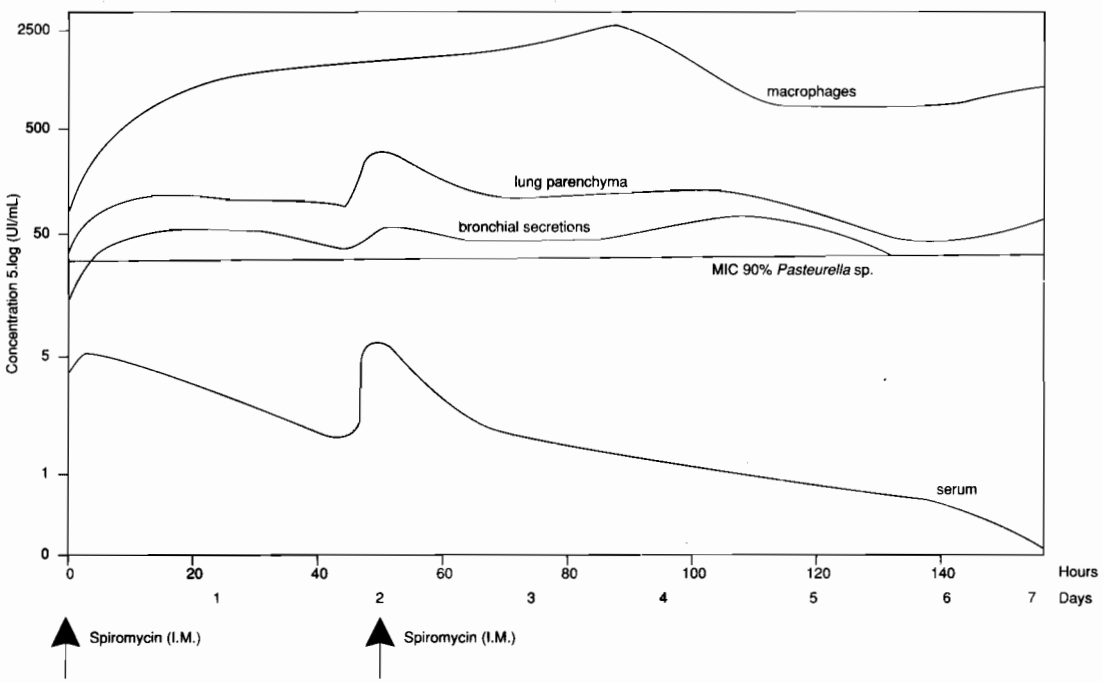

Fig. 3. Efficient tissue concentrations of spiramycin over 7 days.

In the groups receiving two injections of spiramycin 72 hours apart, a marked improvement in clinical symptoms was observed on the 3rd day; i.e. before the second antibacterial injection. The experiment supports, then, a relative efficiency for 3 days after one single injection of spiramycin at a rate of 100 000 IU/kg. This result is of particular importance in areas where extensively raised cattle cannot be collected twice in a 48-hour period, provided bronchopneumonia symptoms are not too severe. The value of such a protocol should, however, be discussed in the light of additional trials involving different types of cattle management.

Conclusions

This experiment supports pharmacological (Table 5, Fig. 3) and clinical studies (Alzieu et al. 1988; Espinasse et al. 1990) in proving the validity of two injections of spiramycin (100 000 IU/kg) 48 hours apart on cattle affected by severe infectious enzootic bronchopneumonia, of which pasteurellosis is an important component.

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The Use of Ceftiofur in the Treatment of Cattle Experimentally Infected with *Pasteurella multocida* Type B

S. Chandrasekaran[1]

Abstract

The efficacy of cephalosporins was investigated for the treatment of haemorrhagic septicaemia (HS) in cattle. Ten healthy, unvaccinated cattle aged about 18 months and free of naturally acquired immunity to *Pasteurella multocida* type B were separated into two groups of four animals (groups one and two) and one group of two animals (group three). All the cattle were infected with a virulent strain of *P. multocida* (C82) by injecting 3×10^6 colony-forming units of the organism subcutaneously. When clinical signs became evident, the cattle in groups one and two were treated parenterally with ceftiofur, a recently developed cephalosporin, at dose rates of 1 and 2 mg/kg/day respectively. The treatment was given for 5 consecutive days. The cattle in group three served as the controls. All the cattle in group one survived, whereas two animals in group two and all the controls succumbed to the infection. Other possible causes (such as viral) of the deaths of the two cattle in group two were not investigated. Based on these findings, it would seem that ceftiofur can be used effectively to treat clinical cases of HS. However, the optimum dose of the drug was not determined in this experiment.

HAEMORRHAGIC septicaemia (HS), caused by *Pasteurella multocida* type B, is enzootic in many parts of Asia and Africa. Bain (1957) estimated that annual deaths due to HS exceeded 100 000 in Asia alone. HS regularly and insidiously occurs in all Southeast Asian countries, except Singapore. In these countries outbreaks of HS have been kept under control primarily by vaccination, which has markedly reduced mortalities (Joseph 1979). In Malaysia, however, only about 25% of cattle and buffalo are vaccinated annually, and outbreaks continue to occur in pockets of untreated animals.

Chemotherapy in clinically sick animals has been attempted by other workers. Chong and Cheah (1963) treated buffalo experimentally infected with HS with sulphamezathine (Sulphadimidine Sodium, ICI England) and immune serum. Immune serum was found to be of no value. The authors claimed that while sulphamezathine was effective in treating infected animals, an adequate initial dose was required to maintain a bactericidal concentration. Later, in a field outbreak of HS, Siew et al. (1970) observed that clinically sick animals did not respond to treatment with the drug. The treatment of in-contact animals with antibiotics and broth bacterin simultaneously is still being practised in Malaysia. However, a controlled study has not been conducted to assess the effectiveness of this treatment.

Cephalosporins have been claimed to be effective against pasteurellae and have apparently been effective in treating animals with shipping fever. The aim of the trial reported here was to determine the effectiveness of cephalosporins, in the form of ceftiofur sodium, in treating cattle with clinical signs of HS.

Materials and Methods

Animals

Ten cattle, aged about 18 months and completely free of antibodies against *P. multocida*, were purchased from a private farmer. The antibody-free status was ascertained by gel diffusion precipitation, passive mouse protection, indirect haemagglutination, and slide agglutination tests. The cattle were brought to the Veterinary Research Institute (VRI), Ipoh, weighed, and separated into two groups of four animals (groups one and two) and one group of two animals (group three). They were fed with grass and commercial feed pellets.

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Experimental infection

Acute HS was experimentally reproduced by the method routinely used at VRI. Each animal was inoculated subcutaneously (in the left neck region) with about 3×10^6 colony-forming units (CFU) of a virulent strain of *P. multocida* type B (C82).

Clinical examination

The cattle were observed for 10 days from the day of infection. They were examined twice daily (at 8.30 am and 3.30 pm) for clinical signs of disease, and rectal temperatures were recorded once each morning.

Treatment

The cattle in groups one and two were injected intramuscularly with ceftiofur sodium (Excenel, Upjohn Company, Orangeville, Ontario) at rates of 1 and 2 mg/kg/day respectively. Treatment was initiated 10 hours post-infection (i.e. at the pyrexic stage of the disease and after collection of blood for bacteriological culture) and continued daily for 5 consecutive days. The controls were left untreated.

Blood and organ culture

About 5 mL of blood was collected from each animal aseptically (when pyrexia was evident) and inoculated into separate bottles containing 50 mL of an enrichment medium. The bottles were incubated at 37 °C for 6 hours, then 0.5 mL of the content was subcultured onto blood agar. Similarly, bacteriological isolations were made from heart, lungs, liver, kidney, spleen, tonsil and retropharyngeal, mediastinal and left prescapular lymph nodes of cattle that succumbed to infection.

Results

Infection

All cattle showed signs of malaise, anorexia, serous nasal discharge, dullness and pyrexia. The fluctuations in rectal temperatures of the individual animals during the course of the trial are given in Table 1.

All cattle developed oedema at the site of inoculation. The swelling extended down to the brisket in two animals in group two.

At 1 week post-challenge, two animals in group one (1 mg/kg/day) developed a circumscribed swelling at the site of challenge. This became larger with time and, at 18 days post-challenge, measured about 12 cm in diameter. These two animals were slaughtered for disease investigation.

Bacteriological culture

The 10 hours post-infection blood culture was positive in each of two animals in groups one (1 mg/kg/day), two (2 mg/kg/day) and three (controls). There was profuse, pure growth of *P. multocida* type B from all the organs of the control animals. However, in the two animals from group two that succumbed to infection, the bacteriological picture varied. In one, there was scanty, mixed growth from the kidney, heart, liver, tonsil and retropharyngeal lymph node. Isolated colonies of *P.*

Table 1. Record of daily temperatures of the cattle in groups one, two and three, and the outcome of infection.

Animal number	Days post-infection											Remarks
	0	1	2	3	4	5	6	7	8	9	10	
1	38.3	41.2	39.0	38.8	38.7	39.3	38.4	38.9	38.4	38.5	38.3	
2	38.5	40.9	39.3	38.0	39.4	38.8	38.3	39.3	39.3	39.1	39.1	
3	38.4	41.4	39.1	38.8	38.5	39.3	38.4	38.7	38.8	38.8	38.5	Abscess at s.o.i.
4	38.3	40.2	39.2	39.2	39.1	38.9	39.1	39.0	39.0	39.0	38.2	Abscess at s.o.i.
5	38.7	41.1	38.7	39.1	38.8	38.3	37.8	39.0	38.5	38.5	38.0	
6	38.3	39.7	39.5	39.5	39.7	38.7	38.5	39.1	39.3	39.5	38.5	
7	38.6	40.2	38.0	38.0	38.7	*						Died on 5th day p.i.
8	38.2	40.1	38.2	*								Died on 3rd day p.i.
9	38.4	40.9	*									Died on 2nd day p.i.
10	38.2	41.2	*									Died on 2nd day p.i.

Group one Nos 1–4 p.i. – post infection
Group two Nos 5–8 s.o.i. – site of infection
Group three Nos 9–10

multocida type B were present in these mixed growths. *P. multocida* could not be isolated from the lungs or the spleen.

The swellings on both animals slaughtered for disease investigation were abscesses containing greenish-yellow, cheesy material. A swab of this cheesy material, when subcultured onto blood agar, yielded profuse, pure growth of *P. multocida* type B.

In the other cattle, the swelling at the site of injection subsided on the 3rd day of treatment and that in the brisket region subsided after the 5th day of treatment.

Discussion

Acute infection was successfully reproduced by exposure of cattle to live *P. multocida* type B, as evidenced by clinical signs after approximately 10 hours. The oedematous swelling formed at the site of injection was probably associated with the rapid multiplication of the organisms. This was smaller by the time of the 3rd antibiotic treatment on day 3, and the animals looked normal by the 5th treatment on day 5. However, the drug did not eliminate the organisms completely in all the animals. As a result of this, two developed abscesses at the site of injection. These appeared to be single lesions, probably due to encapsulation. Had they been left unchecked, however, they may have ruptured, disseminating organisms into the environment.

Other studies have shown that apparently healthy animals can be carriers of virulent *P. multocida* type B (Omar et al. 1962; Chandrasekaran et al. 1981). The bacteria normally lodge in the tonsils and lymph nodes, especially the retropharyngeal lymph nodes, of potential carriers (De Alwis et al. 1990). A breakdown in the resistance of these animals may trigger multiplication of the organisms, precipitating HS (Joseph 1979).

The reason for the death of the two animals in group two was not determined. The Upjohn Company, producers of ceftiofur sodium, claim to have conducted tolerance and toxicity studies with the drug. Normal feeder calves apparently tolerated 55 times (55 mg/kg/day) the recommended dose of 1.0 mg/kg/day for 5 consecutive days. In other toxicity studies, calves were injected intramuscularly with 22 times the normal dose of 1.0 mg/kg/day for 5 consecutive days with no adverse effects. Therefore, it is highly unlikely that the animals died of toxicity caused by the drug. Viral or other aetiological possibilities were not investigated.

Acute infection occurs in susceptible cattle inoculated subcutaneously with 3×10^6 CFU of *P. multocida* type B, and they usually die within 36–48 hours (Chandrasekaran, unpublished). This trial showed that ceftiofur sodium can be used effectively against acute HS if treatment is initiated sufficiently early; i.e. at the pyrexic stage of the disease. A further trial using a larger number of animals would be necessary in order to determine an ideal effective dose.

Acknowledgments

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Efficacy of Cefquinome Against *Pasteurella haemolytica* In Vitro and In Vivo, and Against *Pasteurella multocida* In Vitro

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Abstract

In in vitro experiments, a concentration of 1.8 μg/mL cefquinome showed an obvious bactericidal efficacy against *Pasteurella haemolytica*. For *P. multocida* (types A, B and D), the bactericidal concentrations were between 0.1 and 15 μg/mL.

Administration of cefquinome in concentrations of 5, 2.5 and 1.25 mg/kg body weight to calves of 45–50 kg, repeated three times, protected them from pneumonia triggered by *P. haemolytica* A1.

RESPIRATORY diseases of calves continue to be very important, particularly when animals of different origin are brought together. Severe clinical diseases of the respiratory tract are likely to occur during the subsequent 2 weeks. In Europe, under these circumstances, *Pasteurella haemolytica* A1 and *P. multocida* types A and D are the primary bacteriological agents involved.

Because of the various species and serovars involved, and the age of the calves, immunoprophylactic measures are unsatisfactory. However, therapy with antibiotics is indispensable. This paper describes the efficacy of cefquinome in in vitro and in vivo experiments.

Materials and Methods

In vitro investigations

After opening the lyophilised ampoules, *P. haemolytica* and *P. multocida* strains to be tested were raised in partially defined medium (Flossmann et al. 1974), passaged again in the same nutrient substrate for 18 hours, and purity controlled on solid nutrient medium. After renewed passage, they were available for inoculation of tubes containing decreasing concentrations of cefquinome. The bacterial concentration of the liquid cultures was 1–3 × 10^9 pasteurellas/mL.

The cefquinome dilution series was always prepared freshly with phosphate buffered saline. The cefquinome concentrations were within the range of 240–0.01 μg/mL, with intermediary stages of 60, 30, 15, 10, 5, 2.5, 1.0, 0.5 and 0.1 μg/mL.

The tubes with a decreasing cefquinome content were inoculated with 0.1 mL broth culture in duplicate and incubated at 37 °C for 18 hours. Subsequently, 0.2 mL of the culture–cefquinome mixtures of each concentration were transferred to a solid nutrient medium. After incubation at 37 °C for 18 hours, the plates were evaluated under a stereo microscope (magnification × 15) for colony formation.

In vivo investigations

Calves of the black-spotted dairy race of both sexes, aged 5–7 weeks, were used to test the efficacy of the cefquinome preparation for protection from *P. haemolytica* A1 infection.

