CHARACTERISATION OF CELL-SURFACE COMPONENTS OF *PASTEURELLA HAEMOLYTICA* IN RELATION TO VIRULENCE

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DECLARATION

DEDICATION

This thesis is dedicated to my family for their help and encouragement during my undergraduate and postgraduate studies.

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ABBREVIATIONS

Abbreviation	Term
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
cfu	colony forming units
EDDA	ethylenediaminedihydroxyphenyl-aceticacid
FIA	Freund's incomplete adjuvant
FCA	Freund's complete adjuvant
g	gram
h	hour
kDa	kilodalton
LPS	lipopolysaccharide
ug	microgram
mg	milligram
ml	millilitre
mM	millimolar
min	minute
OMP	outer-membrane protein
PBS	phosphate-buffered saline
SDS-PAGE gel electrophoresis	sodium dodecyl sulphate - polyacrylamide

SUMMARY

This investigation was designed to characterise the surface components, namely the OMPs and LPS, of *P. haemolytica* and to further the current understanding of the role of these components in the pathogenesis of *P. haemolytica* infections.

Initially, OMP and LPS profiles of 29 *P. haemolytica* isolates were examined and compared by SDS-PAGE and Western blotting. The isolates used were of known serotype, had a known origin and were from a host whose disease status was known. Eight distinct OMP profiles and three LPS profiles were demonstrated within this population of isolates. An association was found between certain OMP types, LPS types, species of origin and disease status of the host. Thus, the results demonstrated variation in OMP and LPS profiles and provided evidence that these differences may be useful in epidemiological studies of *P. haemolytica.*

A 39.5 kDa outer-membrane protein of *P. haemolytica* was shown to be heat-modifiable and was identified in all *P. haemolytica* isolates examined. The protein occurred in one of two molecular-weight forms depending on the solubilisation temperature used, and thus resembled the Omp A protein of *E. coli.* Western blot analysis, using a rabbit monospecific antiserum to the *P. haemolytica* 39.5 kDa protein, indicated a serological cross-reactivity with the Omp A protein and N-terminal amino acid sequence analysis of the *P. haemolytica* protein also indicated a close homology between these proteins.

The property of serum resistance in 22 *P. haemolytica* isolates and its possible correlation with known surface components was also investigated. Isolates were defined as being serum resistant, partially serum sensitive or serum sensitive, based on the % survivor value obtained in the serum bactericidal assays. Of the 22 isolates examined, 7 isolates proved resistant to serum killing, 6 were highly serum sensitive and the remaining 9 isolates were partially serum sensitive. Although a relationship between smooth-type LPS and serum resistance was identified, there was no correlation of OMP type or capsular type with resistance

to serum killing.

In vivo studies of *P. haemolytica* included virulence tests on 7 isolates and *in vivo* growth in intraperitoneal implant chambers. The virulence tests involved intratracheal inoculation of calves with approximately 10^{11} cfu diluted in sterile PBS and, after 3 days, expressing the virulence as a lung lesion score. Three of the 7 isolates produced virtually no lesions while the remaining 4 produced lesion scores ranging from 1.7 to 40.5. Comparison of the virulence of the isolates in relation to their LPS type, serum resistance and leukotoxin activity suggested that although leukotoxin production is important, the other factors also play a part in the virulence of *P. haemolytica.*

To supplement the *in vitro* studies of *P. haemolytica* in this investigation, *in vivo*-grown cells were examined. Studies on bacteria placed within a intraperitoneal implant chambers showed a steady decline in numbers over the 16 day period of the experiment. The reason for this decline, was not determined although preliminary experiments indicated the absence of an antibody and complement-mediated mechanism of killing within the chamber. The possible role of a cell-mediated killing mechanism is a potential area for further investigation. Although the bacterial numbers declined inside the chamber, an adequate number were recovered for OMP analysis. Differences were found between *in vivo* and *in vitro*-grown bacterial cells with respect to OMPs. These findings highlight the importance of considering *in vivo* studies of bacteria when investigating virulence determinants and their role in the pathogenesis of bacterial infections.

In summary, this study has provided important information relating the surface components of *P. haemolytica* to the virulence of this species and points to the need for further studies of these components as possible protective antigens against *P. haemolytica* infections.

1. INTRODUCTION

1.1 *PASTEURELLA HAEMOLYTICA* - HISTORICAL INTRODUCTION

Pasteurella haemolytica is a member of the family *Pasteurellaceae*, a family consisting of three genera, namely *Pasteurella*, *Haemophilus* and *Actinobacillus* (Mannheim, 1984). The members of this family have been recognized as important pathogens of both animals and man since very early in the history of medical bacteriology (Zinnemann, 1981).

During the period 1880-1900, organisms which were later identified as members of the genus *Pasteurella* were first isolated. Louis Pasteur in 1880 was the first to identify an organism of this type when he isolated the causative agent of fowl cholera, a serious disease of his time. In 1885, Kitt isolated similar organisms from other host species, including cattle, horses and pigs, and the name "septicaemia haemorrhagia" was proposed by Huppe in 1886 to include all of these bacteria. However, in the following, year the name *Pasteurella* was given to this group of organisms by Trevisan, in commemoration of the early work of Louis Pasteur (Mannheim, 1984; Carter, 1981).

Further characterisation of this genus led to the identification and classification of different species (Mannheim, 1984; Carter, 1981) from a wide spectrum of vertebrate hosts, each causing different clinical syndromes. The period of 1950-1960s saw the most significant advances in research of *Pasteurellas* and the advancements made both during this period and more recently have been extensively reviewed (Carter, 1981; Mannheim, 1984; Wilkie & Shewen, 1988; Biberstein, 1990; Woolcock, 1993).

1.2 NATURE OF THE PATHOGEN

1.2.1 Bacteriological characteristics

The principal characteristics of members of the genus *Pasteurella* are: small, Gram-negative rods or coccobacilli, non-motile, facultatively anaerobic, catalase and oxidase positive, fermentative producing acid but no gas from a number of carbohydrates (Mannheim, 1984; Carter, 1981).

Like other *Pasteurella* species, *P. haemolytica* grows well on blood agar between 25^{o}C and 40^{o}C with the optimum temperature being 37^{o}C (Mannheim, 1984). Selective media have been described (Morris, 1958; Weissman, 1966) for this organism but they are generally not used in routine laboratory work due to the ease with which the bacterium grows on complex media. The colonies on blood agar are surrounded by a zone of beta-haemolysis, a distinctive property of *P.haemolytica* in comparison to the other *Pasteurella* species (Carter, 1981).

Preservation of *P. haemolytica* in the lyophilised state using conventional suspending media or storage for long periods at -70^{o}C is possible, as for all *Pasteurella* species.

1.2.2 Taxonomy of the organism

Two main approaches have been utilized in defining the species *P. haemolytica*. Firstly, the biochemical and cultural properties have been used (Smith, 1961) to distinguish two main biotypes within the species, namely biotypes A and T representing arabinose and trehalose fermenting isolates, respectively. In Table 1, other differential characteristics often used to distinguish the biotypes are listed.

Biotypes A and T may be distinguished by their colonial morphology, their

Table 1. Differential characteristics of *P. haemolytica* biotypes

CHARACTERISTIC	BIOTYPE	
	A	T
CARBOHYDRATE FERMENTATION		
Arabinose	+	-
Trehalose	-	+
COLONIAL MORPHOLOGY	small, grey	large, dark brown
GROWTH CURVE	die out rapidly	survive longer
PENICILLIN SENSITIVITY	sensitive	resistant
LOCALISATION IN NORMAL HOST	nasopharynx	tonsils
PRINCIPAL DISEASE ASSOCIATION	-pneumonia in cattle and sheep -septicaemia in newborn lambs	-septicaemia in lambs > 3 mth

Adapted from Biberstein (1978)

growth curves and also their susceptibility to penicillin (Smith, 1961). However these characteristics do not always provide clear-cut differences and thus carbohydrate fermentation tests have been relied on most heavily for the sub-division of the biotypes.

More recently, it has been proposed that the A and T biotypes actually be regarded as distinct species. Sneath and Stevens (1990), based on the data from numerical taxonomic studies, suggested that biotype T isolates of *P. haemolytica* be renamed *Pasteurella trehalosi* .

Smith (1961) found an association of each biotype with different disease syndromes in a variety of host species. For example, isolates of the T biotype tend to be associated with septicaemia in feeder lambs while those of the A biotype are the predominant isolates from pneumonic sheep and calves. The finding that A biotype isolates were also more frequently found in the nasopharyngeal carrier state than the T biotypes (Smith, 1961) led Biberstein and Thompson (1966) to suggest that biotype T isolates, being less frequent and extremely rare in healthy animals, and more often associated with clinical disease, have a greater pathogenic potential and a lesser ability to adapt to the commensal state. Hence, these early investigations, sub-dividing the species into biotypes, provided important information for future research on this species.

In addition to two distinguishable biotypes within this species, a second scheme, based on the antigenic composition of individual isolates was developed by Biberstein *et al.* (1960) and led to the serotypic classification of this species. The technique was initially based on soluble, presumably surface antigens either of polysaccharide (Cameron, 1965) or lipopolysaccharide (Carter, 1967; 1975) nature and, currently, differentiation of the serotypes, based on the capsular antigen, identified using indirect haemagglutination assays (Biberstein, 1978), is the most widely practised method of serological classification of *P.haemolytica.*

To date, 16 serotypes have been identified (Biberstein *et al.*, 1960; Fodor *et al.*, 1988). The work of Biberstein and Gills (1962) investigated the

Table 2. Association of biotypes and serotypes of *P. haemolytica*

BIOTYPE	SEROTYPE
A	A1 A2 A5 A6 A7 A8 A9 A11 A12 A13 A14 A16
T	T3 T4 T10 T15
UT[1]	

Adapted from Frank (1989)

[1]UT = untypable

distribution of the 16 serotypes among the two biotypes and revealed a distinct association of certain serotypes with either biotype A or T. Confirmation of this was seen by Shreeve *et al.* (1970). This relationship of biotype to serotype is seen in Table 2. In addition to these identifiable serotypes, Quirie *et al.* (1986) found that 10% of *P.haemolytica* isolates were untypable by the usual typing mechanisms possibly suggesting the presence of a different capsular type on these isolates or the absence of a capsule.

It is now understood that certain serotypes are more predominant in certain hosts and cause different clinical syndromes, as discussed by Biberstein (1978) and De Alwis (1993). For example, isolates of serotype A1 tend to be the predominant isolates from septicaemic young lambs and especially from cases of epidemic pneumonia in cattle (Fox *et al.*, 1971; Frank & Smith, 1983), whereas serotype A2 isolates appear to be associated mainly with pneumonia in sheep and other ruminants (De Alwis, 1993).

From the preceding information it is evident that sub-division of *P. haemolytica* into biotypes and serotypes is important with respect to epidemiological and virulence studies. However, in other bacterial species the sub-division does not stop at serotypes and many investigators in this field have found that isolates within a certain serotype of a bacterial species may be further sub-divided into "sub-types" based on various characteristics of the isolates. For example, outer-membrane protein (OMP) and /or lipopolysaccharide (LPS) profiles obtained by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) have often been utilised. Such "sub-typing" schemes can be found in many Gram-negative species including *Escherichia coli* (Achtman *et al* ., 1983), *Haemophilus influenzae* (Loeb & Smith, 1980), *Actinobacillus pleuropneumoniae* (Rapp *et al* ., 1986) and *Pasteurella multocida* (Lugtenberg *et al* .,1984). With this in mind, the possibility exists of using such schemes for the typing of *P. haemolytica* isolates and requires further investigation.

1.3 PATHOGENESIS OF BOVINE PNEUMONIC PASTEURELLOSIS

1.3.1 Clinical disease

Pasteurella haemolytica is an important pathogen causing pneumonia and systemic infections primarily in sheep and cattle (Biberstein & Thompson, 1966; Wessman & Hilker, 1968) although similar organisms have been isolated from other non-ruminant hosts (Biberstein *et al.*, 1960).

Pneumonic pasteurellosis of sheep and cattle is caused by *P. haemolytica* isolates of biotype A. In sheep, the predominant isolates associated with pneumonic pasteurellosis are of serotype A2, whereas in calves serotype A1 is the predominant serotype (Frank, 1989). *Pasteurella haemolytica* biotype T isolates (*P. trehalosi*) are associated with systemic pasteurellosis of sheep, a disease less common on a worldwide scale than pneumonic pasteurellosis, but still a major concern in Britain (Gilmour, 1993). A common feature in all these disease syndromes is the occurence of asymptomatic carriers of the bacterium, which play an important role in the pathogenesis of the disease. Pathogenesis is apparently multifactorial in nature, involving other factors besides bacterial infection (Gilmour & Gilmour, 1989; Gilmour, 1993).

Pneumonic pasteurellosis is the most economically important disease caused by *P. haemolytica*. In North America this respiratory disease in calves is referred to as "shipping fever" while in Europe "transit fever" is the term more commonly used to describe it (Frank, 1989). Both these terms refer to the association of bacterial infections with the rearing procedures of the calves in causing pneumonic pasteurellosis. The disease is believed to involve various factors such as stress or viral infection in addition to *P. haemolytica* infection (Jensen *et al*, 1976; Frank, 1986; 1989; Wilkie & Shewen, 1988) and, based on this multifactorial aetiology, is often referred to as a "disease complex". However, it is generally thought that *P. haemolytica* infection is the major factor in pneumonic

pasteurellosis and previous investigators (Friend *et al.*, 1977; Gibbs *et al.*, 1984; Ames *et al.*, 1985), have reported the production of pneumonic pasteurellosis experimentally in normal animals by infection with *P. haemolytica* alone.

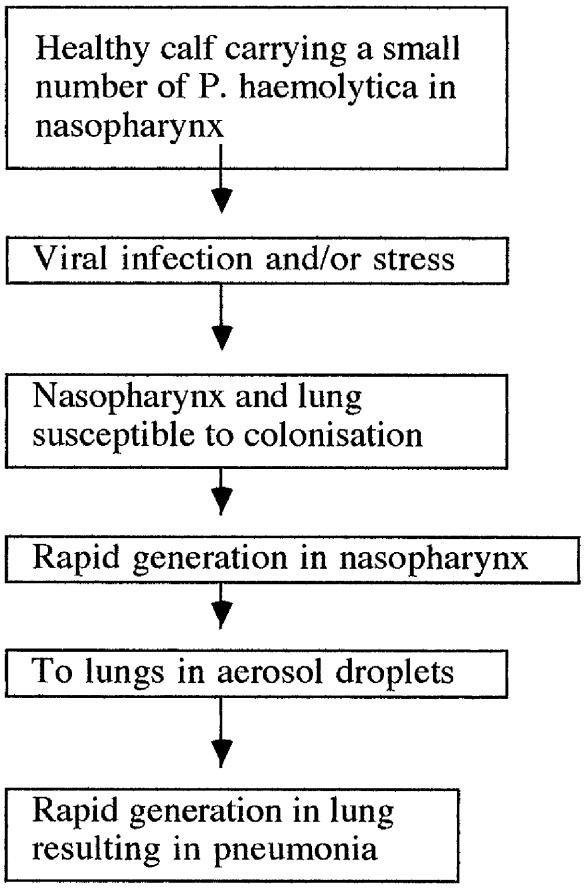
1.3.2 Carrier state in cattle

Pasteurella haemolytica A1 is often carried in the nasopharynx and is also found in the tracheal air of healthy calves. However, since the numbers present comprise only a small proportion of the bacterial flora it is difficult to detect their presence (Grey & Thomson, 1971; Frank & Smith, 1983) thus making epidemiological studies and control of this disease difficult. These commensal bacteria are one of the two sources of *P. haemolytica* which ultimately causes pneumonic pasteurellosis, the other source being passage from infected calves (see Figure 1). As yet, the reasons why healthy carriers convert to cases of clinical disease remain unknown. However, conditions of stress and viral infections, which cause the immune system of the host to become compromised, are considered important predisposing factors for the eventual illness caused by *P. haemolytica* (Gilmour, 1993). Some investigators, however have shown *P. haemolytica* serotype A1 to cause pneumonic pasteurellosis in the absence of such predisposing factors (Friend *et al*, 1977; Newman *et al*, 1982; Gibbs *et al.*, 1984; Ames *et al.*, 1985). Thus, the question remains as to the necessity of predisposing factors for the development of the clinical disease of pneumonic pasteurellosis.

1.4 VIRULENCE ATTRIBUTES OF THE BACTERIUM

Pasteurella haemolytica, like the other pathogenic members of the family *Pasteurellaceae*, must fulfil a number of pathogenicity requirements in order to

Figure 1. Pathogenesis of bovine pneumonic pasteurellosis



Adapted from Frank (1986)

cause disease (Nicolet, 1990). After infection of the host, the bacterium must colonize the mucosal surface, survive and multiply, and interfere with the host defence mechanisms, before ultimately causing the damage to the host which results in clinical disease. The steps leading to pneumonic pasteurellosis in calves are seen in Figure 1.

To fulfil all of these requirements, the bacterium possesses a number of virulence factors which are presumed to be involved at the various stages of the disease (Biberstein, 1990; Nicolet, 1990). These virulence determinants are either surface-associated or produced extracellularly by the bacterium and are presented diagrammatically in Figure 2.

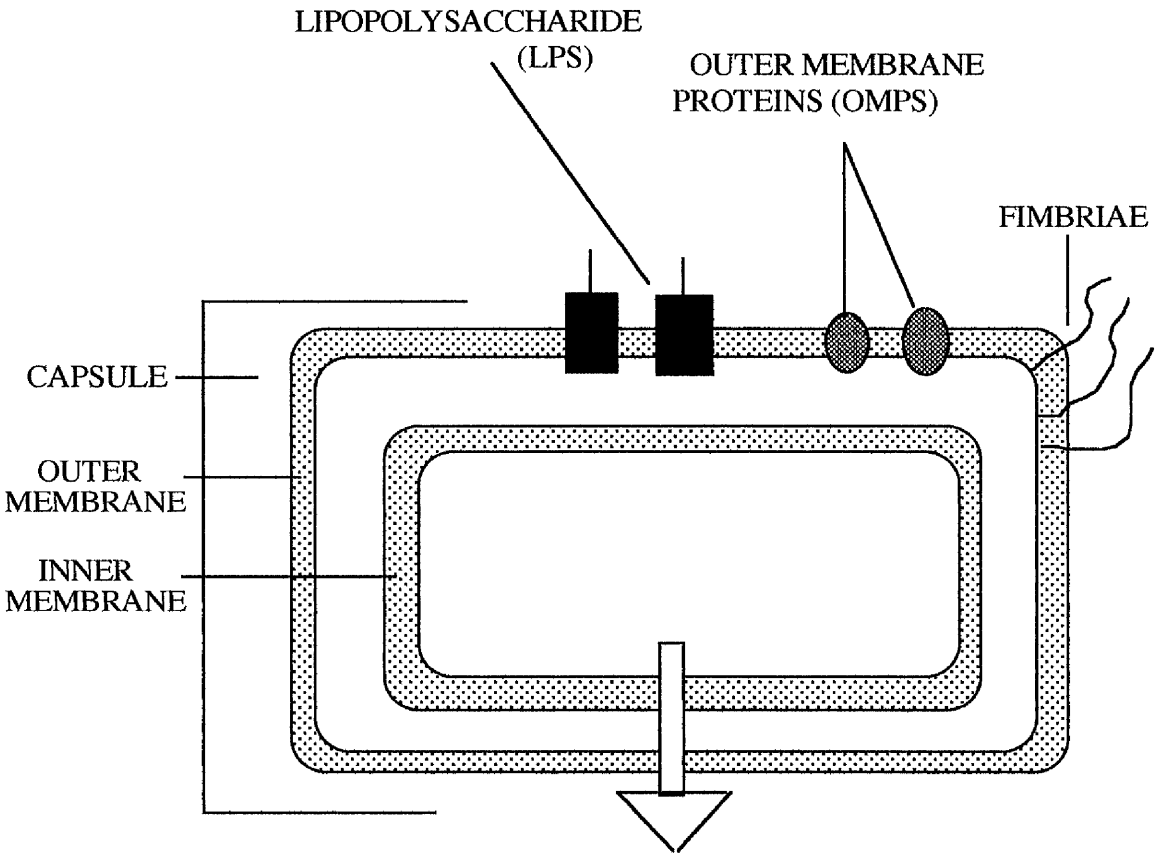
1.4.1 Surface structures of the bacterium

1.4.1.1 Fimbriae

Fimbriae of a number of Gram-negative bacteria have been shown to serve as adhesins, causing the bacteria to adhere to eukaryotic cell surfaces, namely the mucosal surfaces of the host species. Fimbriation of various members of the *Pasteurellaceae* has been observed - *H. influenzae* (Guirma *et al*, 1982), *A. pleuropneumoniae* (Tomak *et al*, 1988), *P. multocida* (Trigo & Pijoum, 1988) and *Actinobacillus actinomycetemcomitans* (Scannapieco *et al*, 1983).

In 1987, Morck *et al.* demonstrated two types of fimbriae on the surface of a *P. haemolytica* serotype A1 isolate grown *in vitro*, these being a large rigid type (10nm wide) and a smaller flexible type (5nm wide). Support for their presence *in vivo* was later seen in organisms from lung lavage fluid (Morck *et al.*, 1988) and also in organisms seen adhering to tracheal epithelial cells (Morck *et al*, 1989). As yet the precise role of fimbriae in the pathogenesis of *P. haemolytica* is unknown. In the light of the role of fimbriae in other Gram-negative bacteria, they are most

Figure 2. Diagrammatic representation of the putative virulence determinants of *P. haemolytica*



Adapted from Confer et al (1990)

likely to be involved in colonization of the upper respiratory tract (URT), the first step in the pathogenesis of the disease.

1.4.1.2 Capsular polysaccharide

The presence of polysaccharide capsules is a common feature among the members of the family *Pasteurellaceae* (Inzana, 1990). These structures confer various properties on the bacterium. Firstly, they often form the basis of sub-division of the bacterial species into serotypes and, in the case of *P. haemolytica* (also including the new species representing biotype T isolates, *P. trehalosi*) 16 distinct polysaccharide capsules have so far been identified (Biberstein, 1978; Adlam, 1989) representing the 16 serotypes. The second role of the capsule is in the pathogenesis of *P. haemolytica* infection.

In *P. haemolytica*, the polysaccharide capsule is produced during the logarithmic phase of growth and has been visualized in organisms grown *in vitro* (Gilmour *et al* .,1985) and *in vivo* (Morck *et al*, 1988). It is believed to serve as a means of attachment to the mucosal epithelial cells during the early stages of disease (Morck *et al*, 1988) and also to inhibit host immune defenses, phagocytosis and complement-mediated killing (Chae *et al.*, 1990).

The chemical composition of the capsule of a number of the serotypes is known (Adlam, 1989) and there appears to be a very close relationship to the capsules of other Gram-negative pathogens (Adlam *et al.*, 1984; 1987) not only in composition but in virulence properties. For example, the *P. haemolytica* serotype A1 capsular polysaccharide structure is similar to the enterobacterial common antigen - a polymer of N-acetyl-D-mannosaminuronic acid linked1-4 to N-acetyl-D-glucosamine (Adlam *et al.*, 1984). The polysaccharide caspsule of serotype A2 isolates is identical to the capsule of *Neisseria meningitidis*, group B and *E.coli* K1, i.e. colaminic acid (Adlam *et al.*, 1987).

1.4.1.3 Lipopolysaccharide (LPS)

Lipopolysaccharide is a prominent surface component of all Gram-negative bacteria and constitutes approximately 12-25% of the dried cell weight of *P. haemolytica* (Keiss *et al.*, 1964).

Structurally, LPS of *P. haemolytica* is similar to that of other Gram-negative bacteria in that it may be composed of three main units:- a lipid A region, a core oligosaccharide region and an antigenic polysaccharide chain of repeating sugar units (O-antigen repeats). In *P. haemolytica*, the composition of the LPS, as analysed by SDS-PAGE, has been found to differ both between the serotypes (Perry & Babuik, 1984; Leitch & Richards, 1988) and even within serotypes (Davies *et al.*, 1991; Ali *et al.*, 1992; Utley *et al*, 1992; McCluskey *et al.*, in press). SDS-PAGE profiles of LPS consist of a series of low-molecular-mass bands which represent the lipid A-core oligosaccharide unit and a "ladder pattern" of high-molecular-weight bands representing different numbers of the O-antigen repeats linked to the core region of the LPS (Davies *et al.*, 1991). Rough-type LPS lacks the high-molecular-weight chains, the O-antigen side chains, while smooth-type LPS possesses them.

Although all isolates of biotype A were initially thought to possess rough-type LPS (lacking the O-antigen repeat units) while those of biotype T possess smooth-type LPS (O-antigens present) (Adlam, 1989), more recent investigations have demonstrated the presence of O-antigen side chains in some isolates of the A biotype (Davies *et al.*, 1991; Utley *et al*, 1992; Lacroix *et al.*, 1993). By SDS-PAGE, these workers were able to show the presence of the high-molecular-weight bands in the profiles of biotype A isolates. Additionally, variation in the lipid A-core oligosaccharide region has been reported (Davies *et al.*, 1991; Ali *et al.*, 1992), where the mobilities of the low-molecular-weight bands differed between isolates of the same or different serotypes. Thus it appears that microheterogeneity of LPS, as seen in other Gram-negative bacteria, also exists in

the LPS of *P. haemolytica*.

As mentioned in section 1.2.2, this type of variation in LPS is often used as the basis of a sub-typing scheme which can be of great value in epidemiological and virulence studies of bacterial species (Inzana, 1983; Inzana & Pichichero, 1984; Achtman *et al.*, 1983; Achtman & Plusche, 1986), and suggests the need for further investigation of *P. haemolytica* LPS.

1.4.1.4 Outer-membrane proteins (OMPs)

Only limited investigations of the outer-membrane proteins of *P. haemolytica* have been carried out and their role in the pathogenesis of *P. haemolytica* infection is still poorly understood. However, analysis of OMPs by SDS-PAGE has indicated similarities to those of other Gram-negative bacteria in that the outer membrane of *P. haemolytica* contains approximately 15-20 minor proteins and about 4-5 major proteins (Squire *et al.*, 1984; Knights *et al.*, 1990; Davies *et al.*, 1992).

The profiles obtained by SDS-PAGE are different between the two biotypes A and T (Adlam, 1989; Woolcock, 1993) and to a lesser extent between the serotypes (Knights *et al.*, 1990) but, until the present study, no variation had been reported within the serotypes. Since variation in the OMP profiles has been identified within individual serotypes of other bacterial species and often used to develop a sub-typing scheme for the species (see section 1.2.2), it was possible that similar variation would exist within *P. haemolytica* populations and was therefore an area which required investigation.

As to the actual role of the OMPs in the pathogenesis of *P. haemolytica*, little is known due to the limited investigations which have been carried out. However, Donachie and Gilmour (1988) examined the SDS-PAGE OMP profiles of *P. haemolytica* serotype A2 isolates and identified the expression of certain high-molecular-weight proteins in organisms obtained from pleural fluid, which did

not appear to be expressed by *in vitro*-grown organisms. Similarly, other investigations have examined these high-molecular-weight proteins, namely the 71, 77 and 100 KDa proteins (Deneer & Potter, 1989; Morck *et al.*, 1991; Murray *et al.*, 1991; Davies *et al.*, 1992) and have found that increased expression of these proteins occurs *in vitro* when the bacteria are grown under iron-restricted conditions. Thus, it is generally thought that these iron-restricted OMPs are involved in survival of *P. haemolytica in vivo*, possibly in the assimilation of iron. Similar findings have been observed in *E.coli* (Finn *et al.*, 1982) and *Haemophilus ducreyi* (Trees *et al.*, 1991). Although investigation of the OMPs of *P. haemolytica* has been limited, it does appear that they are generally similar to those of other Gram-negative species.

The study of OMPs by SDS-PAGE has demonstrated the property of "heat-modifiability" of certain proteins in a number of Gram-negative bacteria (Beher *et al.*, 1980). This property results in the respective protein being present in one of two molecular weight forms depending on the temperature used to solubilise the protein sample prior to electrophoresis. The reasoning behind this phenomenon has been described in detail previously (Nakamuru & Mizushima, 1976) and states that at the lower solubilisation temperature the heat-modifiable OMP appears at the lower-molecular-weight due to an excess binding of SDS to the protein molecule, which is predominantly in the conformation of beta-sheets, the excess binding of SDS causing the protein to run faster in the gel. Solubilisation of the protein at high temperatures causes a conformational change of the protein molecule, such that it exists in the alpha-helical form, which binds less SDS causing the protein to run slower in the gel. Consequently, the lower-molecular-weight form of the protein is observed with low solubilisation temperatures and the higher-molecular weight form is seen when higher solubilisation temperatures are applied.

