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Pathophysiological Investigations of Pneumonic Pasteurellosis due to Pasteurella multocida Type A:3 in Calves

© Aileen Aisha Dowling

For the degree of Doctor of Philosophy

Department of Veterinary Clinical Studies

University of Glasgow Veterinary School

Research performed at

Moredun Research Institute, Edinburgh

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ABSTRACT

Pneumonia in young ruminants, caused by the Gram-negative bacterium *Pasteurella multocida*, world-wide is an infectious respiratory disease of major economic and welfare importance. *P. multocida* infection is on the increase, for unknown reasons, as the serotype A:3 that causes the majority of pneumonic disease has not been fully characterised. Greater knowledge of *P. multocida* A:3 may reveal the underlying mechanisms of pathogenesis that have not yet been elucidated. The aim of the work presented in this thesis was to expand knowledge of *P. multocida*, specifically with respect to serotype A:3. There are several published attempts to develop a suitable animal model of *P. multocida* infection but prior to this investigation none were reliable, reproducible or closely mimicked the field disease. As a consequence, progress towards a vaccine has been slow and there is currently no vaccine available to protect against disease caused by *P. multocida* A:3.

In these studies the lipopolysaccharide (LPS) from *P. multocida* A:3, a potentially important virulence factor, was characterised and found to be of the rough form, indicating loss of the O-antigen responsible for variation in the bacterial isolates. This discovery may facilitate the development of vaccines targeted against LPS. Pulsed-field gel electrophoresis of DNA cut by enzymes with infrequently predicted restriction sites revealed the potential for much genetic diversity. An experimental model of pneumonic pasteurellosis caused by *P. multocida* A:3 was established to help determine mechanisms of pathogenesis. In this model, 300 ml of PBS containing 10^9 cfu of *P. multocida* produced progressive clinical and pathological responses comparable to those observed in field cases providing for the first time a model suitable for future experimental studies (Dowling *et al.*, 2002). Furthermore haptoglobin and serum amyloid-A were found to be useful markers for the disease. Subsequent work confirmed the reliability and reproducibility of the model in terms of the clinical and pathological responses. Host-pathogen interactions at various stages of disease were assessed by 2-dimensional electrophoresis of bronchoalveolar lavage fluid isolated from calves infected with live *P. multocida* A:3 which revealed changes in antimicrobial peptides, annexins, antioxidant protein and apolipoprotein-1 precursor.

Host responses to both live and killed *P. multocida* A:3 challenge were compared. Calves received either formalin killed *P. multocida* or saline, followed, at day 21, by challenge with live *P. multocida*. There were marked changes in both clinical scores and acute phase protein concentrations in responses to both treatments, with the highest values associated with formalin-killed *P. multocida*. Calves given formalin-killed *P. multocida* appeared to cope well with the bacterin challenge indicating a possible method for generating protection against exposure to a

homologous challenge. The responses in calves given homologous live challenge after exposure to the bacterin were more severe than those calves given only the homologous challenge, suggesting that the bacterin compromised the animals' defences rather than enhancing protection.

The *in vitro* interaction of macrophages with *P. multocida* was shown to be independent of opsonisation and the pathogen could survive in these cells for up to 1 hour, possibly by disabling the bactericidal function of the macrophage which may be significant to pathogenesis in the host.

The work reported in this thesis led to a successful, progressive and reproducible animal model that has provided a detailed picture of the clinical and pathological responses following *P. multocida* infection. This study has provided a better understanding of the disease process and the host interaction with *P. multocida* A:3 and from these novel findings more informed approaches to vaccine development should be possible.

AUTHOR'S DECLARATION

I hereby declare that the work presented in this thesis is original and was performed solely by the author, except where assistance of others is acknowledged. I also hereby certify that no part of this thesis has been submitted previously in any form to any university for the award of a degree, but has been and will be published as either scientific abstracts or as papers.

Aileen Aisha Dowling

July 2003

This thesis is dedicated to my soul mate and partner Alexandros Astaras for his patience, devotion, support and encouragement without which I could never have undertaken such an arduous task.

ΕΥΧΑΡΙΣΤΩ ΑΓΑΠΗ ΜΟΥ

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ABBREVIATIONS

AGP	α_1 acid glycoprotein
ANOVA	analysis of variance
AP-PCR	arbitrarily primed polymerase chain reaction
APP	acute phase proteins
APR	acute phase response
AUC	area under curve
BAL	bronchoalveolar lavage
BALF	bronchoalveolar lavage fluid
BALT	bronchial associated lymphoid tissue
BAM	bronchoalveolar macrophages
BPP	bovine pneumonic pasteurellosis
b-SAA	bovine serum amyloid-A
BVDV	bovine viral diarrhea virus
CHCA	α-cyano-4-hydroxycinnamic acid
CL	chemiluminescence
cfu	colony forming units
°C	centigrade (degree)
Da	dalton
2-DE	2-dimensional electrophoresis
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
DW	distilled water
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FcR	Fc-receptors
FCS	foetal calf serum
g	gram
h	hours
H_2O_2	hydrogen peroxide
HBSS	hanks balanced salt solution
HO	home office
Hp	haptoglobin
HRP	horseradish peroxidase
HS	haemorrhagic septicaemia
IBRV	infectious bovine rhinotracheitis virus
IEF	isoelectric focusing
IgA	immunoglobulin-A
IgG	immunoglobulin-G
IHA	indirect haemagglutination
IL	interleukin
IFN-γ	interferon-gamma
i.m	intramuscular
i.p	intraperitoneal
IPG	immobilised pH gradient
IROMP	iron-regulated outer membrane proteins
IRP	iron-regulated proteins
i.v.	intravenously

k	kilo
kg	kilogram
kDa	kilodalton
l	litre
LAP	lingual antimicrobial peptide
LBP	lipopolysaccharide binding protein
LDCL	luminol-dependent chemiluminescence
LPS	lipopolysaccharide
LRT	lower respiratory tract
LV	lavage
MALDI-TOF	matrix assisted laser desorption ionisation time of flight
MBP	mannose-binding protein
MDA	maternal derived antibody
MDF	macrophage deactivating factor
mg	milligram
MHC	major histocompatibility complex
min	minutes
ml	millilitre
mm	millimetre
mM	millimole
MMP	matrix metalloproteases
MPO	myeloperoxidase
M_r	molecular radius
MRI	Moredun Research Institute
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MSDB	Mass Spectrometry Database
NB	nutrient broth
ng	nanogram
NO	nitric oxide
OD	optical density
OMP	outer membrane proteins
ONPG	*O*-nitro-phenyl-D-galactopyranoside
OPD	*O*-phenylenediamine
PAF	platelet activating factor
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween 20
p.c.	post-challenge
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
p.i.	post-infection
pI	isoelectric points
PI-3	parainfluenza virus type-3
PK-C	protein kinase-C
PM	post-mortem
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear neutrophils
PMSF	phenylmethylsulfonyl fluoride
RBCs	red blood cells
REA	restriction endonuclease analysis
REML	residual maximal likelihood
REP-PCR	repetitive extragenic palindromic polymerase chain reaction
RNI	reactive nitrogen intermediates
ROI	reactive oxygen intermediates
rpm	revolutions per minute
RSV	respiratory syncitial virus