Cefquinome was administered subcutaneously on 3 successive days, 4 hours prior to the experimental infection. The dosage was as follows: seven animals 5, seven animals 2.5 and seven animals 1.25 mg/kg body weight cefquinome; and seven control animals 2 mL phosphate-buffered saline. The intratracheal infection (10 mL broth culture of *P. haemolytica*) was administered on 3 successive days. The total bacterial count per dose was 1 × 10^{10} bacteria per animal per day of infection.

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The calves were euthanased on the 2nd day after the last infection. Necropsy and macroscopic evaluation, in particular of the lungs, were done immediately afterwards. The superficially visible regions of pneumonic changes were plotted on squared paper approximately true to scale using a stamped pattern of the lung. Specimens of tonsils, bronchial lymph node, trachea, main bronchus and lungs were taken for bacteriological examination.

Results and Discussion

In in vitro experiments, a concentration of 1.8 μg/mL cefquinome completely suppressed the growth of the *P. haemolytica* type and field strains tested. Fifty percent of the strains investigated did not grow at a concentration of 0.9 μg/mL. As *P. haemolytica* ranks among the most important aetiological agents in connection with respiratory disease in calves, this result signifies that cefquinome treatment is likely to be effective.

The bactericidal concentrations of *P. multocida* (types A, B and D) were between 0.1 and 15 μg/mL.

Before infection in the in vivo experiment, all animals were clinically normal. On the days of infection, four control calves exhibited fever (> 39.9 °C) for 7 days. The calves that had received 5 and 2.5 mg/kg body weight cefquinome did not show temperatures above this value. One calf in the 1.25 mg/kg cefquinome group displayed fever for 1 day.

The pathological-anatomical findings revealed a distinct difference between the control groups and the experimental groups. Four control, but no test, animals showed severe pneumonic lesions with multiple abscess formation. On the basis of the surface determination of the superficially changed areas of the lungs, a variable could be established. This variable served as a basis for ranking according to the extension of pneumonia. The differences were statistically confirmed by the method of the standardised difference of ranks (Steinbach 1988). In three control animals, but no experimental animals, extended subacute pneumonia of lobar or heavy-lobular, confluent extension was progressing.

In the case of the experimental calves, re-isolation of the bacterial strain used for the infection was successful only from tonsils; whereas in the control animals, *P. haemolytica* A1 could be isolated from all organs examined.

On account of the results obtained in in vitro and in vivo experiments, cefquinome can be considered as a highly effective preparation to control pasteurellosis.

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Antimicrobial Susceptibility of *Pasteurella multocida* Isolates

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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 33S 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.

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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),

and distilled water (1). 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 10^3–10^4 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 oxytetracycline (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.

Antimicrobial agent	Number of isolates responded (27)			Range of concentration (μg/mL)		
	Sensitive	Intermediate	Resistant	Sensitive	Intermediate	Resistant
Recommended for cattle and buffalo:						
Penicillin	27	—	—	⩽1	2–8	>9
Ampicillin	27	—	—	⩽2	4–16	>16
Oxacillin	14	3	10	⩽1	—	>1
Cephalothin	27	—	—	⩽4	8–16	>16
Enrofloxacin	27	—	—	⩽0.5	—	>0.5
Streptomycin	15	9	3	⩽8	16–32	>32
Spiramycin	14	2	11	⩽16	—	>16
Clindamycin	9	8	10	⩽1	2–4	>4
Oxytetracycline	25	1	—	⩽1	2–4	>4
Trim-sulpha	23	2	2	⩽0.5	1–8	>8
Sulfamethoxazole	4	8	15	⩽16	32–128	>128
Not recommended for parenteral use:						
Erythromycin	8	17	2	⩽0.5	1–4	>4
Neomycin	26	1	—	⩽8	16–32	>32
Fusidin	7	—	20	⩽2	—	>2
Gentamicin	26	1	—	⩽4	8–16	>16
Chloramphenicol	27	—	—	⩽8	—	>8
Nitrofurantoin	27	—	—	⩽32	—	>32

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

Antimicrobial agent	Number of isolates sensitive (%)	Concentration of drug (μg/mL)	Reference
Penicillin	5/10	0.4 iu/mL	Chang and Carter 1976
	112/144 (77.7)	—	Fales et al. 1982
	4/7	—	Allan et al. 1985
	(80)	⩽2.0	Karlsson and Nystrom 1962
	(100)	—	Bandopadhyay et al. 1991
Ampicillin	116/144 (80.5)	—	Fales et al. 1982
	(96.7)	⩽0.25	Cox et al. 1981
	6/7	—	Allan et al. 1985
	(100)	—	Bandopadhyay et al. 1991
Streptomycin	(81.3)	0.4–100	Chang and Carter 1976
	61/145 (42)	—	Fales et al. 1982
	5/7	—	Allan et al. 1985
	(44)	⩽16	Karlsson and Nystrom 1962
	(37.5)	—	Bandopadhyay et al. 1991
Cephalothin	(96.7)	1.0	Cox et al. 1981
	(100)	—	Bandopadhyay et al. 1991
Chlortetracycline	(25.1)	0.4–50	Chang and Carter 1976
Oxytetracycline	5/7 (94)	1	Karlsson and Nystrom 1962
Chloramphenicol	(1.1)	0.4–1.6	Chang and Carter 1976
	(100)	⩽0.5	Cox et al. 1981
	7/7	—	Allan et al. 1985
	(92)	⩽4	Karlsson and Nystrom 1962
	(100)	—	Bandopadhyay et al. 1991
Triple-sulpha	(12.5)	—	Fales et al. 1982
Trim-sulpha	(100)	0.5	Cox et al. 1981
	4/7	—	Allan et al. 1985
	(12.5)	—	Bandopadhyay et al. 1991
Sulphonamide	(12.5)	—	Bandopadhyay et al. 1991
Erythromycin	119/140 (85)	—	Fales et al. 1982
	(52)	⩽5	Karlsson and Nystrom 1962
	(100)	—	Bandopadhyay et al. 1991
Gentamicin	142/145 (97)	—	Fales et al. 1982
	(100)	1.0	Cox et al. 1981
	(100)	—	Bandopadhyay et al. 1991
Nitrofurantoin	138/144 (96)	—	Fales et al. 1982
	(100)	⩽64	Cox et al. 1981
	(100)	⩽10	Karlsson and Nystrom 1962
	(87.5)	—	Bandopadhyay et al. 1991
Neomycin	90/140 (64)	—	Fales et al. 1982
	(78)	⩽16	Karlsson and Nystrom 1962
	(93.75)	—	Bandopadhyay et al. 1991
Clindamycin	32/142 (22.5)	—	Fales et al. 1982

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.

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SESSION 4: PASTEURELLA: LABORATORY TECHNIQUES FOR TYPING AND DIAGNOSIS OF INFECTION

Pasteurella: Laboratory Techniques for Typing and Diagnosis of Infection

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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 septicaemic 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*.

Test	*P. multocida*	*P. haemolytica*
Fermentation (glucose)	+	+
MacConkey agar growth	−	+
Oxidase	+	+
Haemolysis	−	+
Indole	+	−
Urease	−	−
Mannitol	+	+
Sorbitol	+	+/−
Arabinose	−/+	+/−
Trehalose	+/−	+/−

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

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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 Heddleston et al. (1972). The Heddleston 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 (Rimler 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 (Rimler 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 on the membranes can be reprobed with different antibodies and exact replicates can be made from isolation media for other test purposes. The method has been used for enumeration of *P. multocida* in the respiratory tract of swine, and for recognition of more than one serotype affecting an animal (Rimler, unpublished).

Antibodies labelled with peroxidase and fluorescein isothiocyanate have been used experimentally for detection of pasteurellae in tissue specimens, and for study of their pathogenic mechanisms. These tests are simple and highly sensitive. Their full potential has not been realised, partly because of equipment costs and partly due to unavailability of group- or type-specific antibodies.

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.

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).

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.

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

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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

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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.

Isolate	History, location	Serotype	HS ELISA*
0432	HS, Central Luzon	B	209 (+)
0433	HS, Cagayan Valley	B	201 (+)
0434	HS, Cagayan de Oro	B	199 (+)
0435	HS, Negros Occidental	B	198 (+)
0436	HS, Bicol Region	B	206 (+)
0437	HS, Ilocos Region	B	193 (+)

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

HEMOBAC vaccine was produced by Riverdale Biological Laboratories Inc. from local isolates (0432–0437). It contains 4×10^9 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.

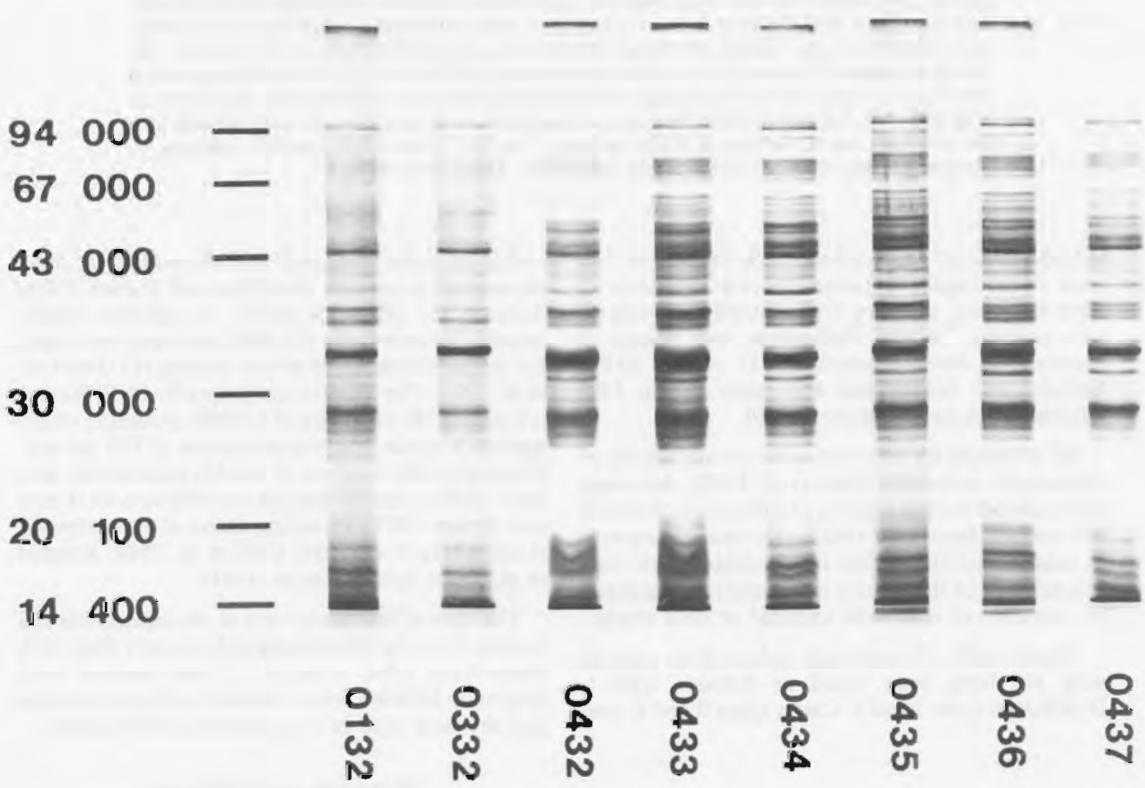

Fig. 1. Coomassie blue-stained PAGE profiles of lysates of eight *P. Multocida* HS isolates. From the left, the lanes contain lysates from the following strains: 0132, 0332, 0432, 0433, 0435, 0436, 0437. Low molecular weight standards are listed to the left of the figure (Pharmacia: lactalbumin [14 400]; trypsin inhibitor [20 100]; carbonic anhydrase [30 000]; ovalbumin [43 000]; albumin [67 000] and phosphorylase b [94 000]).

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,

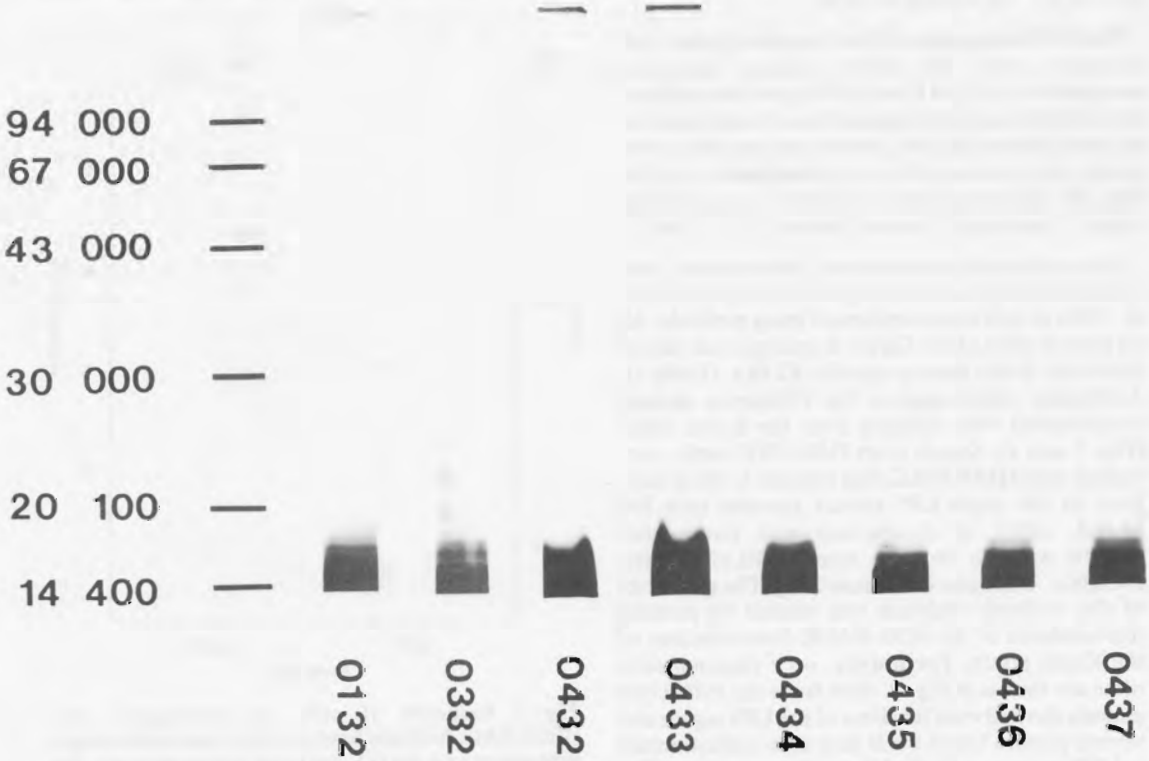

Fig. 2. Silver-stained PAGE profiles of lysates of eight *P. multocida* HS isolates. From the left, the lanes contain lysates from the following strains: 0132, 0332, 0432, 0433, 0434, 0435, 0436, 0347. Low molecular weight standards are listed to the left of the figure (Pharmacia: lactalbumin [14 400]; 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).