The work of Beher *et al.* (1984) demonstrated this property for the Omp A protein of *E. coli*, and showed similar proteins in other Gram-negative species.

More recently, in the *Pasteurellaceae* family, an Omp A-like protein has been identified in *Haemophilus somnus* (Tagawa *et al.*, 1993), *H. ducreyi* (Spinola *et al.*, 1993) and *A. actinomycetemcomitans* (Wilson, 1991) by SDS-PAGE and also by amino acid sequence comparisons of the protein with that of *E. coli*. Until now, no such studies have been reported for *P. haemolytica* .

1.4.2 Extracellular bacterial products

1.4.2.1 Leukotoxin

An extracellular virulence factor produced by some members of the *Pasteurellaceae* is a heat-labile cytotoxin with the ability to lyse leukocytes (Nicolet, 1990; Lo, 1990). The leukotoxin of *P. haemolytica* is target-cell specific in that it acts only on ruminant leukocytes (Woolcock, 1993) and has been implicated as an important virulence determinant which contributes to the pathogenesis of pneumonic pasteurellosis by disrupting the lung defence mechanisms and consequently the immune response to the bacterium within the lung (Shewen & Wilkie, 1982; Lo, 1990). The toxin is a protein of approximately 105 KDa (Lo *et al.*, 1985; Chang *et al.*, 1986) which shares both structural and genetic sequence homology to the alpha-haemolysin of *E. coli* (Strathdee & Lo, 1987) and also to the other similar toxins which form the family of toxins known as the RTX toxins (Welch, 1991; Coote, 1992). The genes involved in the successful production and secretion of the toxin from the bacterial cell form an operon with four individual genes, a feature common to all the RTX toxins. These four genes encode the toxin itself and the proteins involved in its activation and secretion (Welch, 1991).

Currently, investigations of the leukotoxin continue, and further information on and understanding of this protein and its exact role in the pathogenesis of *P. haemolytica* infection is likely to become evident in the near

future.

1.4.2.2 Haemolysin

Surprisingly little information is available on this virulence factor of *P. haemolytica* which gives rise to the species name, even after 30 years of research. It is known that this bacterial product is not encoded on plasmid DNA like similar products of other bacteria (Chang *et al.*, 1987) but its role as a virulence factor is not clear. A recent report (Forestier & Welch, 1990) suggested that the haemolysin of *P. haemolytica* may be identical with the leukotoxin, although, confirmation of this is required.

1.4.2.3 Neuraminidase

Neuraminidase production in *P. haemolytica* was first demonstrated by Scharmann *et al.* (1970) and was later confirmed by Frank and Tabatabai (1981). This virulence factor is not common to the other members of the family *Pasteurellaceae* (Nicolet, 1990) although a neuraminidase has been shown to be produced by *P. multocida* isolates (Scharmann *et al*, 1970). Its role in pathogenesis is not fully understood but, as neuraminidases are present in other bacterial pathogens which are able to survive on mucosal surfaces (Adlam, 1989), it may reduce the protective barrier effect of epithelial mucous membranes of the host (Adlam, 1989). Thus, further investigations are obviously required in order to fully understand the precise role of this determinant in the virulence of *P. haemolytica*.

1.4.2.4 Protease

A protease specific for sialoglycoproteins was demonstrated by

Otulakowski *et al.* (1983). However, since then, little has been done to characterise its role in the pathogenicity of *P. haemolytica* and further investigations are required.

Thus, this section has summarised the bacterial components which possibly have a role in the pathogenesis of *P. haemolytica* infections. Although some of these determinants have attracted great interest, namely the capsule, LPS and leukotoxin, other components, particularly the OMPs, should not be disregarded in future studies, as most researchers in this field believe that a successful vaccine against this pathogen will consist of a number of different components of the bacterium, including the surface proteins.

1.5 *PASTEURELLA HAEMOLYTICA - IN VIVO*

As is evident from the information given in section 1.4 regarding the virulence determinants of the bacterium, most of what is currently known relates to organisms grown *in vitro*, although a few studies discuss *in vivo*-grown organisms (Donachie & Gilmour, 1988; Morck *et al*, 1988; 1989; 1991; Sutherland *et al.*, 1991; Confer *et al.*, 1992;).

The necessity of studying organisms grown *in vivo* relates to the variation which may occur in the expression of bacterial products or virulence determinants under *in vivo* growth (Brown & Williams, 1985; Brown *et al.*, 1988). This variation is of major importance when bacterial components, which may be susceptible to variation, are actually being considered as vaccine candidates (Brown *et al.*, 1988).

Bacteria from lung lavage fluid of chronically-diseased calves have been used in the investigations by Morck *et al.*, (1988,1989) while Donachie & Gilmour (1988) in addition used organisms isolated from pleural fluid as representatives of *in vivo*-grown organisms. Organisms obtained from chambers implanted into the

peritoneal cavity of rabbits (Morck *et al.*, 1991) and also chambers implanted subcutaneously into the flanks of calves (Confer *et al.*, 1992) were examined. These studies generally reported variation in certain properties, especially in SDS-PAGE OMP profiles, between *in vivo* and *in vitro* grown organisms, and the expression of certain high-molecular-weight OMPs (descibed previously - section 1.4.1.4). Investigations of this kind have been reported in other bacterial species such as *E. coli* (Finn *et al.*, 1982), *H. ducreyi* (Trees *et al.*, 1991), and *Pseudomonus aeruginosa* (Kelly *et al.*, 1987; 1989) where implanted chambers were used for obtaining *in vivo*-grown cells (Pike *et al.*, 1991; Day *et al.*, 1980). Until the present study, however, there has been only two reports of such examinations in *P. haemolytica* (Morck *et al.*, 1991; Confer *et al.*, 1992).

1.6 HOST IMMUNE RESPONSE AGAINST *P. HAEMOLYTICA*

Although pneumonic pasteurellosis is thought of as a multifactorial disease, many researchers in this field believe that control of *P. haemolytica* infection would result in a significant decrease in the severity and prevalence of the disease and the economic losses caused by it (Confer *et al.*, 1988; Roth, 1988; Mosier 1993). Thus a detailed understanding of the immune mechanisms which enhance immunity to *P. haemolytica* and a knowledge of the important bacterial antigens which stimulate these mechanisms is required before a more efficacious vaccine can be developed.

As with other bacterial infections, more than one immune mechanism may be involved in producing resistance to infection. The most widely studied mechanism is the humoral immune response, which involves a number of complex interactions. Humoral immunity against *P. haemolytica* involves production of antibodies to specific surface components of the bacterium in addition to leukotoxin-specific antibodies (Confer *et al.*, 1987; Gentry *et al.*, 1988). Such antibodies to surface components of other Gram-negative bacteria have often been

associated with resistance to further infection (Winter, 1979; Gilleland & Matthewsgreer, 1987). The antibodies produced may have several potential actions, namely, inhibition of attachment and colonization, agglutination and opsonisation of the bacterial cells to enhance phagocytosis, neutralization of leukotoxin activity and, activation of the classical complement pathway by binding to bacterial surface components (Roth, 1988; Confer *et al*, 1988). However, as yet, the important protective antigens of *P. haemolytica* have still to be defined.

The host cell-mediated immune response, involving T-lymphocytes which can act directly on the bacterial cell or enhance macrophage killing of intracellular bacteria (Confer *et al.*, 1988) is an important protective mechanism of a number of hosts, although little research has been documented to characterise this particular response to *P. haemolytica.* However, as the cell-mediated immune response enhances the function of alveolar macrophages in the lung and also neutrophil activity (Roth, 1988; Czuprinski & Sample, 1990) it is likely that induction of cell-mediated immunity would be beneficial to the *P. haemolytica*- infected host.

1.6.1 Bactericidal action of host serum against *P. haemolytica*

The bactericidal activity of serum against Gram-negative bacteria, including *P. haemolytica*, is generally believed to represent an important defence mechanism of the host. This subject has been extensively studied and reviewed in the last decade (Taylor, 1983, 1988; Joiner *et al.*, 1984; Crokaert *et al.*, 1992).

The bactericidal activity of serum is largely due to the complement system, a series of proteins which, when activated by one of two pathways, form a membrane-attack complex capable of inserting into the outer membrane of Gram-negative bacteria causing cell lysis to occur (Taylor, 1983; Frank *et al.*, 1987). When activated by antigen-antibody complexes the pathway is referred to as the Classical complement pathway whereas activation in the absence of antibody represents the Alternative complement pathway. This latter pathway may be

activated by a number of substances including bacterial cell wall constituents (Taylor, 1983). Consequently, the ability of bacterial cells to resist killing by the complement system is a major determinant of pathogenicity and has been identified in many Gram-negative species including *A. pleuropneumoniae* (Rycroft & Cullen, 1990), *Neisseria* species (Schneider *et al.*, 1982; Schneider, 1985) and *E. coli* (Kim *et al.*, 1986; Cross *et al.*, 1986). The actual mechanism responsible for this resistance has been extensively studied and is thought to have a multifactorial basis involving bacterial components such as capsular polysaccharides (Kim *et al.*, 1986; Cross *et al.*, 1986), LPS (Cross *et al.*, 1986; Grossman *et al.*, 1987; Merino *et al.*, 1992) and OMPs (Blaser *et al.*, 1987; Weiser & Gotschlich, 1990).

Currently, the studies of bactericidal activity of serum against *P. haemolytica* have been limited (MacDonald *et al.*, 1983; Sutherland, 1988). These reports have shown that isolates of *P. haemolytica* may be susceptible to killing by adult bovine serum or lung washings from infected sheep by antibody and complement. However the exact reason for the sensitivity of individual isolates to this bactericidal effect remains unknown and thus future investigations are required to enable us to understand the importance of this defence mechanism in combating *P. haemolytica* infection and also the role of serum resistance in the pathogenicity of the bacterium.

OBJECT OF RESEARCH

The overall aim of this investigation was to provide a greater understanding of the species *Pasteurella haemolytica* with particular emphasis on the surface components of the organism and their possible role in virulence. The study was divided into five distinct areas, although they were all designed to contribute to fulfilling the aim described above. Each section involved unique investigations which had not previously been performed with this species. The five main sections were entitled:

(1) An epidemiological study of *P. haemolytica*;

(2) Characterisation of a heat-modifiable outer-membrane-protein in *P. haemolytica*;

(3) Serum sensitivity in relation to surface properties;

(4) Virulence tests in calves;

(5) Investigations of *P. haemolytica* grown *in vivo*.

Section (1) involved analysis of OMPs and LPS of 29 *P. haemolytica* isolates by SDS-PAGE and Western blotting. The types identified with these techniques were then compared with the other known details of each isolate.

A heat-modifiable OMP of *P. haemolytica* was identified by SDS-PAGE in section (2) of this study. Further analysis involving Western blotting and N-terminal amino acid comparisons was performed to investigate the degree of relatedness of this protein with heat-modifiable proteins in other bacteria.

In section (3) the serum sensitivity of 22 *P. haemolytica* isolates was assessed using a serum killing assay. The results were assessed in relation to the serotype and LPS type of the isolates in an attempt to correlate serum resistance or sensitivity with surface properties.

The final two sections of this investigation involved examinations of this species *in vivo* . Virulence tests were the basis of section (4). The tests involved

intratrachael inoculation of calves with the bacteria and scoring the number of lung lesions produced after three days. The lung lesion score was taken as a measure of virulence and related to the other known properties of the isolate. In the last section (5), four *P. haemolytica* strains were placed within intraperitoneal implant chambers and their viability determined at intervals. The OMP profiles of the *in vivo*-grown bacteria were compared with those of *in vitro*-grown cells.

2. MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

A total of 39 *P. haemolytica* isolates and one *E.coli* strain, Lilly, were used. The *P. haemolytica* isolates comprised 24 of serotype A1, 14 of serotype A2 and 1 untypable isolate. Further information regarding the date of isolation, farms of origin and disease status of the host can be found in Tables 3 and 4.

2.2 GROWTH CONDITIONS

After primary isolation, isolates were stored at -70^{o}C in brain heart infusion broth (BHIB; Oxoid) containing 50% glycerol. They were subcultured routinely at 37^{o}C on brain heart infusion agar (BHIA; Oxoid) containing 5% sheep's blood.

2.2.1 Growth of *P. haemolytica* for OMP preparations

Bacteria were grown overnight at 37^{o}C in 25 ml volumes of BHIB, with shaking at 120 rpm, and 1 ml of these cultures were used to inoculate 500 ml volumes of fresh BHIB, which was incubated under the above conditions, until late logarithmic phase (approximately 6-7 h; see Figure 3). At this point the cells were harvested by centrifugation at 9000 x g for 30 min and used for OMP preparations as described in section 2.3. Purity checks were carried out on all cultures used for OMP preparations by inoculating 0.1 ml from the 500 ml culture onto BHIA (containing 5% defibrinated sheep blood), incubating them overnight at 37^{o}C and observing the purity of the growth as judged by the colony morphology.

In some cases the bacteria were required to be grown under conditions of iron limitation to obtain expression of the iron-regulated proteins (Davies *et al.*, 1992). For this, the iron chelator ethylenediamine dihydroxyphenyl-acetic acid

Table 3. ***Pasteurella haemolytica*** **isolates used in this study**

Strain designation[1]	Date of isolation	Site of isolation[2]	Disease status	Serotype
PH2 (SC82/1)	-	LRT	DISEASED	A1
PH4 (GA83/5)	-	LRT	DISEASED	A1
PH8 (V965B)	-	L	DISEASED	A1
PH48 (FA1)	-	L	DISEASED	A1
PH162	2.84	L	DISEASED	A1
PH164	12.84	L	DISEASED	A1
PH166	12.84	L	DISEASED	A1
PH168	10.82	L	DISEASED	A1
PH170	11.82	L	DISEASED	A1
PH172	11.83	L	DISEASED	A1
PH174	11.83	L	DISEASED	A1
PH220	-	L	DISEASED	A1
PH26 (GA83/1)	-	NP	HEALTHY	A1
PH30 (WD83/4)	-	NP	HEALTHY	A1
PH176	2.84	NP	HEALTHY	A1
PH178	2.84	NP	HEALTHY	A1
PH180	2.84	NP	HEALTHY	A1
PH182	2.85	NP	HEALTHY	A1
PH184	2.85	NP	HEALTHY	A1
PH186	2.85	NP	HEALTHY	A1
PH188	2.85	NP	HEALTHY	A1
PH190	2.85	NP	HEALTHY	A1
PH192	2.85	NP	HEALTHY	A1
PH194	2.85	NP	HEALTHY	A1
PH42	-	L	DISEASED	A2
PH72 (B664)	-	L	DISEASED	A2
PH196	12.84	L	DISEASED	A2
PH198	12.84	L	DISEASED	A2
PH200	12.84	L	DISEASED	A2
PH202	12.84	L	DISEASED	A2

Table 3. continued.....

PH204	12.84	L	DISEASED	A2
PH44 (GT85/15)	-	NP	HEALTHY	A2
PH208	1.85	NP	HEALTHY	A2
PH210	1.85	NP	HEALTHY	A2
PH212	1.85	NP	HEALTHY	A2
PH214	3.85	NP	HEALTHY	A2
PH216	3.85	NP	HEALTHY	A2
PH218	3.85	NP	HEALTHY	A2
UT3	-	L	DISEASED	UT

[1]Strain designations used by other workers are given in brackets.

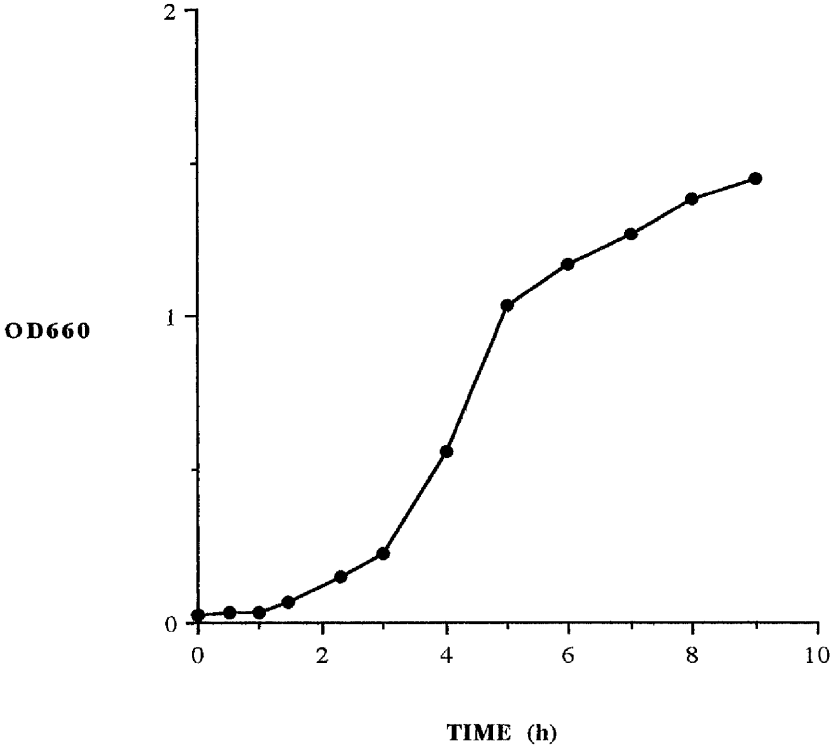
[2] LRT = lower respiratory tract; L = lung; NP = nasopharynx.

All isolates were provided by D. H. A. Gibbs (Glasgow University Veterinary School)with the exception isolates PH8, PH48, PH72 and UT3, which were obtained from Dr. W. Donachie (Moredun Research Institute).

All isolates were of bovine origin except isolates PH8, PH48 and PH72, which were of ovine origin.

Figure 3. Typical growth curve of *P. haemolytica* (isolate PH176) in BHIB

Pasteurella haemolytica was grown in 25 ml of BHIB at 37^{O}C, with shaking, for 9h and the OD660 was recorded at intervals.


OD660
2
1
0
0
2
4
6
8
10
TIME (h)

(Sigma) was added to the 500 ml of BHIB at an optimal concentration for expression of the respective proteins but also to allow suitable growth of the bacteria. Typical growth curves of the bacterium (isolate PH2) in BHIB containing different EDDA concentration are shown in Figure 4. The concentration chosen was that which inhibited growth (as a result of iron-limitation) to some extent when compared to the control , but which yielded sufficient cell numbers to allow for OMP preparation. Thus, in this experiment a concentration of 20 μM EDDA was chosen.

2.2.2 Growth of *P. haemolytica* for serum bactericidal tests

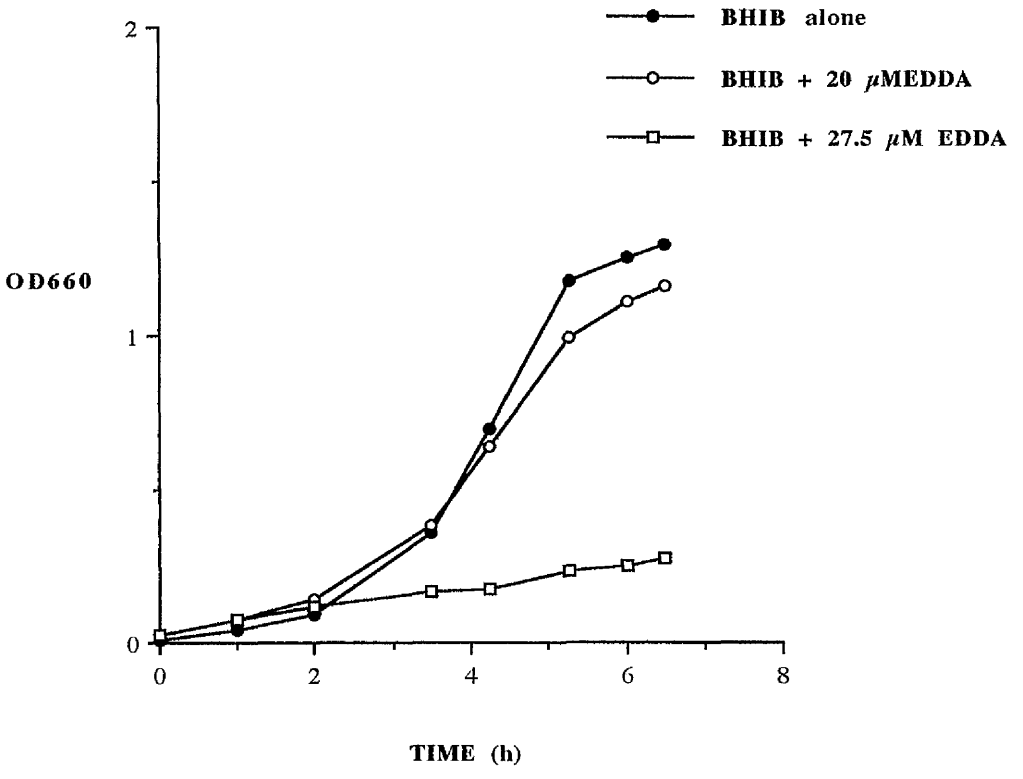
For the serum bactericidal tests, described in detail in section 2.9, bacteria were grown in 25 ml of BHIB at 37°C, with shaking at 120 rpm, until mid-logarithmic phase (approximately 4-5 h - see Figure 3) then harvested by centrifugation at 3,000 x g for 15 min.

2.2.3 Growth of *E. coli* for cross-absorbing antiserum

Monospecific antiserum against the heat-modifiable OMP of *P. haemolytica* (as described in section 2.5.2) was cross-absorbed with *E. coli* Lilly. *E. coli* was grown in 500 ml of BHIB at 37°C, with shaking, until mid-logarithmic phase then harvested by centrifugation at 9,000 x g for 30 min. The cells obtained were used for cross-absorbing, as described in section 2.5.3.

Figure 4. Growth of *P. haemolytica* (isolate PH2) in BHIB containing different concentrations of EDDA

Growth curves obtained as described in the legend to Figure 3, except that filter-sterilised EDDA was added to the media at the concentrations indicated in the figure. Growth of the bacterium in BHIB alone was included as a control.


BHIB alone
BHIB + 20 μMEDDA
BHIB + 27.5 μM EDDA
OD660
2
1
0
0
2
4
6
8
TIME (h)

2.2.4 Growth of *P. haemolytica* for chamber inoculations

For *in vivo* studies, an intraperitoneal implant chamber was used as described in section 2.11. *P. haemolytica* isolates PH2, PH30, PH42, and PH48 were used for inoculating the chambers. These were grown in 500 ml of BHIB at 37°C with shaking, until late-logarithmic phase (approximately 6-7 h; see Figure 3) then harvested as described in section 2.11. Purity checks were performed on all cultures prior to harvesting.

2.2.5 Growth of *P. haemolytica* for virulence tests in cattle

Pasteurella haemolytica isolate PH2 was used for standardisation of the virulence tests, described in detail in section 2.10. Bacteria were grown in 500 ml of BHIB at 37°C with shaking, until late logarithmic phase, and then harvested as described in section 2.10.

The isolates being compared for virulence (PH2, PH8, PH10, PH30, PH42 PH44 and PH72) were grown as described above. Purity checks were carried out as usual.

2.3 PREPARATION OF OUTER-MEMBRANE FRACTIONS

The outer-membrane fractions of the isolates were obtained by Sarkosyl extraction using the method described by Davies *et al.*, 1991. After growth of the cells as described in section 2.2.1, they were harvested by centrifugation at 10,000 x g for 30 min, washed in 20 ml of 20 mM Tris-hydrochloride buffer (pH7.2) and finally resuspended in 10 ml of the same buffer. The cells were disrupted, on ice, by sonication continuously for 10 min (MSE sonicator, 4 - 5 μm amplitude, 20kHz). Unbroken cells were removed by centrifugation at 10,000 x g for 30 min at 4°C and the total cell envelope pelleted by centrifugation at 50,000 x g for 60 min at

4°C. The inner-membrane fraction was solubilised by resuspending the pellet in 0.5% (w/v) sodium lauroyl sarcosine (Sarkosyl) for approximately 30 min at room temperature (Filip *et al.*, 1973) and the insoluble outer-membrane fraction was obtained by centrifugation at 50,000 x g for 60 min at 4°C. The outer-membrane fraction was washed in 20 mM Tris-hydrochloride buffer (pH7.2), centrifuged at 50,000 x g for 60 min at 4°C, resuspended in the same buffer to a protein concentration of 2.0 mg/ml, and stored at -20°C.

The procedure used for obtaining outer-membrane fractions from the *in vivo* -grown cells was identical to the above except that the initial cell number was lower and consequently the final amount of protein obtained was less. Outer-membrane fractions from isolates obtained from lung washings were provided by Dr. R. L. Davies and used in section 2.11.4. The method for obtaining OMP fractions from the bacteria was essentially as described above.

2.3.1 Quantitation of protein

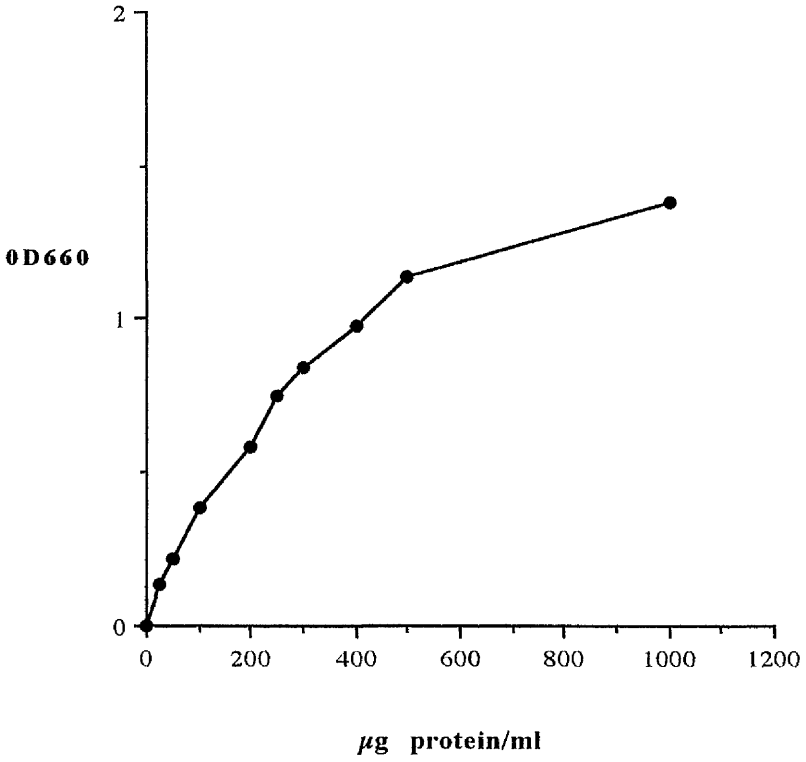
The protein concentration for each outer-membrane fraction was determined using the modified Lowry procedure of Markwell *et al.* (1978). A standard curve of ug protein/ml (using bovine serum albumin (BSA; Sigma) as the standard) versus optical density at 610 nm was obtained (see Figure 5) and used for determining the protein concentration of each outer-membrane sample.

2.3.2 Solubilisation of protein

After the outer-membrane fraction was adjusted to a protein concentration of 2 mg/ml with 20 mM Tris-hydrochloride (pH7.2), a chosen volume was diluted in an

Figure 5. Standard curve for determining protein concentrations of outer-membrane preparations

The method of Markwell *et al.* (1978) was used with bovine serum albumin (BSA) as the standard protein, and the curve opposite was used to determine the protein concentration in each outer-membrane preparation.


OD660
2
1
0
0
200
400
600
800
1000
1200
μg protein/ml

equal volume of x 2 sample buffer (see Appendix 1) and solubilised by heating at 100°C for 5 min.

2.3.3 Effect of different solubilisation temperatures on SDS-PAGE profiles

In section 3.2 the heat modifiability of certain *P. haemolytica* proteins was examined. Outer-membrane fractions were treated at different temperatures - room temperature, 50°C, 60°C, 70°C, 85°C and 100°C, usually for 5 min. In some experiments, the time of solubilisation was extended to 10 or 20 min. All samples were stored at -20°C.

2.4 PREPARATION OF LPS FRACTIONS

2.4.1 Proteinase K digestion of outer-membrane fractions

To obtain LPS fractions for analysis by SDS-PAGE, outer-membrane fractions were digested with proteinase K as described previously by Davies *et al.*, 1991. Ten microlitres of proteinase K (Sigma - adjusted to 2.5 mg/ml in x 1 sample buffer - see Appendix 1) were added to 100 ul of solubilised outer-membrane fraction (100 ug protein) and the mixture heated for 60 min at 60°C. All samples were stored at -20°C.