RT	room temperature
s	seconds
SAA	serum amyloid A
SBA	sheep blood agar
s.c	subcutaneous
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of mean
TAP	tracheal antimicrobial peptide
Tbp	transferrin binding protein
TEC	tracheal epithelial cells
$TCID_{50}$	tissue culture infectious dose
TCS	total clinical score
TFA	trifluoroacetic acid
TGF-β	transforming growth factor-β
Tlr	toll-like receptor
TMB	3,3,5,5-tetramethylbenzidine solution
TNF-α	tumour necrosis factor-α
U.C	universal container
U	units
URT	upper respiratory tract
µ	micro
µg	microgram
µl	microlitre
µm	micrometers
µmol	micromole
VIDA	veterinary investigation diagnosis analysis
VLA	veterinary laboratories agency
v/v	by volume
w/v	by weight

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Chapter 1. General Introduction

Pneumonic pasteurellosis is an infectious bacterial disease of great economic importance and welfare and affects young ruminants. The disease is often initiated by a predisposing infection or stress, and the bacterial species isolated from clinical cases are *Pasteurella multocida* and *Mannheimia haemolytica* serotypes A. Historically, *M. haemolytica* was the predominant isolate from cases of pneumonic pasteurellosis and consequently much research was focused on this organism. This has led to well defined animal models (Panciera *et al.*, 1984a, Jones *et al.*, 1989, Porter *et al.*, 1995, Highlander *et al.*, 2000) describing the host response, subsequently assisting breakthroughs in vaccine technology (Gilmour *et al.*, 1991, Sreevatasan *et al.*, 1996, Kraabel *et al.*, 1998, Hodgins and Shewen., 2000). However, more recently, official figures retrieved from Veterinary Investigation Diagnosis Analysis (VIDA), collated within the UK, for the period 1992 to 2001 show that the incidence of *P. multocida* A:3 is on the increase and is now as or more important than *M. haemolytica* in causing disease. Whereas much is known regarding *M. haemolytica* much less information is available on the pathogenic process of pasteurellosis initiated by *P. multocida*. Several attempts have been made to develop a suitable animal model of *P. multocida* infection but there is no reliable, reproducible model that mimics closely the field situation. As a consequence, progress towards a vaccine has been slow and there is currently no vaccine available against a *P. multocida* infection.

This general introduction seeks to review what is known about *P. multocida* A:3, and to address the gaps in our knowledge on host-bacterial interactions and a reliable and realistic disease model to assist vaccine development and characterisation of the organism itself. The introduction will be divided into two parts, the first part will discuss the organism and the second will focus on the host response to infection with *P. multocida*, with specific reference to bovine pneumonic pasteurellosis.

1.1. *Pasteurella multocida*

1.1.1. History and nomenclature

P. multocida is an opportunistic pathogen in wild and domesticated animals, as well as in man, and has been recognised as an important pathogen by the veterinary profession for over a century. Pasteurellosis caused by this organism was described initially in the bovine by Bollinger in 1878

and *P. multocida* was first isolated a year later by Toussaint (Mutters *et al.*, 1989). The organism has been given many names beginning with *Micrococcus gallicidus* designated by Burrill in 1883.

In 1887 the generic name, Pasteurella, was assigned by Trevisan who wanted to commemorate Louis Pasteur's discovery of *P. multocida* as the causative agent of fowl cholera in turkeys (Mutters *et al.*, 1985).

For a period, organisms with a variety of species names were assigned to the Pasteurella genus on the basis of bipolar staining or on the host species from which the organism was isolated, for example *P. bovicida*. Other names are listed in Index Bergeyana (Buchanan *et al.*, 1966). After approximately 40 years of nomenclature changes for *Pasteurella* spp., Rosenbach and Merchant in 1939 proposed the *epithet*, *multocida* (Rosenbach and Merchant, 1939). Until 1932, the genus Pasteurella consisted only of *P. multocida*, a situation that changed when the genus expanded to include *Pasteurella haemolytica*, on the basis of a lengthy classification study of 114 isolates. This study showed the presence of two distinct types of organism differing in their phenotypic characteristics and warranted their classification into separate species, as suggested by Newsom and Cross, (1931) who gave the species name *Pasteurella haemolytica* to the atypical, avirulent group composed of strains of bovine and ovine origin, leading to the separation of the closely related *P. multocida* in the typical group (Newsom and Cross, 1931).

1.1.2. Determination and classification of *P. multocida*.

P. multocida represents a heterogeneous taxon consisting of a single species and subspecies, and is associated with a range of diseases in a variety of hosts (Mohan and Pawandiwa, 2000). Phenotypic and genotypic methods have been employed to differentiate *P. multocida* from closely related species. Among the phenotypic methods used are colony morphology, biotyping, capsular and somatic typing and these allow us to determine bacterial antigens or biochemical properties that may identify biochemical varieties (Blackall *et al.*, 1995 and Aye *et al.*, 2001). Genotyping currently involves pulsed-field gel electrophoresis (PFGE) (Lainson *et al.*, 2002) and polymerase chain reaction (PCR) (Townsend *et al.*, 2000, 2001 and Rocke *et al.*, 2002) providing a more rapid, reproducible and sensitive means of strain differentiation. The three subspecies within *P. multocida* (*P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica* and *P. multocida* subsp. *gallicida*) were assigned to the genus *Pasteurella* sensu stricto by DNA-DNA hybridisation studies that determined the genetic relationships amongst species and their positions within the family *Pasteurellaceae* Pohl 1981 (Mutters *et al.*, 1985).

1.1.2.1. Phenotypic Methods

1.1.2.1.1 Colony morphology and capsular antigen.

P. multocida is a small, non-motile, nonspore-forming, Gram-negative rod or coccobacillus 0.2-0.4 x 0.6-2.5 μm, which exhibits a typical bipolar staining with Leishman or methylene blue stain in fresh cultures and animal tissues. Cells are arranged singly or in pairs and occasionally as chains or filaments if cultures are grown under unfavourable conditions. When *P. multocida* is grown on blood agar at 37°C the colony shape and size may vary considerably, ranging from circular, convex, discrete colonies (1 to 2 mm) to large mucoid colonies (3 to 5 mm) which are translucent and greyish in colour with a mucoid appearance characteristic of capsular type A *P. multocida*. (Rimler and Rhoades, 1989). Variation in colony morphology is due to differences in the size and chemical composition of the capsule, which is primarily composed of carbohydrate with some hyaluronic acid and is somewhat hydrophilic (Carter, 1972).

Colonies of *P. multocida* capsular groups B and E may also vary in size depending on the degree of capsulation. They can range from large greyish colonies when freshly cultured to small smooth colonies that give a yellowish-or bluish-green iridescence when viewed in transmitted light. Capsular type D and F strains may also produce mucoid colonies that are generally smooth in appearance and display a pearl-like iridescence in oblique transmitted light. The capsular material of serogroups D and F was identified by the action of glycosidases and subsequent decapsulation as heparin or chondroitin, respectively (DeAngelis *et al.*, 2002).