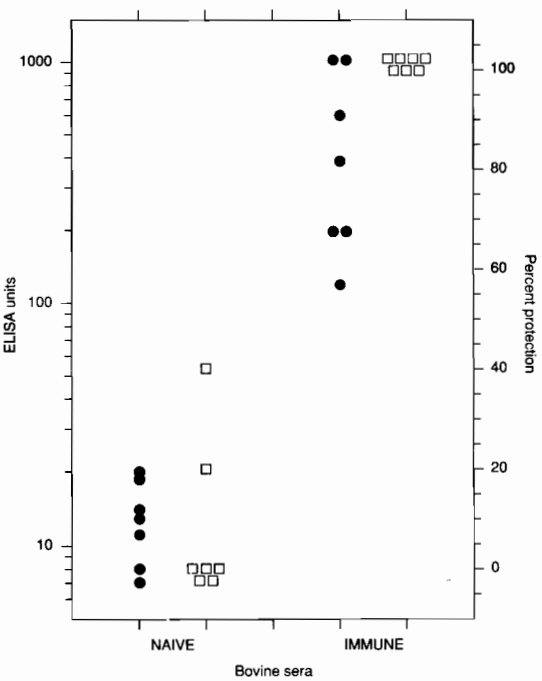

Fig. 3. Response of cattle to vaccination with HEMOBAC. Antibody levels to Katha heat-stable antigen were measured in NAIVE and IMMUNE bovine sera using the HS Antibody ELISA (●). The level of protection generated was assessed from the protection afforded to recipient mice by the passive transfer of 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

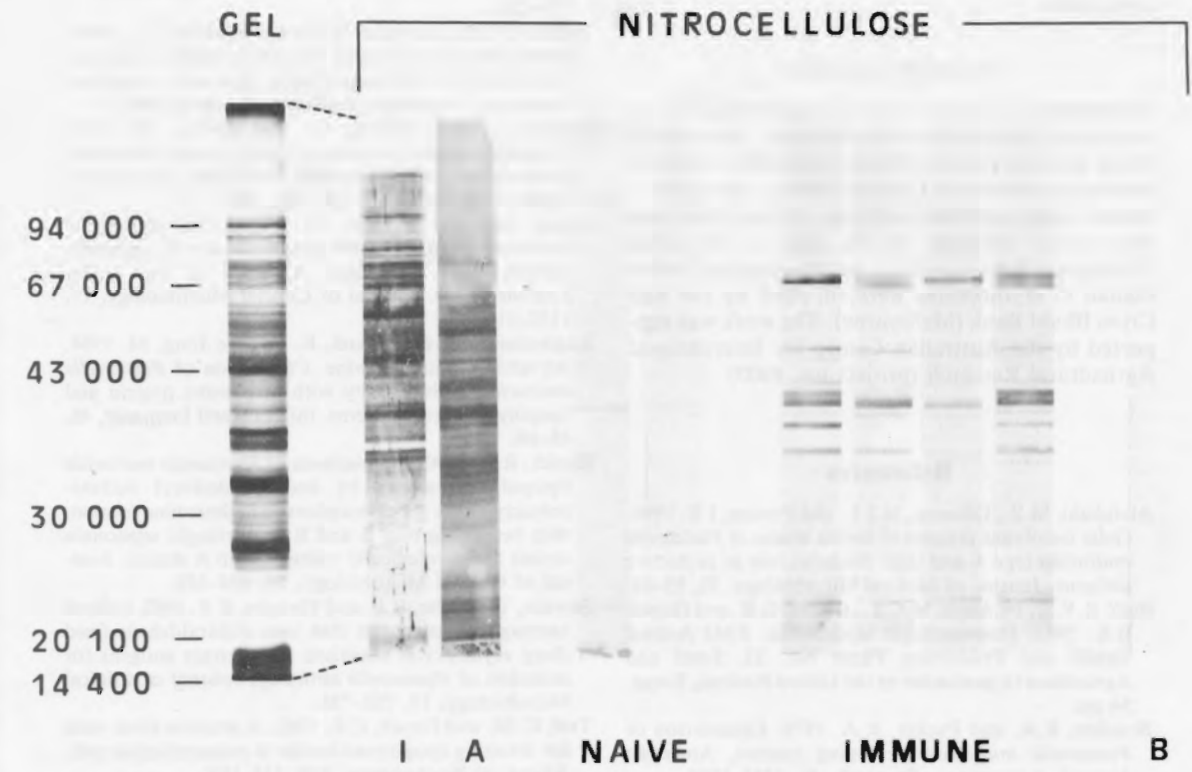

Fig. 4. Comparison of the antibody specificities in NAIVE and IMMUNE bovine sera. Relevant controls include low molecular weight markers (Pharmacia) and Katha lysate fractionations on acrylamide gels stained with Coomassie blue and nitrocellulose strips stained with Indian ink (I) and amido black (A). 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 *P. 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 *P. 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

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

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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 1, two to type 6, and one each to types 4 and 12. The remaining isolate was untypeable by Heddleston's method. Analysis of protein profiles of whole cells of the latter 16 isolates by sodium dodecyl sulphate polycrylamide-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.

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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. Suspicious 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

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 floculation 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 floculation 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 1.27×10^{10} organisms, and the other (Jersey) received an inoculum of 8.4×10^{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.

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

* Optical density of positive control was 0.604; negative control 0.001

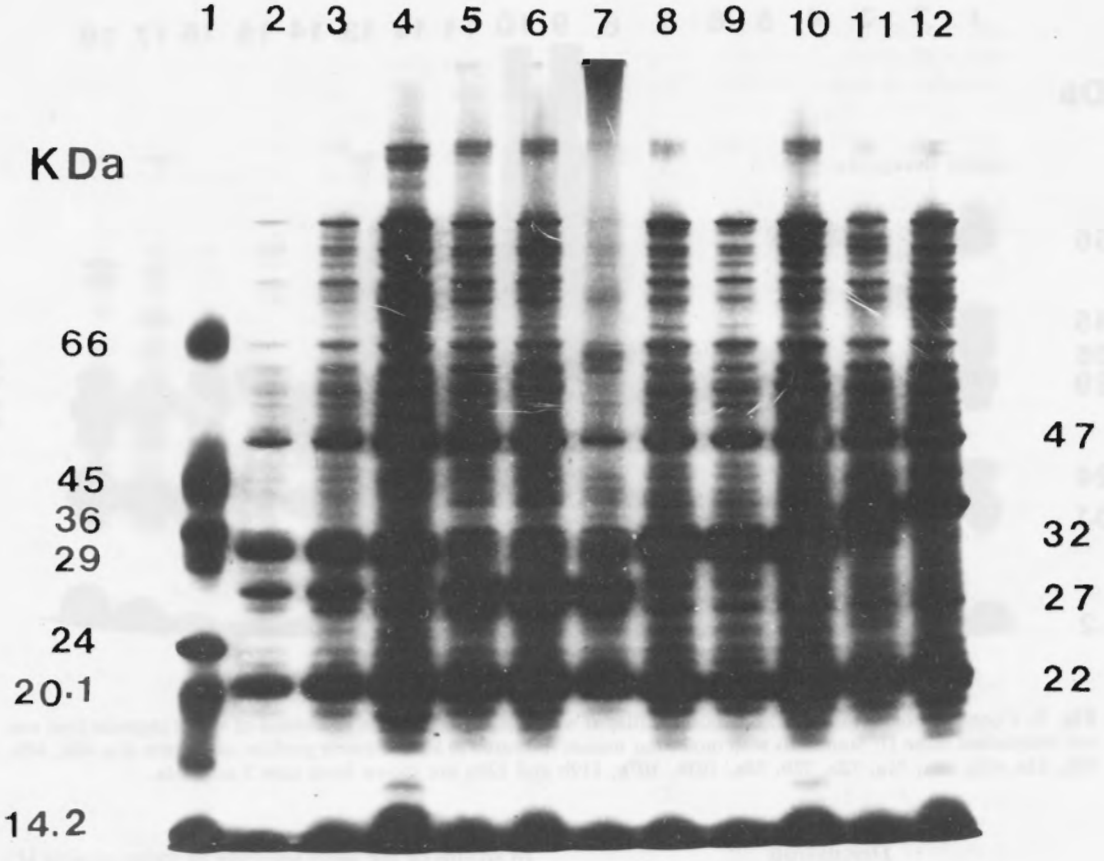

Fig. 1. Coomassie blue-stained SDS–PAGE profiles of whole-cell lysates of capsular type B isolates of *Pasteurella multocida* (lane 1). Standards with molecular masses indicated in kDa. Protein profiles of isolates 37a, 37b, 38a, 39a, C82 (B:2 Malaysian), 41b, 45b, 81a, 88a and 92b are shown from lane 2 onwards.

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 acriflavine 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 8.4×10^{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.)

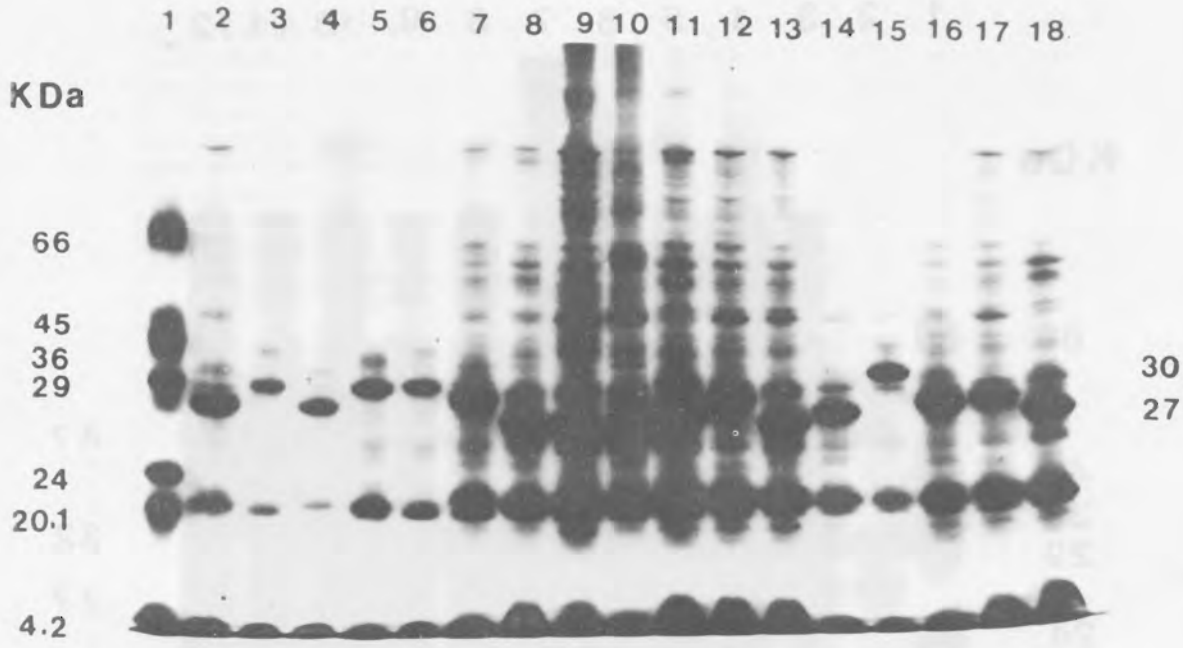

Fig. 2. Coomassie blue-stained SDS–PAGE profiles of whole-cell lysates of the 16 isolates of which capsular type was not determined (lane 1). Standards with molecular masses indicated in kDa. Protein profiles of isolates 41a, 48a, 48b, 50a, 53a, 67a, 69a, 71a, 72a, 72b, 74a, 103b, 107a, 119b and 120a 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 B, 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 B-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 floculation 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-

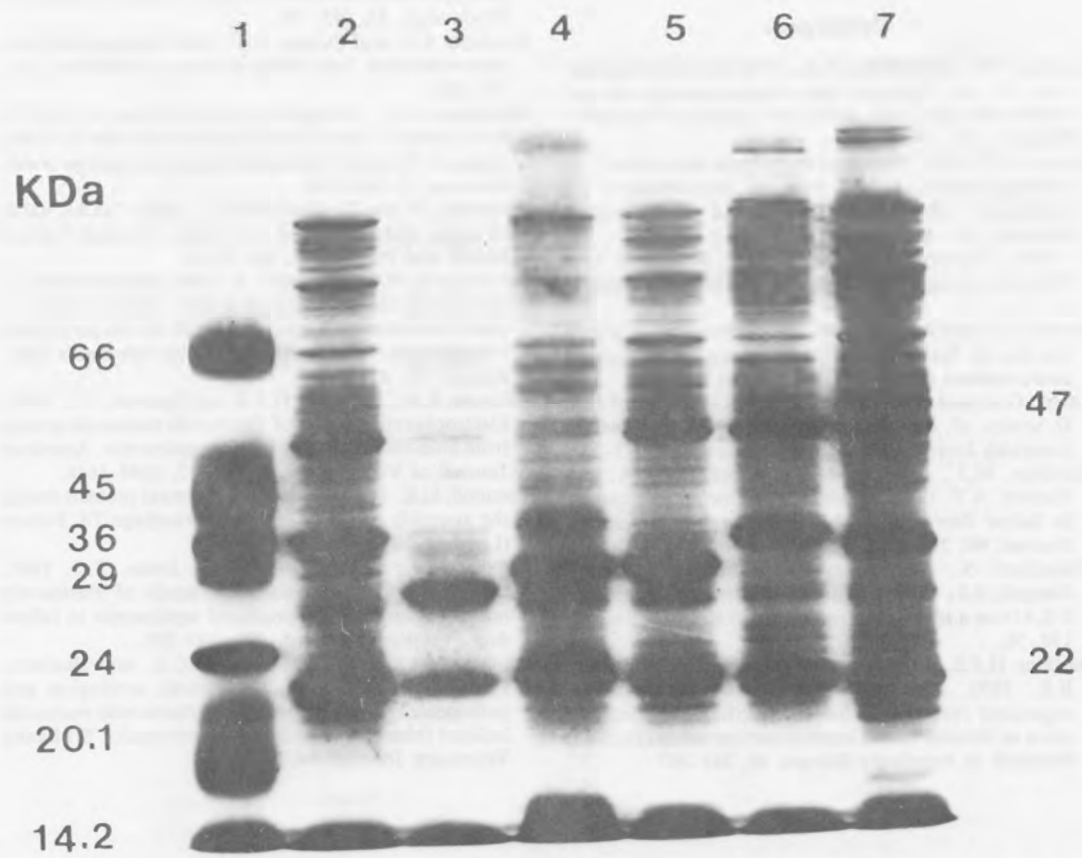

Fig. 3. Coomassie blue-stained SDS–PAGE profiles of whole-cell lysates of a *P. multocida* isolate used to infect calves (lane 1). Standards with molecular masses indicated in kDa. Protein profiles of 7908 (E:2), C82 (B:2 Malaysian), 120 (:3 before passage through calf), 120 (:3 after passage through calf), 1672 (type D) and W674 (type A) are shown from lane 2 onwards.

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.

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

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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.

THE passive mouse protection test (PMPT) has been recommended for evaluating immunity against haemorrhagic septicaemia in either vaccinated ruminants or those with naturally acquired immunity (Bain et al. 1982). During studies on the duration of protection in buffalo immunised with HS vaccines, the relationship was investigated between active protection observed on challenge of immunised buffalo and antibody measured by the the PMPT, the indirect capsular haemagglutination (IHA) test and enzyme-linked immunosorbent assay (ELISA).