2.5 ANTISERA PRODUCTION

For this part of the study, the animal work, i.e.the inoculation and bleeding of the animals, was performed by Drs. H. A. Gibbs (Department of Veterinary Medicine, University of Glasgow) and R. L. Davies (Department of Microbiology, University of Glasgow).

2.5.1 Anti whole-cell sera

Two antisera to *P. haemolytica* whole cells were used in this study, namely antisera 32 and 10. Antiserum 32, a convalescent bovine serum raised against *P. haemolytica* isolate PH2, was provided by Dr. Q. Ali (PhD Thesis, 1993). Production of this antiserum involved experimental intratracheal infection of a calf with live cells, as described by Gibbs *et al.* (1984). Antiserum 10, a hyperimmune bovine serum raised against isolate PH2 was provided by Dr. R. L. Davies and produced by an initial intramuscular injection of a calf with formalin-killed organisms mixed with Freund's incomplete adjuvant (FIA; Difco) and an additional subcutaneous injection after a period of 27 days.

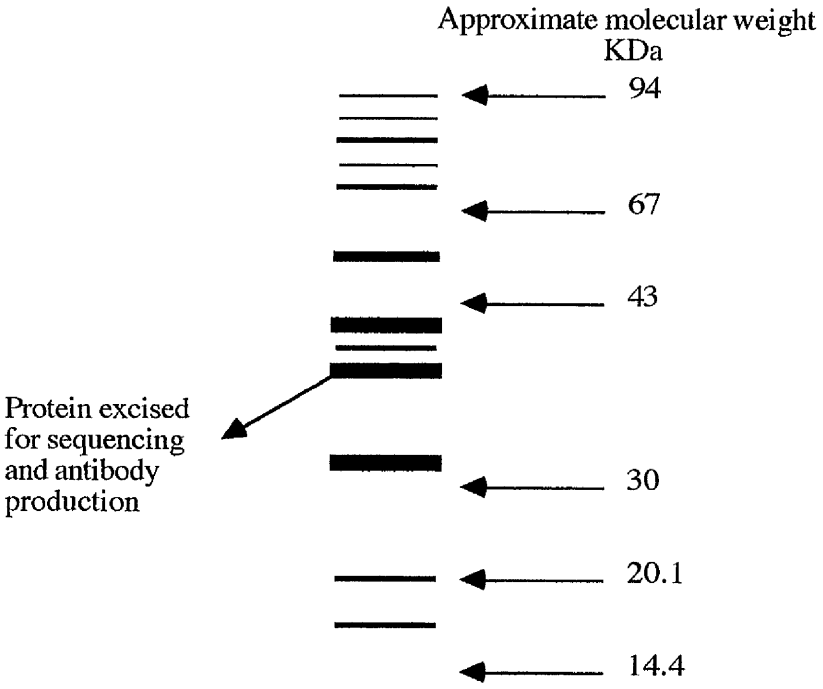
2.5.2 Anti-OMP antisera

Attempts were made to raise rabbit monospecific anti-OMP antibodies to the protein indicated by the arrow in Figure 6. The reasons for choosing this specific protein will become evident later.

The OMP sample of isolate PH2 was subjected to electrophoresis as described in section 2.6, except that a preparation gel was used instead of the usual 15 well gel (i.e. one large well of a single sample, resulting in complete strips of the individual proteins across the whole gel). The gel was stained for 5 min using Coomassie blue and destained for no more than 20 min to enable the appropriate protein band to become visible. The acidity of the gel (due to the acetic acid in the staining reagents) was neutralised by washing in PBS (pH7.2), to which was added 1.0 M sodium hydroxide (NaOH) until the pH stabilised at 7. The inoculum was prepared by excising the strip containing the appropriate band from the gel, crushing the gel in 1 ml of sterile PBS and mixing with 1 ml of Freund's complete adjuvant (FCA; Difco) for the initial intramuscular inoculation or 1 ml Freund's

Figure 6. Diagramatic representation of the OMP profile of PH2

A simplified representation of the OMP profile of *P. haemolytica* isolate PH2 as visualised on a SDS-polyacrylamide gel or after Western blotting on a Problott membrane after staining with Coomassie blue stain. The protein excised was used in sections 2.5.2 and 2.8 of this study.


Approximate molecular weight
KDa
94
67
43
30
20.1
14.4
Protein excised
for sequencing
and antibody
production

incomplete adjuvant (FIA) for subsequent subcutaneous inoculations. This 2 ml antigen/adjuvant mixture was injected into a New Zealand rabbit at four different sites. The antibody response to the protein was monitored by Western Blotting and the procedure terminated when a significant response was observed. The antiserum obtained was stored at -20^{O}C.

2.5.3 Cross-absorption of antiserum

The rabbit anti-OMP serum was cross-absorbed with *E. coli* Lilly (see section 3.2.3). The bacterial cells used for cross-absorption were grown as described in section 2.2.3 and harvested by centrifugation at 9,000 x g for 30 min. Bacterial cells pelleted from 100 ml of BHIB cultures were resuspended in a 5 ml mixture of antiserum diluted in PBS (pH7.2) at a ratio of 1:5. The mixture was incubated at 37^{O}C, with rotating, for 1 h, after which the bacterial cells were removed by centrifugation at 3,000 x g for 15 min, leaving the cross-absorbed antiserum. This procedure was repeated twice more to ensure complete absorption.

2.6 SDS-PAGE

OMPs (prepared as described in section 2.3) were separated by SDS-PAGE using the SDS discontinuous system of Laemmli (1970) as described by Davies *et al.*, 1992. Briefly, 20 ul (20 ug protein) of protein sample were loaded into each well (or 450 ul into one well in the case of a preparation gel) of a 4% (w/v) acrylamide stacking gel and separated in a 12% (w/v) acrylamide resolving gel (see Appendix 1). Electrophoresis was carried out in a vertical slab gel apparatus (Protean II; Bio Rad) in buffer comprising 25 mM Tris-hydrochloride, 192 mM glycine and 0.1% (w/v) SDS (pH 8.3), at a constant current of 20 mA per gel through the stacking gel and 30 mA per gel through the resolving gel.

Molecular weight standards (Pharmacia) used were phosphorylase b (94 KDa), bovine serum albumin (67 KDa), ovalbumin (43 KDa), carbonic anhydrase (30 KDa), trypsin inhibitor (20.1 KDa), and alpha-lactalbumin (14.4 KDa). Proteins were visualised by staining overnight with 0.1% (w/v) Coomassie Brilliant Blue (Sigma) in 10% (v/v) acetic acid and 45% (v/v) methanol in deionised water, and destaining for approximately 5 h with several changes of the destaining solution (ee Appendix 1).

The apparent molecular weights of individual proteins were estimated from a calibration curve of Rf values (distance travelled by protein band from top of the gel/distance from top to the bottom of the gel) plotted against the $\log_{10}$ molecular weight of the standard proteins (Figure 7). Thus, by calculating the Rf value of the unknown protein and by interpolation on the standard curve, the apparent molecular weight could be obtained.

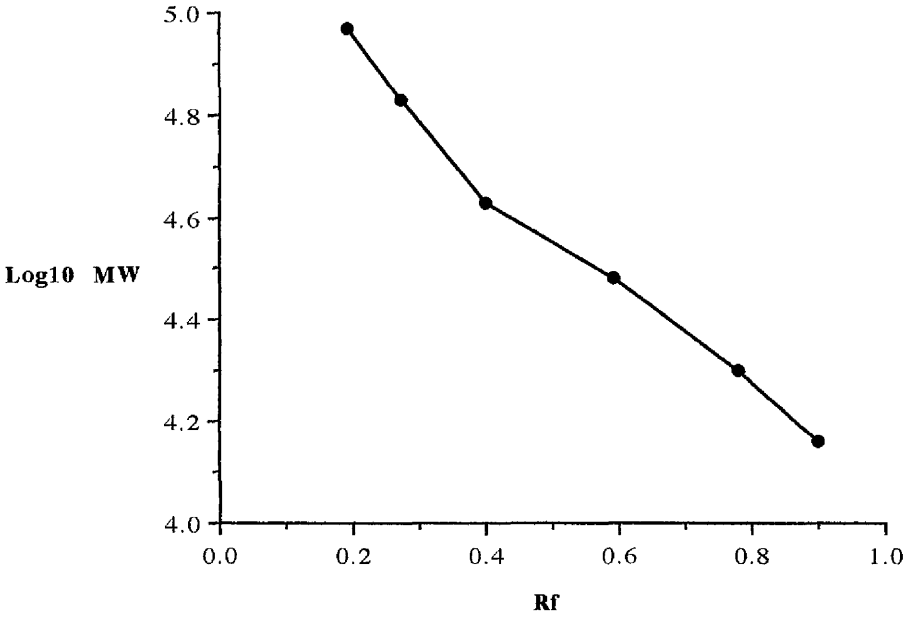
LPS samples (prepared as described in section 2.4.1) were analysed essentially as for OMPs with the exception that the resolving gel contained 15% (w/v) acrylamide and 4 M Urea (Davies *et al.*, 1991) - see Appendix 1. Five microlitres of the proteinase K-treated sample (approximately 5 ug LPS) were loaded per well and the separated LPS visualised by silver staining (Appendix 2) as described by Tsai & Frasch (1982).

2.7 WESTERN BLOTTING

Western blotting was performed as described previously (Davies *et al.*, 1994. OMPs and LPS were transferred to nitrocellulose (Schleicher & Schuell) in a Bio-Rad Trans-Blot Cell with plate electrodes (Bio-Rad, Richmond, CA 94804) following the manufacturer's instructions. Transfer of OMPs was carried out overnight at a constant voltage of 15 V in a buffer containing 25 mM

Figure 7. Standard curve for estimating the apparent molecular weights of individual proteins in SDS-PAGE profiles

The $\log_{10}$ MW of each of the standard proteins phosphorylase b (94 KDa), bovine serum albumin (67 KDa), ovalbumin (43 KDa), carbonic anhydrase (30 KDa), trypsin inhibitor (20.1 KDa), and alpha-lactalbumin (14.4 KDa) were plotted against the Rf value for the protein i.e. the distance travelled by the protein band from the top of the gel/the distance from the top to the bottom of the gel, to obtain the calibration curve opposite. By calculating the Rf value of any protein band and interpolation on this curve, the apparent molecular weight of the protein was obtained.


5.0
4.8
4.6
4.4
4.2
4.0
Log10 MW
0.0
0.2
0.4
0.6
0.8
1.0
Rf

Tris-HCl, 192 mM glycine (pH 8.3). LPS was transferred similarly but in a buffer with the addition of 20% (v/v) methanol. After transfer, the blotting membranes were washed in 20 mM Tris-hydrochloride, 500 mM sodium chloride buffer , pH 7.5 (TBS), for 5 min. Non-specific binding was blocked by incubation for 1 h in TBS containing 3% (w/v) gelatin (Bio Rad). The nitrocellulose membranes were washed (two 5 min washes) in 0.05% (v/v) Tween 20 (Sigma) in TBS (TTBS) and incubated overnight in primary antibody diluted 1 in 200 in 1% (w/v) gelatin in TTBS (antibody buffer). After two 5 min washes in TTBS, the membranes were incubated for 4 h with horseradish peroxidase-conjugated anti-rabbit or -bovine IgG antibodies diluted 1 in 2000 in antibody buffer. The membranes were washed in TTBS (two 5 min washes) followed by one wash in TBS (5 min) and developed in substrate solution. The substrate solution for OMPs contained 0.05% (w/v) 4-chloro-1-naphthol (Sigma) (dissolved in 20 ml ice-cold methanol) and 0.05% (v/v) hydrogen peroxide in 100 ml of TBS, while that for LPS contained 0.025% (w/v) diamino benzoic acid (DAB; Aldrich) dissolved in 100 ml PBS, 2% (v/v) cobalt chloride and 0.05%. Development was stopped by immersing the nitrocellulose in deionised water for 10 min. The transfer efficiency for OMPs was tested by staining a section of the membrane with 0.1% (w/v) amido black (Sigma). All reagents used in Western blotting are given in Appendix 3.

Different primary antibodies were used in the different sections of this study. Bovine antiserum 32 was used in sections 3.1.1 and 3.2.3; rabbit anti-OMP antiserum was used also in sections 3.2.3 and 3.2.4. The horseradish peroxidase-conjugates (SEROTEC) were specific for either bovine or rabbit IgG antibodies depending on the source of the primary antibody used.

2.8 PROTEIN SEQUENCING

N-terminal amino acid sequencing was performed for a particular *P. haemolytica* outer-membrane protein, was performed. Tricine SDS-PAGE was used for preparing the protein samples for N-terminal sequencing, according to the method provided by Dr. M. Cusack (Department of Geology and Applied Geology, University of Glasgow).

This procedure was essentially the same as SDS-PAGE with a few alterations: the stacking gel contained 4 % acrylamide and the resolving gel 10 % acrylamide (Appendix 1); electrophoresis was performed overnight (using the same apparatus as previously) in a cathode buffer containing 0.1 M Tris, 0.1 M Tricine and 0.1% (w/v) SDS and an anode buffer containing 0.2 M Tris-HCl (pH8.9) (Appendix 1), at a constant current of 20 mA. OMPs were then transferred from tricine polyacrylamide gels to a Problott membrane (Applied Biosystems) as described in section 2.7, with a few alterations. Transfer was carried out overnight at a constant voltage of 20 V in a buffer containing 10 mM 3 - [cyclohexylamino] - 1 - propane sulfonic acid (CAPS; Sigma) adjusted to pH 11 with 1 M sodium hydroxide (NaOH). After transfer, the Problott was washed briefly in milliQ water, stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 (Sigma) in 10% (v/v) acetic acid and 45% (v/v) methanol in milli Q water for 1 min, destained with 50% (v/v) methanol and washed once more in milli Q water. The protein band to be sequenced (see Figure 6), having been identified by brief staining with Coomassie blue stain, was excised from the membrane.

The sequencing was carried out by Dr. M. Cusack (Department of Geology and Applied Geology, University of Glasgow) using an ABI 477A pulse-liquid protein sequencer and an ABI 420-H amino acid analyser. Computer printouts and interpretations of the sequence were provided.

2.9 SERUM BACTERICIDAL ASSAYS

2.9.1 Assay procedure

Initially, optimal conditions for the demonstration of bactericidal activity were determined with respect to serum concentration, bacterial inoculum size and incubation period of the reaction. These initial results are given in section 3.3.1.

The assay was an adaptation of the method of Davies (1991) and was performed in 96-well, tissue culture-grade, U-bottomed microtiter plates (Dynatech) into which 180 ul of diluent (PBS, pH7.2) was delivered into 8 wells with a multi-channel pipette. The reaction mixture was set up, on ice, and contained 10 ul of bacterial cells and 90 ul of serum or, in some assays, a greater total volume with the same overall ratio of cells and serum. The reaction mixture was incubated at 37^{o}C for the required incubation period. At time 0 h, (or any other time at which the viable cell numbers was to be determined) two 20 ul volumes were removed from each reaction mixture, loaded into the first two wells of the microtiter plate and serially-diluted (1 in 10) using a multi-channel pipette through 6 wells. Twenty microlitres from each well were inoculated onto BHIA containing 5% defibrinated sheep's blood and incubated overnight at 37^{o}C. The mean cfu ml^{-1} for each assay was calculated from each duplicated count. The percentage of bacterial inoculum remaining after the incubation period, i.e. % survivors, was calculated using the formula: mean cfu ml^{-1} after incubation / mean cfu ml^{-1} before incubation x 100.

2.9.2 Serum bactericidal activity against *P. haemolytica*

A number of *P. haemolytica* isolates, representing those possessing different capsular, OMP and LPS types, were examined for serum sensitivity or resistance. The assay was performed as described above using bovine

hyperimmune antiserum 10 raised against isolate PH2 (see section 2.5.1). *P. haemolytica* isolate PH8 and *E.coli* Lilly were used as controls, since both were known to be serum sensitive. Decomplementation of the serum by heating at 56°C for 30 min was also used as a negative control. The definitions of serum resistance or sensitivity were based on the percentage of survivors where a value of ⩾100 % survivors represented serum resistance, a value < 0.1 % survivors represented serum sensitivity and a value of 0.1-99.9 % survivors represented partial serum sensitivity.

All sera used for screening the isolates was stored at -70°C in aliquots to maintain complement activity.

2.10 VIRULENCE TESTS IN CALVES

The virulence tests were in collaboration with Dr. H. A. Gibbs (Department of Veterinary Medicine, University of Glasgow). The tests involved intratracheal inoculation of the calves which were then sacrificed after three days and the lungs removed for lung scoring.

2.10.1 Standardisation of virulence tests

Standardisation of these tests, prior to screening different *P. haemolytica* isolates, involved 21 calves divided into 7 groups of 3. Variables in each group were the size of the bacterial inoculum and the diluent used. Two of the groups were control groups, receiving one of the diluents alone.

P. haemolytica isolate PH2 was grown as described in section 2.2.5 in 4 x 500 ml BHIB broth cultures and pooled together prior to inoculum preparation. In order to vary the cell numbers of the inocula, the volume of bacterial culture used for the initial harvesting differed between the groups: 25 ml, 250 ml, and 1150 ml were used to provide low, medium and high inocula, respectively. Cells were

harvested by centrifugation at 9,000 x g for 30 min and resuspended in 20 ml of either sterilised PBS or filter-sterilised (Acrodisc, 0.2μm) culture supernate. Two control groups of PBS or culture supernate alone were included in this standarisation process.

2.10.2 Comparison of virulence of various isolates

The virulence tests involved 4 calves per isolate tested, these being PH2, PH30, PH10, PH8, PH42, PH44 and PH72. Each isolate was grown as described in section 2.2.5. Two 500 ml cultures of each isolate were pooled together, mixed and dispensed into 4 x 250 ml volumes (representing the medium inoculum size - see previous section). After harvesting by centrifugation at 9,000 x g for 30 min, the cell pellets were resuspended in a total volume of 20 ml of sterile PBS to produce the final inocula. Calves were inoculated intratracheally as described previously.

Determination of the virulence of the individual isolates involved lung scoring in addition to assessment of various physiological factors throughout the experiments. This work was carried out by Dr. H. A. Gibbs who provided a tabulated form of the results, as seen in section 3.4.

2.11 *IN VIVO* STUDIES OF *PASTEURELLA HAEMOLYTICA*

2.11.1 Intraperitoneal implant chambers in calves

Chambers implanted into a number of different animals have been used as a means of obtaining *in vivo*-grown bacterial cells. Bacteria obtained from such a system were examined in this work. The chamber was designed by Dr. R. L. Davies (Department of Microbiology, University of Glasgow) and the operations to insert the chamber intraperitoneally into the calves were performed by Dr. H. A.

Gibbs. The chamber was essentially a modification of that described by Pike *et al.* (1991), an external sampling port which allowed continuous sampling. More details of both the design and the insertion of the chambers will be given elsewhere. My involvement in this work was in preparing the bacterial inocula for the chambers and in processing the chamber fluid and organisms obtained.

2.11.2 Preparation of inocula and sampling of the chamber

After insertion, the chambers, which had an internal volume of approximately 100 ml, were filled with PBS then left for 3 days to allow host peritoneal fluid to diffuse into the chamber. The bacterial isolates to be examined were then inoculated into the chambers.

P. haemolytica isolates PH2, PH30, PH42, and PH48 were grown as described in section 2.2.4 and harvested by centrifugation at 9,000 x g for 30 min. Cells were resuspended in PBS (pH7.2) to an OD_{610} of 1.0, representing approximately 10^9 cfu/ml. Inoculation of 1 ml of this cell suspension into the chamber, resulted in a initial concentration of approximately 10^7 cfu/ml in the chamber. Viable counts were performed to confirm the actual numbers of cells inoculated and to check the purity of the cultures.

At daily intervals, 2-3 ml samples were removed from the chamber and bacterial counts were determined. Samples of filter-sterilised fluid removed from the chamber were stored at -70°C.

2.11.3 Effect of chamber fluid on *in vitro*-grown *P. haemolytica*

The effect of chamber fluid on the viability of *in vitro*-grown cells was assessed. *P. haemolytica* isolates PH2, PH30, PH42 and PH48 were grown in 25 ml of BHIB at 37°C, with shaking at 120 rpm, for approximately 4 h, after which

they were harvested and resuspended to an OD610 of 1.0 which contained approximately 10^9 cfu/ml. Fifteen microlitres of each suspension were inoculated into 135 µl of homologous chamber fluid, i.e., fluid in which the same isolate had been growing *in vivo* . Cell viability in this fluid was examined over a period of 2 h by performing bacterial counts in duplicate and the % survivor value calculated as described in section 2.9. The *in vitro* growth of the bacteria was assessed in chamber fluid removed at days 0, 9 and 16 of the experiment. The antibody content of the chamber fluids was also examined by Western blot analysis using *P. haemolytica* OMPs as target antigens.

2.11.4 OMP preparations from *in vivo*-grown cells

Outer-membranes from the *in vivo* -grown cells were obtained essentially as described in section 2.3. On the day prior to removal of the chamber from the calf, a 5 ml cell suspension (containing 10^{10} cfu/ml) was inoculated into the chamber. The following day the chamber was removed and the fluid collected and centrifuged at 9,000 x g for 30 min to pellet the cells for outer-membrane preparations.

3. RESULTS

3.1 AN EPIDEMIOLOGICAL STUDY OF *PASTEURELLA HAEMOLYTICA*

In this investigation, SDS-PAGE and Western blotting were used to demonstrate variation in OMP and LPS profiles of 29 *P. haemolytica* isolates, 18 of serotype A1 and 11 of serotype A2. The variation observed enabled the division of these isolates into distinct groups possessing different OMP and LPS types. In addition, information on the disease status of the host from which the isolates were obtained, and the origin of each isolate, were also known and considered.

3.1.1 Identification of different OMP profiles in *P. haemolytica*

The OMP profiles of serotypes A1 and A2 of *P. haemolytica*, as visualised by Coomassie blue staining, consisted of three or four major proteins and approximately 15 - 20 minor proteins (Figure 8). The profiles of both serotypes were very similar but not identical. There were a number of differences, particularly in the minor proteins, although the mobility of the upper major protein was different between the serotypes. These differences are indicated by the arrrows in Figure 8. The OMP profiles observed within the 18 serotype A1 isolates were very similar when examined by Coomassie blue staining and could not readily be distinguished (Figure 9a), while the profiles of the 11 serotype A2 isolates, as seen on the Coomassie blue-stained gels, could be distinguished more readily on the basis of variation in the mobilities of individual proteins (Figure 9b). Differences in the mobilities of the high molecular weight proteins were observed between serotype A2 isolates PH196, PH198 and PH200 (lanes 1-3, arrows), isolates PH202 and PH204 (lanes 4 and 5, arrows) and isolate PH214 (lane 9, upper arrows). This latter isolate also differed from the others in the mobility of

Figure 8. Typical OMP profiles of *P. haemolytica* serotypes A1 and A2 obtained by SDS-PAGE analysis

Coomassie blue-stained SDS-PAGE OMP profiles of *P. haemolytica* isolates of serotype A1 (PH188) and serotype A2 (PH210), in lanes 1 and 2, respectively. Arrows indicate the differences in OMP profiles (see text). Molecular weight standards (kDa) are shown in lane 3.

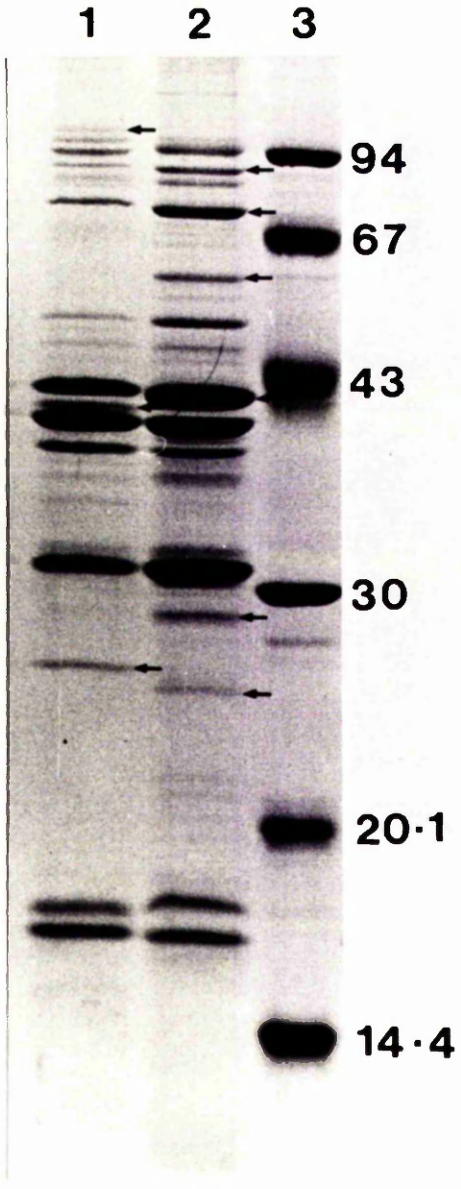

1
2
3
94
67
43
30
20·1
14·4

Figure 9a. SDS-PAGE OMP profiles of *P. haemolytica* isolates of serotype A1

Coomassie blue-stained SDS-PAGE OMP profiles of *P. haemolytica* seroytpe A1 disease isolate, PH162 (OMP type 1.1), PH164 and PH166 (OMP type 1.2), PH 168 and PH170 (OMP type 1.3), and PH172 and PH174 (OMP type 1.2), and non-disease isolates PH180 and PH184 (OMP type 1.2) and PH176, PH178 and PH182 (OMP type 1.4), in lanes 1-12, respectively. Molecular weight standards (kDa) are shown in lane 13.