Rough colonies, with a circular, slightly dry appearance, are often produced by old cultures, due to the loss of capsular material, a process known as dissociation. The capsule can be regained by passaging these rough cultures through the natural host or through laboratory animals such as mice.

Colony morphology is related to virulence, while dissociation is related to the loss of virulence and antigenicity. This was shown in a study whereby acapsular bacteria were readily taken up by murine peritoneal macrophages while capsular *P. multocida* were significantly resistant to phagocytosis (Boyce and Adler, 2000). Encapsulated cultures have been associated with the production of effective vaccines, as the capsule contains an array of antigens that can stimulate the production of an efficient immune response against these foreign invaders (Adler *et al.*, 1999). However, the relationship between capsule and virulence is not absolute as an acapsular B:2 strain of *P. multocida* was still able to stimulate protective immunity against a *P. multocida* challenge (Boyce and Adler, 2001), indicating that some noncapsulated strains have the ability to be virulent.

1.1.2.1.2 Capsular typing

To study the pathogenesis and epidemiology of disease agents it is essential that isolates are classified in order to arrive at a concise diagnosis. This is of particular importance when applied to *P. multocida* organisms that have the ability to infect a broad spectrum of hosts (Brogden and Packer, 1979). Serotyping of *P. multocida* using an indirect haemagglutination (IHA) test was able to identify four serological groups (A, B, D and E) based on capsular antigens (Carter, 1967 and Rimler and Rhoades, 1987). The IHA test was based upon the agglutination of human 'O' red blood cells (RBCs) sensitised by capsular antigen from bacterial cultures. The capsular antigen was prepared by heating suspensions of the bacteria at 56°C for 30 m and removing cells by centrifugation. Over the years the IHA test has been modified and now uses fresh sheep RBCs (Sawada *et al.*, 1982). Using this modified IHA, further *P. multocida* type was consistently isolated from turkeys and could not be placed in the existing serogroups, leading to the assignation of a serogroup F (Rimler and Rhoades, 1987). It is worth noting that a disadvantage of capsular typing is the difficulty of inducing antibodies to the specific antigen and, moreover, cross reactions may occur between antibodies against the serogroup antigens.

1.1.2.1.3 Somatic typing

Two systems exist currently for somatic antigen typing of *P. multocida*; Namioka's system based upon tube agglutination tests (Namioka and Murata, 1961a; Namioka and Murata, 1961b) and Heddleston's system based upon a gel diffusion precipitin test (Heddleston *et al.*, 1972). The Namioka tube agglutination test is based on release of core or somatic bacterial components by agglutinating HCl-treated cells with rabbit antiserum. Using this method, 11 somatic types were characterised. A disadvantage of this typing system is that some isolates undergo autoagglutination following HCl treatment and cannot be typed (Rimler and Rhoades, 1989). The Heddleston gel diffusion precipitin test uses the supernatant of culture suspensions heated at 100°C for 1 h as the antigen (Heddleston *et al.*, 1972). Antiserum are prepared in chickens because they respond better to the type specific antigen after a short course of immunisation with a bacterin (Rimler and Rhoades, 1989). This method was able to differentiate 16 somatic types (Heddleston *et al.*, 1972). Brogden and Packer (1979) attempted to equate serotypes among different typing systems and found any correlation unreliable due to the antigenic complexity of *P. multocida* and the type of antigen used in each test.

The most widely accepted and current serotype designation system is a combination of Carter capsular typing and Heddleston somatic typing, whereby the capsular type is expressed first, followed by the somatic type. Using this method the Haemorrhagic Septicaemia (HS) serotypes from Asia and Africa have been designated B:2 and E:2, respectively. This system has consistently identified serogroups that cause diseases of economic significance.

1.1.2.1.4 Non-serological typing

A particular limitation of serological typing of *P. multocida* is the inability of serogroups A, D and F to agglutinate with homologous sera (Rimler, 1994). This phenomenon is associated with a component of the bacterial capsule, namely hyaluronic acid in serogroups A and a mucopolysaccharide, similar to hyaluronic acid, in serogroup D strains. Alternative tests include the addition of acriflavine to concentrated broth cultures, whereby a positive reaction is determined by production of a flocculent precipitate (Carter and Subronto, 1973). This test is used primarily to differentiate D strains of *P. multocida* from other types but some serogroup F strains also react positively. Furthermore, tests based on enzymic removal of capsule using a disc diffusion technique constitute a non-serological means of disease diagnosis. Addition of staphylococcal hyaluronidase to mucoid serogroup A strains causes depolymerisation of hyaluronic acid present in the bacterial capsule resulting in the loss of capsule. The decapsulation of D strains is mediated by the addition of chondroitinase AC and heparinase III; F strains were decapsulated by chondroitinase AC and were unaffected by the other enzymes (Rimler, 1994). It is worth noting that these tests are often used to complement the serological tests rather than replace them.

1.1.2.1.5 Biochemical characteristics of P. multocida

Classification of *P. multocida* isolates into biotypes based on fermentation reaction patterns has been reported and is associated with isolates from a particular host species (Fegan *et al.*, 1995). *P. multocida* can be differentiated from closely-related bacteria on the basis of the following: fermentative growth, positive reaction for oxidase, catalase and indole, negative reaction with urease, lack of growth on MacConkey agar, acid production from hexoses (glucose, mannose, galactose, fructose) and variable fermentation with disaccharides positive for sucrose and negative for maltose and lactose. The subspecies of *P. multocida* can be differentiated by the following reactions: *P. multocida* subsp. *gallicida* ferments arabinose, whereas subsp. *septica* and subsp. *multocida* cannot. *P. multocida* subsp. *multocida* and subsp. *gallicida* ferment sorbitol, whereas subsp. *septica* does not.

1.1.2.2. Genotypic characterisation

With the advent of molecular biology techniques it has been possible to characterise isolates according to their most fundamental and stable property, their genetic information (Hunt *et al.*, 2000), and to identify bacterial pathogens that otherwise would be untypable. These techniques provide a more precise and rapid mode of grouping strains than phenotyping methods (Hunt *et al.*, 2000).

Recently the differentiation of strains of a similar serotype has been achieved using a variety of genotyping methods these include restriction endonuclease analysis (REA) which uses restriction

enzyme digestion of genomic DNA and agarose gel electrophoresis for DNA fragment separation (Zhao *et al.*, 1992 and Rubies *et al.*, 2002), and ribotyping based on REA except that only restriction fragment length polymorphisms are highlighted (Hunt *et al.*, 2000). These techniques are often applied in conjunction with one another, for example to differentiate avian (Blackall *et al.*, 1995) and porcine (Zhao *et al.*, 1992) strains that exhibit considerable genomic heterogeneity. PFGE is a specialised form of REA producing a digest with fewer but larger fragments (Blackall and Miflin, 2000) and which demonstrates greater discrimination than ribotyping for the differentiation and identification of bacterial strains (Kodjo *et al.*, 1999). PFGE has shown the potential to discriminate closely related strains (Lainson *et al.*, 2002) although two limitations exist, the DNA extraction procedure is rather time consuming and the technique requires specialised and expensive equipment which is difficult to acquire in diagnostic laboratories.