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 2.25×10^{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 2×10^{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 (10^8 CFU, 5×10^8 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.

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Challenge of buffalo

Immunised or non-immunised buffalo were challenged subcutaneously with 3×10^6 CFU of *P. 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. 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. 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. 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).

Table 2. Analysis of ELISA antibody titres of immunised buffalo surviving or succumbing to challenge with virulent *P. multocida* type 6:B.

Antigen used in ELISA	Serum sample	Serum antibody titres of immunised buffalo	
		surviving challenge (±SEM)	succumbing to challenge (±SEM)
Formalised organisms	Pre-vaccination	106 ± 20 (n=15)	Not applicable
	Pre-challenge	549 ± 110[a] (n=15)	35 ± 3.8[b] (n=7)

Statistically significant differences: a vs. b ($P<0.001$)

Table 3. Relationship between antibody titre and the passive mouse protection test in the homologous system.

Volume (mL) of immune mouse serum injected	Total ELISA units injected	Number of mice surviving challenge/ total number challenged
0.5	540	4/4
0.25	270	4/4
0.10	108	4/4
0.05	54	4/4
0.025	27	2/4
0.01	10.8	0/4
Controls	0.0	0/4

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.

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

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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 organism must be selected carefully to ensure its purity and pathogenicity;
- 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.

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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 ×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 LD_{50} doses of virulent *P. multocida* was significant.

The challenge dose for PMPT has varied from 10 LD_{50} (Bain 1955) to 100 LD_{50} (Bain et al. 1982) and 10 LD_{50} (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 5×10^1 increase in injected serum required a 10^4 increase in the challenge dose to produce a 40% protection level (Roberts 1947); whereas a 10^2 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 LD_{50} (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 LD_{50} (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.

Group (ELISA units)	Prevaccination		1 month post-vaccination	
	Number/ ELISA unit[1]	Number/ PMPT[2] (%)	Number/ ELISA unit[1]	Number/ PMPT[2] (%)
0–<70	110	110	87	86
	37 ± 15	18 ± 23	306 ±323	63 ± 35
70–88	14	12	12	12
	76 ± 5	41 ± 31	481 ± 265	78 ± 23
>88	151	151	130	128
	440 ± 337	75 ± 30	701 ± 276	95 ± 13
Control	15	15	11	10
	36 ± 20	0	103 ± 171	10 ± 17

[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.

ELISA units	Collection 1 Number (%)	Collection 2 Number (%)	Collection 3	
			1 vaccine dose Number (%)	2 vaccine dose Number (%)
0–70	113 (41%)	19 (8%)	6 (6%)	4 (7%)
71–88	9 (3%)	11 (5%)	2 (2%)	2 (3%)
>88	153 (56%)	196 (87%)	87 (92%)	55 (90%)
Total	275	226	95	61

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

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

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

Number of samples	PMPT % protection	ELISA units[1]
74	0	38 ± 28
48	20	62 ± 28
55	40	100 ± 48
57	60	182 ± 128
42	80	441 ± 269
223	100	663 ± 298

[1] ELISA units mean and standard deviation.

Two hundred and twenty-six samples were collected from the test group 1 month after the first vaccination. The distribution of samples according to ELISA units and PMPT is also shown in Tables 2(a) and 2(b). At the time of the second collection, cattle in the two-vaccine group were given a second dose of vaccine.

One hundred and fifty-six samples were collected from the test groups 3 months after the first vaccination. The distribution of samples according to their ELISA unit values is shown in Table 2(a).

The correlation of ELISA units with PMPT titre is shown in Table 3. The calculated correlation coefficient between ELISA and PMPT titre was 0.76, with 524 degrees of freedom. The correlation was significant ($P<0.01$).

The sensitivity of the ELISA was 75.6% on the assumption that a PMPT protective value of 20% or greater indicated immunity, while the specificity of the ELISA was 95.7% (Table 4). The sensitivity and specificity were calculated on the basis of exclusion of all suspect reactors. If all suspect reactors were regarded as negative, then the sensitivity of the ELISA was 87.9% and the specificity 92.1%.

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.

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Table 4. Correlation of ELISA units and PMPT (% protection).

ELISA group	Passive mouse protection test PMPT Positive (%)						
	PMPT Negative (0%)	20	40	60	80	100	Total positive
negative	67	35	20	7	0	1	63
suspect	3	4	5	3	1	1	14
positive	3	11	30	47	42	221	351
Total	73	50	55	57	43	223	428

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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

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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 LD_{50} 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, 45.0% 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).

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COUNTRY REPORTS

Indonesia

A.A.G. Putra[1]

Geography, vegetation and agriculture

The Republic of Indonesia stretches from 6°08′ north latitude to 11°11′ south latitude, and from 94°45′ to 141°05′ east longitude. The estimated area of the Republic of Indonesia is 5 193 250 km², which consists of a land territory of 2 027 087 km² and a sea territory of 3 166 163 km². Indonesia has two main weather seasons, the dry season from June to September and the rainy season from December to March. The temperature varies between 21 °C and 33 °C, with an average of about 25 °C.

The rich flora of Indonesia contains many unique examples of tropical plant life and abounds in useful timber species. About 60% of Indonesia is under forest and about 8% of the total area is used for agriculture. Of the arable land, 30% is used for rice paddy, 21% for food/vegetable gardens and the remainder for house compounds, plantations, tree-crop cultivation and fallow land.

The importance of agriculture in the economy

Agriculture continues to be the dominant sector of the Indonesian economy, with a growth rate of about 4.2% annually. The sector contributed about 23.5% of total GDP between 1983 and 1985. Food-crop production was the most important contributor to the GDP, followed by the production of commercial crops by small farm holders. Food-crop production accounts for about 63% of the agricultural output, while livestock and livestock products contribute about 10%.

The livestock industries

Livestock numbers have increased each year for the past 4 years, due to an increasing demand for meat, eggs and milk and a major improvement in purchasing power.

Animals are used primarily as a store and source of wealth and a source of draught power. Most farmers have only two or three animals and use traditional management systems such as zerograzing or the cut-and-carry system.

The major animal product is meat, with an annual production of more than 1.2 million tonnes. It is considered that the demand for meat will grow, that supply will not keep pace with demand, and that there will be an upward movement of prices for both meat and livestock. The increasing demand for meat and draught power should ensure that farmers' wealth will grow in the short and medium terms.

Animal health systems

The three main objectives of animal health systems in Indonesia are to provide:

- healthy animals in order to optimise production and reproduction;
- a healthy farming situation by controlling contagious diseases of economic importance; and
- healthy livestock products that are safe for human consumption.

The Indonesian Government maintains 338 Pos Kesehatan Hewan (Animal Health Centres) throughout the country, with at least one Animal Health Centre in each District. The task of the centres is to diagnose and to treat sick animals and to control animal disease. To support this activity there are 7 A-type Veterinary Laboratories (Disease Investigation Centres) that cover particular regions, 18 B-type laboratories at the Province level, and 14 C-type laboratories at District level. The Government recruits personnel (veterinarians and veterinary assistants) to man the animal health centres and laboratories and supports improvement of their skills through the provision of training, either locally or abroad.

Apart from the diagnostic laboratories, the Government has established a veterinary drug assay laboratory with the task of controlling the safety and efficacy of veterinary drugs and biological products

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before they are used in the field. A biological production facility (Pusat Veterinary Farma — PusVetma) has also been established to produce vaccines and some biological reagents.

Integrated animal health services are provided through the laboratory network and the animal health centres under the coordination of the Provincial/District Veterinary Services. An animal health information network and an early warning system have recently been introduced to support the Veterinary Services.

Animal diseases of importance

At least 24 animal diseases are of economic importance. Some occur sporadically, while others are endemic throughout the country. The Government supports the control of these diseases through vaccination programs. Animal movement control and strict quarantine procedures have restricted some diseases to certain islands and kept other areas free of some diseases.

Pasteurellosis

Pasteurellosis, particularly haemorrhagic septicaemia (HS), is one of the most economically important diseases affecting livestock in Indonesia. In 1973, losses due to the disease were estimated at about 5.4 million rupiahs (Anon. 1977). HS affects buffalo, cattle, pigs and, sometimes, goats and sheep.

In the field, the disease is easily diagnosed clinically by experienced personnel at the Animal Health Centres or by District Veterinary Officers. In most situations, sample collection materials and bacterial transport media are not available in the field, and clinical diagnosis is supported by histopathology.

Saman and Mappeasse (1985) isolated *Pasteurella pneumotropica* from a pig. The identification was based on the organism's biochemistry. *P. multocida* has been isolated from clinically affected animals with HS and through abattoir surveys.

The annual incidence of HS throughout the country is about 0.1%. This figure varies according to the geographical area and the proportion of immune animals in a population. In some areas, the disease tends to be epidemic, while in other parts of the country HS is reported only sporadically. It has been observed that, in extensive farming systems, if herd immunity is greater than 70% the disease occurs sporadically. This is not so in intensive (or mini-ranch) farming systems (Putra 1992). Field cases of HS are usually treated with oxytetracycline.

HS, because it is so economically important in cattle in Indonesia, is controlled by the central Government through a routine vaccination program. The Government aims to vaccinate 60% of susceptible animals. A pilot project has been undertaken to control HS on the island of Lombok, in the Province of West Nusa Tenggara, by vaccinating all susceptible animals for 3 consecutive years. A similar program is being introduced in the neighbouring island of Sumbawa.

An oil adjuvant (paraffin/lanolin) vaccine — made from *P. multocida* Katha strain — is used widely in Indonesia. Under field conditions, up to 99.6% of cattle vaccinated against HS are protected (Setiawan et al. 1983). Animals older than 5 months are vaccinated every 6 months, with 3 mL administered intramuscularly.

Four million doses of HS vaccine are produced annually by PusVetma for use in Government vaccination programs. Recently, a private company also began producing a HS vaccine, which has a cost per dose of approximately Rp300, or about US$0.15.

In the past, most of work done on HS has concentrated on the evaluation of the effectiveness of vaccines. Other work has included abattoir surveys for the isolation of *P. multocida* from buffalo, cattle and pigs (Prodjoharjono et al. 1977; Sudana et al. 1981). While a number of strains of *P. multocida* have been isolated, only a few have been typed.

Priorities for pasteurella work in the future include:

- the development of techniques to replace the passive mouse protection test, which is presently used to detect the presence of protective antibody against HS; and
- epidemiological studies on HS to optimise control and eradication programs for the disease.

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India

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Geography, vegetation and agriculture

The Government of India has divided the country into 15 regions for agricultural planning purposes. For livestock purposes, however, the agro-climatic regions are divided as follows:

- Himalayan region up to 1000 mm rainfall
- North-west dry region 1000 mm rainfall
- Eastern region 1000–1800 mm rainfall
- Central plateau 1000 mm rainfall
- North-eastern region heavy rainfall, 1800 mm
- Western region heavy rainfall, 1800 mm
- Southern region 1000–1800 mm rainfall

The main crops grown depend on the climate, and include rice, sugarcane, jute, maize, wheat, bajra, gram, potato, jowar, ragi, cotton, groundnut, tobacco, bananas and coconut.

The livestock industries

Cash crops and grain production are the major agricultural activities in India. Livestock production is integrated with these enterprises, providing draught power and manure.

Animal husbandry plays a significant role in the rural economy by providing employment, particularly to small/marginal farmers and agricultural labourers. Cattle, buffalo and goats are used primarily for milk production rather than for meat, because of religious beliefs. It is estimated that 60% of the income from animals is from milk and milk products, 11.5% from meat and meat products, and 7.8% from dung used for fuel.

In 1990, the estimated ruminant population was 197 million cattle, 75 million buffalo, 110 million goats and 54 million sheep. Extensive or free-range grazing systems in rural areas, and semi-intensive and intensive systems in organised farms, are used in cattle, buffalo, sheep and goat husbandry.

Livestock genetic improvement is being made through artificial insemination and through embryo transfer programs. Fine-wool sheep are being introduced to meet the local and export demand for wool, and improved breeds of poultry are being developed to increase egg and broiler meat production.

The introduction of dairy development programs has led to better utilisation of land, water and human resources. Recent improvements in agriculture and animal husbandry have made a perceptible change in livestock farming systems. Agro-industrial by-products such as crop and forest residues, oil seed cakes, by-products of vegetable and fruit farming, marine wastes and water weeds are being used for livestock feed, and animals are given little access to grazing.

Animal health systems

Animal health care is provided through various polyclinics, hospitals, dispensaries, veterinary aid centres and diagnostic laboratories. The Government is placing emphasis on the prophylactic use of vaccines and drug therapy and has established control and eradication programs for major notifiable diseases, as well as disease surveillance units and quarantine stations. Due to the variation in the agro-climatic conditions in India, it is difficult to implement health programs in a uniform manner throughout the country.

India has 30 000 veterinarians engaged in animal health activities. On average, 1000 veterinarians graduate from a number of veterinary colleges every year.

Animal diseases of importance

Under the Animal Disease Surveillance Program, epidemiological information in respect of 37 major diseases of livestock and poultry is collected from strategically placed epidemiological units. The information is compiled and circulated to the States for guidance, and to allow veterinarians to take appropriate control measures.

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Pasteurellosis

Haemorrhagic septicaemia (HS) occurs throughout India. The disease is seasonal, with major outbreaks occurring just before the onset of, and during, the monsoon season. During the 1940s and 1950s the annual mortality rate due to HS was about 60 000 in cattle and buffalo. With the adoption of newer vaccines and drugs, the incidence of disease and the mortality rate have been reduced considerably. In addition, effective antimicrobial agents such as antibiotics and sulphonamides are being used to control mortality. In some areas, pre-monsoon outbreaks of pasteurellosis have been recorded in livestock, especially in poultry. The pneumonic form of pasteurellosis occurs in sheep and goats.

The mortality rate in pasteurellosis varies with factors such as the species and age of the animal, its immune status and history of pre-exposure to the disease, and the sanitary status of the area. Younger animals tend to be more susceptible.

P. multocida P52 strain is used to produce vaccines to control HS in cattle and buffalo. Almost all Government-owned biological production centres manufacture an alum-precipitated vaccine, whereas the oil adjuvant vaccine is manufactured in only a few units.

The alum-precipitated vaccine provides only 3–4 months immunity, whereas the oil adjuvant vaccine provides 6–9 months protection, after primary vaccination. In organised farms, oil adjuvant vaccine is used, whereas under field conditions the alum-precipitated vaccine is preferred. Exotic cross-bred cattle and buffalo are vaccinated at 4–6 months of age using 3 mL of oil adjuvant vaccine initially, followed by a booster vaccination in 3–6 months time and annual vaccinations thereafter. Vaccination is carried out at the onset of the rainy season. Sick animals are treated with drugs.

Efforts are being made to manufacture a HS vaccine that may increase the immune response and immunity period. Immunostimulators are also being used for developing vaccines that may be helpful in increasing the humoral and cell-mediated immune responses.