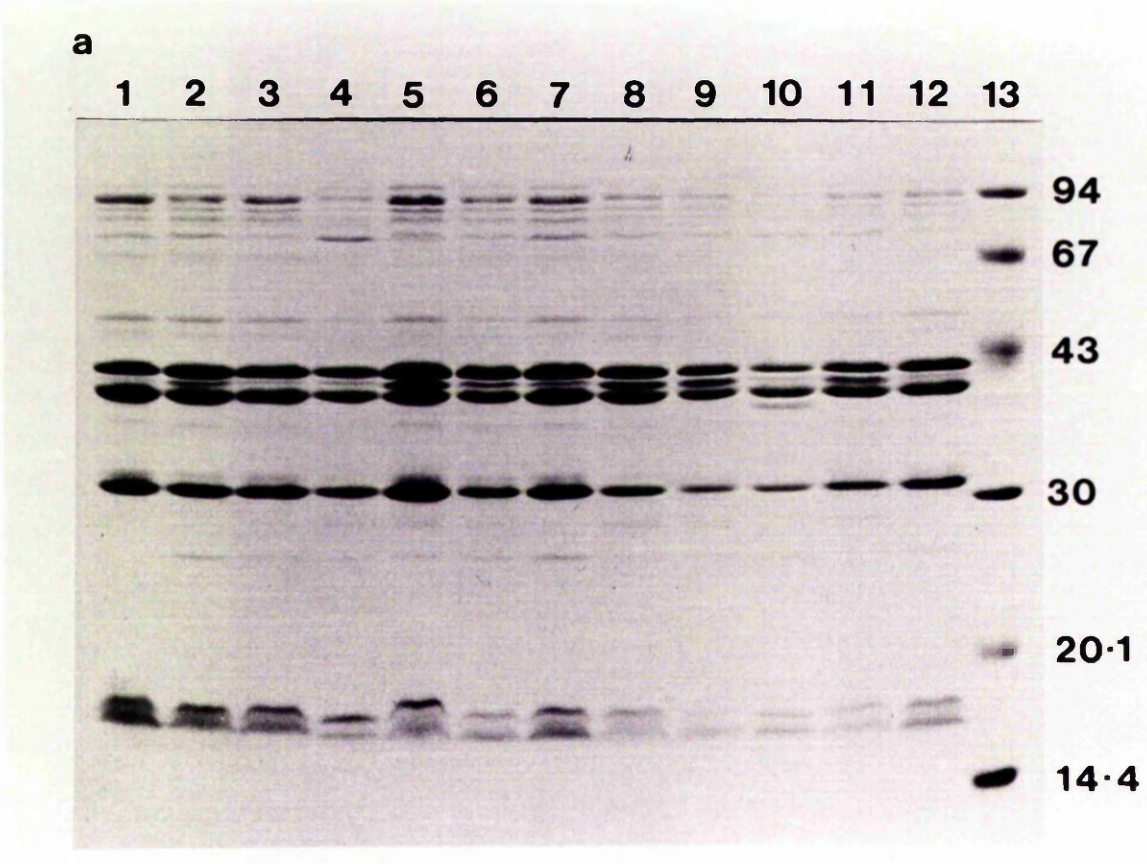
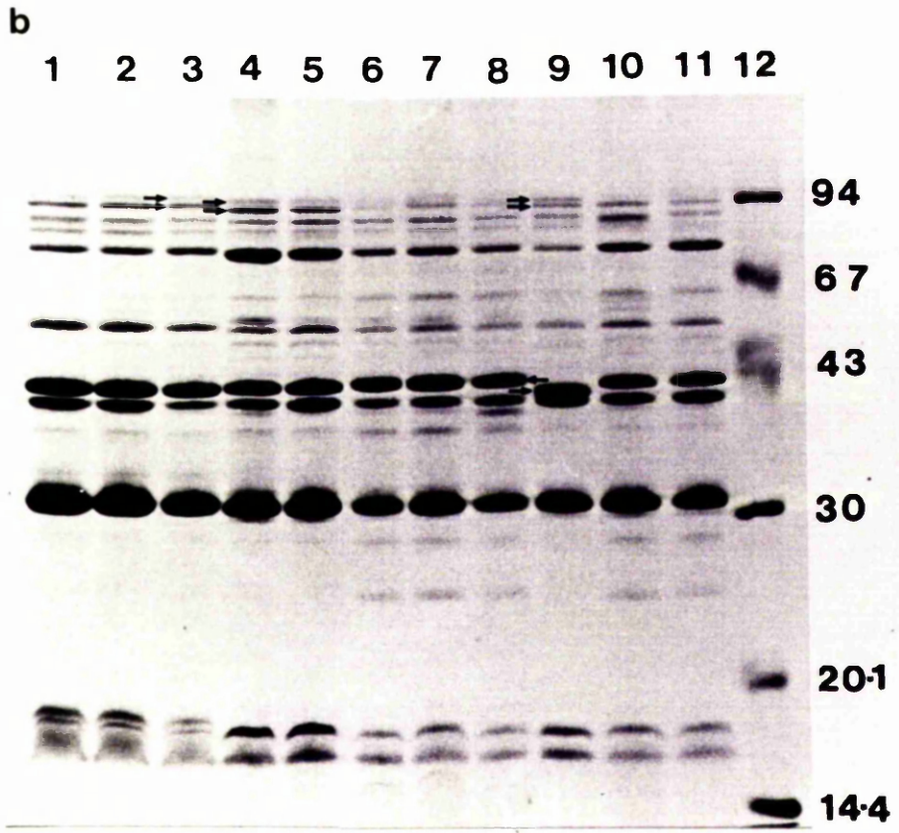

a
1 2 3 4 5 6 7 8 9 10 11 12 13
94
67
43
30
20·1
14·4

Figure 9b. SDS-PAGE OMP profiles of *P. haemolytica* isolates of serotype A2

Coomassie blue-stained SDS-PAGE OMP profiles of *P. haemolytica* serotype A2 disease isolates PH196, PH198 and PH200 (OMP type 2.1), and PH202 and PH204 (OMP type 2.2) and non-disease isolates PH212, PH210 and PH208 (OMP type 2.3), PH214 (OMP type 2.4) and PH216 and PH218 (OMP type 2.2), in lanes 1-11, respectively. Arrows indicate differences in OMP profiles (see text). Molecular weight standards (kDa) are shown in lane 12.


b
1 2 3 4 5 6 7 8 9 10 11 12
94
67
43
30
20·1
14·4

the upper major protein (lanes 8 and 9, lower arrows). Thus, within each of the serotypes, particularly serotype A1 it was difficult to distinguish between individual OMP types by Coomassie blue-staining. However, by supplementing these results with those from Western blotting, variation was more clearly evident and the OMP types could be distinguished.

Western blotting with bovine convalescent serum 32 identified differences within the serotype A1 isolates which had previously been unrecognised and also more clearly differentiated the isolates of serotype A2. These differences, identified by Western blotting, enabled four distinct OMP types to be distinguished in each of the serotypes. Within the 18 serotype A1 isolates, the OMP types were designated OMP types 1.1, 1.2, 1.3, and 1.4 and these differed in the immunological recognition of various minor proteins (Figure 10a, indicated by arrows). The 11 A2 isolates also consisted of four distinct OMP types, 2.1, 2.2, 2.3 and 2.4 (indicated in Figure 10b). Again, this variation was based on differences in the immunological recognition of various minor proteins as indicated by the arrows (lanes 4, 6 and 9). In OMP type 2.1 a wider range of proteins, in addition to a high background staining were recognised. Thus, based mainly on the Western blotting results, four individual OMP types could be distinguished in each of the serotypes.

3.1.2 Identification of different LPS profiles in *P. haemolytica*

The LPS SDS-PAGE profiles of *P. haemolytica* serotypes A1 and A2 exists in either the smooth or rough form, depending on the presence or absence of O-antigen side-chains, respectively (Davies *et al* ., 1991; Ali *et al.*, 1992). The O-antigen side-chains are seen as a series of high-molecular-weight bands which form a ladder pattern (Davies *et al.*,1991). In previous studies, the presence of two smooth and three rough LPS types (types 1, 2 and 3, 4 or 5, respectively) in

Figure 10a. Western blot showing the antibody recognition patterns of bovine antiserum 32 against the OMPs of *P. haemolytica* A1 isolates

Western blot showing the reaction of a 1 in 200 dilution of bovine antiserum 32 against the OMPs of *P. haemolytica* serotype A1 disease isolates PH162 (OMP type 1.1), PH164 and PH166 (OMP type 1.2), PH168 and PH170 (OMP type 1.3), PH172 and PH174 (OMP 1.2), and non-disease isolates PH180 and PH174 (OMP type 1.2), and PH176, PH178 and PH182 (OMP type 1.4), in lanes 1-12, respectively. Approximate molecular weights (kDa) indicated on the right. OMP types are indicated along the bottom; arrows indicate differences between OMP types (see text).

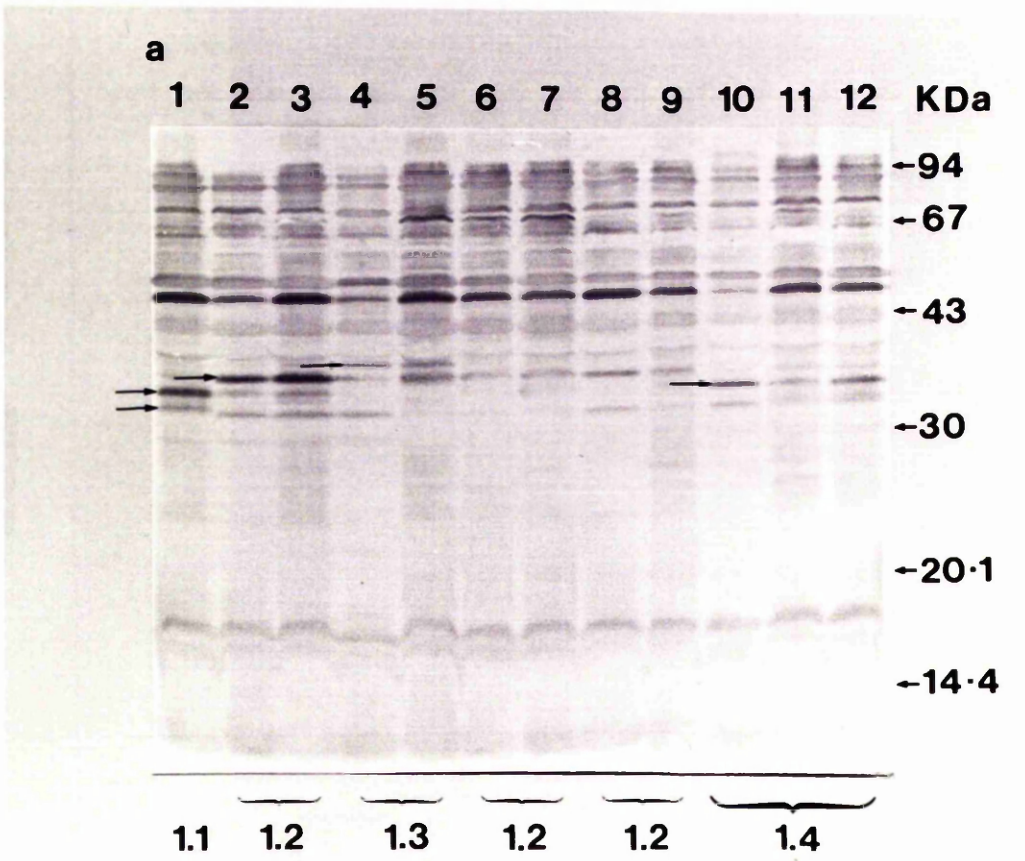
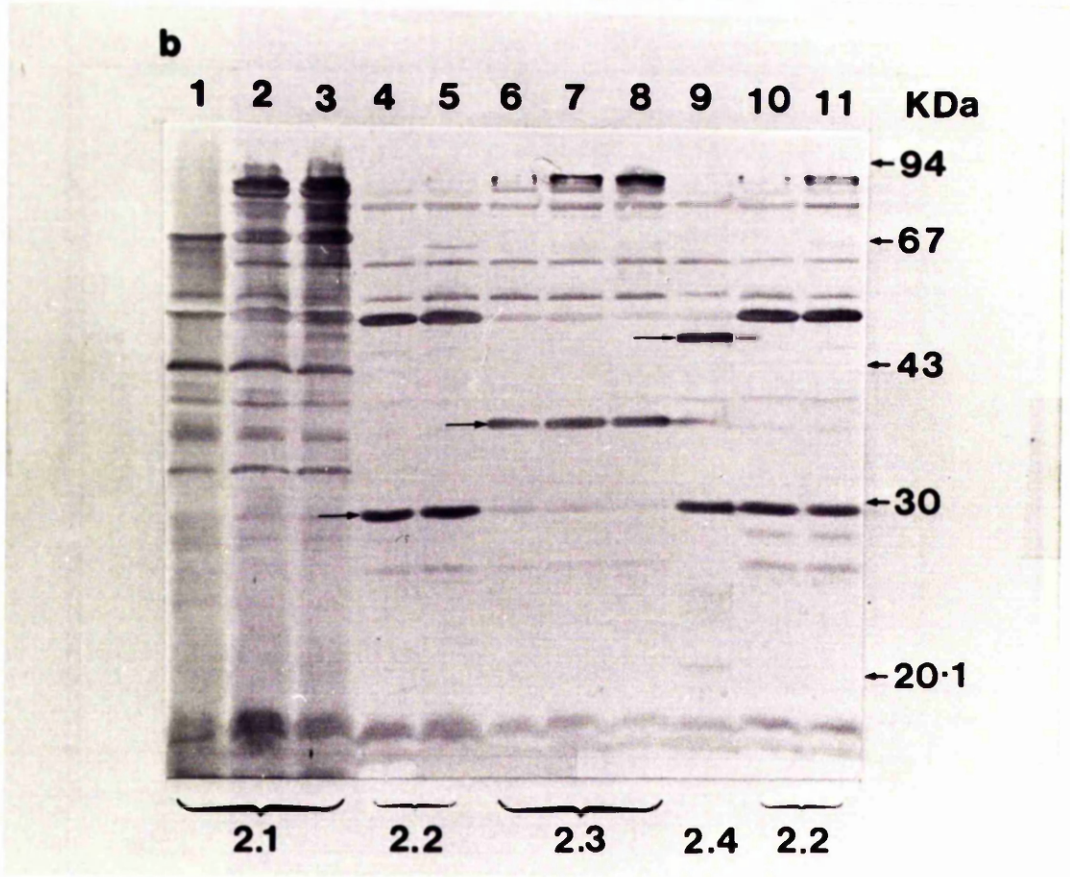

a
1 2 3 4 5 6 7 8 9 10 11 12 KDa
94
67
43
30
20·1
14·4
1.1 1.2 1.3 1.2 1.2 1.4

Figure 10b Western blot showing the antibody recognition patterns of bovine antiserum 32 against the OMPs of *P. haemolytica* A2 isolates

Western blot showing the reaction of a 1 in 200 dilution of bovine antiserum 32 against the OMPs of *P. haemolytica* serotype A2 disease isolates PH196, PH198 and PH200 (OMP type 2.1) and PH202 and PH204 (OMP type 2.2), and non-disease isolates PH212, PH210 and PH208 (OMP type 2.3), PH214 (OMP type 2.4) and PH216 and PH218 (OMP type 2.2), in lanes 1-11, respectively. Approximate molecular weights (kDa) are indicated on right. OMP types are indicated along the bottom. Arrows indicate differences between OMP types (see text).


b
1 2 3 4 5 6 7 8 9 10 11
KDa
94
67
43
30
20·1
2.1
2.2
2.3
2.4
2.2

serotypes A1 and A2 isolates have been described (Ali *et al.*, 1992). The LPS profiles identified in this study were compared with those previously described and were demonstrated as either LPS types 1, 3 or 5 (Figure 11).

Within the 18 serotype A1 isolates, only one LPS profile was identified. This was a smooth type LPS, type 1 (Figure 11, lanes 2 and 3). The isolates of serotype A2 consisted of one of two rough LPS types, type 3 or 5. These differed from each other in the mobilities of the low-molecular-weight bands which represent the core-oligosaccharide region. LPS type 3 was found in three isolates (Figure 11, lanes 6 and 7), whereas the remaining 8 isolates possessed LPS type 5 (Figure 11, lanes 10 and 11). The variation in the low-molecular-weight bands is indicated by arrows (lanes 6 and 7).

3.1.3 Relationship of disease status of the host, the geographical location and date of the isolation with OMP and LPS types

Information regarding the disease status of the host from which the isolate was obtained, the geographical location where the isolation was made and the date of the isolation, was available for each isolate. The relationship of these to serotype, OMP type and LPS type is shown in Table 4.

Serotype A1 disease isolates, all of which possessed LPS type 1, were obtained from farms 1, 2, 3 and 4 and could be differentiated on the basis of their OMP types, i.e. types 1.1, 1.2 or 1.3. Serotype A1 non-disease isolates, all of which possessed LPS type 1, were obtained from farm 5 and the majority were of OMP type 1.4. Two isolates were of OMP type 1.2. The situation was more complex within the serotype A2 isolates because two different LPS types were present, i.e. types 3 and 5. Isolates of OMP type 2.1 were associated only with LPS type 3; the remaining isolates possessed LPS type 5 but could be distinguished by having one of three different OMP types, i.e. types 2.2, 2.3, or 2.4. Serotype A2 disease isolates were obtained from farm 6 and formed two

Figure 11. SDS-PAGE LPS profiles of *P. haemolytica* isolates

Silver-stained SDS polyacrylamide gels comparing the LPS profiles of *P. haemolytica* isolates used in the present study (isolates PH188, PH190, PH196, PH198, PH204 and PH208) with those of the 5 previously described LPS types (isolates PH2, PH30, PH8, B664, and PH44). Isolates and LPS types are as follows: PH2, PH188 and PH190 (LPS type 1), PH30 (LPS type 2), PH8, PH196 and PH198 (LPS type 3), B664 (LPS type 4), and PH44, PH204 and PH208 (LPS type 5), in lanes 1-11, respectively. Arrows indicate the O-antigen side chains in LPS type 1.

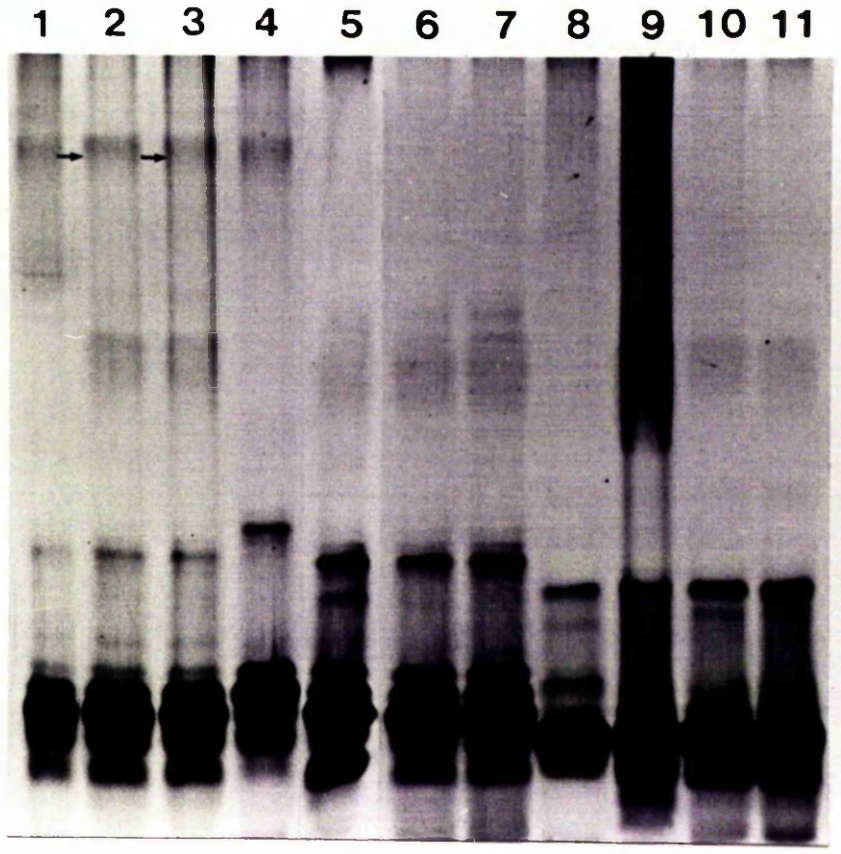

1 2 3 4 5 6 7 8 9 10 11

Table 4. Comparison of serotype, OMP type, LPS type for each *P. haemolytica* isolate with the origin of the isolate and the disease status of the host

Laboratory designation	Date of isolation	Origin of isolate		Disease status of host	Serotype	OMP type	LPS type
		Location[1]	Site of isolation[2]				
PH162	2.84	1	L	PNEUMONIC	A1	1.1	1
PH164	12.84	2	L	PNEUMONIC	A1	1.2	1
PH166	12.84	2	L	PNEUMONIC	A1	1.2	1
PH168	10.82	3	L	PNEUMONIC	A1	1.3	1
PH170	11.82	3	L	PNEUMONIC	A1	1.3	1
PH172	11.83	4	L	PNEUMONIC	A1	1.2	1
PH174	11.83	4	L	PNEUMONIC	A1	1.2	1
PH220	NK[3]	NK[3]	L	PNEUMONIC	A1	1.2	1
PH176	2.85	5	NP	HEALTHY	A1	1.4	1
PH178	2.85	5	NP	HEALTHY	A1	1.4	1
PH180	2.85	5	NP	HEALTHY	A1	1.2	1
PH182	2.85	5	NP	HEALTHY	A1	1.4	1
PH184	2.85	5	NP	HEALTHY	A1	1.2	1
PH186	2.85	5	NP	HEALTHY	A1	1.4	1
PH188	2.85	5	NP	HEALTHY	A1	1.4	1
PH190	2.85	5	NP	HEALTHY	A1	1.4	1
PH192	2.85	5	NP	HEALTHY	A1	1.4	1
PH194	2.85	5	NP	HEALTHY	A1	1.4	1
PH196	12.84	6	L	PNEUMONIC	A2	2.1	3
PH198	12.84	6	L	PNEUMONIC	A2	2.1	3
PH200	12.84	6	L	PNEUMONIC	A2	2.1	3
PH202	12.84	6	L	PNEUMONIC	A2	2.2	5
PH204	12.84	6	L	PNEUMONIC	A2	2.2	5
PH208	1.85	5	NP	HEALTHY	A2	2.3	5
PH210	1.85	5	NP	HEALTHY	A2	2.3	5

Table 4. continued....

PH212	1.85	5	NP	HEALTHY	A2	2.3	5
PH214	3.85	5	NP	HEALTHY	A2	2.4	5
PH216	3.85	5	NP	HEALTHY	A2	2.2	5
PH218	3.85	5	NP	HEALTHY	A2	2.2	5

[1] 1-6 indicate the different farms the isolates originated from.

[2] L = lung, NP = nasopharynx

[3] NK = not known

groups which could be clearly distinguished by both their OMP and LPS types, i.e. OMP/LPS types 2.1/3 and 2.2/5. Serotype A2 non-disease isolates were obtained from farm 5. They were all of LPS type 5 and could be differentiated by their OMP types, i.e. types 2.2, 2.3 or 2.4. When serotype A1 and A2 isolates from farm 5 were considered together, at least 5 groups of isolates were present, i.e.OMP/LPS types 1.2/1, 1.4/1, 2.2/5, 2.3/5 and 2.4/5. However , these isolates were obtained on three separate occasions (1.85, 2.85 and 3.85) and, as can be seen from Table 4, the various OMP/LPS types were isolated on only one of the three occasions.

3.2 CHARACTERISATION OF A HEAT-MODIFIABLE OMP IN *PASTEURELLA HAEMOLYTICA*

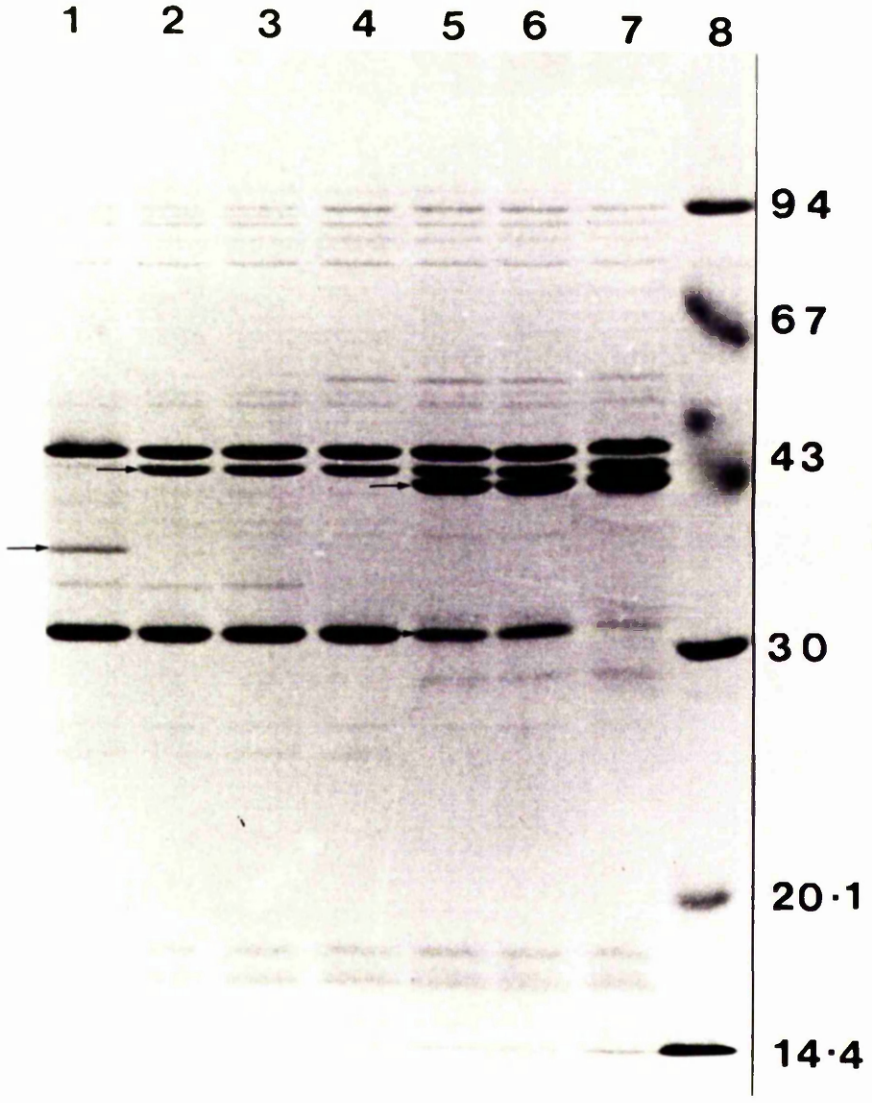
SDS-PAGE and Western blotting were used in this study to demonstrate the presence of a heat-modifiable OMP in *P. haemolytica*. This property results in a change in the apparent molecular weight of a particular protein, as seen on stained gels or in immunoblots, when the protein is solubilised at different temperatures in sample buffer.

3.2.1 Identification of a heat-modifiable OMP in *P. haemolytica*

The SDS-PAGE profiles of Sarkosyl-insoluble OMP preparations of *P. haemolytica* isolate PH188 treated at different solubilisation temperatures and also different times, are shown in Figure 12. As the solubilisation temperature was increased from room temperature to 50^{o}C, a protein band of apparent molecular weight 40.5 kDa appeared while a lower-molecular-weight protein of disappeared (lanes 1 and 2, indicated by arrow). Although this finding suggested that the

Figure 12. SDS-PAGE OMP profiles of *P. haemolytica* after solubilisation at different temperatures

Coomassie blue-stained SDS-PAGE OMP profiles of *P. haemolytica* isolate PH188 after solubilisation at room temperature, 50°C, 60°C, 70°C, 85°C and100°C for 5 min (lanes 1-5, respectively), and 100°C for 10 and 20 min (lanes 6 and 7, respectively). Arrows indicate differences between profiles at different solubilisation temperatures (see text). Molecular weight standards (kDa) are shown in lane 8.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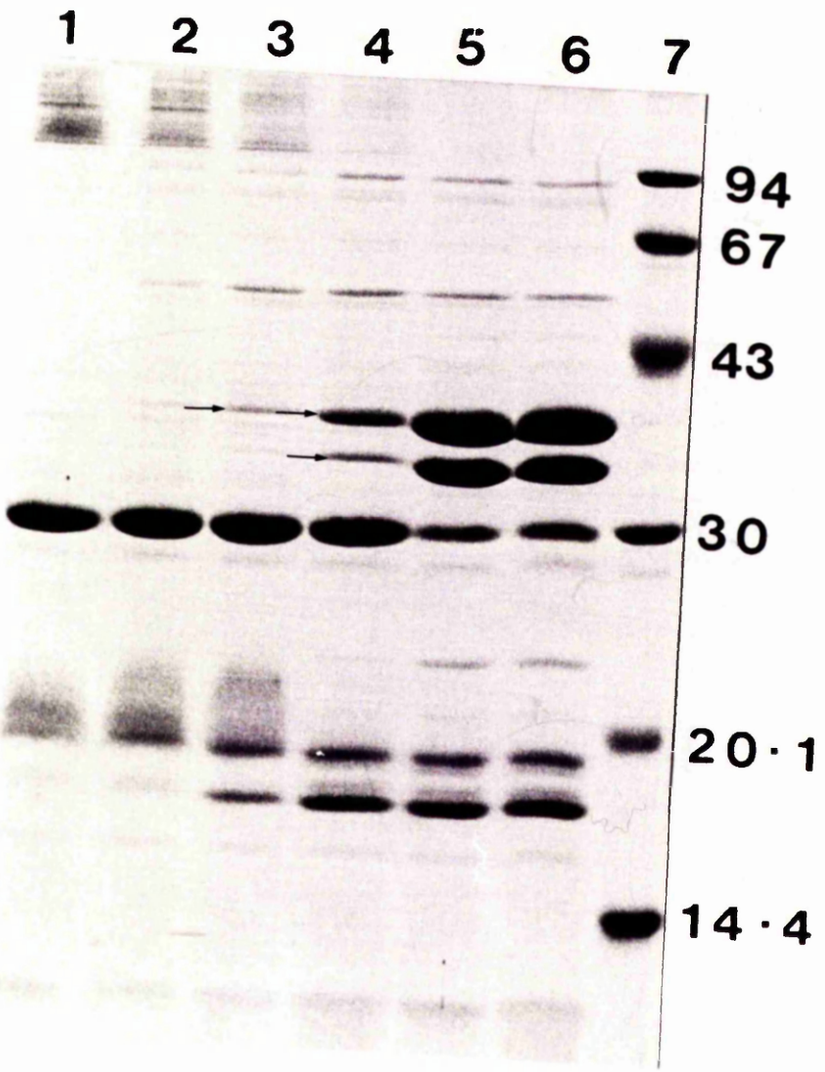
lower-molecular-weight protein, after solubilisation at 50°C, appeared at the higher-molecular-weight position, this required confirmation, especially since this heat modifiablity at the lower temperatures does not appear to have been reported with other Gram-negative species. However, as in other species, a heat-modifiable protein of approximately 30-40 kDa appeared after solubilisation temperatrures of 100°C. As the solubilisation temperature is increased from 85°C to 100°C (lanes 4 and 5) a protein band of apparent molecular weight 39.5 kDa becomes evident with a decrease in a band of 31 kDa. This shift from 31 kDa to 39.5 kDa is observed more clearly in lanes 6 and 7, where the OMP samples were solubilised at 100°C for 10 and 20 min instead of the usual 5 min. Confirmation of this shift was made by excising from the gel the lower-molecular-weight band from a sample solubilised at room temperature, re-solubilising the band at 100°C for 5 min and finally re-examining it on an SDS-polyacrylamide gel (results not shown). Solubilisation of the excised low-molecular-weight 31 kDa protein at 100°C caused it to appear at the higher molecular weight when re-examined. Thus, *P. haemolytica* appears to possess a 39.5 kDa protein which is heat-modifiable, characteristically shifting from a low to high-molecular-weight at 100°C. This finding was also confirmed by Western blotting with a monospecific antiserum raised against the high-molecular-weight form of the protein (described in section 2.5.2). The results obtained are described below and shown in Figures 16 and 17.