Several studies have shown the benefit of PCR fingerprinting for the differentiation of *P. multocida* isolates (Townsend *et al.*, 1997, 1998). Arbitrarily primed PCR (AP-PCR) was effective in the discrimination of post-vaccination *P. multocida* isolates from turkeys (Hopkins *et al.*, 1998) and was used to detect DNA fragments that distinguish serotype 1, the causative agent of avian cholera in wild waterfowl, from the other 15 serotypes of *P. multocida* (Rocke *et al.*, 2002). Repetitive extragenic palindromic PCR (REP-PCR) exhibits a high degree of discrimination as shown in a recent study of the epidemiological relatedness of avian and swine *P. multocida* isolates, wherein REP-PCR compared favourably with PFGE (Gunawardana *et al.*, 2000; Amonsin *et al.*, 2002). Furthermore, a PCR assay using primers derived from the 23S rRNA gene sequence of *P. multocida* has been developed and applied to the rapid identification of 144 *P. multocida* isolates of avian and porcine origin (Miflin and Blackall, 2001).

PCR assays amplify target sites of the organism's genome that may be responsible for their genetic diversity or similarity and are sensitive, rapid and reproducible with a high level of differentiation. Following the identification and sequence analysis of the capsular biosynthetic loci which are highly specific for each *P. multocida* capsular serogroup, a multiplex PCR assay has been designed to identify serological differences between strains (Townsend *et al.*, 2001). This assay provides an alternative to the present capsular serotyping system which tends to be rather laborious and often unable to produce definitive results. However, for accuracy and reliability, newly developed molecular typing techniques should be used in conjunction with other conventional methodologies with at least two distinct typing methods incorporated in any epidemiological study.

1.1.3. Virulence factors of *P. multocida*

P. multocida, in common with other pathogens, has the potential to colonise, invade the mucosal surface, proliferate within host tissues and evade the host defence system resulting in irreversible

damage to the host (Brown and Williams, 1985). Key virulence factors include lipopolysaccharide (LPS), capsule, fimbriae and outer membrane proteins (OMP) (Fig 1.1) and these factors, in combination with cellular and secreted host components, contribute to the characteristic clinical and histopathological changes associated with pneumonic disease (Confer, 1993). However, a better understanding of the chemical determinants of pathogenicity responsible for adherence, colonisation, invasion, and virulence in various hosts is required for the development of novel methods for treatment and control (Rimler and Rhoades, 1989).

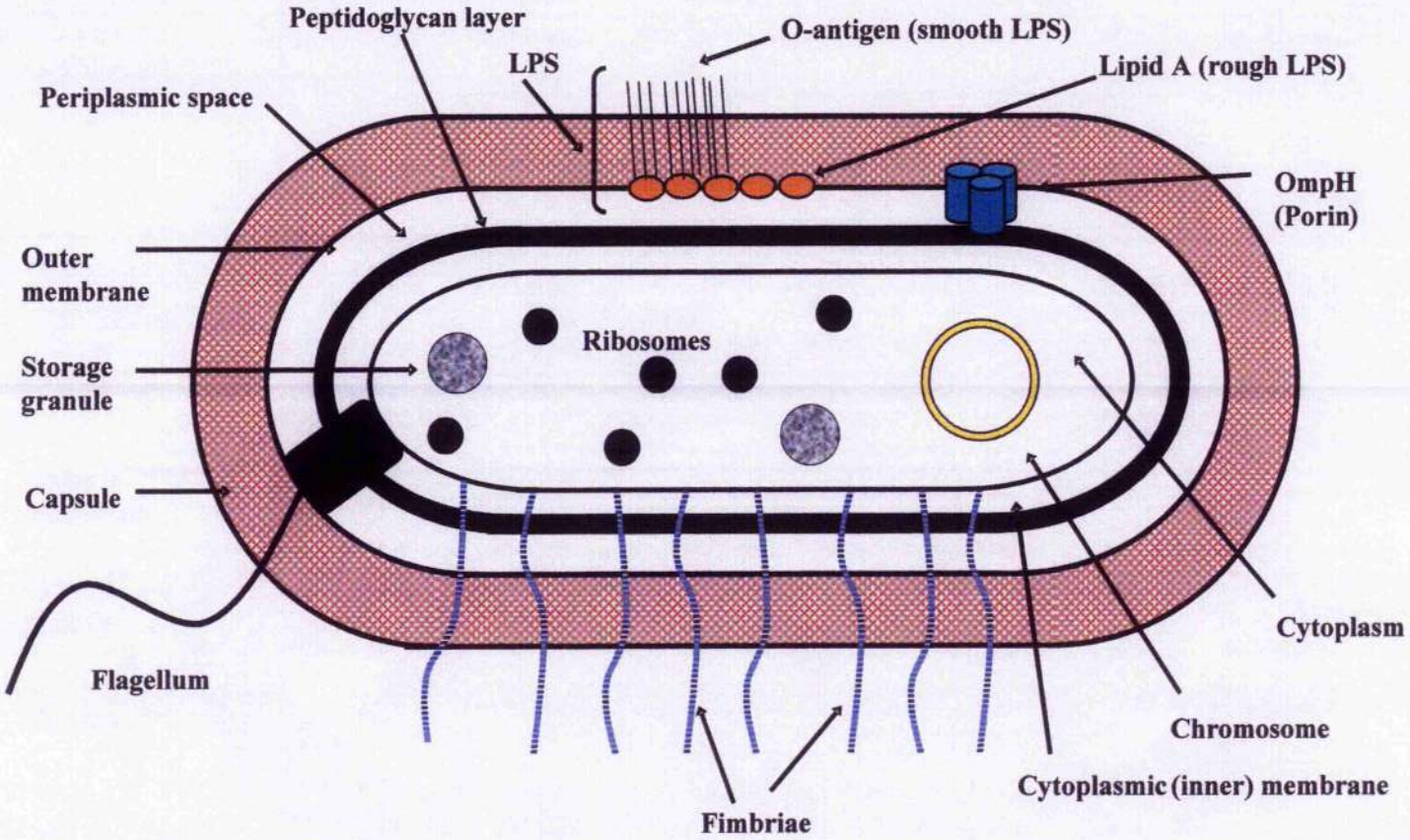


Fig. 1.1. A schematic diagram of a typical Gram-negative bacterium with surface virulence determinants of interest.

1.1.3.1. Lipopolysaccharide

LPS is an integral component of the outer membrane of Gram-negative bacteria. It forms a hydrophobic barrier which restricts the entry of digestive enzymes and certain antibiotics, and enables the bacteria to evade many innate host defence factors including complement, lysozyme and cationic proteins (Luderitz *et al.*, 1983; Brogden, 2000).