Thailand

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Geography, vegetation and agriculture

The Kingdom of Thailand has a land area of 513 115 km^2, shares land boundaries with Myanmar, Laos, Kampuchea and Malaysia, and has a southern sea boundary with the Gulf of Thailand. The country can be divided into four regions based on geography, topography and climatic conditions. These regions are the Central Plain, the North, the North-East and the South. Bangkok metropolis is the capital city and the principal port of Thailand.

Thailand is a tropical country with an average temperature of 28 °C and a yearly rainfall of 1400–2400 mm. In most regions, there are three seasons, the rainy season from June to October, the cool season from November to February, and the dry season from March to May.

Most of the land in the Central, the North and the North-East comprises fertile rice-growing areas. In the South, tree-crops such as rubber, oil palm and coconut are grown. Crops such as cassava, sugarcane, sorghum, soybean and cotton are grown in the North-East, the North and the Central regions, while maize, mungbean and groundnuts are grown in all regions of the country. In the North, farmers mainly practice agronomiculture and horticulture, while some maintain small numbers of livestock.

In the North-East region, most of the farms have native poultry and cattle or buffalo, and usually grow rice as the major crop. In the Central Plain, farmers have developed cash-crop cultural technologies, animal husbandry, aquaculture, orchards and woodlots. Around irrigated areas, farmers grow vegetables or high-value crops for cash after rice harvesting. In the South, farmers produce rubber, oil palm, coconut, coffee, fish, and livestock on a small-scale.

The importance of agriculture in the economy

Agricultural production in Thailand consists of crops (73%), livestock (13%), fisheries (9.2%) and forestry (4.7%). Agricultural production employs directly or indirectly almost 70% of the Thai population and accounts for 23% of the national income. The average income of farmers is less than 1000 baht (US$40) per month, which is less than that of workers in other sectors of the economy.

Livestock and livestock products contribute approximately 20% of total agricultural output and in 1985 accounted for about 5% of the national income.

During the 1980s, Thailand achieved considerable growth in cereal, coarse grain and cash-crop production. The country is a net exporter of rice, maize and cassava. Exports of agricultural products provided 69% of the value of Thailand's total export and an annual export surplus over agricultural imports of almost 85 000 million baht (US$3400 million), which makes agriculture the most important sector in the balance of trade. Most Thai farmers are small-holder farmers, with the average size of farm holdings in the Kingdom being 22.3 rai (3.5 ha).

The livestock industries

In 1990, Thailand's livestock population was approximately 5.6 million cattle, 4.6 million buffalo, 7.3 million pigs, 107.5 million chickens, 18 million ducks, 518 000 geese, 162 000 sheep, 120 000 goats, 19 000 horses and 3000 elephants. Sheep and goat populations are too small to be significant in the national production.

Large-scale cattle farms using open-range growing are situated in some areas of the Central and North-East regions. In some feedlots, cattle are housed in shelters with open sides. Dairy cattle are usually housed, and are turned outside to pasture or drylots between milkings. Dairy cows are fed with hay and concentrate feeds.

Traditionally, cattle and buffalo have been raised mainly for draft purposes. Meat and milk production has been of secondary importance. Small-scale farms depend on natural fodders, weeds and crop residues for feed, due to the limited grazing areas

[1] Northern Veterinary Research and Diagnostic Center, Department of Livestock Development, Thailand

and a shortage of natural fodders and weeds in the field. Supplemental feeding with forages and concentrates is rarely practiced in rural areas. In these areas, most farmers take cattle and buffalo to weekly auction markets.

Animal health systems

The Department of Livestock Development is responsible for animal health services. The Department is under the Ministry of Agriculture and Cooperatives. The Government is mandated to prevent, control and eradicate several types of diseases, therefore most small-scale farmers are provided with animal health services such as vaccination, deworming and diagnostic tests. Four divisions of the Department are involved in different aspects of animal health, namely the Division of Disease Control, the Division of Veterinary Services, the Division of Veterinary Biologics and the Division of Veterinary Research.

The Division of Veterinary Research has a central diagnostic laboratory located at the National Animal Health and Production Institute. The Division also has three regional veterinary research and diagnostic centres in the North, the North-East, and the South.

The commercial sector is primarily concerned with swine and poultry health and maintains large intensive production units that provide animal health services for farm customers. This includes the supply of vaccines and pharmaceuticals and veterinary supervision of stock.

Animal diseases of importance

Epidemic diseases such as foot-and-mouth disease, swine fever, Newcastle disease, haemorrhagic septicaemia (HS), fowl cholera and duck plague are important diseases of livestock in Thailand. Less-debilitating diseases, such as mastitis of dairy cattle, helminthiasis of cattle and brucellosis of dairy cattle cause chronic ill-health and therefore significant economic losses.

Sporadic outbreaks of the following infectious diseases have been recorded: Aujeszky's disease, pasteurellosis, atrophic rhinitis, enterotoxigenic *Escherichia coli* infection, enzootic pneumonia, fowl pox, chronic respiratory disease, infectious coryza, infectious bronchitis, infectious bursal disease and infectious laryngotracheitis. Zoonoses such as anthrax, rabies, trichinosis, salmonellosis and tuberculosis constitute important public health problems in some areas of the country.

Pasteurellosis

Haemorrhagic septicaemia (HS) in cattle and buffalo has been recognised in Thailand since 1960. Regional veterinary diagnostic centres are currently undertaking laboratory surveillance of the incidence of pasteurella infections. The prevalence of pasteurella infections in sheep and goats is not high, but is often damaging to small-scale farms. Pasteurellosis is usually diagnosed by field staff on the basis of clinical signs, and only some cases are investigated by the regional veterinary centres.

An epidemiological survey of two outbreaks of HS in the North-East indicated that, for village buffalo, there was about a 2% morbidity rate, a 1.5–1.8% mortality rate, and a case-fatality rate of from 72% to 100%.

HS has been classified as a notifiable disease since 1956. When it breaks out in a village, a government veterinarian has by law to consider an epidemic zone to control the extent of spread. Although the Department of Livestock Development has strongly recommended that at least 70% of village cattle and buffalo

Table 1. Pasteurellosis under laboratory surveillance by year, Thailand (1982–86).

Year	Host	Region**	No. cases
1982	Cattle	N	1
	Buffalo	N	1
	Swine	NE	1
		NE	10
		NE	12
		C	2
		C	6
		S	1
1983	Cattle	N	8
	Buffalo	NE	1*
	Goat	NE	1*
	Swine	NE	1
		NE	4 + 1*
		C	3
1984	Sheep	N	1
	Swine	C	1* + 4
1985	Buffalo	N	6
	Goat	C	4
	Swine	S	1
		S	2*
		S	11
1986	Cattle	N	2
	Buffalo	N	9
	Sheep	C	3*
	Goat	C	7
	Swine	S	1
		S	1*
		S	2* + 1
		S	7

* cases of *P. haemolytica* infection (all other cases are of *P. multocida* infection)

** N, North; NE, North-East; C, Central; S, South

be vaccinated with aluminium hydroxide gel vaccine every 6 months, HS still occurs sporadically in animals that have, for various reasons, not received regular vaccination. HS is seen most frequently at the start of the rainy season.

Table 2. Pasteurellosis under laboratory surveillance by year, Thailand (1987–91).

Year	Host	Region**	No. cases
1987	Buffalo	N	1
	Sheep	NE	1
	Goat	C	1*
	Swine	C	14
		S	4*
		S	0
1988	Cattle	N	1
	Buffalo	N	1
	Goat	N	1
	Swine	NE	1
		C	18
		S	1*
		S	2* + 1
		S	113
1989	Cattle	N	1
	Buffalo	N	1
	Sheep	N	4
	Goat	NE	5
	Swine	NE	5
		C	1
		C	1
		C	13
		S	2*
		S	3
1990	Cattle	N	2
	Buffalo	N	1
	Goat	N	3
	Swine	NE	1
		NE	6
		NE	5
		C	13
		S	1*
		S	2*
		S	4
1991	Cattle	N	2
	Buffalo	N	4
	Goat	NE	3
	Swine	NE	4
		NE	1
		C	4
		C	8
		S	6*
		S	7

* cases of *P. haemolytica* infection (all other cases are of *P. multocida* infection)
** N, North; NE, North-East; C, Central; S, South

Diagnosis of HS is based on a history of non-vaccinated animals, a rapid course, and oedematous swelling in the throat, cervical and parotid regions. Clinical diagnosis can be confirmed by examining blood smears from moribund or recently dead animals and by sending samples for laboratory culture. Definitive diagnosis of HS can only be made on the basis of isolation and identification of *P. multocida*, with subsequent serotype identification (B:2 or E:2) by an indirect haemagglutination test. The other pasteurella infections can be identified from morphology and characteristics of bacteria by conventional methods.

Disease in animals that do not show the typical clinical signs may be attributed to anthrax, pneumonic pasteurellosis (other serotypes) or acute salmonellosis.

In Thailand, prophylactic vaccination is the policy of choice to control HS. Aluminium hydroxide gel vaccine is preferred, probably because of the ease of production and the ease of injection. An oil adjuvant vaccine may not be practical for use in Thai villages — it may cause swelling of the site of injection, which would not be accepted by villagers. Besides vaccination, good management and husbandry practices can be helpful in controlling the disease.

Aluminium hydroxide gel vaccine is produced at the Veterinary Biologics Center, under the policy of the Department of Livestock Development. It is formulated from a broth bacterin to which aluminium hydroxide gel has been added. The vaccine is standardised to contain 3×10^{10} CFU/dose of *P. multocida* serotype B:2, have a dry weight of 2–5 mg/dose and a turbidity equal to MacFarland's tube number 7. The recommended dose is 3 mL, given subcutaneously.

Recently, a new HS vaccine in oil adjuvant has been studied in an agricultural technology transfer project of the Ministry of Agriculture and Cooperatives. The Veterinary Biologic Center at Pakchong was concerned with formulating different kinds of emulsion using various emulsifying agents and light mineral oils. The composition of a selected formulation (by weight) is as follows: 55 parts of Marcol 52; 4 parts of Arlacel A (HLB 4.3); 1 part of Tween 80 (HLB 15); 40 parts of broth bacteria. A preliminary study using the oil adjuvant vaccine with the above formula indicated good protection lasting up to 12 months in experimental cattle and buffalo.

The Department of Livestock Development currently produces about 7 million doses of HS aluminium hydroxide gel vaccine a year. The cost per dose could not be estimated officially, but a

cost/benefit study of HS disease control determined that it costs 70 baht to vaccinate an animal in Thailand. In our HS control program, the villagers eagerly cooperate in vaccination, indicating that they recognise HS as a danger that can be prevented. The support of the villagers is recognised as essential in any successful disease control program.

Established research projects on pasteurella are as follows:

- A study to determine minimum inhibition concentration of various antibiotics for *P. multocida* isolates
- A study to determine the serotype of *P. multocida* causing fowl cholera
- Serotyping of *P. anatipestifer* isolated from ducks.

The Government has not prioritised areas of research on pasteurellosis because of unreliable information on the actual incidence of pasteurella infections. Most Thai researchers judge the importance of pasteurella infections on the basis of their experience, gained from case studies.

Pasteurella research planned for the future includes:

- Studies on the efficacy of a whole-cell oil adjuvant fowl cholera bacterin in ducks
- Studies on the efficacy of a purified protective antigen of *P. multocida* produced by saline extraction
- Serotyping of *P. haemolytica* in the South
- Analysis of the antigenicity of *P. haemolytica* isolates.

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Malaysia

R. Jamaludin[1]

Geography, vegetation and agriculture

Malaysia has a total land area of 329 758 km^2 and a population of about 18.6 million people. The country is situated in the wet tropics and has an average annual rainfall of 2540 mm. Eighty percent of Malaysia is covered by tropical rain forest and 35% of the total land area is suitable for agriculture.

The importance of agriculture in the economy

In 1979, agriculture (including forestry and fisheries) contributed 24% of the GDP. This proportion has fallen steadily, to 17.3% in 1991. Presently the agricultural sector provides 35% of total national employment. Plantation crops — mainly rubber, oil palm and cocoa — are the major crops grown in the country. Paddy, coconut fruits, pepper, vegetables and tobacco are other crops grown in large areas.

The livestock industries

The livestock sector contributes 3% to the GDP, 13% to the overall agriculture production and 33–35% to the value of food produced in the country. Cattle, buffalo, goats, sheep, poultry and swine are the main animals reared. The system of ruminant production is gradually changing from subsistence to intensive operations. Presently, 200 000 small farmers rear cattle and buffalo, however there is a gradual decrease in the number of farmers who have larger herds. Specialisation in farm operations is being adopted, especially feedlotting and cow-calf operations. In the poultry subsector, there is specialisation in egg, broiler and breeder production. For sheep and goats, integration with plantation crops is expanding. Nutrition, breeding and management continue to be of concern in ruminant production.

[1] Regional Veterinary Laboratory, Johor Bahru, Malaysia

Animal health systems

Diagnostic Services in Peninsular Malaysia are provided by seven Regional Veterinary Laboratories and the Veterinary Research Institute (VRI), all of which are strategically located.

Diseases of importance

Malaysia is free of major exotic diseases such as rinderpest, African swine fever, contagious bovine pleuropneumonia, anthrax, sheep pox, goat pox, scrapie, peste des petits ruminants, vesicular stomatitis, African horse sickness and fowl plague.

Foot-and-mouth disease occurs sporadically and is confined to the northern border. Similarly swine fever is rarely encountered and exclusively confined to backyard operations, which are being phased out.

The main diseases of economic importance are haemorrhagic septicaemia (HS), babesiosis, brucellosis, myiasis and tuberculosis in cattle; helminthiasis, caseous lymphadenitis, clostridial diseases, melioidosis and pasteurellosis in sheep and goats; Aujeszky's disease in pigs; and Newcastle disease, infectious bursal disease, pullorum, duck viral hepatitis, duck plague and duck pasteurellosis in poultry.

Pasteurellosis

HS is a disease of major economic importance in buffalo and cattle. Ovine pasteurellosis is becoming increasingly important. About 90% of cases of pasteurellosis in buffalo are caused by *Pasteurella multocida* Carter's Type B, while in cattle type B is isolated in only 22% of cases. Joseph (1979) reported outbreaks and losses during 1967–76 in 3605 cattle and buffalo worth M$2 million. The total loss for the period 1980–89 was M$2.25 million (Abdul Rahim 1991). HS is enzootic in several states of Malaysia. Other forms of pasteurellosis in cattle are usually characterised by pneumonia and are caused by *P. haemolytica* (39% of cases) and *P. multocida*

type A or D (61% of cases). Most isolations are from animals less than 1 year old.

Pasteurellosis is also one of the most common infectious bacterial diseases of sheep and goats. Unlike the case in cattle, the disease is caused mainly by *P. haemolytica* (70%) and less commonly by *P. multocida*. The two main biotypes of *P. haemolytica* in sheep are A and T, while the predominant *P. multocida* serotype is Carter's type D (86%). Cases are usually coexistent with other diseases such as helminthiasis, melioidosis, enterotoxaemia, malnutrition and other stress-related conditions. All ages are affected and the disease is seen throughout the year.