Since heat-modifiable OMPs have been described in other Gram-negative bacteria, including *Escherichia coli* (Beher *et al.*, 1980), the findings reported in this study for *P. haemolytica* were compared with those seen in *E. coli*. In Figure 13, the SDS-PAGE profiles of Sarkosyl-insoluble OMP preparations of *E. coli*, solubilised at different temperatures are shown. Comparison of these profiles shows the appearance of the characteristic porin proteins of *E. coli* (in the molecular weight range of 30-43 kDa), at solubilisation temperatures of 60°C and

Figure 13. SDS-PAGE OMP profiles of *E.coli* after solubilisation at different temperatures

Coomassie blue-stained SDS-PAGE profiles of *E. coli* after solubilisation at room temperature, 50°C, 60°C, 70°C, 85°C and 100°C for 5 min in lanes 1-6, respectively. Arrows indicate differences in profiles at different temperatures (see text). Molecular weight standards (kDa) are shown in lane 7.


1
2
3
4
5
6
7
94
67
43
30
20·1
14·4

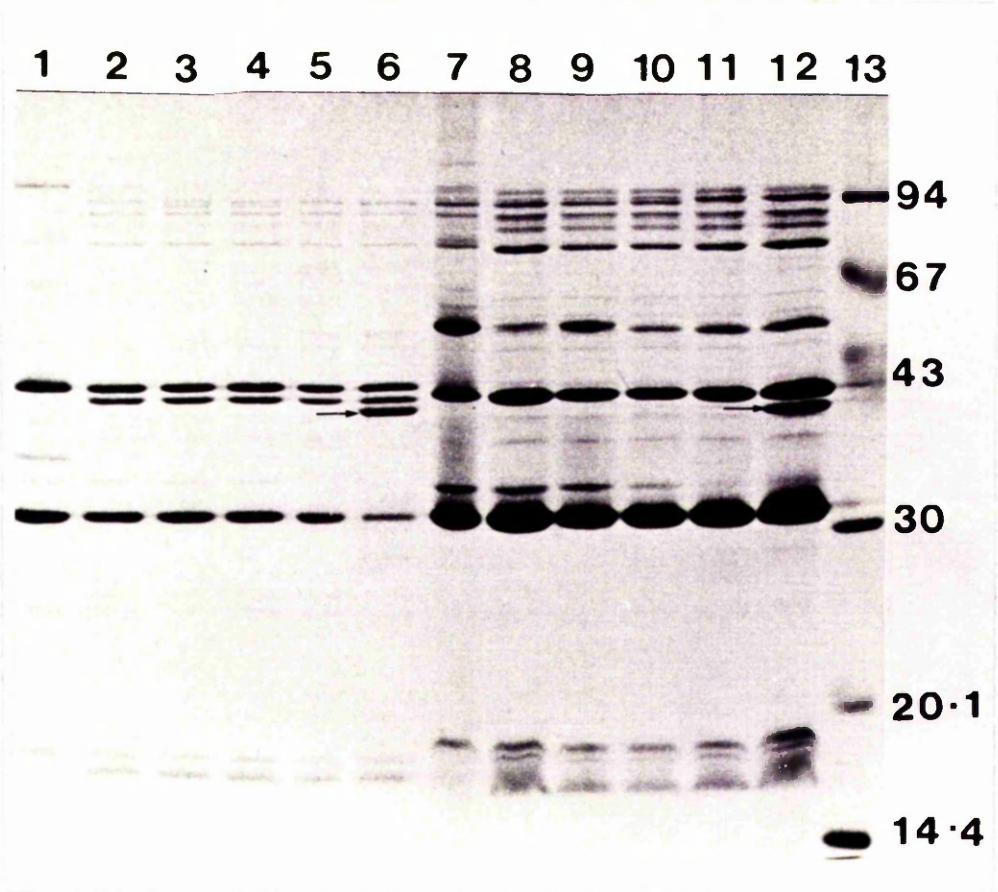
above. The disappearance of a high-molecular-weight protein and appearance of the upper major protein (Figure 13, lane 3; lower arrow), after solubilisation at 60°C possibly represents the peptidoglycan-associated-protein of *E. coli* (Beher *et al.*, 1980), although confirmation of this is required. The heat-modifiable OMP of *E. coli* was present in the high molecular weight form following solubilisation temperatures of 70°C and above (Figure 13, lanes 4-6, indicated by the arrows) and this is in contrast to the heat-modifiable protein of *P. haemolytica* which appeared in the high-molecular-weight form only after solubilisation at 100°C. The apparent molecular weights of the high- and low-molecular-weight forms of the heat-modifiable protein of *E. coli* also differ from those of the *P. haemolytica* protein, those of the former being 30 and 33 kDa in comparison to 31 and 39.5 kDa in *P. haemolytica*. The heat-modifiable proteins of both these species are also compared in section 3.2.4.

3.2.2 The presence of a heat-modifiable OMP in *P. haemolytica* serotypes A1 and A2

The occurrence of a heat-modifiable OMP in 9 *P. haemolytica* isolates, representing both serotypes A1 and A2 (4 isolates and 5 isolates, respectively), was demonstrated by SDS-PAGE (results not shown). Representative profiles of the OMPs from an A1 isolate, PH188, and an A2 isolate, PH210 (lanes 1-6 and 7-12, respectively), solubilised at different temperatures, are shown in Figure 14. In lanes 6 and 12, the heat-modifiable OMP, following solubilisation at 100°C for 20 min is indicated by the arrows. The change in apparent molecular weight took place between 85°C and 100°C. This finding was common to all 9 isolates examined in this study.

Figure 14 . SDS-PAGE OMP profiles of *P. haemolytica* isolates of serotypes A1 and A2 after solubilisation at different temperatures

Coomassie blue-stained OMP profiles of *P. haemolytica* isolates PH188 (serotype A1) and PH210 (serotype A2), lanes 1-6 and 7-12, respectively, after solubilisation at room temperature (lanes 1 and 7), 50°C (lanes 2 and 8), 60°C (lanes 3 and 9), 70°C (lanes 4 and 10), and 85°C (lanes 5 and11) for 5 min and 100°C (lanes 6 and 12) for 20 min. Arrows indicate the heat-modifiable OMP (see text). Molecular weight standards (kDa) are shown in lane 13.


1 2 3 4 5 6 7 8 9 10 11 12 13
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14·4

3.2.3 Immunogenicity of the heat-modifiable OMP

3.2.3.1 Analysis with bovine convalescent antiserum

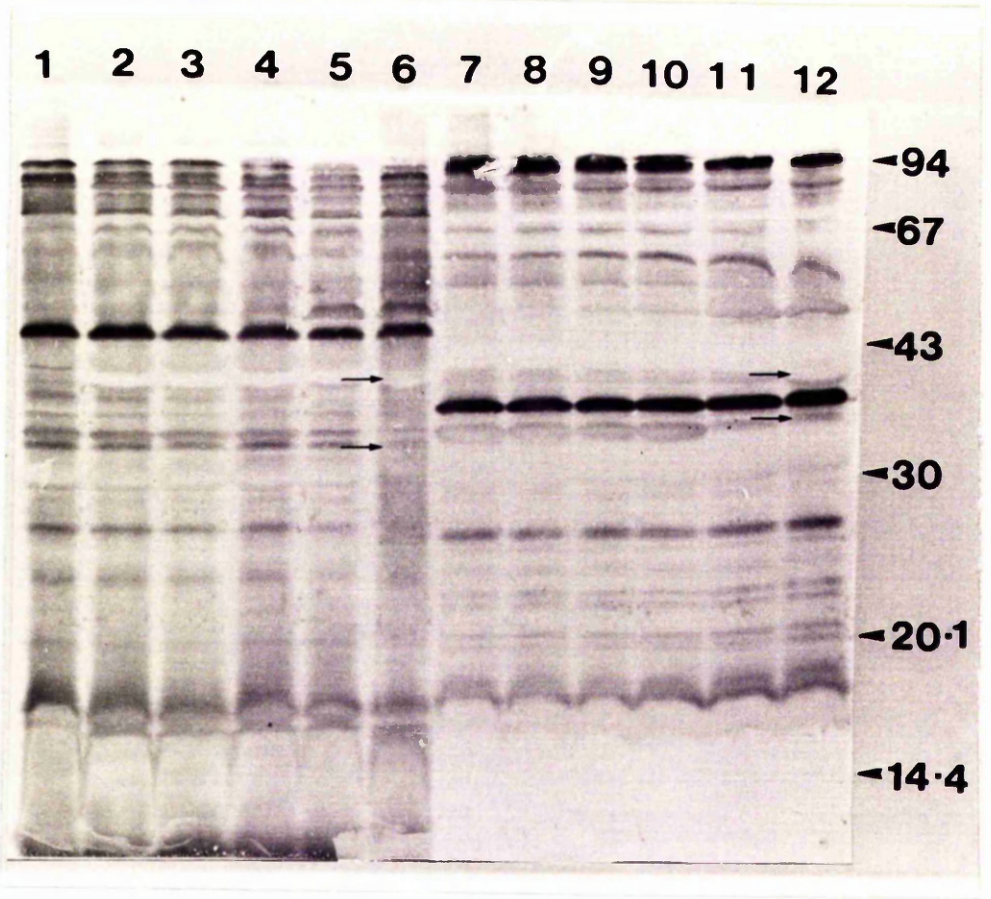
To determine the immunogenicity of the heat-modifiable OMP in either of the two molecular weight forms, serum obtained after experimental infection of a calf with whole cells of *P.haemolytica* (bovine serum 32 - see section 2.5) was analysed for the presence of specific antibodies. This involved immunoblot analysis, where the antigen source was Sarkosyl-insoluble OMP preparations of *P. haemolytica* solubilised at different temperatures. The reactivity of the serum against *P.haemolytica* isolates PH188(serotype A1) and PH210 (serotype A2) is shown in Figure 15 (lanes 1-6 and 7-12, respectively). A number of findings were evident. Firstly, the antibody recognition patterns were different for the two isolates. However, this finding has been discussed previously and is due to the two distinct OMP types in these two strains (section 3.1). Secondly, no significant antibody recognition was demonstrated at the approximate positions of the heat-modifiable OMP as indicated by the arrows (lanes 6 and 12). These approximated positions for the heat-modifiable OMP are further confirmed and discussed below (section 3.2.3.1). Thirdly, the immunoblot profiles of each isolate did not vary significantly with the solubilisation temperature, further indicating that no heat-modifiable OMP were being recognised. Thus, the heat-modifiable OMP appears to be poorly immunogenic in calves in both the low and high molecular weight forms. However, a number of other factors could account for these findings and these will be discussed later (see section 4.2).

3.2.3.2 Analysis with rabbit monospecific antiserum

In an attempt to raise antibodies specific for the heat-modifiable OMP of *P. haemolytica*, the isolated protein (obtained as described in section 2.5.2) was

Figure 15. Western blot demonstrating the immunogenicity of the heat-modifiable OMP using bovine convalescent antiserum 32

Western blot showing the reaction of a 1: 200 dilution of bovine antiserum 32 against the OMPs of *P. haemolytica* isolates PH188 (serotype A1) and PH210 (serotype A2) in lanes 1-6 and 7-12, respectively, after solubilisation temperatures described in Figure 14. Arrows indicate the approximate position of the heat-modifiable OMP in both strains. Approximate molecular weights (kDa) are indicated on the right.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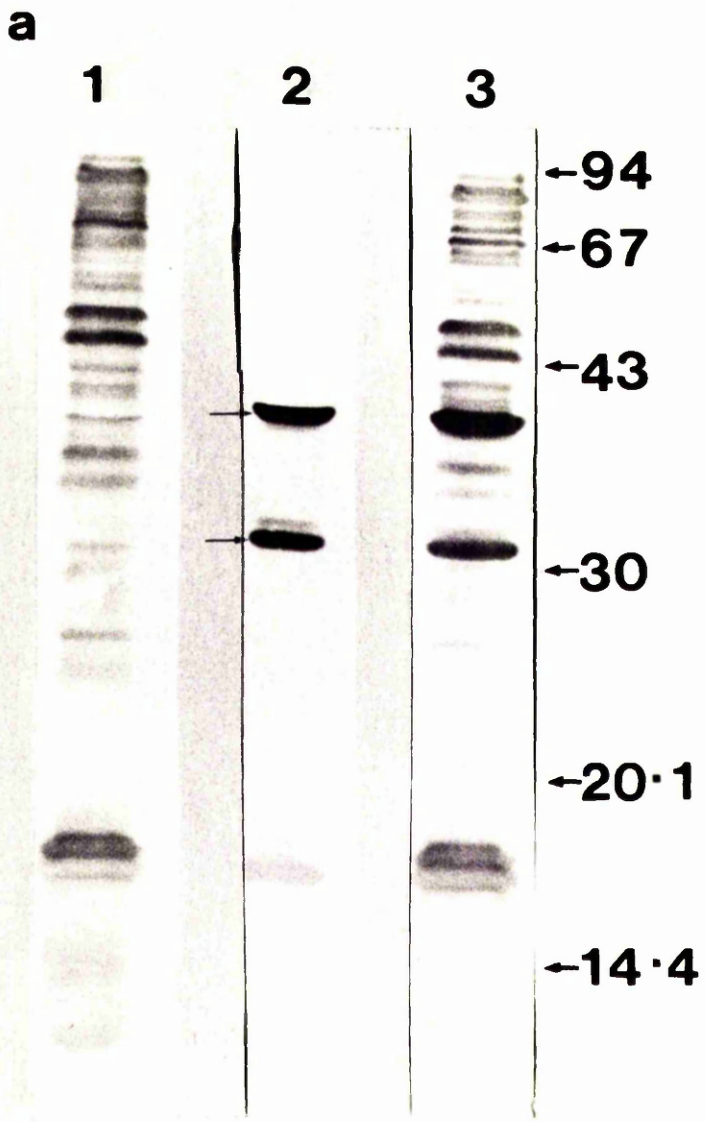
injected into rabbits, which were bled at intervals and the antibody response examined by Western blotting. There was a low but detectable antibody response to the heat-modifiable OMP in the pre-immune serum and a progressive increase following immunisation with the protein and boosting, as described in section 2.5.2. (results not shown). The specificity of this monospecific antiserum for the heat-modifiable OMP of *P. haemolytica* can be seen in Figure 16 where the serum was reacted with OMP preparations (solubilised at 100^{o}C for 20 min) of *P. haemolytica* isolates PH188 (serotype A1) and PH210 (A2 isolate) (Figure 16a and b, respectively, lane 2). These results indicated that although the antiserum was raised against the isolated protein from a serotype A1 strain, the antibodies cross-reacted with the protein of an A2 strain, an indication of the similarities of the protein in both serotypes. The results also demonstrated that recognition of both molecular weight forms of the protein occurred even though the high molecular weight form was used for immunisation.

Direct comparisons of bovine antiserum 32 and the monospecific antiserum to the heat-modifiable OMP of *P.haemolytica* isolates PH188 and PH220, are shown in Figures 16a and b, respectively. In lanes 1 and 2 of each figure, recognition of the antigen by the convalescent (whole-cell) antiserum and the monospecific antiserum, respectively, are compared. It is evident that a strong antibody response to the heat-modifiable protein of both the A1 and A2 isolate occurs with the monospecific antiserum but not with the whole-cell antiserum (indicated by the arrows). In lane 3 of both figures, the probing antiserum used was a mixture of equal parts of both the bovine antiserum 32 and the monospecific antiserum, and shows the antibody recognition pattern of the monospecific antiserum superimposed on the antibody recognition pattern of the whole-cell antiserum. This highlights the positions of the heat-modifiable protein on the latter pattern as described in the previous section, 3.2.2, and confirms that bovine antiserum 32 did not recognise the heat-modifiable OMP of either of the two serotypes.

16a. Comparison of the reactivity of bovine antiserum 32, rabbit monospecific anti-heat modifiable protein serum and a mixture of both, against the OMPs of *P. haemolyitca* (serotype A1)

Western blot showing reaction of different antisera against the OMPs of *P. haemolytica* isolate PH188 (serotype A1), solubilised at 100^{O}C for 20 min. The antisera used were as follows: 1 in 200 dilution of bovine antiserum 32 (lane 1); 1 in 2000 dilution of rabbit monospecific anti-heat modifiable protein serum (lane 2); and a 1 to 1 mixture of both of the above diluted antisera (lane 3). Arrows indicate the position of the heat-modifiable OMP. Molecular weights (kDa) are indicated on the right.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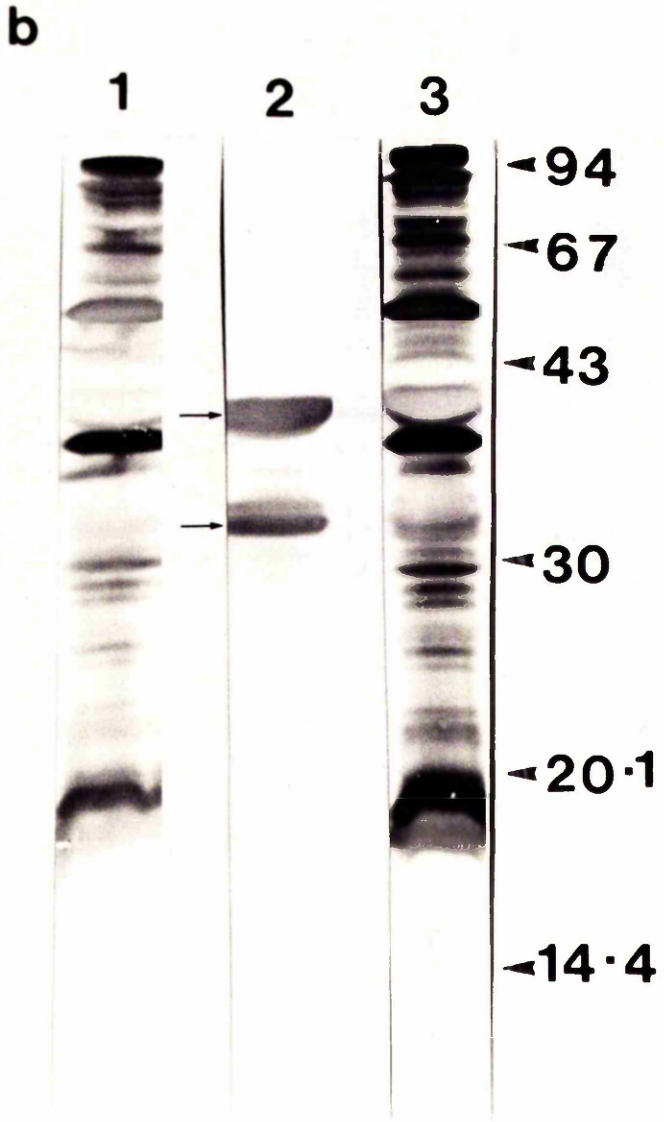
14·4

Figure 16b. Comparison of the reactivity of bovine antiserum 32, rabbit monospecific anti-heat modifiable protein serum and a mixture of both, against the OMPs of *P. haemolyitca* (serotype A2)

Western blot showing reaction of different antisera against the OMPs of *P. haemolytica* isolate PH210 (serotype A2), solubilised at 100^{O}C for 20 min. The antisera used were as follows: 1 in 200 dilution of bovine antiserum 32 (lane 1); 1 in 2000 dilution of rabbit monospecific anti-heat modifiable protein serum (lane 2); and a 1:1 mixture of both of the above diluted antisera (lane 3). Arrows indicate the position of the heat-modifiable OMP. Molecular weights (kDa) are indicated on the right.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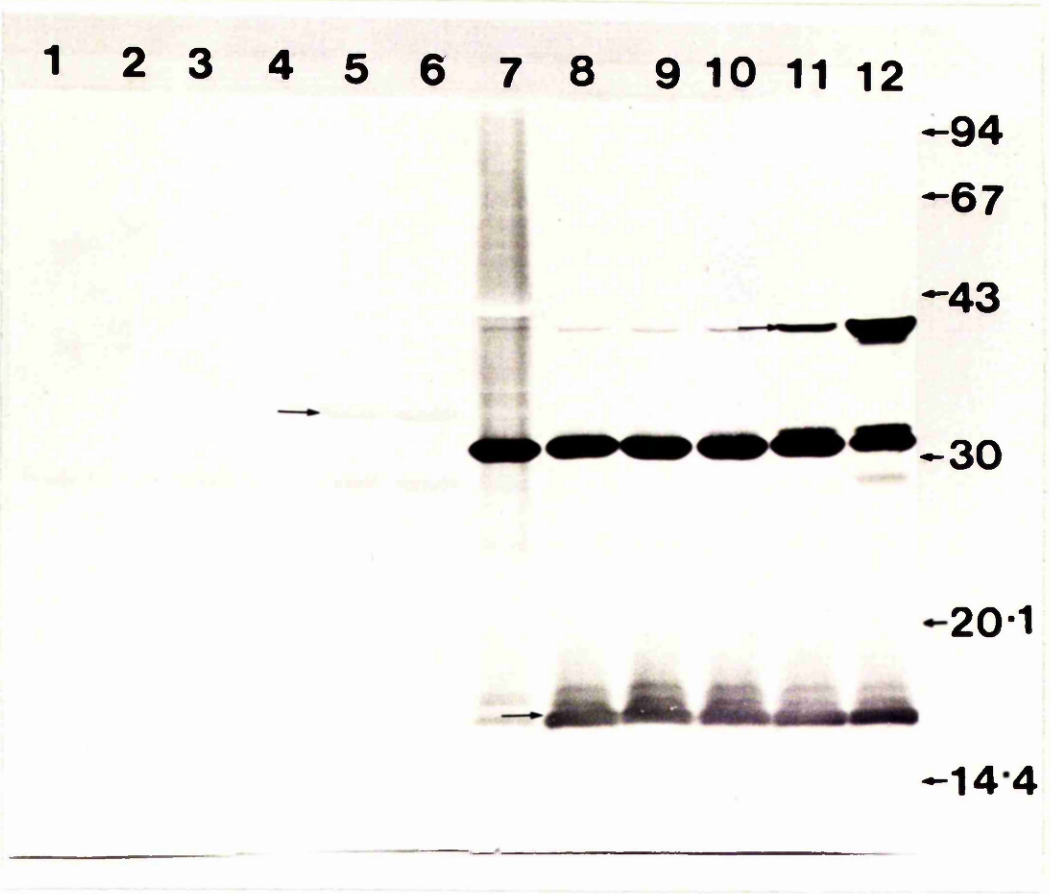
The monospecific antiserum discussed above was also used to demonstrate the molecular weight switch, characteristic of the heat-modifiable OMP. In Figures 17 and 18, lanes 7-12, the antibody recognition patterns with Sarkosyl-insoluble OMP preparations of PH188 (serotype A1), solubilised at various temperatures, are shown. In addition, Figures 17 and 18 also shows the cross-reactivity of the antiserum with OMP preparations from *E.coli* Lilly (lanes 1-6), as will be described below (section 3.2.4.1). The difference between these blots was due to the antiserum used, i.e., the antiserum used in Figure 17 was antiserum prior to cross-absorption with *E.coli*, whereas the antiserum used in Figure 18 had been cross-absorbed with *E. coli*. Likewise, in Figures 19a and b, respectively, the recognition patterns of both the unabsorbed and cross-absorbed monospecific sera against the OMP preparations of a serotype A2 isolate (PH210) solubilised at different temperatures are shown. In Figure 17, significant recognition of the high-molecular-weight form of the heat-modifiable OMP was observed at solubilisation temperatures of 85°C and 100°C (lanes 11 and 12, arrow), while recognition of the low-molecular-weight form was seen at all temperatures (lanes 7-10). Non-specific antigen recognition was evident in the low-molecular-weight region between 14.4 and 20.1 kDa (lane 8, arrow). However, this was successfully removed from the antiserum by cross-absorbing the serum with *E. coli* (Figure 18, lanes 7-12). In contrast to the patterns seen when the cross-absorbed antiserum was reacted with PH188 (serotype A1), where all non-specific antibodies were removed (Figure 18, lanes 7-12), non-specific antibodies which cross-reacted with isolate PH210 (serotype A2) remained in the antiserum, even after cross-absorption with *E. coli* (Figure 19b, indicated by the arrow), suggesting that further cross-absorption, possibly with a different bacterial species is required to completely remove these cross-reacting antibodies.

Figure 17. Western blot demonstrating the immunoreactivity of the heat-modifiable OMP of *P. haemolytica* and *E. coli* with rabbit monospecific anti-heat-modifiable protein antiserum

Western blot showing reaction of a 1 in 2000 dilution of monospecific anti-heat-modifiable protein antiserum against the OMPs of *E. coli* Lilly and *P. haemolytica* isolate PH188 (serotype A1) in lanes 1-6 and 7-12, respectively, after solubilisation at room temperature (lanes 1 and 7), 50°C (lanes 2 and 8), 60°C (lanes 3 and 9), 70°C (lanes 4 and 10), 85°C (lanes 5 and 11) for 5 min and 100°C for 20 min (lanes 6 and 12). Arrows indicate the heat-modifiable protein in addition to other differences (see text). Molecular weights (kDa) are indicated on the right.