The structure of LPS is shown in diagrammatic form in Fig. 1.2. The outermost part of the LPS molecule consists of repeat units of covalently linked oligosaccharides, called O-antigen, that are structurally and antigenically diverse and play a major role in the serotypic classification system of *P. multocida* (Adler *et al.*, 1999). Internal to the O-side chains is the core oligosaccharide region that is structurally similar in common Gram-negative bacteria. To the core portion is linked a highly conserved lipid region, Lipid A that is embedded in the outer membrane of the bacterium and which is responsible for the toxicity of LPS, but which exerts its lethal effects only when the bacteria lyse. This general structure is typical of the smooth or S-form chemotype LPS and is typical of *Escherichia coli*. When S-form LPS is analysed by polyacrylamide gel electrophoresis and stained with silver a ladder pattern appears, with the number of rungs denoting the number of repeat O-antigen units. Mutants that have lost the ability to synthesise an O-side chain are described as rough (R-form chemotype) (Poxton, 1995).

1.1.3.1.1 Lipid A and O-side chains are determinants of virulence

An intact O polysaccharide is involved in the expression of virulence by a number of possible mechanisms. For instance the presence of an O-chain allows bacteria to adhere to certain tissues, resist serum killing and provide protection against damaging reactions with antibodies and complement. When rough forms are exposed to antibody, interaction with antigens in the core region enables complement to lyse the bacteria and contributes to a loss of virulence, as observed with rough *P. multocida* A:3 isolates (Confer, 1993). However many rough strains of bacteria can be protected from the lytic action of serum due to the presence of capsular polysaccharides (Poxton, 1995)

LPS plays a vital role in the interaction of the pathogen with its host; for example LPS may be involved in adherence leading to colonisation, resistance to phagocytosis, or antigenic shifts that determine the course and outcome of infection (Adler *et al.*, 1996). The injection of purified lipid A into experimental animals mimics the effects of LPS, causing an array of non-specific physiological reactions such as fever, intravascular coagulation, hypotension, shock and death (Cullor, 1992). Thus lipid A is the toxic factor and is able to act when released from the outer membrane during growth or following cell lysis as a result of killing by phagocytes, attack by the membrane attack complex of complement or by antibiotics such as tetracycline.

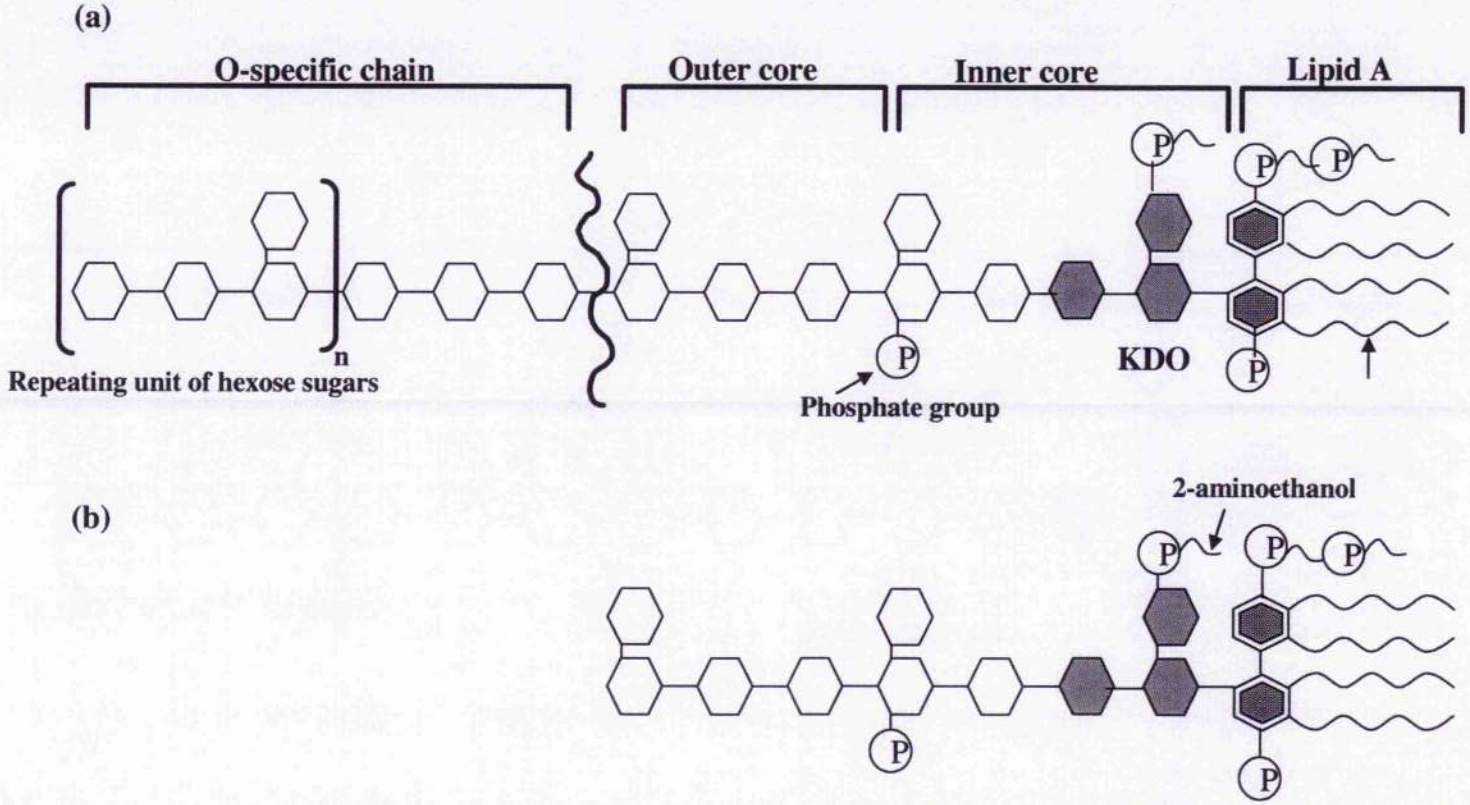


Fig. 1.2. Diagrammatic structure of LPS exhibiting both (a) smooth and (b) rough forms. KDO = 3-deoxy-D-manno-octulosonic acid

LPS released into the blood stream interacts with phagocytes to trigger the complement and coagulation cascade and release inflammatory cytokines, which constitute an integral part of the host defence against invaders.

P. multocida LPS has shown to be antigenic in birds and is essential for the development of an immune response against *P. multocida* infection in turkeys. However, exposure of cattle or rabbits to LPS from *P. multocida* has failed to confer protection against experimental exposure with *P. multocida* (Adler *et al.*, 1999). Nevertheless, an investigation using a bactericidal monoclonal antibody against LPS was found to completely protect mice against a homologous challenge of live *P. multocida* (Wijewardana *et al.*, 1990), though only partial protection was observed in mice using opsonic monoclonal antibodies to LPS (Ramdani and Adler, 1991).

The range and role of LPS chemotypes present in *P. multocida* have not been defined for both healthy and diseased animals. It is unknown whether *P. multocida* adopts the rough or smooth form of LPS, whereas the rough form of *M. haemolytica* LPS has shown to be more virulent than the smooth form on *in vitro* phagocytosis by ovine alveolar macrophages (Hodgson *et al.*, 2000). Such studies will elucidate the mechanisms of pathogenesis and assist vaccine development.