In rabbits 86% of cases of pneumonic pasteurellosis are caused by *P. multocida* Carter's Type A, while in deer the main serotype is Carter's Type D.

Diagnosis of pasteurellosis is based on pathology, bacteriology and histopathology. The serotyping of *P. multocida* is carried out by the slide agglutination test or the passive mouse protection test.

The types of pasteurella vaccines produced in Malaysia and their estimated cost per dose are:

- HS alum-precipitated vaccine (APV) M$1.05
- HS oil adjuvant vaccine (OAV) M$0.84
- Sheep pasteurellosis vaccine (OAV) M$1.10
- Duck pasteurellosis vaccine (APV) M$0.05

HS APV is recommended for in-contact animals in an area of outbreak of HS. All susceptible animals within a radius of 5 km of the focus of an outbreak are also vaccinated with APV. Animals are revaccinated with HS OAV within 2–3 weeks, using a dose of 5 mL subcutaneously.

The HS OAV is used for prophylaxis. All susceptible animals in government farms are vaccinated. In endemic HS areas, animals are vaccinated with OAV at least 1 month before the next anticipated outbreak. Currently it is advised that 5 mL of both HS OAV and APV be given subcutaneously, with annual revaccination.

Sheep pasteurellosis OAV is recommended for use in the control of pneumonic pasteurellosis, especially in areas or on farms where there is a very high concentration of sheep and goats. Animals 3 months or older are given 3 mL of the vaccine intramuscularly, then a booster dose followed by annual boosters. Imported combined vaccine against clostridial diseases and pasteurellosis is also used, because of concurrent disease problems.

No major problems have been encountered with any of these vaccines, except for swelling at the site of administration and lameness with uneventful recovery in about 10% of sheep. Abscess formation occurs when contaminated needles are used. The OAV vaccines are very viscous and have poor injectability. It is recommended that they are left at room temperature for 1–2 days before use.

Sulphamezathine and long-acting tetracyclines have been used in the treatment of HS. In ovine pasteurellosis, long-acting tetracyclines have been employed in disease control with varying success.

The VRI, in collaboration with ACIAR, is developing an improved vaccine based on the most potent immunogenic fraction of *P. multocida*. Field studies on the epidemiology of HS in cattle and buffalo are being undertaken by the Agriculture University. Priority is being given to HS research in buffalo. There is also a need to elucidate the aetiology of sheep pneumonia, as pasteurella may not necessarily be a primary pathogen. Field reports indicate that the present vaccine is only about 60% effective in crossbreds, and does not protect purebreds adequately.

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Vietnam

P.T. Phuong[1]

Geography, vegetation and agriculture

Vietnam borders China in the north, Laos and Cambodia in the west, and the sea in the east. The country has a mainland area of 331 689 km^2. The climate is tropical to sub-tropical with frequent monsoons, and the average temperature is 22–27 °C. Hanoi, the capital of Vietnam, is the political, economic and cultural centre of the country.

Three-quarters of the total land area of Vietnam is occupied by mountains and hills. Fifteen million hectares (45.7%) are covered by forest, and 5 million ha (15%) are cultivated. The country can be divided into seven ecological regions according to geography, climate and crops grown.

- The northern mountainous and midland region (rice, corn, cassava, soybean, coffee, tea, timber trees and bamboo)
- The Red River delta (rice, maize, sweet potato and vegetables)
- The northern part of the centre (rice, cassava, sweet potato, peanut and timber trees)
- The highland (cassava, coffee, rubber, maize, soybean)
- The coastal region of the centre (rice, cassava, sweet potato, pepper, coconut, timber trees)
- The eastern part of the south (rice, peanut, coffee, rubber, maize and cashew nuts)
- The Mekong River delta (rice).

The importance of agriculture in the economy

The economy of Vietnam is based mainly on agriculture. The agricultural income is around 50% of total national income. Food crops and vegetable production represent 75% of the agricultural output, and animal production, 25%.

Rice is the main crop cultivated, especially in the delta regions of the Mekong and Red Rivers. The second most important crop is maize, which is an important component of the Vietnamese diet. Maize is also a staple food for animals. Other rootcrops such as sweet potato and cassava are grown for animal feed. The production of annual crops has increased in recent times.

Fish production is a major agricultural component in Vietnam. The country has 2000 km of sea border and 309 760 ha of water used for aquaculture. The gross production of fish in 1991 was 1 767 800 dong (compared with 967836 dong in 1986).

Vietnam's 15 million ha of forest produces round wood, firewood, big bamboo and bamboo, slender bamboo, and special bamboo for paper production.

Table 1. Structure of national income (%), Vietnam.

Agriculture	50.6
Industry	20.2
Trade, material supply	17.6
Construction	4.3
Others	7.3
Total	100.0

Table 2. Structure of gross production of agriculture (%), Vietnam.

Vegetable production	75.5
— Food crops	52.8
— Vegetable and beans	3.3
— Industrial crops	12.7
— Fruit trees	5.3
Animal production	24.5
— Livestock	15.7
— Livestock production without slaughter	3.6
— Aquatic products	1.8
— Poultry	3.4
Total	100.0

[1] National Veterinary Research Institute, Hanoi, Vietnam

Table 3. Production of major annual crops (thousand tons), Vietnam.

	1976	1991
Food (in rice equivalent)	13 493	21 718
— Spring rice	3 730	6 788
— Autumn rice	1 531	4 761
— Winter rice	6 566	7 877
Other cereals (rice equivalent)	1 666	2 291
— Maize	387	652
— Sweet potatoes	1 485	2 104
— Cassava	1 816	2 390
— Potatoes	260	281
Industrial crops		
— Cotton	3	5
— Jute	28	27
— Rush	63	50
— Sugarcane	2 986	5 940
— Peanut	100	212
— Tobacco	16	29

The livestock industries

Approximately 2 929 000 buffalo and 3 282 700 cattle are used in Vietnam, mainly for draught power and to a lesser extent for milk and meat. The average milk yield per cow per year is around 1800–2000 kg. For buffalo, the figure is 1400–1500 kg.

Pigs number 12 582 000 head, with the Vietnamese breeds Y and Mong cai predominating. Imported breeds such as Landrace, Yorkshire, Duroc and Edel are also kept for pig meat for local consumption and for export. Pigs are reared on the less fertile agricultural land.

There are approximately 110.1 million poultry in Vietnam. Eggs and poultry meat are produced by state poultry enterprises and by village-based peasant families.

Goat farming occurs, but it is not important in terms of the gross national income. Sheep are not farmed in Vietnam.

Animal health systems

The Animal Health and Production Department is responsible for government veterinary activities. The Department has the following components:

- Provincial Veterinary Departments
- District Veterinary Stations
- animal health staff in the field
- a National Centre for Veterinary Diagnosis and Training, with six sub-centres
- a National Veterinary Control Drug and Vaccine Centre
- a National Veterinary Inspection Office, with six sub-centres.

Animal diseases of importance

- hog cholera (pigs)
- pasteurellosis (cattle, buffalo, pigs and poultry)
- trypanosomiasis (cattle, buffalo)
- Newcastle disease (poultry)
- leptospirosis (pigs, cattle)
- pullorum (poultry)
- Gumboro disease (poultry).

Pasteurellosis

Cattle, buffalo, pigs and poultry are affected by pasteurellosis, but buffalo, in particular, and cattle sustain major losses. Recovery in the absence of early treatment is rare. Pasteurellosis in cattle and buffalo is caused by *Pasteurella multocida* Carter's type B. Incidence of the disease is greatest during the rainy season, although isolated cases may occur at any time during the year. Epizootics occur in many areas, and in 1991 the number of recorded deaths due to pasteurellosis in cattle and buffalo increased.

Preference has been given to the use of an inactivated vaccine for the control of haemorrhagic septicaemia (HS) in Vietnam. Since 1957, the aluminium hydroxide-absorbed HS vaccine for cattle and buffalo has been used. However, this vaccine has low potency and a short duration of immunity, even after a relatively large dose (20 mL).

Three other types of pasteurella (HS) vaccine are now used in Vietnam. The vaccines are prepared from fermentor-produced bacterin, and cost approximately 150–200 dong per dose. They are:

- an alum-precipitated vaccine, 2 mL per dose, two injections per year
- an aluminium hydroxide absorbed vaccine, 2–3 mL per dose, two injections per year
- an oil adjuvant vaccine, 2 mL per dose, one injection per year.

Annual vaccination for HS is timed to take place just prior to the rainy season in March–April. If necessary, revaccination is undertaken in September, when cattle and buffalo are grazed.

Current pasteurella research activities and priorities are:

- studies on the immunogenicity and antigenicity of *P. multocida* types isolated in Vietnam and the typing of the isolates
- the preparation of an oil adjuvant vaccine for pasteurellosis and the evaluation of the immunoefficiency of the vaccine.

Priorities for future pasteurellosis work include:

- studies on modern methods such as ELISA, restriction endonuclease analysis (REA) and radiolabelling techniques for the diagnosis of pasteurellosis in cattle, buffalo, pigs and poultry
- introduction of the oil adjuvant vaccine on a large scale to eradicate the disease.

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Sri Lanka

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Geography, vegetation and agriculture

The Republic of Sri Lanka is an island situated in the tropics between the northern latitudes of 5°55′ and 9°50′ and eastern longitudes of 79°42′ and 81°51′. Its land area of 65 609 km^2 has a maximum north-south length of 435 km and a maximum east-west width of 225 km. The island is separated from the southern tip of India by a distance of 29 km at the nearest point. It has a mountainous area in the south-central region rising to a peak of 2500 m. This area is surrounded by plains — narrow in the western, southern and eastern sides, but widening into a vast expanse of land north of the mountains.

The mean temperature in the plains is 27–28 °C, and in the hill countries 10–24 °C. The annual rainfall differs considerably between regions, and ranges from less than 1000 mm in the driest plains to 5000 mm in the south-western slopes of the central hills. The rainfall is dependent on two monsoons, the south-west monsoon from April to September, and the north-east monsoon from October to March. The central hills, and the south-western parts of the island benefit from both monsoons, and comprise the wet zone. Those areas receiving rain only from the north-east monsoon constitute the dry zone. A combination of the elevation (hence temperature) and the rainfall give rise to a number of climatic zones (Table 1).

Sri Lanka has a human population of nearly 17 million.

The importance of agriculture in the economy

Agriculture accounts for approximately 25% of GDP and 60% of export earnings. Rice — the staple food — is grown in the wet regions; and also in the dry zone, with artificial irrigation. Tea, rubber and coconut are the major export crops. Tea is grown mainly in the hill and mid country, rubber in the wet plains, and coconut in the coastal areas. The contribution of the different agricultural commodities to agricultural GDP is shown in Table 2.

The livestock industries

There are approximately 1 783 000 cattle, 964 000 buffalo, 534 000 goats, 29 000 sheep, 86 000 pigs and 7 638 000 chickens in Sri Lanka.

The types and distribution of cattle and buffalo in Sri Lanka are shown in Table 1. Cattle are kept mainly for dairy and draught purposes. There is no organised beef industry, beef being provided from dairy bull calves and indigenous animals. Buffalo are used for milk, and for draught power in the rice fields.

The goat population is scattered around the country, with a higher density in the dry plains. Goats are used mainly for meat. A small percentage of the population, which has been upgraded by cross-breeding, is milked. Sheep are used for meat.

Pig husbandry is confined to the western coastal belt. Pig meat finds a ready market only in the main cities, and the industry is linked to processing plants and to tourist hotels around the city of Colombo.

Egg and broiler production are growing industries. There are 33 registered, large-scale commercial poultry breeders that supply chicks to poultry farmers.

Animal health systems

Animal health policy is determined by the Central State Department of Animal Production and Health and is implemented by the Provincial Departments of Animal Production and Health. Thus, animal health services have been devolved to the provinces. The work is carried out through 118 veterinary ranges.

Research, diagnostic services and production and quality control of animal vaccines are Central Government functions, mediated through a research institute, two vaccine-production laboratories and five regional veterinary investigation centres.

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Table 1. Different climatic zones in Sri Lanka, distribution of cattle and buffalo, and husbandry practices.

Zone	Elevation	Rainfall	Temperature	Type of cattle/buffalo	Husbandry practice
Wet zone					
Hill country	Above 1000 m	Over 2000 mm	10–24 °C	Pure exotic* breeds and crosses	Zero grazing Small herds
Mid country	300–1000 m	1875–5000 mm	21–32 °C	Pure exotic* breeds and crosses exotic breeds	Zero grazing Small herds
Low country	0–300 m	1875–2500 mm	24–35 °C	Crosses of exotic* breeds with zebu types. Indigenous cattle and buffalo	Limited grazing Medium-sized herds
Dry zone					
Hill country	Above 90 m	1250–1500 mm	10–30 °C	Crosses of exotic* breeds with zebu types. Indigenous cattle and buffalo	Limited grazing Medium-sized herds
Mid country	300–900 m	1250–1500 mm	21–32 °C	Crosses of exotic* breeds with zebu types. Indigenous cattle and buffalo	Limited grazing Medium-sized herds
Low country	0–300 m	1000–1750 mm	21–38 °C	Indigenous cattle, zebu cattle and their crosses. Buffalo	Free grazing Large herds

* Friesian, Jersey, Ayrshire and Shorthorn

Table 2. GDP in the agricultural sector, Sri Lanka, 1990.

Commodity	Agricultural GDP (%)
Tea	13.7
Rubber	2.2
Coconut	8.2
Other export crops	5.0
Rice	20
Other food crops	30.2
Forestry	4.0
Tobacco	0.2
Miscellaneous products	4.0
Fisheries	8.0
Livestock	4.5

Table 3. Reported deaths due to haemorrhagic septicaemia, and doses of HS vaccine used, 1984–91, Sri Lanka.

Year	Reported deaths	Vaccine use (doses)
1984	224	664 700
1985	383	562 900
1986	125	747 700
1987	480	648 000
1988	115	424 100
1989	409	465 800
1990	269	829 900
1991	379	450 200*

* Figure for North and Eastern Provinces not included

Animal diseases of importance

The following diseases are most important in Sri Lanka:

Cattle and buffalo	haemorrhagic septicaemia (HS) foot-and-mouth disease brucellosis black-quarter disease babesiosis (in exotic cattle only) rinderpest (now controlled)
Goats	parasitism enterotoxaemia
Sheep	parasitism
Pigs	swine fever
Poultry	Newcastle disease Marek's disease fowl pox fowl cholera

Pasteurellosis

HS is endemic in cattle and buffalo. Sporadic outbreaks of pneumonic pasteurellosis, caused by *Pasteurella haemolytica*, occur in goats where intensive rearing is practised.

The number of reported deaths, and the number of vaccinations carried out annually for HS for the past few years, are given in Table 3. The disease occurs mainly in the dry plains during the monsoon, and following a long dry spell. In 1991, outbreaks were recorded in 13 of the 118 veterinary ranges. The

case mortality rate was around 82%. However, since HS is a disease of poorly managed, free-roaming herds, it is likely that the recorded cases and deaths are underestimates.