1 2 3 4 5 6 7 8 9 10 11 12
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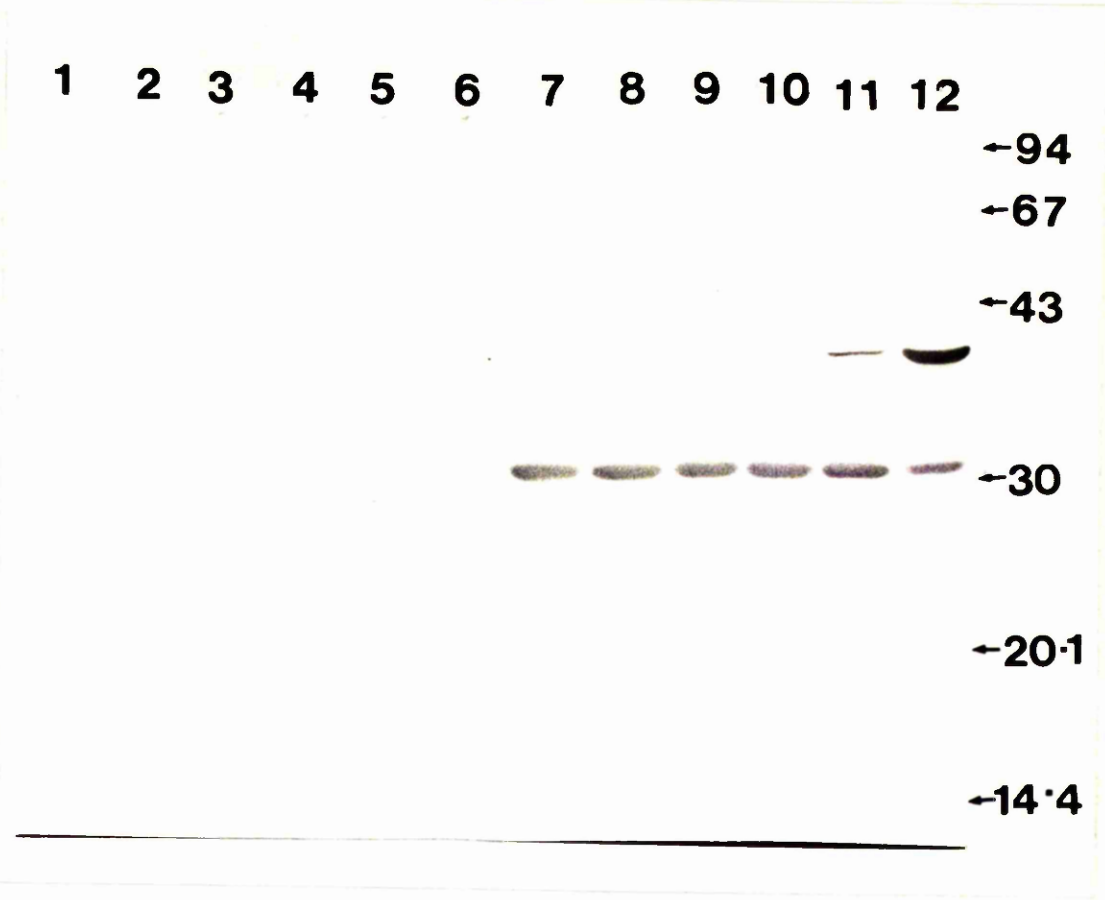
14·4

Figure 18. Western blot demonstrating the immunoreactivity of the heat-modifiable OMP of *P. haemolytica* and *E.coli* with cross-absorbed monospecific anti-heat-modifiable protein antiserum

Identical to Figure 17, except the antiserum used had previously been cross-absorbed with *E.coli* Lilly as described in section 2.5.3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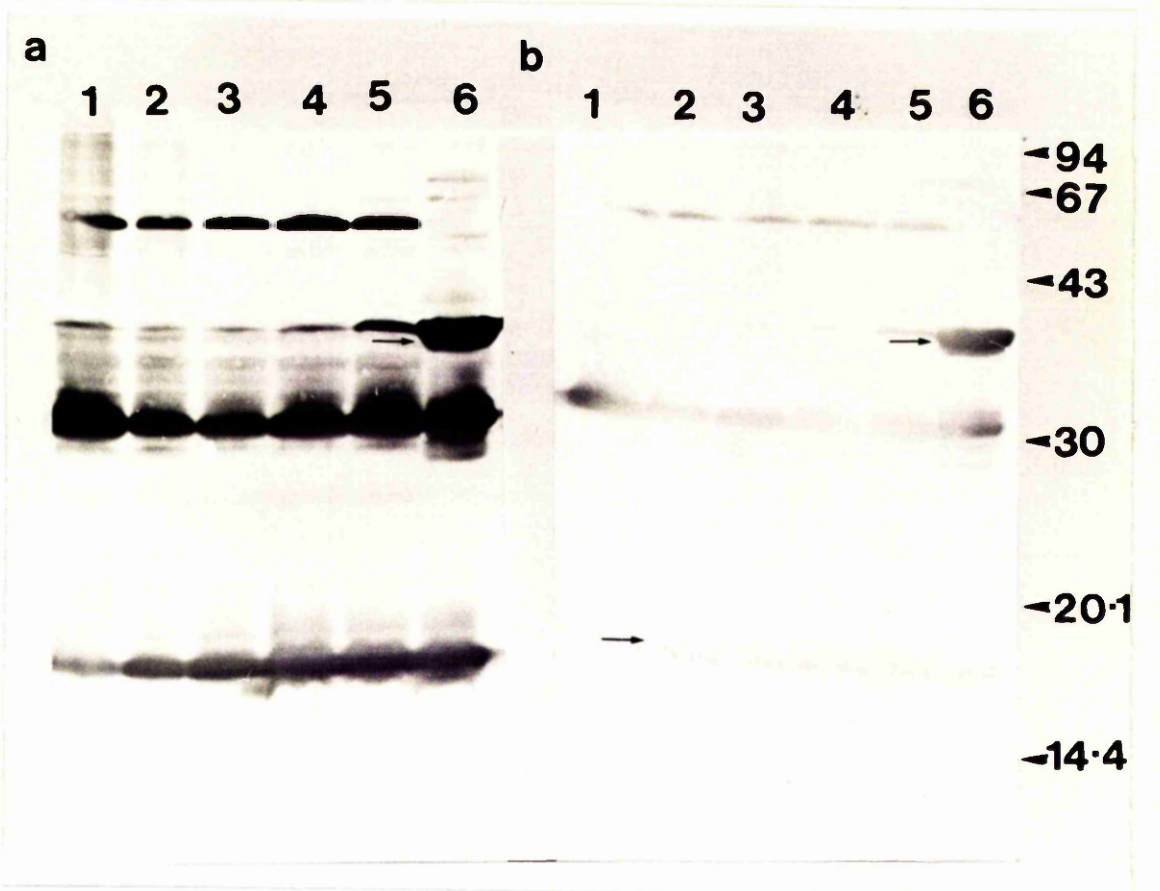
12
94
67
43
30
20·1
14·4

19a and b. Western blot demonstrating the immunoreactivity of the heat-modifiable OMP of *P. haemolytica* isolate PH210 with unabsorbed and cross-absorbed monospecific anti-heat-modifiable protein antiserum

Western blots showing the reaction of a 1: 2000 dilution of unabsorbed (Figure 19a) and cross-absorbed (Figure 19b) monospecific anti-heat-modifiable protein antiserum against the OMPs of *P. haemolytica* isolate PH210 after solubilisation at room temperature (lane1), 50°C (lane 2), 60°C (lane 3), 70°C (lane 4), 85°C (lane 5) for 5 min and 100°C (lane 6) for 20 min. Arrows indicate the heat-modifiable OMP and other differences (see text). Molecular weights (kDa) are indicated on the right.


a
1 2 3 4 5 6
b
1 2 3 4 5 6
94
67
43
30
20·1
14·4

Thus, the monospecific antiserum raised in rabbits against the heat-modifiable protein isolated from *P. haemolytica* and absorbed with *E.coli* proved to be specific for the heat-modifiable OMP of both serotypes and was useful for further demonstrating the characteristic molecular weight change which occurs in this protein. This antiserum was also used in demonstrating the homology of the protein of *P. haemolytica* with proteins in *E. coli* and other Gram-negative species, as described below (section 3.2.4.1).

3.2.4 Comparison of the heat-modifiable OMP of *P. haemolytica* with that of *E.coli* and other Gram-negative species

3.2.4.1 Western Blottting

Previous workers (Beher *et al.*, 1980; Wilson, 1991; Tagawa *et al.*, 1993) have shown that heat-modifiable OMPs are common in Gram-negative bacteria and appear to have very similar properties. The findings reported in this investigation demonstrate the similarities of the heat-modifiable OMP of *P. haemolytica* with that of *E. coli* and other Gram-negative species, first by Western blotting and second by N-terminal amino acid sequence.

Figure 17 (lanes 1-6), show the patterns observed when the monospecific anti-heat-modifiable protein serum is reacted with Sarkosyl-insoluble OMP preparations of *E.coli* Lilly solubilised at different temperatures. It was evident that this antiserum contained antibodies which cross-reacted weakly with the *E.coli* heat-modifiable OMP (Fig 17, lanes 1-6) previously visualised by Coomassie blue staining (Figure 13). To further confirm the presence of cross-reacting antibodies to the heat-modifiable protein of *E. coli* in this monospecific antiserum, the serum was cross-absorbed with *E.coli* , whereupon no recognition of the heat-modifiable protein of *E.coli* was observed (Figure 18, lanes 1-6), although, recognition of the heat-modifiable protein in *P. haemolytica* isolates PH188 and PH210 remained

(Figure 18, lanes 7-12 and Figure 19b, respectively). Thus, cross-absorbing the monospecific antiserum with *E. coli* removed the cross-reacting antibodies, leaving only the *P. haemolytica*-specific antibodies.

3.2.4.2 N-terminal amino acid sequencing

In addition to the results obtained above, which have indicated a similarity in various properties of the heat-modifiable OMP of *P. haemolytica* with those in *E. coli*, analysis of the N-terminal amino acid sequence of *P.haemolytica* (as described in section 2.8) was performed. The results further supported the homology of this protein with that in other species. The N-terminal amino acid sequences of the heat-modifiable OMP of a number of Gram-negative species have been published (Tagawa *et al.*, 1993; Spinola *et al.*, 1993) and in Figure 20 the homology of these sequences with that found for the *P. haemolytica* protein in this study is seen. The sequence for the *P. haemolytica* protein is identical with those of *Haemophilus somnus* and *Actinobacillus actinomycetemcomitans*, while it differs from the *E. coli* sequence in four amino acids (underlined in Figure 20).

3.3 SERUM SENSITIVITY IN RELATION TO SURFACE PROPERTIES

Twenty two isolates of *P. haemolytica* were examined for their susceptibility to killing by the bactericidal activity of bovine hyperimmune serum 10. The isolates were chosen to represent those with different capsular types, LPS types and OMP types, with the aim of correlating the degree of susceptibility to serum killing with these particular properties. This investigation involved a micro-titration plate assay, described previously by Davies (1991), but modified accordingly. Preliminary experiments were carried out to standardise the procedure, prior to its application in screening the different isolates.

Figure 20. Comparison of the N-terminal amino acid sequence of the heat-modifiable OMP of *P. haemolytica* with those of other Gram-negative species.

Bacterial species	N-terminal amino acid sequence
P. haemolytica	**A P Q A N T F Y A G A K**
E. coli	**A P K D N T W Y T G A K**
Haemophilus somnus	**A P Q A N T F Y A G A K**
Actinobacillus actinomycetemcomitans	**A P Q A N T F Y A G A K**

Adapted from Beher *et al.*, 1984; Tagawa *et al.*, 1993; and Spinola *et al.*, 1993.

3.3.1 Standardisation of the assay

Before utilising this procedure to screen the different isolates, it was important to consider the variable factors which may have had an effect on the results and to standardise them accordingly.

Initially, a standard curve of optical density of the *P. haemolytica* suspension against log10 viable count was prepared to enable the adjustment of the bacterial inoculum to a known concentration (colony forming units/ml; cfu ml^{-1}). This calibration curve is shown in Figure 21 and was used for all assays. *P. haemolytica* isolate PH8 was used initially, as this proved to be serum-sensitive and was a suitable positive control in all future assays. Also, the antiserum source used in all assays was antiserum 10 (see section 2.5.1), a hyperimmune antiserum raised in calves against *P. haemolytica* isolate PH2. This antiserum had previously been shown by Dr. R. L. Davies to have significant bactericidal activity against serum-sensitive isolates. In addition, the serum-sensitive *E. coli* strain Lilly was incorporated into certain assays as another positive control. A further control with heat-inactivated serum (treated at 56°C for 30 min) was also used as a negative control in all assays.

The factors considered for standardisation of the assay were as follows: the concentration of serum in the reaction mixture; the incubation period of the reaction; and the size of the bacterial inoculum. Each of these were examined and optimised. Figures 22 and 23 demonstrate the killing effect of different concentrations of serum on isolate PH8 and *E. coli* Lilly over a 2 h period. From Figure 22 it can be seen that the extent of killing (that is, the decrease in log10[viable counts]) increased with increasing serum concentration. Concentrations of 80 or 90% (v/v) serum resulted in complete killing of the

Figure 21. Calibration curve for estimating cell numbers - Log_{10} viable count vs OD610

Pasteurella haemolytica isolate PH188 was grown in 25 ml BHIB at 37°C, with shaking for 4 h. After harvesting (centfrifugation at 3,000 x g for 15 min), the bacterial pellet was resuspended in approximately 5 ml of sterile PBS (pH 7.2). Six two-fold serial dilutions were performed and the OD610 recorded. Viable counts were performed, in duplicate, and the mean log10 viable count calculated for each dilution.

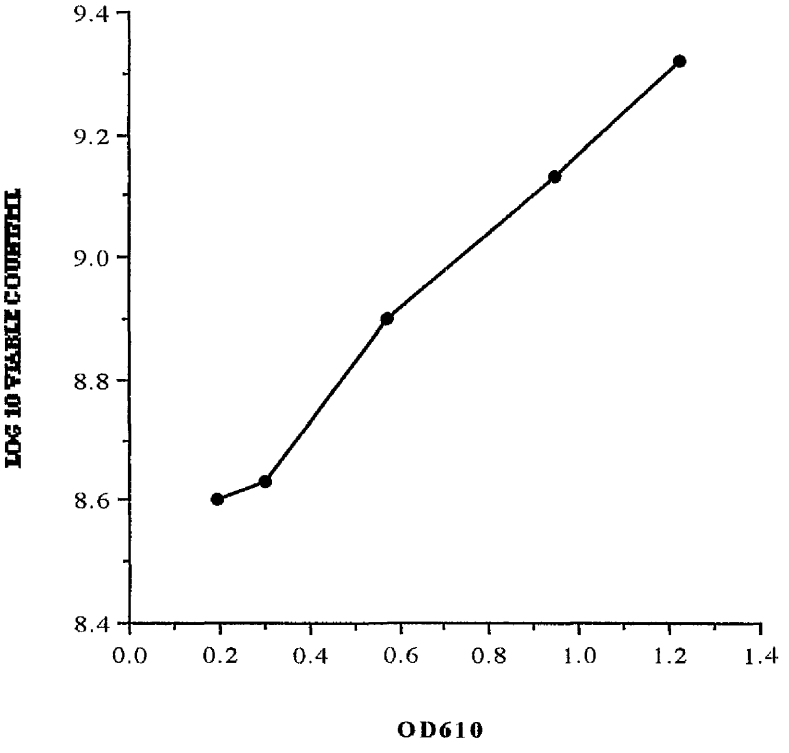

LOG 10 VIABLE COUNT/ML
9.4
9.2
9.0
8.8
8.6
8.4
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
OD610

Figure 22. Effect of different incubation periods and serum concentrations on the bactericidal activity of serum 10 against *P. haemolytica* isolate PH8 and *E. coli* Lilly

The bactericidal activity of different serum concentrations (as indicated by key in figure) against PH8 and *E.coli* Lilly was assessed at 1 and 2 h.

(C-) in key represents the decomplemented serum control, i.e., serum treated at $56^{o}C$ for 30 min.

PH8 alone represents PH8 suspended in PBS (pH 7.2).

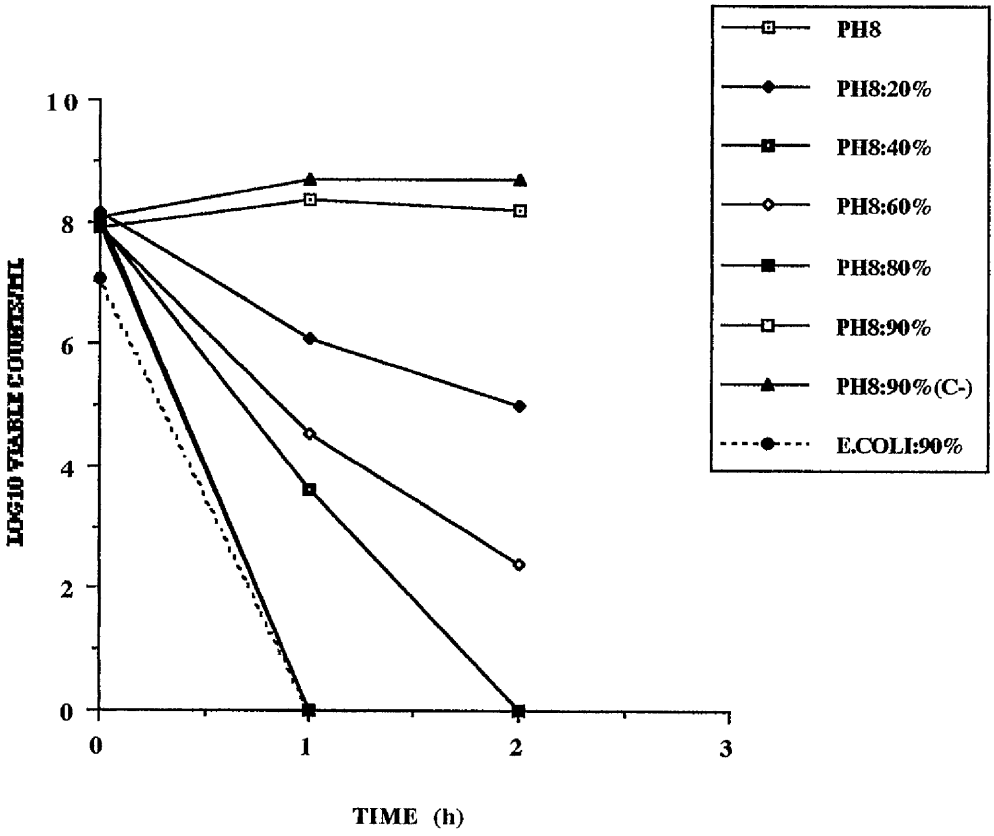
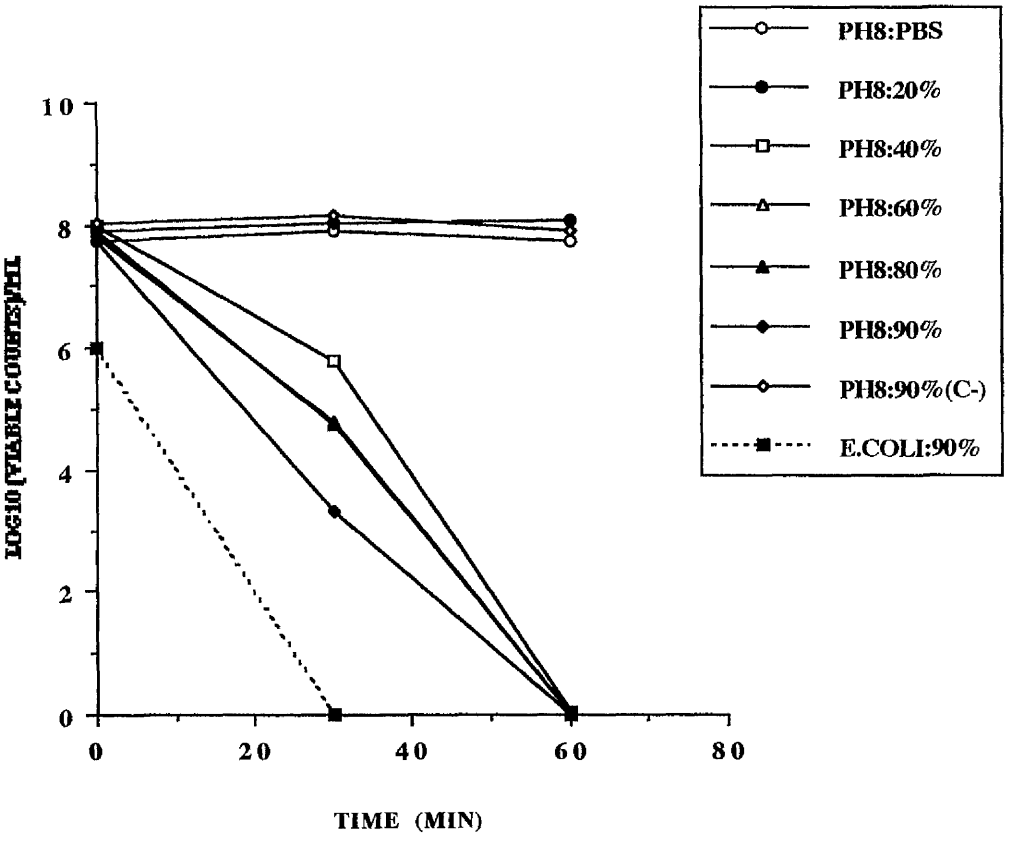

PH8
PH8:20%
PH8:40%
PH8:60%
PH8:80%
PH8:90%
PH8:90%(C-)
E.COLI:90%
LOG10 VIABLE COUNTS/ML
10
8
6
4
2
0
0
1
2
3
TIME (h)

Figure 23. Effect of short incubation periods and serum concentrations on the bactericidal activity of serum 10 against *P. haemolytica* isolate PH8 and *E.coli* Lilly

The experiment was similar to that described in Figure 22 where different serum concentrations were examined for bactericidal activity against PH8 and *E. coli*. However, the viability was assessed at 30 and 60 min.


PH8:PBS
PH8:20%
PH8:40%
PH8:60%
PH8:80%
PH8:90%
PH8:90%(C-)
E.COLI:90%
LOG10 [VIABLE COUNTS/ML]
10
8
6
4
2
0
0
20
40
60
80
TIME (MIN)

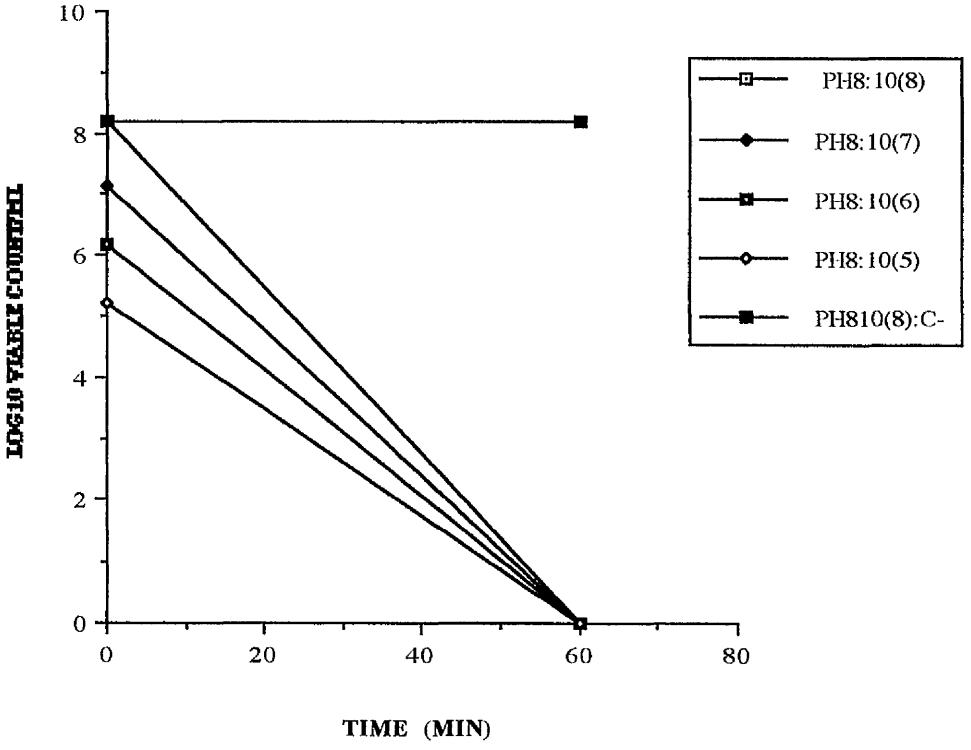
bacterial cells within 1 h and hence, further incubation using either of these concentrations was not required. Heat-inactivated serum at 90% had no effect on viable counts and was therefore a satisfactory negative control, while the serum-sensitve *E. coli* Lilly was killed completely within 1 h (Figure 23). In all subsequent assays, a serum concentration of 90% was used to ensure that the serum was not a limiting factor in the reaction and also because it was more like the conditions which actually occur *in vivo*. Although Figure 22 suggested that an incubation period of 1 h was sufficient to produce complete killing of the bacterial inocula, it was of interest to determine if a shorter period of incubation would also produce complete killing. As shown in Figure 23, the shorter incubation period of 30 min demonstrated only partial killing of the *P. haemolytica* even at the higher serum concentrations, although serum-sensitive *E. coli* Lilly was completely killed after 30 min. It appeared, therefore, that in order to demonstrate complete killing of serum-sensitive *P. haemolytica* isolates the minimum incubation period required was 1 h.

In the above assays, the bacterial inocula consisted of approximately 10^9 cfu in 10 ul of PBS (pH 7.2) added to a final reaction mixture volume of 100 ul to give a concentration of 10^8 cfu ml^{-1}. However, a comparison of different sized inocula in the reaction mixture was performed to determine whether this affected the results. This test involved growing *P. haemolytica* isolate PH8, as described in section 2.2.2, harvesting and adjusting the cells to an OD_{610} of 1.1 in PBS (pH7.2), representing approximately 10^9 cfu ml^{-1}. Ten-fold serial dilutions were made to give final concentrations of 10^8 cfu ml^{-1}, 10^7 cfu ml^{-1} and 10^6 cfu ml^{-1}. The suceptibility of each of these different inocula to the bactericidal activity of serum 10, is represented in Figure 24 where the number of viable cells was recorded after a 1 h incubation period. The results suggested that the inoculum size did not have any significant affect on the final

Figure 24. Effect of inoculum size on the bactericidal activity of antiserum 10

The bactericidal activity of antiserum 10 was examined with different inocula of *P. haemolytica* in the reaction mixture. Final concentrations of 10^8, 10^7, 10^6, and 10^5 cfu/ml were used (as indicated by key in figure).

(C-) in key represents the decomplemented serum control.


LOG10 VIABLE COUNT/ML
10
8
6
4
2
0
0
20
40
60
80
TIME (MIN)
PH8:10(8)
PH8:10(7)
PH8:10(6)
PH8:10(5)
PH810(8):C-

results. That is, complete killing of the bacteria after an incubation period of 1 h was observed, regardless of the inoculum size used. Thus it was decided that the highest inoculum would be the standard inoculum for all future assays and would give a good range for detecting sensitivity i.e., ranging from 0.01 - 100 % survivors, a value described in section 2.9.2.

Hence, from these preliminary experiments the assay was standardised to use a serum concentration of 90% (v/v), a bacterial concentration of 10^8 cfu ml^{-1} and an incubation time of 1 h. Such conditions, using isolate PH8 as a positive control, would enable the determination of the serum sensitivity or resistance of a large number of isolates in a relatively short period of time with consistency in the results obtained.

3.3.2 Serum sensitvity of different isolates of *P. haemolytica*

The assay was standardised as described above. In each assay, *P. haemolytica* isolate PH8 was included as a positive control together with an assessment of the effect of heat-inactivated serum on each isolate tested - effectively a negative control.

The range of isolates examined where chosen to represent those possessing different capsular, LPS and OMP types as described previously in section 3.1 and also by Ali *et al.* (1992).

The results obtained for the various isolates are shown in Table 5. The definition of serum sensitivity or resistance was based on the % survivor value obtained for each isolate, i.e., a value >100 % survivors represented serum resistance, a value < 0.1 % survivors represented a high degree of serum sensitivity and values of 0.1-99.9 % survivors represented partial sensitivity. The % survivors observed for the control tests, i.e. tests in which heat-inactivated antiserum was used, is given in brackets in Table 5, alongside the % survivors

Table 5. Relationship between serum sensitivity, serotype, LPS and OMP type for the 22 *P. haemolytica* isolates examined

Bacterial isolate	serotype	LPS type	OMP type	%survivors[1]	Serum sensitivity R/S/PS[2]
PH2	A1	1	-	152 (168)	R
PH4	A1	1	-	45.5 (106)	PS
PH162	A1	1	1.1	104 (127)	R
PH164	A1	1	1.2	174 (170)	R
PH180	A1	1	1.2	103 (138)	R
PH168	A1	1	1.3	98 (73)	PS
PH176	A1	1	1.4	127 (138)	R
PH188	A1	1	1.4	234 (361)	R
UT3	UT	1	-	101 (111)	R
PH26	A1	2	-	95 (400)	PS
PH30	A1	2	-	96 (575)	PS
PH8	A1	3	-	0.01(400)	S
PH48	A1	3	-	0.01(100)	S
PH42	A2	3	-	0.07(150)	S
PH196	A2	3	2.1	12 (403)	PS
PH198	A2	3	2.1	12 (107)	PS
PH72	A2	4	-	0.008(50)	S

Table 5. continued...

PH44	A2	5	-	0.04 (292)	S
PH202	A2	5	2.2	6 (89)	PS
PH216	A2	5	2.2	0.7 (215)	S
PH208	A2	5	2.3	3 (50)	PS
PH214	A2	5	2.4	2 (155)	PS

[1] % survivors calculated using the formula described in section 2.9.2 i.e., cfu/ml at 1 h / cfu/ml at time 0 x 100. All % survivor results are mean values of duplicate results. Control results, using heat-inactivated antiserum are indicated in bracket.

[2]R = serum resistant; S = serum sensitive; PS = partially serum sensitive, as defined in section 2.9.2

UT = untypable

using the complement-preserved sera. As is evident, a majority of these control groups show $\geqslant 100\%$ survivors, the expected results as the bactericidal activity of the serum was destroyed by heat treatment. Although there were some controls which actually showed a decrease in cell numbers, the reason for this was not entirely clear and requires further investigation. However, these control results were particularly useful when interpreting the results observed for the partially serum sensitive isolates, PH4, PH26, PH30, PH196, PH198, PH202, PH208 and PH214. The values obtained for the controls of these results were significantly greater than the actual test, thus indicating that these isolates were sensitive to the bactericidal activity of the serum, although to different degrees as seen by the range of results from 2% to 98% survivors.