1.1.3.2. Capsule

The capsule of Gram-negative bacteria is located outside the outer membrane and is composed of hydrated polyanionic polysaccharides. Bacterial capsule plays a significant role in preventing the entry of particular molecules through the membrane, resists dehydration and mediates adherence of the bacteria to epithelial surfaces. In addition capsule promotes the survival and subsequent proliferation of the pathogen by interfering with opsonisation and phagocytosis and reducing complement-mediated killing (Boyce *et al.*, 2000). These observations indicate that capsule is likely to be a virulence determinant, and studies have shown that capsulated isolates of *P. multocida* in mice and piglets are more virulent than non-capsulated isolates (Jacques *et al.*, 1993). However, capsulated *P. multocida* strains were unable to adhere to respiratory tract mucus *in vitro* because the capsule may have masked any surface components (adhesins) involved in adherence. It has been demonstrated that capsules of most *P. multocida* strains express sialic acid units on their surfaces to mimic the sialyl-mucin layer coating epithelial cells, which is believed to have a nutritional function and which may be responsible for the ability of *P. multocida* to colonise and invade mucosal surfaces (Mizan *et al.*, 2000, Vimr and Lichtensteiger, 2002).

Growth conditions may also influence capsule production, as *P. multocida* grown under iron-depleted conditions *in vitro* or *in vivo* produces significantly less capsule and expresses a greater affinity for the respiratory tract mucus than cells grown in iron-replete conditions (Jacques *et al.*, 1994).

Aside from its association with virulence, capsule structure has been used to distinguish the serogroups of *P. multocida* for epidemiological studies (Rimler, 1994). Capsules are poor immunogens but once recognised by the host can provide an effective target for antibodies, thereby contributing to protection.

1.1.3.3. Fimbriae

Colonisation of host cell surfaces by Gram-negative bacteria is mediated by numerous adhesins, such as polymeric filamentous appendages known as type 4 fimbriae (Strom and Lory, 1993 and Abraham *et al.*, 1998). Type 4 fimbriae have been identified and characterised in serogroups A, B and D of *P. multocida* (Ruffolo *et al.*, 1997). An increase in expression of fimbriae in *P. multocida* was observed under the microaerophilic conditions that bacteria colonising the mucosal surfaces generally encounter (Ruffolo *et al.*, 1997). The ability of fimbriae to exist in large numbers tends to facilitate cell-cell or cell-substrate adhesion. The ability of fimbriated *P. multocida* serogroup A strains to adhere to host epithelial cells has been reported in rabbits (Glorioso *et al.*, 1982; Al-Haddawi *et al.*, 2000).

A type 4 subunit protein (PtfA) from *P. multocida* A:1 strain was isolated and characterised (Ruffolo *et al.*, 1997) and the *ptfA* gene has subsequently been cloned which may lead to work exploring its role as a candidate vaccine antigen (Doughty *et al.*, 2000).

1.1.3.4. Outer Membrane Proteins

OMPs of *P. multocida* can elicit protective immunity in experimental animal models (Confer, 1993). This has been demonstrated in rabbits whereby antibody responses were detected against 5 *P. multocida* OMPs (Lu *et al.*, 1988). Furthermore, rabbits were protected against homologous challenge following vaccination with *P. multocida* outer membranes and an antibody response was raised against OMPs (Lu *et al.*, 1988). A monoclonal antibody prepared against 37.5 kDa OmpH, protected both mice and rabbits against a live *P. multocida* challenge (Lu *et al.*, 1991a; Lu *et al.*, 1991b). However, Abdullahi *et al.*, (1990) found no correlation between protection in mice exposed to either a homologous challenge or to a 37.5 kDa OMP antigen from bovine isolates of *P. multocida*

The OmpH of *P. multocida* was identified as a porin (Chevalier *et al.*, 1993) and its potential to confer protection, as an immunogen was shown experimentally (Luo *et al.*, 1999). A study to characterise the effects of a major *P. multocida* porin on bovine neutrophils demonstrated an increase in functional activity of the cells as assessed by stimulation of chemotaxis and oxidative burst measurement (Galdiero *et al.*, 1998).

Several different OMPs of *P. multocida* type A:3 may stimulate an antibody response against the organism in cattle (Confer *et al.*, 1996) and OMP from serotype B:2 protected buffalo calves against a homologous challenge and could be used in vaccines against haemorrhagic septicaemia (Pati *et al.*, 1996). Vaccination studies in chickens showed that a cyclic synthetic peptide was able to mimic a region of the *P. multocida* OmpH and induce partial homologous protection (Luo *et al.*, 1999).

Following the construction of a genomic library for *P. multocida* A:1 and screening with rabbit antiserum against whole membrane preparation, a gene encoding the Oma87 OMP was cloned (Ruffolo and Adler, 1996) and exhibited 75 % amino acid identity with D15 protective OMP of *H. influenzae* (Flack *et al.*, 1995). Subsequently, Oma87 was found to be present in all 16 LPS serotypes of *P. multocida* and Oma87 homologues were found in a number of Gram-negative bacteria. The protective properties of Oma87 were demonstrated, whereby mice passively immunised with rabbit antiserum raised against recombinant Oma87 were protected against a lethal challenge dose of the homologous A:1 strain (Adler *et al.*, 1999; Hunt *et al.*, 2000). However, no protection was observed against a heterologous strain A:3 due possibly to the large amount of capsule in this strain masking Oma87 from antibody (Adler *et al.*, 1999). Recent findings have demonstrated also a lack of protection in chickens against challenge with a virulent *P. multocida* serotype A following vaccination with a GST-Oma87 fusion protein (Mitchison *et al.*, 2000). Further work is required to determine the expression of the protective epitopes of Oma87 and its role in immunity to pasteurellosis.

1.1.3.5. Iron-regulated proteins

Iron is an essential nutrient for most organisms through its central role in the metabolic electron transport pathways (Paustian *et al.*, 2001). However, *in vivo* the free ionic concentration of iron is too low to support microbial growth due to the presence of iron-binding glycoproteins such as transferrin and lactoferrin. Bacteria have addressed this problem by developing a mechanism of sequestering iron from these protein carriers (Ruffolo *et al.*, 1998). The first such system was identified in the supernatant of *P. multocida* A:3 grown in iron-depleted conditions and involved a growth-enhancing factor called multocidin that functioned as a siderophore, and acted as a low molecular weight iron chelating agent (Hu *et al.*, 1986). It was later shown that the same organism expressed high molecular weight iron-regulated OMP (IROMP) when grown under iron-restricted conditions (Ikeda and Hirsh, 1988). It was demonstrated that these IROMP, with a molecular mass ranging from 52 kDa to 99 kDa, were also expressed by bacteria grown *in vivo*, suggesting a possible role for linking the multocidin complex with the organism (Keumhwa *et al.*, 1991).

These novel antigens can raise an antibody response against both homologous and heterologous serotypes of *P. multocida*, a possible role for these antigens in cross-protective immunity (Choi *et al.*, 1989). Expressed OMP were later identified as the receptor proteins involved in the uptake and transport of the iron-siderophore complex across both outer and cytoplasmic membranes. It was reported recently that IR-OMP could induce cross-protective immunity in mice whereas OMP grown in iron surplus conditions induced protection only to a homologous challenge (Ruffolo *et al.*, 1998).