Laboratory confirmation is by direct culture, mouse inoculation and isolation of pure culture, and biochemical and serological tests. Carter's 'capsular' typing by the indirect haemagglutination test and Heddleston's 'somatic' typing by the gel diffusion test are done routinely. Namioka and Murata's 'somatic' typing is done occasionally. ELISA, SDS-PAGE and immunoblotting are done for research purposes. Control of HS is by routine prophylactic vaccination in endemic areas, and ring vaccination in the face of outbreaks.

Serotype 2 of *P. haemolytica* predominates in cases of sporadic pneumonic pasteurellosis in intensively reared goats. In a serological study of 88 isolates — 50 from pneumonic lungs and 38 from the nasopharynx of normal animals — 55% belonged to serotype 2, 14% to serotype 11 and the rest to serotypes 1, 6, 7, 8, 9 and 12. Diagnosis is by culture and serology. In outbreak areas, autogenous vaccines are prepared and used.

Other pasteurella infections occurring occasionally are diagnosed in the laboratory by culture, serology, and non-serological tests such as hyaluronic acid decapsulation (for type A) and the acriflavine flocculation test (for type D).

Approximately 1 million doses of HS oil adjuvant vaccine are prepared annually. The vaccine consists of a dense bacterin, which, until 1992, was grown in a 40-L vessel with aeration. A 100-L fermentor is now used. The bacterin is emulsified with an equal volume of light mineral oil, with 4% anhydrous lanoline, BP grade.

Ideally, cattle and buffalo are vaccinated at 4–6 months of age and given a booster 3–6 months later. Thereafter, vaccination should be carried out on an annual basis. A 3-mL dose, containing approximately 2.25 mg dry bacteria, is administered for each vaccination.

The cost of production of vaccine is US$0.035 per dose. This costing includes materials, wages, services and depreciation on capital costs of equipment. It excludes the cost of buildings and land used. In the early years of vaccination, obtaining the farmers' cooperation was a problem. At the present time, in areas where the disease has been controlled effectively, farmers' response to HS vaccination is good.

Current pasteurella research activities and priorities are directed towards two broad areas:

- production of better vaccines
  - — investigation of the important immunogens of HS-causing *P. multocida* and investigations on in vitro antigens
  - — improvements to culture systems for obtaining denser cultures in the fermentor
  - — experiments with thinner emulsions or other adjuvants
  - — development of a reliable laboratory method for assessment of immunity
  - — investigations on avirulent variant strains for use as potential live vaccines
- further studies on epidemiology
  - — determination of the factors that are likely to convert a latent carrier to an active state, and their role in the initiation of an outbreak.

Cambodia

K. Kral,[1] M. Maclean[1] and S. San[1]

Geography, vegetation and agriculture

Cambodia covers a land area of 181 035 km^2 in Southeast Asia and shares its borders with Thailand, Laos and Vietnam. The majority of Cambodia's people live on a low-lying plain around the Tonle Sap and upper Mekong deltas. Phnom Penh, the capital city, is located at the junction of the Mekong, Bassac and Tonle Sap Rivers. In the southwest, a highland region formed by the Cardamom Mountains and the Elephant Mountains separates the rest of the country from a heavily forested lowland strip along the southern coast.

Cambodia's climate is tropical monsoon, with two distinct seasons — the rainy season from June to October and the dry season from November to May. Rainfall, often uneven and irregular, results in periodic droughts or floods. The heaviest rainfall occurs on the coast between the mountains and the Gulf of Thailand. Cambodia's temperature range is 21–35 °C, with the highest temperatures occurring between March and April at the end of the dry season.

In the central lowland area there are rice paddies, crops such as corn and tobacco, tall grass areas, and thinly wooded areas. The transitional plains are mostly covered with savanna grasses. Throughout the country there are evergreen, deciduous and mangrove forests as well as rainforests. Because firewood remains Cambodia's principal source of energy, a great deal of deforestation has taken place in recent years. Sugar palm trees are abundant, especially in the central lowland areas, where they are used in construction and to make sugar, medicine, vinegar and wine.

[1] American Friends Service Committee, Phnom Penh, Cambodia

The importance of agriculture in the economy

Cambodia has five main agricultural ecosystems:

- forests and mountains, where, in addition to timber, a number of crops are grown (rubber, cotton, coffee, fruit, and upland rice)
- river banks, where high yields are achieved due to the availability of water and the yearly deposit of silt by the receding floodwaters (vegetables, beans, maize and fruit)
- lowland floodplains (dry-season rice cultivation)
- upper terraces, which, except for the clay soils of Battambang province, have poor, sandy and acid soils (wet season rice)
- great lake floodplains, which border the Tonle Sap (floating and deep-water rice).

Economically, the 2 500 000 cattle and 780 000 water buffalo are the most important animals in Cambodia. The animals are used for draught power to prepare and cultivate rice fields and in transportation of goods to the market. Pigs are raised to be sold at the markets or eaten by the family. Many farmers keep small flocks of chickens for food. The eggs are eaten by the family or sold to the market. Most of the horses are small and are used to pull carts. There are very few sheep and goats in the country.

Eighty-five percent of the population is engaged in agricultural production, mainly in the cultivation of rice. Other important crops include rubber, sweet potato, maize, sugarcane, beans, fruits, vegetables and tobacco. Although the best soils are found in the central plains, including those areas flooded by the Mekong and Tonle Sap Rivers, two-thirds of the paddy acreage is situated on sandy soil, the poorest type in the country.

The average farmer has one cow, one pig and five chickens on 1 ha of land. During the rainy season, when the rice is planted and harvested, cattle are kept on the property and fed using a cut-and-carry system. During the dry season, cattle and water buffalo are allowed to graze freely. Their nutritional status deteriorates significantly during this period, before heavy field work is resumed.

In addition to rice and other crop production, farmers also engage in additional agricultural and subsistence activities such as sugar palm tapping, sugar processing, aquaculture and fishing, animal husbandry, silk-weaving and basket- and mat-making.

Animal health services

The Ministry of Agriculture has 16 departments, including Agronomy, Animal Health and Production, and Fisheries. The Department of Animal Health and Production, with assistance from the Lutheran World Service (LWS), operates one pig-breeding and two cattle cross-breeding stations. There is a chicken research station in Kandal province. In conjunction with American Friends Service Committee (AFSC), the Department has operated a laboratory producing vaccine against haemorrhagic septicemia (HS) since 1986.

The Department of Animal Health and Production cooperates with Church World Services (CWS) in the operation of the Veterinary National Diagnostic Laboratory in Phnom Penh and a provincial diagnostic laboratory in Battambang. The Department receives technical and financial assistance from CWS, LWS, AFSC, and the Food and Agriculture Organisation of the United Nations (FAO) for maintaining stocks of animal vaccines and cold-chain equipment.

Chamcar Daung Agricultural Institute and Prek Leap Agriculture College instruct students in agronomy, veterinary science, animal husbandry, fishery and forestry. Working with departmental staff, development and relief officers provide multiple 2-week training courses for vaccinators in the provinces during the dry season. People from the districts, subdistricts, and villages are taught principles of vaccination and storage and administration of vaccines, and how to construct chutes in which to confine animals to be vaccinated, identified and treated for certain diseases.

A cold-chain system has been developed to ensure that the vaccines will not lose their potency before reaching the provinces. The vaccines are stored in large cold rooms at the Department in Phnom Penh. They are then transported either in trucks or buses to the provinces, where they are kept in solar refrigerators, at least one of which is operated by each province. Small cold boxes attached to bicycles or motorbikes facilitate transport of the vaccine to the vaccination clinic site.

Animal diseases of importance

The major animal diseases of importance are: HS (*Pasteurella multocida*), foot-and-mouth disease, anthrax and blackleg in cattle and buffalo; hog cholera, swine plague (*P. multocida*), salmonella infection and erysipelas in pigs; and Newcastle disease and fowl cholera (*P. multocida*) in poultry.

Pasteurellosis

HS is seen in cattle and water buffalo. The animals often die within 12–24 hours of the onset of symptoms. In 1992 to date, HS has affected 2000 animals in 11 of the 21 provinces of Cambodia, and 939 have died. The disease is usually diagnosed in the field on the basis of clinical symptoms, and treated where possible.

The Ministry of Agriculture, through the Department of Animal Health and Production, has organised a program of vaccination against pasteurella infection in cattle, water buffalo and pigs. Vaccine produced in the laboratory in Phnom Penh (for draught animals) or vaccine imported from Laos is transported to the provinces, where vaccination clinics are held.

In 1991, the HS vaccine production laboratory in Phnom Penh produced nearly 1 million doses, of which 66% was in an oil adjuvant base and 33% in alum. In addition, 300 000 doses of oil adjuvant HS vaccine were imported from Laos. Due to problems associated with the difficulty in obtaining laboratory supplies, only 264 000 doses of vaccine (66% oil adjuvant) have been produced by the Phnom Penh laboratory thus far in 1992, and 250 000 doses have been imported from Laos.

The optimal time for vaccination of draught animals against HS is at the end of the dry season in February–March, before the period of highest productivity and susceptibility, the rainy season, begins. The alum adjuvant vaccine requires twice-yearly inoculations, involving difficulties in timing and transportation of animals to vaccine clinics. Some farmers in mountainous areas allow the cattle to range far from home during the dry season and the movement of the animals back to the fields leaves no time for vaccination. There are also difficulties associated with transporting vaccine (including lack of vehicles and fuel), with keeping vaccine cold, and with availability of trained vaccinators.

No research is being done on pasteurella in Cambodia at the present time.

Future plans include:

- increasing the proportion of HS oil adjuvant vaccine to eventually eliminate the need for twice-yearly injections;
- a feasibility study to increase production at the Cambodia laboratory, thus decreasing the need for importation of vaccines;

- training courses for vaccinators and education of farmers.

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Myanmar

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Geography, vegetation and agriculture

Myanmar is the largest country on the mainland Southeast Asia, with a land area of 676 568 km^2. It shares international borders with Bangladesh and India on the north-east and Laos and Thailand on the south-east. Myanmar is a mountainous country with three parallel chains of mountain ranges running from north to south: the Rakhine Yoma, the Bago Yoma and the Shan Plateau. These mountain chains divide the country into three river systems: the Ayeyarwady, the Sittoung and the Thanlwin. The Ayeyarwady (length is 2170 km) is the most prominent river system in the country. Its major tributary, the Chindwin, is 960 km long. The delta area of the Ayeyarwady is vast (240 km by 210 km) and is the main rice-growing area of the country. About 48% of the total land area is covered with forests, whereas 15% of the total land surface is used for cultivation. The principal crop-growing areas are located in the river basins of central Myanmar, the Delta and the coastal areas of lower Myanmar.

The importance of agriculture in the economy

The economy of Myanmar is dominated by the rural sector, which comprises 85% of the country's population. Agriculture contributes 38% of GNP and almost 60% of employment.

Most land is farmed by small holders having an average holding of 2 ha. Rice is the most important crop, accounting 48% of the total land sown. Oil seed crops, such as groundnuts and sesame, rank second in importance. Industrial crops such as cotton, jute, sugarcane and tobacco account for less than 5% of the total sown acreage. Myanmar also grows chillies, garlic, onions, tea, coffee, rubber and many kinds of fruits and vegetables. These account for 10% of the sown acreage.

Myanmar maintains 102 600 km^2 of reserved forests. Teak, various kinds of hard wood and cane are the major export items, which earn around 28% of export revenues.

Livestock and fisheries account for 7.7% of GNP. Livestock form an integral part of the agricultural economy and they provide almost all the draught power for land cultivation and rural transport. They also provide milk and meat, and the manure is a valuable fertiliser for field crops.

The livestock industries

There are 11.5 million cattle/buffalo, 1.3 million sheep/goats, 2.4 million pigs, 24.9 million poultry and 3.7 million ducks in Myanmar. The livestock industry contributes 6.3 million draught animals for agriculture and produces around 0.2 million tons of meat, 0.6 million tons of fresh milk and 875 million eggs per year.

Cattle and buffalo are raised mainly for draught purposes. Buffalo are mainly located in the high rainfall areas of the Delta, Northern and Eastern Myanmar. Cattle, while widely distributed, are mainly concentrated in Middle and Upper Myanmar. Livestock production is widely dispersed in the rural areas and is integrated with crop production. A typical farm family would raise two or three cattle or buffalo for draught purposes, a few pigs, and 6–12 head of poultry.

The livestock-rearing systems vary according to the region. In the Delta, a cut-and-carry system is practised during the rice-production season, when the cattle are mostly confined in sheds. Once rice production has finished, the animals are sent to the natural grasslands. In Middle and Upper Myanmar, where cattle predominate, the animals are driven to the natural grasslands in the morning and brought back to the villages in the afternoon.

Sheep and goats are reared mainly in the dry zone of Middle Myanmar. The animals are kept mainly for meat production. About 80% of the flocks comprise less than 20 animals and are kept near the villages. Larger flocks of a few hundred to 2000 head are raised in the natural grasslands far away from

[1] Livestock Breeding and Veterinary Department, Insein, Union of Myanmar

the villages. The herdsmen drive their animals from one grazing area to another throughout the year.

Animal health systems

The Livestock Breeding and Veterinary Department (LBVD) is the Government body responsible for animal health. LBVD has its main laboratory in Insein, with offices in each of the 14 States/Divisions and in the townships. The veterinarians in the townships are assigned to work in different village tracts (sub-townships).

The LBVD provides vaccination services free of charge for cattle and buffalo. Vaccines are produced by the Biologics Production Division of the Department and distributed to the townships in accordance with vaccination programs of the LBVD.

Facilities for post-mortem examination are provided at the township. One central and three regional diagnostic laboratories, Mandalay (Upper Myanmar), Taunggyi (Shan State/Eastern Myanmar) and Pathein (the Delta), provide laboratory diagnostic services.

Animal diseases of importance

Important diseases include:

- foot-and-mouth disease (in cattle, buffalo, pigs)
- haemorrhagic septicaemia (HS) (in cattle, buffalo, pigs and elephants)
- blackleg (in cattle, buffalo and sheep)
- anthrax (in cattle, buffalo and elephants)
- classical swine fever (in pigs)
- brucellosis (in pigs)
- Newcastle disease (in chickens)
- pasteurellosis (in chickens and ducks).

Pasteurellosis

HS is the only type of pasteurellosis recognised, and the most important bacterial disease of livestock in Myanmar. More than 50% of the LBVD's efforts in disease control programs have involved HS. Vaccination for HS has effectively covered up to 70% of the total susceptible animal population in Myanmar in the past 15 years. As a result, death due to HS, especially in the areas of Middle and Lower Myanmar, is now rare. The disease remains endemic in Northern and Eastern Myanmar, particularly in areas of difficult communication and where an open-range system of keeping livestock poses a major constraint for effective disease control programs. In these areas, yearly reports of 20–30 outbreaks with losses of around 1000 cattle or buffalo are recorded. As HS outbreaks now occur particularly in remote areas of the country, the actual losses may be much higher than those officially recorded.