Serum sensitivity or resistance could possibly be related to surface components such as the capsule (serotype), LPS and the OMP. All of the serotype A1 isolates, with the exception of PH8 and PH48, were resistant or partially sensitive to the bactericidal activity of serum 10 (Table 5), while all isolates of serotype A2 were serum-sensitive; the untypable isolate, UT3, was serum-resistant. Significantly, isolate PH2, used for production of the serum 10, was serum-resistant. Possible reasons why this hyper-immune serum had no bactericidal activity on the homologous isolate is discussed below (section 4.3). The LPS type of all the A1 isolates, again, with the exception of PH8 and PH48 were of the smooth types 1 or 2. The LPS of isolates PH8 and PH48 was of type 3, a rough type LPS. This suggests, therefore, that the resistance or sensitivity to the bactericidal activity of the serum was correlated to the LPS type, where all isolates possessing a smooth type LPS (types 1 and 2) were resistant while those with rough type LPS (type 3, 4, and 5) were sensitive. On the other hand, there was no indication that variation in serum sensitivity was due to any particular OMP type. Isolates with OMP types 1.1, 1.2, 1.3 and 1.4 were all resistant to serum killing, while isolates with OMP types 2.1, 2.2, 2.3 and 2.4 were sensitive. However, as described in section 3, isolates possessing the former OMP types also possessed

LPS type 1, a smooth type LPS and thus resistance is likely to be due to this property. Likewise the latter OMP types were identified in isolates possessing only rough LPS types 3 or 5. Hence, although the property of serum resistance or sensitivity is likely to result from a number of factors, this investigation in *P. haemolytica* suggested that this property was associated more so with the LPS type of the isolates than with either the serotype or OMP type.

3.4 VIRULENCE TESTS IN CALVES

Investigation of the virulence of *P. haemolytica* isolates involved intratracheal inoculation of calves after three days, the lungs were removed for scoring of any pathological changes. Initially the test was standardised with respect to the size of the bacterial inoculum and the diluent used. Using the optimal conditions determined in this part of the study, a number of isolates, representing different capsular and LPS types, were compared.

3.4.1 Standardisation of virulence test

Standardisation of the virulence tests involved 21 calves divided into 7 groups as shown in Table 6 and given different size of i nocula in one of two diluents. Two control groups with either diluent alone, were included. Both gave satisfactory results, where the mean lesion scores were minimal and significantly lower than those seen in the tests in which bacterial cells were included.

Comparing groups 1, 2 and 3, all of which were diluted in PBS but differed in the size of the inoculum, an increase is lung lesion scores was evident

Table 6. Standardisation of virulence tests - mean lesion scores compared with the different sized inoculum and diluent used

Group	Size of inoculum[1]	Diluent[2]	Mean lesion scores
1	Low	PBS	7.28
2	Medium	PBS	19.40
3	High	PBS	51.21
4	Low	CS	41.28
5	Medium	CS	51.76
6*	None	PBS	0
7*	None	CS	2.11

[1]Low inoculum = 2.5×10^{10} cfu; Medium inoculum = 2.5×10^{11} cfu; High inoculum = 1.25×10^{12} cfu

[2]PBS = Phosphate buffered saline (pH 7.4); CS = filter-sterilised culture supernate

* Control groups

with an increase in the inoculum size. Likewise in groups 4 and 5 an increase in lesion scores was seen between the low and medium inocula. However when the two diluents, PBS and filter-sterilised culture supernate, were compared it was apparent that the lesions were considerably more extensive with the latter diluent than PBS, for a given inoculum. This may have been due to the presence of leukotoxin in the culture supernate (discussed in further detail in section 3.4.2).

From this standardisation experiment it was decided that the conditions used for group two, i.e., medium inoculum size and diluted in PBS, would be used when comparing various isolates. This inoculum was chosen because it was shown to produce moderately severe lung lesions but did not result in so many that the animal was at risk of being killed. The diluent PBS was chosen to minimise any effects which the leukotoxin may have had on the results of the tests as this study was mainly concerned with the surface properties of the bacterium, namely the capsule and LPS. However, the importance of the leukotoxin in the virulence of each isolate was not ignored.

3.4.2 Comparison of virulence of different *P. haemolytica* isolates

The virulence of *P. haemolytica* isolates PH2, PH8, PH10, PH30, PH42, PH44 and PH72 was assessed as described above using a final bacterial inoculum of 10^{9} cfu diluted in PBS. Four calves were inoculated with each bacterial strain and the mean lesion score for each of the four calves was determined and calculated by Dr H. A. Gibbs. The serotype (capsular type) and the LPS type of each isolate varied is shown in Table 7 alongside the mean lesion scores determined in the virulence tests, the serum sensitivity data (see below) and the leukotoxin activity of each isolate (determined by Mr. M. Saadati, Department of

Table 7. Relationship of lesion score to serotype, LPS type, serum resistance and leukotoxin activity

Isolate	Serotype	LPS type	Mean lesion scores	Mean bactericidal activity (% Survivors)	Leukotoxin activity
PH2	A1	1	8.9	67.1	High
PH10	A1	1	40.5	13.2	High
PH30	A1	2	24.7	120.0	Moderate
PH8	A1	3	1.7	0.0	Moderate
PH42	A2	3	0.5	0.0	Low
PH72	A2	4	0.0	0.0	Low
PH44	A2	5	0.0	0.0	Low

Microbiology, University of Glasgow, using a neutrophil chemiluminesence assay).

The results indicated that serotype A2 isolates, PH42, PH44, and PH72, produced fewer lesions than serotype A1 isolates, with the exception of isolate PH8. This latter isolate, although of serotype A1, caused lesion scores which were more comparable to those observed for the serotype A2 isolates Serotype A1 isolates, PH2, and especially PH10, PH30 produced more extensive lung damage (Table 7). This finding suggests that the capsular type could not be directly related to the virulence of the isolate unlike other properties, namely the LPS type.

The LPS types of isolates PH2 (LPS type 1), PH10 (LPS type 1) and PH30 (LPS type 2) were of the smooth chemotype, whereas isolate PH8 possessed LPS type 3, which as described previously in section 3.1, was a rough-type LPS. The former 3 isolates produced lesion scores significantly higher that PH8 while the 3 serotype A2 isolates, PH42, PH44 and PH72 possessed LPS types 3, 4 and 5, respectively, all of which were of the rough chemotype, and produced lesion scores more similar to PH8. These findings suggested a possible correlation of smooth type LPS with increased virulence and rough type LPS with avirulence or low lesion scores.

The other data shown in Table 7 indicate the serum sensitivity and the leukotoxin activity of the various isolates. The % survivors was the mean value from bactericidal assays with each isolate tested in serum from each of the four calves into which it was inoculated in the virulence test. The assays were performed as described in section 2.9. The findings shown here were similar to those reported in section 3.3.2 where isolates which possessed smooth type LPS (LPS type 1 and 2) in general appeared to be more resistant to the bactericidal activity of the serum than those with rough type LPS (LPS types 3, 4 and 5). Thus, in addition to the possible correlation of LPS type with virulence, the result above suggested that the ability of the bacterium to resist the bactericidal activity of host serum may also be of importance in the overall virulence of the isolate.

The isolates which proved to be avirulent, producing virtually no lesions

in the virulence tests, were also shown to have low leukotoxin activity whereas isolates which caused a greater number of lesions were shown to have moderate or high leukotoxin activity, again with the exception of isolate PH8. This isolate, although having moderate leukotoxin activity similar to that of isolate PH30, caused fewer lung lesions. These isolates, as mentioned previously, differed in LPS types, the former possessing LPS type 3 (rough-type) the latter possessing LPS type 2 (smooth-type). They also differed in their ability to resist the killing activity of host serum, where PH8 was serum sensitive and PH30 resistant to serum killing. These findings confirm the importance of other factors, in addition to the leukotoxin, in determining the virulence of each isolate observed in these tests.

Overall, this study indicated that the LPS type of the isolate could be correlated with the virulence of the isolate rather than with the capsular type. However, other factors such as serum resistance and leukotoxin activity appear to be important.

3.5 INVESTIGATION OF *P. HAEMOLYTICA* GROWN *IN VIVO*

This investigation was primarily concerned with the growth of *P. haemolytica in vivo* and the differences which may be apparent between *in vivo*- and *in vitro*-grown cells. *P. haemolytica* isolates PH2, PH30, PH42 and PH48 were inoculated into intraperitoneal implant chambers (see section 2.11.1) and the growth of these isolates within the chambers was followed by daily sampling of the chamber contents (see section 2.11.2). Bacteria from the chambers were recovered and outer-membrane fractions prepared and analysed by SDS-PAGE and Western blotting.

3.5.1 Growth of *P. haemolytica* in chamber fluid *in vivo*

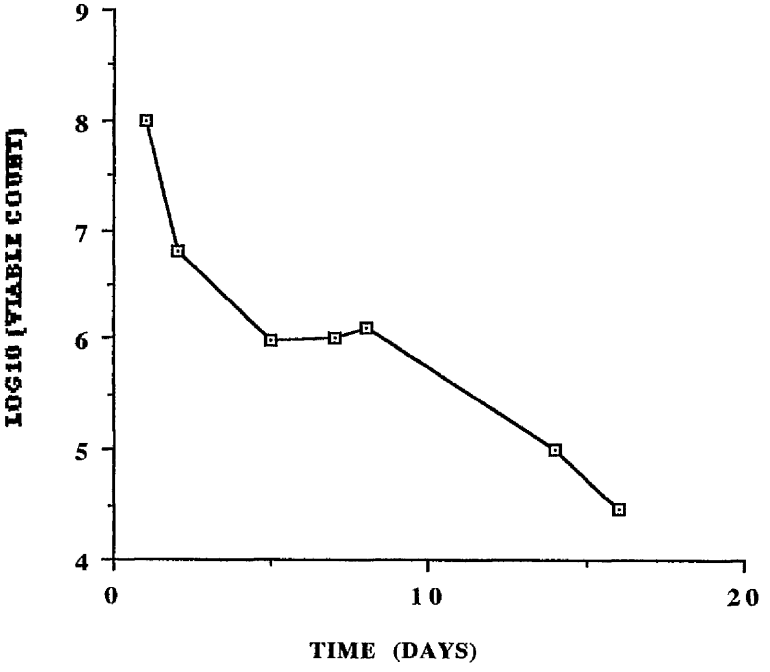
Viable counts were performed on samples removed from the chamber over a period of 16 days. The growth curves obtained for each of the four isolates were similar and thus the curve for isolate PH2 representative of all 4 strains and is shown in Figure 25. The initial count at day 1, was approximately 10^8 cfu ml^{-1}, however this number decreased with time until approximately 10^4 cfu ml^{-1} remained when the experiment was terminated on day 16 and the chamber removed from the calf (Figure 25). These results suggested that the isolates inoculated into the chamber did not multiply, but rather their numbers decreased steadily over the period of the experiment. Preliminary investigations were carried out in an attempt to understand why the bacterial cells were dying *in vivo* .

3.5.2 Investigations into the decline in cell numbers within the implant chamber

In an attempt to understand why the bacterial cell numbers declined inside the implant chamber, various experiments were performed. First, the chamber fluid removed from the four chambers at days 0, 9 and 16 of the experiment were examined for the ability to kill *P. haemolytica in vitro*, using the method described for the bactericidal assays (section 2.9). The chamber fluids, after removal, were filter-sterilised and stored at -70^{o}C to remove and preserve any complement activity which may have been present. The percentage of bacteria surviving in the chamber fluid after 2 h was calculated as described previously and can be seen in Table 8. The results indicated that no significant bactericidal activity was present in any of the chamber fluids which could have accounted for the decline in cell numbers with the exception of day 16 chamber fluid from chambers inoculated with PH2 and PH30. The % survivors were generally greater than 100 indicating bacterial growth, although, the day 16 chamber fluid removed from the chambers

Figure 25. Growth of *P. haemolytica isolate PH2 in vivo* in an intraperitoneal implant chamber

The survival of the bacteria was examined by removing samples of chamber fluid at intervals for viable counts


LOG10 (VIABLE COUNT)
9
8
7
6
5
4
0
10
20
TIME (DAYS)

inoculated with PH2 and PH30 did cause a small decrease in bacterial numbers (see Table 8).

The role of complement activity in the fluid was assessed by incorporating decomplemented chamber fluid controls in all assays (results shown in brackets in Table 8). The results indicated that the absence of complement activity from the chamber fluids did not have a significant effect on the final % survivors but rather, produced results relatively similar to those in which complement activity had been preserved. This suggested that a complement-mediated killing activity was unlikely to be the cause of the decline in bacterial cell numbers.

In addition, the antibody content of the chamber fluids was examined by Western blot anlaysis using *P. haemolytica* (isolate PH2) OMPs as the target antigen. In Figure 26, the recognition patterns obtained for each of the four chamber fluids from which isolates PH2, PH30, PH42 and PH48 were obtained (Fig 26 a, b, c and d respectively) are shown. Chamber fluid removed at days 0, 9 and 16 of the experiment are represented in lanes 1 to 3 of each figure. From this figure it is evident that the antibody contents in each of the four chamber fluids remained virtually the same throughout the period of the experiment and therefore indicates that an increase in specific antibody was not the reason for cell death. Further investigations are required to understand the reason for bacterial cell death within the implant chambers. However, the bacteria which were obtained from the chambers as described in section 2.11.4, were representative of *in vivo*-grown cells and thus examined by gel electrophoresis.

3.5.3 Comparison of SDS-PAGE OMP profiles of *in vivo*-grown *P. haemolytica* with those of *in vitro*-grown isolates

Outer-membrane fractions from each of the isolates from the implant chambers were examined by SDS-PAGE. The OMP profiles of the *in vivo*-grown

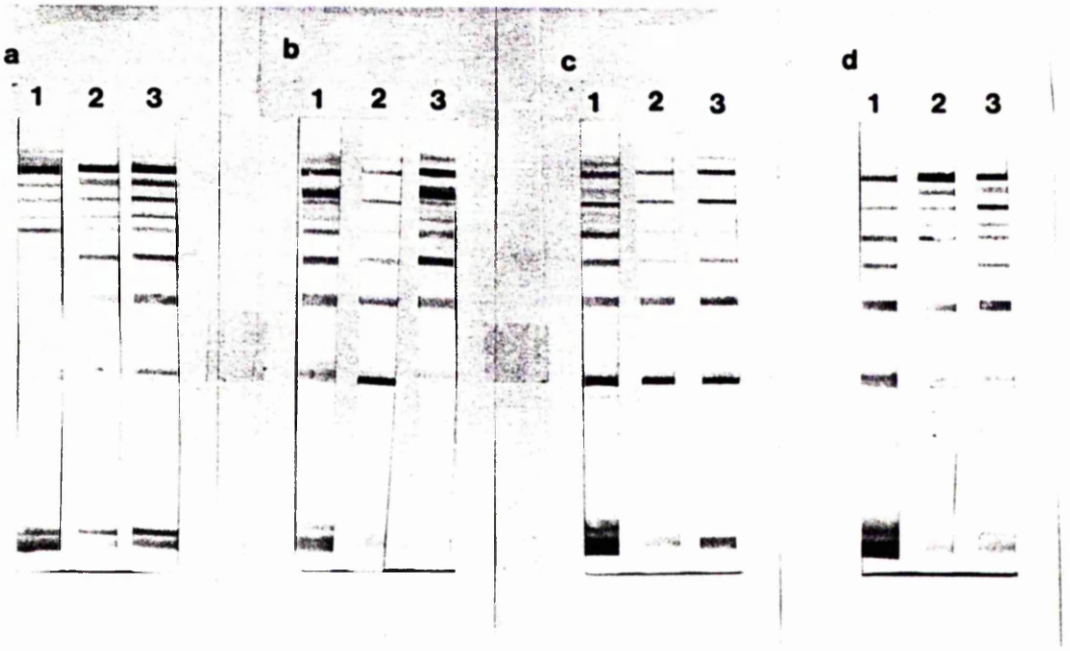
Table 8. Effect of chamber fluid on the *in vitro* growth of *P. haemolytica* isolates PH2, PH30, PH42 and PH48

Isolate	% survivors[1]		
	day 0 chamber fluid	day 9 chamber fluid	day 16 chamber fluid
PH2	224 (223)	153 (153)	82 (244)
PH30	150 (212)	155 (208)	76 (333)
PH42	106 (380)	144 (178)	227 (200)
PH48	651 (233)	108 (60)	216 (187)

[1] Numbers in brackets = results obtained using decomplemented chamber fluid i. e. 56^{o}C for 30 min

Figure 26. Western blot demonstrating the immunoreactivity of *P. haemolytica* (PH2) OMPs wih chamber fluid

Western blot showing reaction of a 1 in 200 dilution of chamber fluid, removed from chambers into which isolates PH2 (a), PH30 (b), PH42(c), and PH48(d) were inoculated, against the OMPs of *P. haemolytica* PH2. The fluids were removed at days 0 (lanes 1), 9 (lanes 2) and 16 (lanes 3).


a
1 2 3
b
1 2 3
c
1 2 3
d
1 2 3

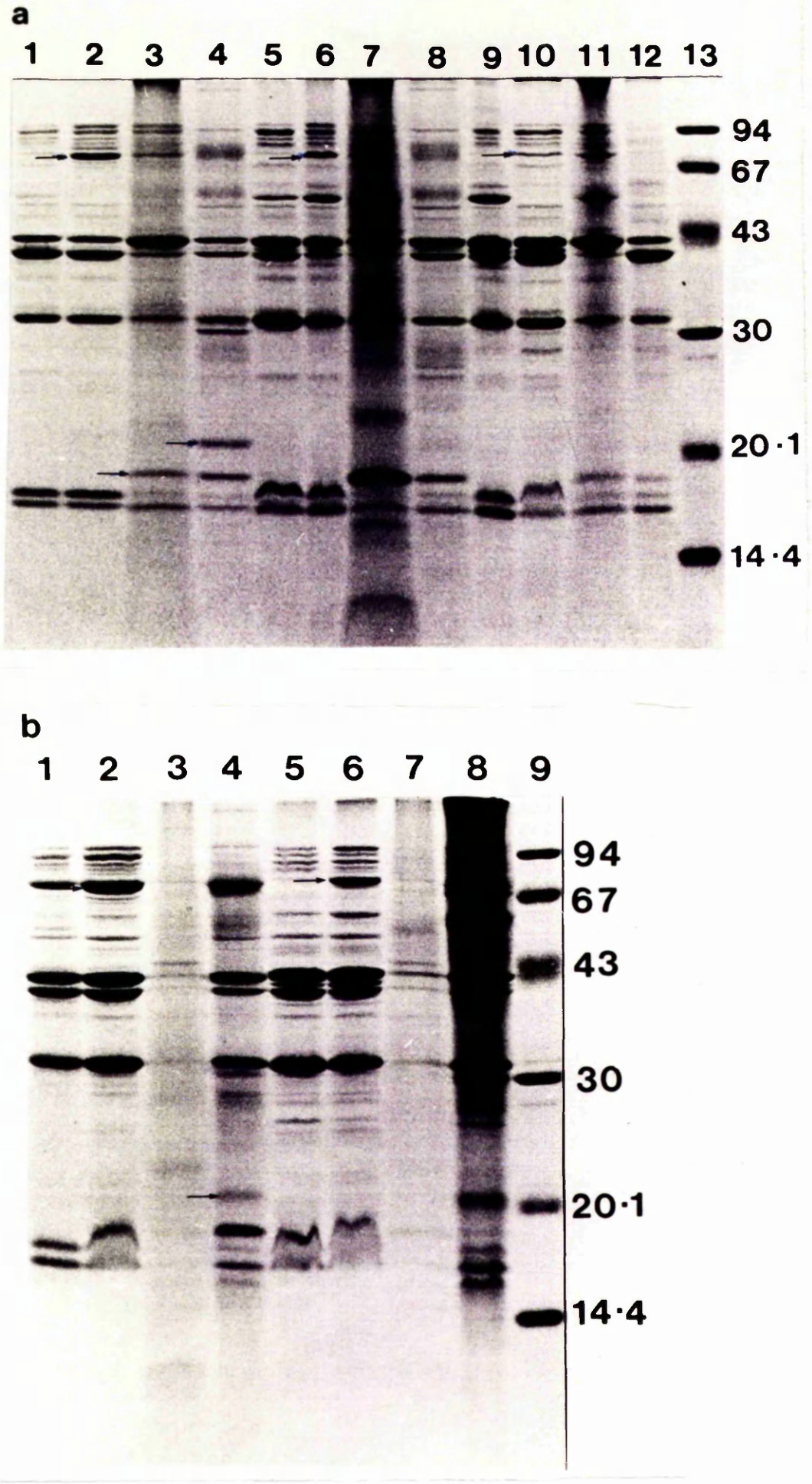
isolates, PH2, PH30, PH42 and PH48, on Coomassie blue-stained gels are shown in Figure 27a lanes 4 and 12, and 27b lanes 4 and 8, respectively. For comparison, the OMP profiles from isolates grown *in vitro* (Fig 27a lanes 1 and 9, and 27b lanes 1 and 5, respectively), or grown *in vitro* under iron limitation (Fig 27a lanes 2 and 10 and 27b lanes 2 and 6, respectively) and from isolates obtained from the lungs of experimentally-infected calves (Fig 27a lanes 3 and 11, and 27b lanes 3 and 7) have been included. These latter two groups of OMP fractions were provided by Dr. R. L. Davies. Lanes 5 to 8 in Figure 27a show the OMP profiles of isolate PH10 where the outer-membrane fractions were again provided by Dr. R. L. Davies.

The variation between profiles of each isolate obtained from diffferent sources was slight although some differences are indicated by the arrows in Figures 27a and b. Variation between isolates grown *in vitro* and under iron limitation has been described previously (Davies *et al.*, 1992) and consists of variation in the expression of certain proteins in the high-molecular-weight region, particularly the 77 kDa protein (Fig 27a, lanes 2, 6 and 10) which was more evident in the latter profiles. Comparison of the profiles of *in vitro*-grown cells with those derived from *in vivo*-grown cells and from cells removed from infected calf lungs has not been reported previously.

In Figures 27a and b (lanes 3,4,7,8,11,12 and 3,4,7,8) the OMP profiles of the lung derived cells (Fig 27a, lanes 3, 7, 11 and 27b, lanes 3 and 7)and the *in vivo*-grown cells (Fig 27a, lanes 4, 8, 12 and 27b, lanes 4 and 8) appear to be similar, although both are different from those of *in vitro*-grown cells (Fig 27a, lanes 1,2,5,6,9,10 and 27b, lanes 1,2,5,6). The variation occurs both in the low-molecular-weight region (Fig 27a, lanes 3 and 4; arrows) and the high-molecular-weight region. These differences appear to be common in all 5 isolates, where the

Figures 27a and b. SDS-PAGE OMP profiles of *P. haemolytica* isolates PH2, PH10, PH30, PH42 and PH48, under different growth conditions

Coomassie blue-stained SDS-PAGE OMP profiles of *P. haemolytica* isolates PH2 (Fig 27a, lanes 1-4), PH10 (Fig 27a, lanes 5-8), PH30 (Fig 27a, lanes 9-12), PH42 (Fig 27b, lanes 1-4) and PH48 (Fig 27b, lanes 5-8) obtained from *in vitro*-grown cells, *in vitro*-grown cells under iron limitation, lungs from infected calves and implant chamber-grown cells in each of the four lanes respectively. Arrows indicate differences in OMP profiles (see text). Molecular weight standards (kDa) in Fig 27a, lane 13 and Fig 27b, lane 9.


a
1 2 3 4 5 6 7 8 9 10 11 12 13
94
67
43
30
20·1
14·4
b
1 2 3 4 5 6 7 8 9
94
67
43
30
20·1
14·4

in vitro-grown cells and cells grown under iron limitation produce profiles which although different from each other in some respects, were more significantly different from the profiles of *in vivo*-grown and lung-derived cells. Likewise the *in vivo*-grown cells and those derived from the lungs of infected calves gave profiles which were more similar to each other than to the *in vitro* profiles.

4. DISCUSSION

4.1 AN EPIDEMIOLOGICAL STUDY OF *PASTEURELLA HAEMOLYTICA*

The objectives of this investigation were to examine and compare the OMP and LPS profiles of a selection of *P. haemolytica* isolates of serotypes A1 and A2, obtained from both pneumonic and healthy cattle, with the aim of detecting differences and similarities which could prove useful in epidemiological and virulence studies. The OMP and LPS profiles of the 29 isolates examined in SDS-polyacrylamide gels were similar to those described in previous studies (Ali *et al.*, 1992; Davies *et al.*, 1992). The OMP profiles consisted of three to four major proteins and approximately 15 - 20 minor proteins and the LPS profiles were either of the smooth or rough chemotypes. Based on variation in the OMP and LPS profiles, the study revealed significant heterogeneity both between and within the serotype A1 and A2 isolates. Thus, the serotype A1 isolates differed from the serotype A2 isolates in the mobilities of a major protein and various minor proteins, as well as in the possession of smooth as opposed to rough LPS. Variation of OMP profiles within the serotype A1 isolates could be detected only by Western blotting but variation within serotype A2 isolates was detected both in stained gels and by Western blotting. Four distinct OMP types were identified within both serotypes by Western blotting, the serotype A2 isolates being more clearly differentiated that the serotype A1 isolates. The LPS profiles were compared to those described previously (Ali *et al.*, 1992) and demonstrated to be of smooth type 1 or rough types 3 or 5. The 18 serotype A1 isolates comprised OMP types 1.1, 1.2, 1.3 and 1.4 and LPS type 1; the 11 serotype A2 isolates comprised OMP types 2.1, 2.2, 2.3 and 2.4 and LPS types 3 and 5.

Previous studies on the OMP profiles of *P. haemolytica* had not demonstrated significant differences either between serotype A1 and A2 isolates or within serotypes. In a study of envelope proteins of isolates representing each of the 16 serotypes of *P.*

haemolytica, Knights *et al.* (1990) were unable to distinguish between individual serotypes. Deneer and Potter (1989) described variation between serotypes in the expression of certain iron-regulated OMPs when cells were grown under iron-restricted growth conditions. However, these authors did not observe significant differences between serotype A1 and A2 isolates.

The present study has confirmed and extended previous findings that serotype A1 and A2 isolates of *P. haemolytica* differ in their LPS profiles (Ali *et al.*, 1992). It has also demonstrated that serotype A1 and A2 isolates differ in the expression of various OMPs. However it should be noted that certain similarities also exist between some isolates of serotypes A1 and A2. For example, the core-oligosaccharide regions of LPS types 1 and 3, which are present in certain serotype A1 and A2 isolates, respectively, are identical (Ali *et al.*,1992). Type 3 LPS associated with the OMPs of serotype A2 isolates of OMP type 2.1/LPS type 3 could account for the enhanced imunological staining of the OMP type 2.1 profiles (Fig 10b, lanes 1-3) when compared to the OMP types 2.2, 2.3, and 2.4 (Fig 10b, lanes 4-11) which have LPS type 5. The primary antiserum was raised against an isolate containing type 1 LPS and would therefore contain antibodies to the homologous core region found only in LPS types 1 and 3. Such an association between OMPs and LPS in Western blots has been described in *Pseudomonas aeruginosa* by Poxton *et al.* (1985). The differences observed in both the OMP and LPS profiles of isolates of serotypes A1 and A2, as well as differences in capsule structure (Adlam *et al.*, 1984; 1987) and host specificity, suggests that isolates of these two serotypes may not be as closely related as previously considered (Frank, 1989). Other work from this laboratory suggests that isolates of other biotype A serotypes are more closely related to isolates of serotype A1 than those of serotype A2 (Davies, R. L., personal communication).

In the present study, OMP and LPS profiles of *P. haemolytica* isolates of serotypes A1 and A2 in stained SDS-polyacrylamide gels were compared and further

investigations were made by Western blotting. Although SDS-PAGE alone was able to differentiate between some isolates of serotype A2, the method was unable to discriminate between isolates of serotype A1. Western blotting, however, was able to differentiate more clearly between isolates of serotype A2 and was also able to distinguish between isolates of serotype A1. Similar findings were reported by Mulligan *et al.* (1988) and Hansman and Lawrence (1993) who found Western blotting to have greater resolving power than SDS-PAGE alone in studies on *C. difficile* and *H. influenzae*, respectively. It was concluded, therefore, that Western blotting is a more useful tool for investigating strain variation within *P. haemolytica*. Although only a single antiserum was used in the present study, this antiserum was still able to differentiate between isolates of either serotype. It is likely that the LPS type, rather than the serotype, of the isolate used to generate the antiserum will have a greater influence on the pattern of protein recognition in Western blots for reasons discussed above. LPS analysis alone was found to be less discriminating than OMP analysis. However, since various LPS types occur within serotype A1 and A2 isolates of *P. haemolytica* (Ali *et al.*, 1992) it is important that LPS analysis should be included in future epidemiological studies of this speices.