Finally, a mechanism of sequestering iron, independent of siderophore, has been identified and recently reviewed, (Ogunnariwo and Schryvers, 2001) and which involves the expression of a bi-partite receptor composed of two proteins TbpA and TbpB. Both proteins exist in most bacterial species; they are surface exposed, iron-regulated and bind transferrin (Ogunnariwo *et al.*, 1991). TbpB is a lipoprotein that binds directly to transferrin and TbpA is a member of a family of TonB-dependent transporters that removes iron from transferrin at the cell surface, where it then migrates into the cytosol of the Gram-negative bacteria (Zhao *et al.*, 1998). Previous studies have documented the presence of transferrin receptors in bovine *P. multocida* strains isolated from cases of pneumonia and haemorrhagic septicaemia (Ogunnariwo *et al.*, 1991; Veken *et al.*, 1996). A single novel receptor protein has been isolated from *P. multocida* strains with immobilised bovine transferrin (TbpA) that is able to acquire iron from bovine transferrin without the need for a second receptor protein (TbpB) (Ogunnariwo and Schryvers, 2001).

The surface proteins involved in the acquisition of host iron have the potential to make effective vaccine candidates as they are essential to the bacteria for overcoming the iron-limiting conditions and are accessible at the cell surface, facilitating the production of antibodies to these antigens. Since the discovery of *in vivo* expressed genes by Hu *et al.*, (1986), iron-regulated proteins (IRP) grown *in vitro* have been the major component of a successful vaccine against *M. haemolytica* infection in lambs (Gilmour *et al.*, 1991). A similar approach may be adopted for calves against *P. multocida* infection, except that with the development of genetic tools, certain genes controlling iron levels within the bacteria could be manipulated to disadvantage the pathogen.

1.1.3.6. Gene products associated with P. multocida virulence

Since the sequencing of the *P. multocida* genome (May *et al.*, 2001) it is now possible to identify genes that may play an indirect role in the pathogenesis of disease caused by this organism. Genes of particular interest with respect to a live attenuated vaccine strategy are *galE*, *recA*, aroA, *fur*, *exbB*, *exbD*, *tonB* and *hgbA*, all of which (except *galE*, *recA* and *aroA*) regulate intracellular iron concentration in the pathogen.

The *galE* gene product catalyses metabolism of galactose, located within the O-antigen of LPS, to glucose (Robertson *et al.*, 1993). Mutants of the gene grown in glucose were unable to produce wild-type LPS, indicating a role for this gene in the virulence of *P. multocida*. Reduced virulence was observed following intraperitoneal inoculation of mice with *P. multocida galE* mutants and led to the development of live attenuated vaccines to protect susceptible animals (Fernandez de Henestrosa *et al.*, 1997).

Studies on the *recA* gene have failed to confirm a specific role in the virulence of *P. multocida*, although a *recA* mutation led to delays in proliferation, which slowed the process of infection and thus reduced virulence (Cardenas *et al.*, 2001). There is potential for use of this *recA* mutant in the development of safe live attenuated vaccines, along similar lines with an aroA mutant which has shown to protect mice against an artificial infection of Haemorrhagic Septicaemia (HS) (Tabatabaei *et al.*, 2002). These mutants have shown to be more effective than inactivated vaccines.

In addition to iron, zinc is also required by bacteria (Ratledge and Dover, 2000) and a zinc-uptake system comprising *znuACB* genes has been identified and cloned in *P. multocida* (Garrido *et al.*, 2003). These genes have found to be under the control of the ferric-uptake regulator (*fur*) gene and are required for virulence of *P. multocida* in a mouse model (Garrido *et al.*, 2003). Moreover, *P. multocida exbB, exbD and tonB* genes regulate the uptake and transport of iron or haem from haemoglobin into the cytoplasm of the organism. These genes are transcribed independently, but all three are required for the organism to cause infection (Bosch *et al.*, 2002a). Finally, a gene product reported to bind haemoglobin *in vitro* was identified and the gene designated *hgbA*. This gene is regulated by iron and is present in all *P. multocida* strains irrespective of their origin or serotype (Bosch *et al.*, 2002b).

The positive association of these gene products with *P. multocida* virulence suggests their potential as targets for the development of safe vaccines and a better understanding of iron uptake and transport mechanisms.

1.2. Bovine pneumonic pasteurellosis

P. multocida is a pathogen affecting a wide range of species, causing serious diseases in man, animals and birds. The bacterium is the primary agent of a disease in cattle known as pneumonic pasteurellosis and which is of great economic significance in Britain and North America, where it has caused losses to the livestock industry for over a century (Dalgleish, 1990). Currently, the associated loss to beef and dairy industries caused by bovine pneumonic pasteurellosis (BPP) in the USA is over one billion dollars per annum (Weekley *et al.*, 1998) and in the UK the annual losses due to BPP and other respiratory infections amounts to over £30 million (Dalgleish, 1989). In the

UK pneumonic pasteurellosis is associated primarily with newly weaned calves after housing or transport to a new herd or premises and the peak incidence of BPP occurs between September and December when the bulk of the calf sales takes place (Dalgleish, 1990). BPP forms the main topic of this thesis and is discussed in detail below.

The primary isolates from clinical cases of BPP are *M. haemolytica* A:1 and *P. multocida* A:3. A decade ago *M. haemolytica* was considered the aetiological agent of BPP while *P. multocida* was isolated only occasionally (Frank, 1989). However, the latest data retrieved from Veterinary Investigation Diagnosis Analysis (VIDA, UK) for the period 1992-2001 indicate that the number of reported cases of BPP attributable to *P. multocida* was 35 % in 1994, with an increase from 42 to 51 % during 1998 to 2001. This data highlights the increasing prevalence of *P. multocida* in the development of BPP compared to that of *M. haemolytica* (Veterinary Laboratories Agency (VLA) 2001). Bennett *et al.* (1999) collated data from published and unpublished sources with regard to the occurrence of BPP and suggested that 10 to 15 % of all finishing beef cattle are at risk at any given time in the UK.

P. multocida is part of the commensal flora of the respiratory tract but is prevented from causing disease by the host's immune system, with a variety of defence mechanisms coming into play. The relationship between host and bacteria is stable in a healthy animal, however this stability can be altered by changing circumstances, such as a predisposing infection with a viral, parainfluenza virus-3 (PI-3) or bacterial agent (*Haemophilus somnus*) or stressful circumstances such as transport and environmental stimuli, that compromise the integrity of the antimicrobial barrier (Griffin, 1997). It is believed that once the immune system becomes compromised, *P. multocida*, along with other bacteria of its genera, may proliferate rapidly within the nasopharynx, migrate to the lung in aerosolised droplets and colonise to an unfavourable degree, causing bronchopneumonia. To date in the literature no distinction has been made between commensal and virulent strains and their involvement in the pathogenesis of disease. There is however, in the case of *M. haemolytica* A:1 evidence for the emergence of a more virulent population than a predominant less virulent *M. haemolytica* A:2 strain (Gonzalez and Maheswaran, 1993). It would be of interest to investigate if a phenotypic switch between commensal and pathogen could take place, from an identical genetic background resulting in the development of pneumonic pasteurellosis.