As field veterinarians have experienced HS for decades, they are able to diagnose the disease by the case history and clinical findings. Blood smears, and sometimes fresh specimens, are sent to laboratories for confirmation of the diagnosis. Laboratory diagnosis is mainly by serotyping of the organism. Only serotype B:2 has been identified in Myanmar.

The LBVD has relied on preventative vaccination to control HS. Animals in areas with a previous history of HS are vaccinated with the alum-precipitated vaccine twice yearly. The vaccination program aims to cover 70% of the animals in the area.

Alum-precipitated vaccine using *Pasteurella multocida* serotype B:2 is the vaccine that has been used during the past decade in Myanmar. The 1991–92 production was 6.5 million doses. Production cost of the vaccine is about US$0.04. The recommended dose is 2 mL per animal. The vaccination expenses are fully subsidised by the LBVD. The difficulty with the use of alum-precipitated vaccine is the requirement for two inoculations per year for each animal. Individual inoculation is always a difficult task, and it is almost impossible for animals in the open range. Difficulty is also encountered in handling young buffalo for vaccination. As these animals are frequently left unvaccinated, they become the main victims of HS.

A HS live vaccine, based on *P. multocida* serotype B:3,4, was introduced recently. It is planned to use the vaccine in the place of the alum-precipitated vaccine. One dose per year is recommended. The use of live vaccine is now practised in high-incidence areas, but it is planned that all HS control programs will employ it in the near future. The cost of production of one dose of live vaccine is estimated at US$0.02. Difficulty in inoculation of individual animals is also encountered with the subcutaneous use of this vaccine. The problem is avoided by intranasal administration, because this can be performed by the farmers, who can easily approach their animals.

Research on the live vaccine is continuing. Emphasis is being placed on the identification of the secretory IgA developed by its intranasal use.

Research is also being done on determination of immunity elicited by a genetically transformed pasteurella that expresses both type B and E antigens. The objective is to develop a bivalent HS vaccine that could prevent both B:2 and E:2 infections.

Priorities for future pasteurella work include:

- continued research on the live vaccine to determine the possible spread of the vaccine strain from

intranasally inoculated animals to other animals in the flock;

- the development of a simple HS diagnostic kit for field veterinarians; and
- the development of recombinant isolates of pasteurella, based on the live vaccine strain, that could lead to a combined FMD–HS live vaccine to be used by the intranasal route.

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Singapore

H. Loh[1]

Geography, vegetation and agriculture

Singapore consists of the island of Singapore and some 58 adjacent islets. It is situated between 1 °09 ′ and 1 °29 ′ north latitudes and 103 °38 ′ and 104 °06 ′ east longitudes, approximately 137 km north of the Equator. The main island is 42 km long, 23 km wide and 574 km^2 in area. The total land area, including islets, is 633 km^2. Singapore is separated from Peninsular Malaysia by the Straits of Johor and from the Indonesian islands to the south by the Straits of Singapore.

Agriculture in Singapore includes activities such as poultry layer farming, poultry breeder farming, meat duck farming, dairy cattle farming, vegetable production, horticulture, aquatic plant growing, ornamental plant and foliage production, marine aquaculture and ornamental fish production. With the phasing out of pig farms in Singapore in 1990, these animals are no longer reared.

The importance of agriculture in the economy

In 1990, agriculture (including fishing) had a 0.3% share in the GDP of Singapore's economy. Financial and business services, manufacturing, commerce, transport and communication together comprised 83.5% of the GDP. Faced with high land costs, and land and labour shortages as a result of urbanisation and industrialisation, farm holding areas had shrunk to 10.8 km^2 in 1990.

All farmland has been developed by the Government into Agrotechnology Parks, which contain a mix of intensive high-technology farms giving the ecological balance to achieve a non-polluting and aesthetically pleasing environment.

The livestock industries

There are currently 14 livestock farms — five poultry layer, one poultry breeder, seven meat duck, and one dairy cattle — in production within the Agrotechnology Parks. These occupy an area of 103 ha and have an annual turnover of S$30 million, thereby providing a rate of turnover of almost S$300 000 per hectare per year. The farms have developed from traditional backyard or semi-intensive farms into multi-million dollar enterprises through intensification, automation, or concentration on high-value niches.

In spite of the limited farmland available for livestock farming, local farms produced 6%, 40% and 60% of poultry, hen's egg and meat duck requirements in 1990 respectively. Local farms are expected to produce 15% of Singapore's poultry, 87% of eggs and 87% of ducks, with a total annual turnover of S$190 million, when the Agrotechnology Parks are fully established. In 1990, there were approximately 1.9 million layers, 523 000 broilers, 63 500 breeders and 420 000 ducks.

Animal health systems

The Primary Production Department is responsible for the control of animal diseases in Singapore; and for the establishment of a system of food inspection that ensures that food supplies are wholesome, fit for consumption, and of the highest quality. Strict quarantine measures on the importation of animals have prevented the introduction of exotic diseases into the country.

Singapore is free from epizootic diseases such as foot-and-mouth disease, rinderpest, African swine fever and rabies. It is also free from major poultry diseases such as fowl plague and duck viral enteritis. Disease monitoring and surveillance is carried out regularly on local livestock farms through clinical inspections and the collection and testing of samples for laboratory diagnosis.

[1] Ministry of National Development, Primary Production Department, Central Veterinary Laboratory, Jalan Serang-gong Kecil 13, Singapore

The Central Veterinary Laboratory is responsible for the diagnosis of animal disease, the quality control testing of locally produced vaccines, and the development of vaccines to meet the needs of farms.

Animal diseases of importance

All major diseases are controlled through vaccination, prophylactic medication, disease eradication programs and farmer education. Occasional cases of chronic respiratory disease, Marek's disease and infectious bronchitis are encountered in chickens. Newcastle disease was diagnosed in two outbreaks in 1992, after an absence of 3 years. The main disease problem in ducks is pasteurellosis caused by *Pasteurella multocida* and *P. anatipestifer*.

Pasteurellosis

Pasteurella infections have not been reported in the sole dairy farm in the Agrotechnology Park.

Pasteurella vaccines for cattle/buffalo and sheep/goats have never been used in Singapore. A locally produced formalin-inactivated fowl cholera vaccine is routinely administered to meat ducks on all seven duck farms.

Currently, research is being undertaken on the serotyping and identification of immunoproteins of *P. anatipestifer*, and on the development of a multivalent vaccine against pasteurellosis in ducks.

The Government has prioritised the development of a combined *P. multocida* and *P. anatipestifer* vaccine for ducks for future work on pasteurella.

Philippines

M.M. Interior[1]

Geography, vegetation and agriculture

The Republic of the Philippines is an archipelago consisting of about 7100 islands and islets. The Philippines has a land area of 300 000 km^2 and is bounded by the Philippine Sea (east), the Celebes Sea (south), and the South China Sea (west and north). The three large islands are Luzon, Visayas and Mindanao.

Much of the land area of the islands is covered with forest, and many of the mountains are densely forested. Much of the vegetation resembles that found in Malaysia, and the vegetation of the coastal areas, including mangrove swamps, is practically identical with similar areas throughout the Malay Archipelago. Himalayan elements occur in the mountains of Northern Luzon, while a few Australian plants are found at various altitudes. However, most plant species are indigenous, and Philippine mahogany often comprises a high percentage of forest species.

The importance of agriculture in the economy

The Philippines has an agricultural economy. The share to Gross Value Added of total agriculture (including forestry) for 1990 was 29.62 billion pesos (US$1.27 billion) (National Statistical Coordination Board). The principal farm products are rice, coconut, sugar, abaca (Manila hemp), tobacco, corn (maize), maguey (used for making ropes etc.), and pineapple. Many tropical fruits are also grown, the most important being banana, mango, lanseh or lanzon (*Lansium domesticum*), orange, papaya, avocado, starapple, atis (*Anona* spp.), grapefruit, jackfruit, guava, santol (*Sandoricum* spp.), and durian (*Durio zibethinus*). Vegetables such as beans, tomatoes, eggplant, Chinese cabbage, mustard, lettuce, sweet potato, sugar beets, cassava and squash are grown for domestic consumption. Cassava and sugar are sources of alcohol that can be mixed with gasoline and used as motor fuel.

Rice — the principal staple crop — is grown throughout the country and much of the total farmland is used for rice growing. The Philippines is one of the world's largest producers of coconuts and a major exporter of coconut products, producing half the world's supply of copra. The area devoted to coconut production is second only to that used for rice. Sugar is the country's third largest export, most of which is exported to the United States. Abaca or Manila hemp, the best hemp fibre available, ranks high among the country's exports. Forest products include dry woods, rattan, tanbarks, gutta-percha, high-quality timber and veneer products, beeswax, rubber, and edible birds' nests.

Fishing is also an important Filipino industry, and fish provides a large proportion of the protein in the Filipino diet. Canned tuna is the principal fish export.

The livestock industries

Total animal population in the Philippines in 1991 was approximately:

Cattle	1 677 000
Buffalo (carabao)	2 647 000
Goats	2 122 000
Chickens	65 480 000
Ducks	7 418 000
Pigs	8 079 000

Livestock and/or poultry are raised by most small farm households, and often provide their only significant assets. Commercially raised pigs, poultry and cattle are a small but significant part of the livestock industry. Cattle and buffalo are raised by small farmers as essential elements of their farming systems. The animals are used for draught power and for cash income from the sale of offspring, cull adults, milk and meat. Animals are fed farm and crop residues and are allowed to graze otherwise unused grasslands. Until the early 1980s, ranching on the country's vast and degraded upland grasslands

[1] Department of Agriculture, Bureau of Animal Industry, Visayas Avenue, Diliman, Quezon City, Philippines

was substantial. A small but significant number of cattle are fattened in commercial feedlots based mainly on the feeding of industrial by-products.

Although 84% of the country's pigs are raised by smallholders, commercial livestock industries have developed dramatically in the past decade and provide a growing proportion of the country's meat requirements. Pigs are fed mainly corn, protein supplements and farm residues. Commercial pigs receive mixed feeds based primarily on corn, imported soybean meal, and both imported and locally produced fishmeal.

Commercial poultry receive diets similar to those of commercial pigs, while village poultry, which make up the majority of the country's chicken population, are scavengers. Like pork production, chicken meat production is increasing in response to a growing demand, mainly from the commercial sector.

Goats and sheep play significant roles in upgrading the poorest of the country's farming systems. They are now being used for meat, milk and breeding purposes. Their numbers are increasing at a high rate in several provinces.

Ducks are also being raised in some parts of Luzon, mainly for egg production, but also for meat. The eggs are sold as salted eggs or embryonated eggs — favourite delicacies of most Filipinos. Horses are used as draught animals in some provinces. They are also kept for breeding or for racing.

Animal health systems

The Department of Agriculture's Regional and Provincial Offices and the National and Regional Diagnostic Laboratories are responsible for the collection of animal health data by means of monthly reports. The field reports are collated from data submitted by agricultural technologists. Since these workers base their diagnoses solely on clinical signs, the accuracy may be suspect. The information produced from the laboratories is more reliable; however, it is limited by the number of samples received, particularly in the regional laboratories. The Animal Health Division of the Bureau of Animal Industry is attempting to improve the quality of data being collected. Verification letters are regularly sent out to the provinces calling the attention of the reporting officers to questionable reports submitted.

There are 13 diagnostic laboratories (12 regional and one national) in the 14 regions in the country. The different regional laboratories are being upgraded through training of personnel and through the provision of some reagents, such as locally prepared antigens and antisera.

Animal diseases of importance

The Department of Agriculture, through the Bureau of Animal Industry, has proposed an Administrative Order on the Classification of Animal Diseases. The first priority diseases are: foot-and-mouth disease (01, A24, C3), haemorrhagic septicaemia (HS), anthrax, blackleg, hog cholera, Newcastle disease and rabies.

The second priority diseases are: swine plague, swine erysipelas, fowl cholera, fowl pox, pullorum, brucellosis, leptospirosis and bovine tuberculosis.

Pasteurellosis

The following forms of pasteurellosis occur in the Philippines:

Cattle/buffalo	*Pasteurella multocida* Carter's types A and B
Goat	*P. haemolytica* (untypeable) biotype A
Swine	*P. multocida* Carter's types A and B

Type B isolates of *P. multocida* cause haemorrhagic septicaemia (HS) in cattle and water buffalo, types A and D cause swine pasteurellosis, while untypeable biotype A of *P. haemolytica* causes pasteurellosis in goats.

Based on the 1990 Animal Health Situation Report, HS affected a total of 17 720 buffalo, 14 331 cattle and 3852 goats/sheep with mortalities of 1725, 1057 and 485 respectively. The disease occurred mainly during the months of May and July and was at its lowest incidence in December. Swine plague caused 3420 deaths, while losses in poultry due to fowl cholera were also reported.

Pasteurellosis is diagnosed on field and laboratory reports and controlled by vaccination. Vaccines used for the control of HS are prepared both by the Government and by private companies. The government vaccine is distributed to the animal health posts in the field, while the commercial vaccine is used mainly by the larger private farms. Killed alum adjuvanted vaccines are at present being used in the country, while oil adjuvanted vaccines are still at the developmental stage. Annual production of government vaccines is approximately 1.2 million doses, while that of vaccines produced by the private sector (two companies) is approximately 1 million doses.

The government HS vaccine is sold for P2.40 per dose (US$0.10), swine plague vaccine is being sold at P2.53 per dose (US$0.11), while fowl cholera vaccine is sold for P0.48 per dose (US$0.02). Commercially produced HS vaccine is sold for P12.00 per dose (US$0.52).

The Bureau of Animal Industry's HS vaccine and one produced by a private company are administered subcutaneously, with 5 mL being given initially. Vaccination is repeated at 4–6 weeks and then again 3–4 months later. Booster vaccination is recommended every 6 months. The HS vaccine produced by the other commercial company is used at a dose rate of 2 mL subcutaneously, with two doses being given 2 weeks apart. The swine plague vaccine is administered at a dose rate of 3 mL in adult pigs and 1–2 mL in piglets. Fowl cholera vaccine is administered at a dose rate of 1 mL and given intramuscularly.

In the latter part of 1991 and early in 1992, three buffalo were reported to have died from hypersensitivity to HS vaccine. The administration of three injections is also a problem, since farmers do not usually want their cattle/buffalo to be vaccinated so many times.

Current pasteurella research activities and priorities include:

- the isolation and serotyping of *Pasteurella* spp.; and
- the use of an in vitro assay to test vaccines prepared from pasteurella isolates that are not pathogenic to laboratory mice and rabbits.

The Government has directed researchers to address the problems of hypersensitivity, accurate field diagnosis and isolation and serotyping of pathogenic pasteurella species, and to develop an oil adjuvant vaccine.

Priorities for future pasteurella work include:

- the development of ELISA for the diagnosis of pasteurella infections and for assay of vaccines;
- field trials on the efficacy of the different pasteurella vaccines;
- studies on the safety of the vaccines; and
- development of an oil adjuvanted vaccine.

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