On the basis of differences detected in OMP and LPS profiles, the present study has demonstrated that serotype A1 and A2 populations of *P. haemolytica* are more diverse than previously thought. In addition, the study has provided evidence to suggest that these differences may be useful in epidemiological studies. For example, the serotype A1 isolates from farms 1, 2, 3 and 4 could be distinguished in terms of their OMP types even though the differences were slight. Isolates obtained from farm 5 on three separate occasions could be distinguished in terms of their serotype, OMP type and LPS types. Although the number of isolates in the present study was relatively small, the examples described above nevertheless demonstrate that differences in the OMP and LPS profiles of serotype A1 and A2 isolates of *P.*

haemolytica do occur, and that this variation can be detected by the methods described. Although there was no clear correlation of a particular OMP or LPS type with virulence in the isolates examined, with the possible exception of serotype A2 isolates of OMP type 2.1/LPS type 3, future investigations on a larger number of isolates may be useful in demonstrating such a relationship.

4.2 CHARACTERISATION OF A HEAT-MODIFIABLE OMP IN *P. HAEMOLYTICA*

Heat-modifiable proteins have been described in a variety of Gram-negative bacteria, including *E. coli* (Beher *et al.*, 1980), and more recently in *H. somnus* (Tagawa *et al.*, 1993), *H. ducreyi* (Spinola *et al.*, 1993) and *A. actinomycetemcomitans* (Wilson, 1991). The latter three species are all members of the family *Pasteurellaceae*. Nakamuru & Mizushima (1976) described the property of heat-modifiablity and related the phenomenon to the structural confirmation of the protein at different solubilisation temperatures, i.e. at higher solubilisation temperatures the protein is predominantly alpha-helical, binding less SDS and consequently running slower on SDS-polyacrylamide gels. Conversely, at lower temperatures, beta-sheets predominate, binding more SDS and running more quickly on gels. Thus at lower temperatures, the low-molecular-weight form of the protein was visualised and vice versa.

This report was concerned with the characterisation of a heat-modifiable outer-membrane protein in *P. haemolytica* and comparing it with those previously reported in other Gram-negative species. Several lines of evidence from this investigation indicate that the species *P. haemolytica* does possess a heat-modifiable protein which is similar to the Omp A protein found in *E . coli* and to heat-modifiable proteins in other species. Firstly, the molecular mass of the heat-modifiable protein of *P.*

haemolytica and the characteristic shift from a low to a high molecular weight at higher solubilisation temperatures resembles the findings observed for *E. coli* Lilly in this study and also the findings previously reported for other bacterial species (Beher *et al.*, 1980; Wilson, 1991; Spinola *et al.*, 1993; Tagawa *et al.*, 1993). Secondly, the immunological cross-reactivity of rabbit monospecific antiserum raised against the 39.5 kDa protein of *P. haemolytica* with the heat-modifiable protein of *E. coli*, indicates the possession of similar epitopes. Thirdly, the N-terminal amino acid sequence of the 39.5 kDa protein of *P. haemolytica*, determined in this investigation,was closely homologous to those of other bacterial species, not only in the same family *Pasteurellaceae* (*H. somnus*, *H. ducreyi* and *A. actinomycetemcomitans*) but also in the *Enterobactericeae*, such as *E. coli*.

The presence of heat-modifiable proteins in *P. haemolytica* and *E. coli* Lilly was demonstrated firstly by SDS-PAGE analysis of outer-membrane fractions of each species and secondly by Western Blotting with rabbit monospecific antiserum. The molecular weights of the high- and low-molecular weight forms of the heat-modifiable protein in each species were 31 and 39.5 kDa and 30 and 33kDa, respectively. This difference in the molecular weights between the two species is not surprising as Beher *et al.* (1980) studied the heat-modifiable protein of many bacterial species and showed that variation did exist, although the range of molecular weights was generally between 27 and 42 kDa. The high-molecular-weight forms of the heat-modifiable proteins of *H. somnus* and *A. actinomycetemcomitans* were 37 and 34 kDa, as reported by Tagawa *et al.* (1993) and Wilson (1991), respectively. The conclusion from this section was that the 39.5 kDa protein of *P. haemolytica* was the heat-modifiable Omp A-like protein, a protein common to other Gram-negative species.

The immunogenicity of this *P. haemolytica* protein was assessed. Using a bovine convalescent antiserum raised against strain PH2, the heat-modifiable protein of both serotype A1 and A2 isolates was shown to be unrecognized by the antibodies

present in this antiserum as observed in Western blots. However, in contrast to this finding, a rabbit monospecific antiserum raised against the isolated 39.5 kDa protein of *P. haemolytica* demonstrated a high immunoreactivity with the heat-modifiable protein. These findings could suggest that the heat-modifiable protein, when present on the surface of intact cells, is less immunogenic than the isolated protein. In the intact cell, other bacterial surface components may conceal the heat-modifiable protein in such a way that it is unable to induce the production of specific antibodies and is therefore poorly immunogenic. It is also possible that the native form of the protein in the intact cells does induce antibodies but which are unable to recognise the denatured form of the protein present in SDS-polyacrylamide gels. Alternatively, this protein may be less immunogenic in calves than in rabbits due to a resemblance to a bovine protein and it would therefore not be recognised as a foreign antigen.

In contrast, Tagawa *et al.* 1993 and Spinola *et al.* 1993, showed the heat-modifiable proteins of *H. somnus* and *H. ducreyi*, respectively, to be significantly immunogenic. These workers also used convalescent sera. Thus, further studies are required to understand the reasons behind the apparent non-immunogenicity of the heat-modifiable protein of *P. haemolytica* in the natural host species.

The monospecific antiserum raised against the isolated 39.5 kDa protein, was also useful in demonstrating the cross-reactivity of the heat-modifiable protein of *P. haemolytica* serotype A1 isolates with a similar protein in serotype A2 isolates of *P. haemolytica* and also in *E. coli*. This cross-reactivity was not surprising as the heat-modifiable proteins found in many Gram-negative species show considerable homology (Beher *et al.*, 1980). In this report the cross-reactivity was demonstrated by Western blotting. The monospecific antiserum was able to recognise the heat-modifiable protein of all *P. haemolytica* strains tested of either A1 or A2 serotype. Unfortunately, time did not permit further studies of this similarity. Such studies might have involved cross-absorbing the antiserum with whole-cells of each serotype and re-

examining the extent of antibody recognition which remained. However this type of examination was performed using antiserum cross-absorbed with *E. coli* antiserum. The results demonstrated that the cross-absorption removed all *E. coli*-specific antibodies but did leave antibodies which recognised the *P. haemolytica* protein. This indicated that although the proteins of both these species were homologous to some degree, there were distinct epitopes on the *P. haemolytica* protein which were not present in the *E. coli* protein. This finding was particularly interesting when the N-terminal amino acid sequences were studied.

The N-terminal amino acid sequence of the 39.5 kDa protein of *P. haemolytica* was determined in this investigation. Comparisons were made with the sequences previously reported for the Omp A protein of *E. coli*, the 37 kDa heat-modifiable protein of *H. somnus* (Tagawa *et al.*, 1993) and the 34 kDa protein of *A. actinomycetemcomitans* (Wilson, 1991). The latter two species belong to the same family as *P. haemolytica.* Comparisons of the first twelve amino acids of the N-termini of the protein revealed that those of *P. haemolytica*, *H. somnus* and *A. actinomycetemcomitans* were identical whereas the *E.coli* sequence differed in four amino acids. This finding agreed with the work of Beher *et al.* (1980) who reported that although most Gram-negative species possess an Omp A-like protein, chemical comparisons of the proteins indicated that their primary structures differed in relationship to their phylogenetic position.

In summary, this part of the study demonstrated that a major 39.5 kDa heat-modifiable OMP of *P. haemolytica* was common to both serotype A1 and A2 isolates and was similar to the Omp A protein of *E. coli* both immunologically. The protein of *P. haemolytica* was shown in this study to be poorly immunogenic in cattle when present in intact cells but was highly immunogenic in rabbits in the isolated form. The *P. haemolytica* heat-modifiable protein appeared to be more closely related to those from species belonging to the same family than to a protein from *E. coli*, as was

evident from the sequence data.

The Omp A protein of *E. coli* has previously been shown to confer stability on the outer-membrane (Sonntag *et al.*, 1978), has been recognised as a surface receptor for certain bacteriophages (Van Alphen *et al.*, 1977) and, more recently, its association with serum resistance and pathogenicity have been reported (Weiser & Gotschlich, 1991). This report has identified the heat-modifiable protein of *P. haemolytica* and assessed its immunogenicity and similarities with the heat-modifiable proteins of other species. Purification and isolation of the protein and determination of the exact functions of the protein is now required and should be the focus of future investigation. This will ultimately lead to a greater understanding of the role of this OMP in pathogenesis and will also determine whether this antigen should be considered in future *Pasteurella* vaccine development studies .

4.3 SERUM SENSITIVITY IN RELATION TO SURFACE PROPERTIES

The bactericidal action of complement, found in extracellular fluids and serum, is an important host defence mechanism against Gram-negative bacteria (Taylor, 1983; Joiner *et al.*, 1984; Crokaert *et al.*, 1992). The proteins which constitute the complement system, after activation by the alternative or classical pathway, form a membrane-attack-complex which ultimately leads to lysis of bacterial cells. The ability of bacteria to resist complement killing is an important virulence attribute and may allow multiplication of the bacteria inside its host species (Taylor, 1983). This property of complement resistance has been documented in a number of Gram-negative species (Schneider, 1985; Kim *et al.*, 1986; Rycroft & Cullen, 1990), although its basis may be multifactorial (Crokaert *et al.*, 1992). Surface components of bacterial cells, namely capsular polysaccharides (Kim *et al.*, 1986; Cross *et al.*, 1986), LPS (Cross *et al.*, 1986; Grossman *et al*, 1987; Merino *et al.*, 1992) and OMPs

(Blaser *et al*, 1987; Weiser & Gotschlich, 1990) have been associated with resistance to killing by host serum and have been investigated in many species including *E. coli*, *Neisseria* species and *Actinobacillus pleuropneumoniae.*

The study of complement bactericidal activity against *P. haemolytica* to date has been limited, although the work of MacDonald *et al.* (1983) and Sutherland (1988) demonstrated that *P. haemolytica* isolates were susceptible to the Classical pathway of complement-mediated killing by bovine serum. Both of these groups of workers showed that killing of the bacteria was observed only when bactericidal antibodies were present , i.e., complement alone was ineffective.

This study set out to identify strains of *P. haemolytica* which were resistant to killing by the Classical complement pathway and attempt to correlate this resistance with the various surface components of the bacteria. Twenty two *P. haemolytica* isolates, differing in their capsular, LPS and OMP types, were examined for sensitivity or resistance to the bactericidal activity of bovine hyperimmune antiserum 10 which has been raised against *P. haemolytica* isolate PH2. The assay used was an adaptation of that of Davies (1991) and was standardised for the purpose of this study. A serum concentration of 90% (v/v) of the total reaction mixture was used, an initial inoculum of 10^7 cfu in a final volume of 100 ul and an incubation period of 1 h were the standard conditions for all assays. The % survivors was calculated for each isolate. Of the 22 isolates examined, seven proved resistant to serum killing, six were highly sensitive to serum killing while the remaining nine were designated partially sensitive. These designations were based on the values >100 % survivors, <0.1 % survivors and 0.1-99.9 % survivors, respectively.

In this assay, the isolate to which the hyperimmune serum was raised, PH2, proved to be resistant to the bactericidal activity of the serum. This finding was unexpected as it suggested that no bactericidal antibodies were present in the serum. However, a similar result has been reported by Rycroft & Cullen (1990) for the

porcine pathogen *Actinobacillus pleuropneumonieae* and these workers proposed that this finding was due to the presence of blocking antibodies in the serum. Such antibodies have been described in relation to other species (Corbiel *et al.*, 1988; Rice & Kasper, 1982). This phenomenon is thought to involve the binding of IgG type antibodies to the bacterial cell surface at sites which prevent the binding of IgM antibodies which are thought to be more efficient bactericidal antibodies (Taylor, 1983), thus inhibiting the Classical complement-mediated lysis of the bacteria. This explanation does not suffice for the results of this study, as other isolates of *P. haemolytica* were successfully killed by this serum.

In addition to isolate PH2, six other isolates proved to be resistant to the bactericidal activity of bovine serum and all seven were of serotype A1 and possessed LPS type 1. These findings, alone, did not prove very useful in determining the basis of serum resistance in *P. haemolytica*. However, when the capsular, LPS and OMP types of these serum resistant isolates were compared with those of the serum sensitive isolates, a number of conclusions could be drawn. The six isolates which were highly serum sensitive were either of serotype A1 (two isolates) or serotype A2 (four isolates). Three different LPS types were found amongst these isolates, LPS 3, 4 or 5 (three, two and one isolates, respectively). Contrary to previous reports in *E. coli* (Kim *et al.*, 1986; Cross *et al.*, 1986) where certain capsular types were found only in serum resistant strains, there was no clear correlation between capsular type and serum resisitance in *P. haemolytica*. On the other hand a more definitive association of LPS types with serum resistance could be made. LPS type 1, found in all serum resistant isolates was of the smooth form whereas LPS types 3, 4 and 5 were all rough type LPS and found only in serum sensitive isolates. This association of serum resistance with smooth forms of LPS has been described previously (Muschel & Larsen, 1970; Schneider *et al.*, 1982; Grossman *et al*, 1987), although not in *P. haemolytica*. Two mechanisms by which smooth type LPS confer resistance have been proposed. Firstly

the long polysaccharide chains present on smooth forms of LPS bind the antibodies which initiate the complement cascade at sites too far from the bacterial membrane to allow insertion of the membrane-attack-complex. Thus, lysis does not take place. Secondly, the extended LPS structures may mask potential antigens found on the surface of the bacteria to which the bactericidal antibodies were produced. Therefore it may be that in *P. haemolytica* the smooth type LPS found in all serum resistant strains is conferring resistance by one or both of the mechanisms above.

The role of OMPs in serum resistance has been described in *E. coli* (Weiser & Gotschlich, 1990) and also in *Campylobacter jejuni* (Blaser *et al.*, 1987). In *P. haemolytica* their role is not clear. Although isolates possessing the different OMP types were examined, no definite conclusions could be drawn. OMP types 1.1, 1.2, and 1.4 were associated with smooth type LPS and found in serum resistant isolates only, whereas OMP types 2.1, 2.2, 2.3 and 2.4, associated with rough forms of LPS, were present in serum sensitive isolates. Thus it seems that the important determinant of serum resistance in *P. haemolytica* is the LPS, although OMPs may have a role which remains to be defined.

In this study, nine of the 22 isolates were designated partially serum sensitive. These isolates, although grouped together, differed quite significantly in the percentage of survivors after incubation. The values ranged from 2 to 98 % and in some cases, particularly those isolates which had a % survivor value of >90 %, it may be that the isolates are resistant when one allows for viable counting errors. Therefore, in considering three of the serotype A1 isolates which possessed LPS type 1 (smooth type LPS) and had % survivor values of 95, 96 and 98 it is possible that these isolates were in fact serum resistant. Further assays with greater replication would help to clarify this situation.

In this investigation, serum resistant isolates of *P. haemolytica* were identified. No definite relationship was observed between capsular or OMP type and serum

resistance, but a correlation of smooth type LPS with resistance was seen. The importance of complement resistance in *P. haemolytica* infections is not clear. *Pasteurella haemolytica* is, primarily, respiratory pathogen. The alveolar macrophages are thought to be the most important line of defence in the lower respiratory tractbut, it is known that *P. haemolytica* secretes a potent leukotoxin which can destroy these immune effector cells (Lo, 1990). Under these circumstances, host defences such as the complement cascade are likely to be important. Thus, resistance to the bactericidal activity of complement would promote survival and colonisation of the respiratory tract under such conditions.

Resistance of bacteria to complement killing is often related to their involvement in septicaemic infections. Isolate PH2, however, and the other bovine isolates of *P. haemolytica* which are serum resistant are not known to cause a septicaemia infection but rather cause a localised pneumonia. Possibly the resistance to complement killing is a property which is of importance in later stages of the lung disease. The increasing production of the leukotoxin destroys a greater number of the macrophages and consequently the humoral immunity becomes increasingly important. Also, the release of inflammatory mediators at the site of infection may cause an influx of serum components including antibodies and complement proteins into the alveoli thus facilitating this humoral immunity. However, it is evident that to fully understand the basis of complement resistance in *P. haemolytica* and its importance in pathogenesis, further investigations are required.

4.4 VIRULENCE TESTS IN CALVES

In this part of the investigation the ability of a number of isolates to cause lung lesions following intratracheal inoculation was assessed. This property was taken to be indicative of the virulence of the strain. The isolates examined were chosen to

represent those with different capsular and LPS types, in an attempt to correlate various types of surface antigens with virulence.

The initial stage of this investigation involved standardisation of the conditions of the tests in terms of the inoculum and the effect of different diluents. When filter-sterilised culture supernate was the diluent, a significantly higher number of lesion scores were observed than when sterile PBS was used. This suggested that there was a component present in the culture supernate which may be involved in producing lung lesions. It may have been due to *P. haemolytica* leukotoxin in the culture supernate which is itself capable of causing lung lesions (Gibbs *et al.*, 1984) or to the presence of LPS. It was decided therefore that sterile PBS would be used when comparing the various isolates so as to avoid any interference which may occur due to leukotoxin or LPS presence.

The inoculum size suitable for the virulence tests was that which gave significant lesions which were reproducible and countable but also would not create a risk of killing the animal. The medium-sized inoculum, approximately 2.5×10^{11} cfu, was chosen as it fulfilled the above requirements. Thus a final inoculum of the above size diluted in sterile 20 ml PBS were the standard conditions applied to the comparative virulence tests.

In these tests a number of isolates differing in serotype (capsular type) and LPS type were examined. Other features of each of the isolates, namely the ability to resist the bactericidal activity of serum and the leukotoxin activity were determined and the results incorporated into this study. Similar studies have been reported in other bacterial species where an association was shown between specific capsular antigens (Achtman *et al.*, 1983; Kim *et al.*, 1986), LPS types (Cross *et al.*, 1986), serum resistance (Pai & DeStephano, 1982; Davies, 1991) and virulence. In this present study the aim was to identify any such properties which correlated with virulent *P. haemolytica* isolates.

No clear-cut association of capsular type with virulence was detected although a correlation of LPS type with virulent strains was shown. That is, isolates with smooth-type LPS appeared to be more virulent than those with rough-type LPS, producing a higher number of lesions in the virulence tests. Similar findings, where smooth-type LPS was associated with increased virulence, have also been reported for other bacterial species (Achtman *et al.*, 1983; Lugtenberg *et al.*, 1984), namely *E. coli* and *P. multocida*, respectively. However as the virulence of a bacterium is known to be multifactorial, other factors in addition to LPS are likely to be contributing to the results observed in these virulence tests.

Serum resistance and leukotoxin activity of the isolates were considered. The results of the bactericidal assays indicated that serum sensitivity could somewhat be correlated with a high mean lesion score. As previously reported, however, smooth-type LPS seems to be associated with serum resistance (Cross *et al.*, 1986; Grossman *et al.*, 1987; Merino *et al.*, 1992). Thus, the importance of LPS in virulence is further stressed.

As for the leukotoxin, this virulence determinant is known to be one of the most important factors in *P. haemolytica* pathogenicity and thus the inclusion of the leukotoxin data for each of the isolates was particularly important even though this investigation was concerned primarily with the surface structures of the bacterium. The results indicated that, in general, isolates which produced low levels of leukotoxin also produced low lesion scores. However, comparison of two isolates, namely, PH8 and PH30, both of which produce medium levels of leukotoxin, showed that other factors were important in determining the results of the virulence tests as they differed significantly in the level of lesions produced.

In summary, this line of the investigation, did provide strong evidence for taking account of determinants such as LPS type and serum resistance properties in studies of virulence mechanisms of *P. haemolytica*.

4.5 INVESTIGATIONS OF *P. HAEMOLYTICA* GROWN *IN VIVO*

Tissue chamber implants in the peritoneal cavity have been used for some time to study the *in vivo* characteristics of bacteria (Finn *et al.*, 1982; Kelly *et al.*, 1989; Trees *et al.*, 1991). Previous investigations of *P. haemolytica* grown in implant chambers have been limited (Morck *et al.*, 1991; Confer *et al.*, 1992) and thus the *in vivo* characteristics of this species are not completely understood.

In this study, the growth of four *P. haemolytica* isolates (PH2, PH30, PH42 and PH48) in intraperitoneal implant chambers was assessed. Organisms recovered from the chambers were used for studying variations between OMP profiles of *in vitro* and *in vivo*-grown cells; LPS profiles of the *in vivo* organisms, unfortunately, were not examined due to time limitation and also a lack of bacterial samples for performing the LPS extractions.

Initially, however, the growth of the organisms within the chambers was examined. Contrary to the findings of Confer *et al.* (1992) where the bacteria grew successfully inside the chambers inserted into the paralumbar fossa of calves, this investigation reported a decrease in the bacterial numbers, a finding similar to the report of Morck *et al* (1991) who also saw a decline in bacterial cell number in chambers inserted into sheep. In this present study the initial inoculum of approximately 10^8 cfu ml^{-1} decreased to 10^4 cfu ml^{-1} during the 16 days of the experiment. Similarly, Morck *et al.* (1991) reported a decline from 10^8 cfu ml^{-1} to 10^5 cfu ml^{-1} over a 14 day period. These findings differ from those with other bacterial species (Finn *et al.*, 1982; Kelly *et al.*, 1987; Trees *et al.*, 1991), namely, *E. coli*, *Pseudomonas aeruginosa*, and *Haemophilus ducreyi*, respectively, where successful growth of the bacteria within the chambers was reported. However Onderdonk *et al.* (1989) showed a decline of 10^6 cfu ml^{-1} to zero when *Bacteroides fragilis* was

inoculated into the implant chambers inserted into the peritoneal cavity of of mice. A number of experiments were performed in an attempt to understand the decline of *P. haemolytica* within the chambers.

The results indicated that there was no change in the level of *P. haemolytica* specific antibodies to OMPs in the chamber fluid during the 16 days of the experiment and also that complement activity was not important for the decline in cell numbers within the chamber; this possibly suggested that an antibody-dependent complement-mediated killing mechanism was not occurring inside the chamber. The report of Onderdonk *et al.* (1989) also supported an antibody-complement independent mechanism of bacterial killing and they suggested that a cellular immune response was the most likely reason for the decline in cell numbers. The role of a cellular immune response in the killing of *P. haemolytica* within the chamber should be considered in future *in vivo* studies of *P. haemolytica.*

Although the cell numbers were relatively low at the end of the experiment, a sufficient number of cells were rescued for outer-membrane preparations, as described previously in section 2.11.4. Examinations of the OMP SDS-PAGE profiles and comparison with OMP profiles obtained from the same isolates grown *in vitro* and derived from the lungs of *P. haemolytica*-infected calves were made. A number of differences were detected although they were slight and their is unknown.

The high-molecular-weight proteins, 71, 77 and 100 kDa, previously reported in *in vivo*-grown cells (Morck *et al.,* 1991) and in cells grown *in vitro* under iron limitation (Donachie & Gilmour, 1988; Deneer & Potter, 1989; Davies *et al.,* 1992), were also detected in the bacteria which had been grown inside the implant chamber. In addition, the differences in the low-molecular-weight region of the profiles detected in this report were similar to those reported by Confer *et al.* (1992) who detected differences in the region < 30 kDa in the profiles of *in vivo* and *in vitro*-grown cells. The importance of these differences remains to be investigated.

Some of the differences between *in vitro*- and *in vivo*-grown cells may be due to contaminating host proteins as was previously discussed by Finn *et al.* (1982). They suggested that host immunoglobulins may contribute to the profiles of the *in vivo*-grown cells. However, to determine the origin of the extra proteins in the *in vivo*-grown *P. haemolytica* cells, further studies would be required. Nevertheless, the results have highlighted some of the changes in bacterial properties which can occur when bacteria are grown *in vivo*. In addition they have stressed the importance of considering the results of *in vivo* studies when interpreting the *in vitro* studies of pathogenic features of bacteria.

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APPENDICES

APPENDIX 1 - Reagents used in SDS-PAGE

SAMPLE BUFFER:	X1	X2
O.5M Tris-HCl (pH6.8)	5 ml	5 ml
glycerol	4 ml	4 ml
10%SDS	8 ml	8 ml
2-beta-mercaptoethanol	2 ml	2 ml
0.05% bromophenol blue	1 ml	1 ml
distilled water	20ml	-

STACKING GEL:

0.5M Tris-HCl (pH6.8)	5.0 ml
10%SDS	0.2 ml
Acrylamide/bisacrylamide (30& T. 2.6%C)	2.6 ml
distilled water	12.2 ml
10%ammonium persulphate	0.1 ml
TEMED	0.02 ml

RESOLVING GEL:

12% acrylamide (for OMP SDS-PAGE):

1.5M Tris-HCl (pH 8.8)	15.0 ml
10% SDS	0.6 ml
Acrylamide/bis,acrylamide	24.0 ml
distilled water	20.1 ml

10%ammonium persulphate	0.3 ml
TEMED	0.03 ml

10% (for tricine gel electrophoresis):

Gel buffer	19.8 ml
(3M Tris, 0.3% SDS)	
Acrylamide/bisacrylamide	12.0 ml
(48%T, 1.5% C)	
glycero	16.0 ml
distilled water	22.2 ml
10%ammonium persulphate	0.45 ml
TEMED	0.045 ml

15% acrylamide/4M urea (for LPS SDS-PAGE):

1.5M Tris-HCl (pH8.8)	15.0 ml
10% SDS	0.6 ml
Acrylamide/bisacrylamide	30.0 ml
Urea	14.4 g

RESERVOIR BUFFER:

Trizma base	13.5 g
Glycine	64.8 g
SDS	4.5 g
distilled water	4500 ml

CATHODE BUFFER (for tricine SDS-PAGE):

Trizma base	12.11 g

Tricine	17.92 g
SDS	1.00 g
distilled water	4000 ml

ANODE BUFFER (for tricine SDS-PAGE):

Trizma base	96.88 g
distilled water	4000 ml

COOMASSIE BLUE STAIN:

Methanol	450 ml
Acetic acid	100 ml
Coomassie blue	1.00 g
distilled water	450 ml

DESTAIN:

Methanol	300 ml
Acetic acid	100 ml
distilled water	600 ml

APPENDIX 2 - Reagents used for silver staining of LPS

FIXING SOLUTION:

ethanol	400 ml
acetic acid	50 ml
distilled water	550 ml

FIXING SOLUTION + 0.7% PERIODIC ACID:

fixing solution	400 ml
periodic acid	2.8 g

STAINING REAGENT:

0.1M sodium hydroxide	56 ml
ammonium hydroxide	4 ml
20% silver nitrate	10 ml
distilled water	230 ml

FORMALDEHYDE DEVELOPER:

5% citric acid	2.0 ml
37% formaldehyde	0.2 ml
distilled water	400 ml

50% METHANOL:

methanol	500 ml
distilled water	500 ml

APPENDIX 3 - Reagents used for Western blotting

TRANSFER BUFFER:

Trizma base	12.12 g
Glycine	57.60 g
distilled water	4000 ml

TRIS BUFFERED SALINE (TBS, pH7.5):

Sodium chloride	58.44 g
Trizma base	4.84 g
Distilled water	2000 ml

TBS +0.05% TWEEN 20 (TTBS, pH7.5):

TBS	1000 ml
Tween 20	0.5 ml

BLOCKING SOLUTION:

TBS	100 ml
Gelatin	3.0 g

ANTIBODY BUFFER:

TTBS	200 ml
Gelatin	2.0 g

COLOUR DEVELOPMENT SOLUTION:

4-CHLORO-1-NAPTHOL:

4-chloro-1-napthol	60 mg

methanol	20 ml
TBS	100 ml
hydrogen peroxide	0.06 ml

DIAMINO BENZOIC ACID (DAB):

PBS	100 ml
DAB	30 mg
1% cobalt chloride	2 ml
hydrogen peroxide	100 ul

PUBLICATIONS

McCLUSKEY, J., GIBBS, H. A., DAVIES, R. L. (1994). Comparison of outer membrane protein and lipopolysaccharide profiles of *Pasteurella haemolytica* isolates of serotypes A1 and A2 obtained from pneumonic and healthy cattle. Microbiology (in press).