The clinical features of BPP include sudden dullness, pyrexia (up to 41.2°C), anorexia and laboured breathing often accompanied by adventitious respiratory sounds that may be heard on auscultation of the anterior pulmonary region. A mucosal discharge from the nostrils, mouth and eyes may be present. Calves that recover remain dull and anorexic for a period after recovery, causing significant economic losses due to retarded growth rates (Gibbs *et al.*, 1984).

At post-mortem (PM) the pulmonary lesion is an acute exudative fibrinous pneumonia confined to the anterior lobes, though in severe cases the pathology may be more extensive and may affect the caudal lobes (Jubb *et al.*, 1993). Areas of affected lung are dark red or purple in colour, consolidated, with areas of fibrinous pleurisy. Histopathologically there is an acute inflammatory reaction where the alveoli become inundated with neutrophils, macrophages and oedema. Subsequently, these pulmonary lesions became walled off by fibrous tissue and became infiltrated by plasma cells and lymphocytes. The interlobular septa are often enlarged with fibrinous exudates and inflammatory cells (Allan *et al.*, 1985).

Current methods of control in regions affected by BPP include managemental and prophylactic schemes. Management procedures involve minimising imposed stress, with weaning, vaccination and castration in place ahead of transportation to the market. Prophylactic schemes practiced by farmers in North America include the in-feed and in-water use of antimicrobials along with immunomodulators on cattle purchased from market (Gibbs, 2001). However, the practice of dosing cattle with antimicrobials as a preventative measure is controversial because of the perceived danger of the emergence of resistant *Pasteurella* spp. strains (Post *et al.*, 1991). Research on this matter is scant and is essential when considering the increase in prevalence of *P. multocida*. Furthermore, evidence for the reduced efficacy of antimicrobials has been demonstrated with oxytetracycline and ampicillin which were unable to remove *M. haemolytica* from the nasopharynx of 20 clinically healthy calves (Shoo *et al.*, 1990). A similar situation has not been demonstrated with *P. multocida*.

In the UK the prophylactic use of antimicrobials in an outbreak of respiratory disease is restricted to housed, in-contact at risk calves and aims to arrest the spread of infection. A study conducted by Jenkins (1985) found that antimicrobials were effective against actively dividing or pathogenic *Pasteurella* spp., indicating the merits of administering antibiotics at the earliest onset of disease. Treatment for BPP recommended by veterinary practitioners is oxytetracycline in conjunction with an anti-inflammatory drug flunixin meglumine.

The mixed success of antimicrobials in controlling BPP has focused research interests on vaccination. New vaccines against the disease caused by *M. haemolytica* A:1 and A:2 are based on inducing antibodies to IRP and these have been successful in providing an acceptable level of protection in sheep (Gilmour *et al.*, 1991). Surprisingly, there are no equivalent vaccines available currently for *P. multocida*-induced bovine pneumonic infection. Following intensified research over the past five years, progress in understanding the pathogenesis of pneumonic pasteurellosis caused by *P. multocida* has been made. The use of bronchoalveolar lavage to recover the cells involved in the first line of defence in the lower respiratory tract (Caldow *et al.*, 2001) has allowed preliminary studies of the interaction between *P. multocida* and the host defence system to be examined *in vitro*

(Mathy *et al.*, 2002). It is anticipated that these advances will provide the incentive to produce an effective vaccine targeted against *P. multocida* infection in cattle.

1.2.1. Host response to bovine pneumonic pasteurellosis

A successful pathogen possesses a variety of virulent characteristics that can either interfere with or even evade the host defence mechanisms. For us to understand the survival strategies of the bacterium we must increase our knowledge of the host protection system and this review will focus on the innate immune system that is relevant to the age of experimental animals used in this thesis.

The innate immune system of the lung comprises a hierarchical system of defence for the recognition, rapid removal and killing, of foreign agents. Each component of the system has a distinct role in the elimination of micro-organisms and in maintaining a sterile lung environment (Bienenstock, 1984). These defences include epithelial barriers, enzymes within saliva and tears, aerodynamic filtration by the nasal cavities, mucociliary apparatus, cough reflex and the inflammatory response that leads to fever, a mechanism by which the immune system inhibits the growth of the pathogen. These are highly effective first line defence mechanisms and prevent the colonisation of pathogens in the upper respiratory tract (URT) and their subsequent entry into the lower respiratory tract (LRT). Bacterial components such as LPS interact with host cells and trigger the release of cytokines and chemokines, which then orchestrate the innate cellular responses of the lung that include complement-mediated lysis, cytotoxicity, and phagocytosis (Tizard, 2000). An array of such responses communicating with each other will stabilise the antimicrobial responses against invading pathogens. Research has shown that these mechanisms are often responsible for the development of host tissue damage if they are not controlled in a co-ordinated manner.

1.2.1.1. Host defences of the upper respiratory tract.

The URT includes the nasal sinuses, pharynx, larynx, trachea and bronchi. Inhaled air encounters resistance to flow in the nasal cavity and induces a turbulent flow pattern. This phenomenon deflects and traps aerosolised particles larger than 10 μm in the inhaled air, against the nasal mucosal lining and these particles are eliminated subsequently by swallowing (Boyton and Openshaw, 2002). Foreign bodies greater than 5 μm but less than 10 μm often escape the defences of the nasal cavity and are deposited on a ciliated epithelial layer that extends from the nasal turbinate to the terminal bronchioles. Within this epithelial layer are glandular cells that are responsible for the release of mucus when stimulated by a turbulent flow of secretion, and trap the inhaled foreign material (Boyton and Openshaw, 2002). Cilia impede the adherence and entry of foreign agents into the LRT by beating in a co-ordinated fashion to propel the inhaled material towards the buccal cavity for swallowing (Zhang *et al.*, 2000).

For bacteria to overcome these host defences and cause tissue damage, attachment to host epithelial cells is vital and this is considered the first step in the production of disease. Recent findings have shown that *P. multocida* attaches to microvilli and the mucus layer of respiratory epithelium through fimbriae (Al-Haddawi *et al.*, 2000). Following attachment, it is thought that *P. multocida* and its toxic secretions rupture the cilia membrane resulting in the deciliation of epithelial cells. This decreases bacterial clearance, promotes colonisation and growth and the progression of disease (Al-Haddawi *et al.*, 1999).

1.2.1.1.1 Molecular responses to colonisation of the upper respiratory tract

Once the functional integrity of the epithelial layer has been surpassed by disruption and inflammation, the immunological barrier is unable to stem the infection. Additional defence mechanisms are required to maintain lung sterility and to prevent access of pathogens to the LRT. Surface secretions from the epithelium act to halt microbial activity and proliferation. These secretions include immunoglobulins (IgA being the most prevalent in the URT), proteases, defensins, cathelicidins, lysozyme, lactoferrin, surfactant proteins (SP-A and D) and lactoperoxidase which generates superoxide free radicals to kill bacteria (Ganz and Weiss, 1997). These antimicrobial factors have a beneficial role in the pathogenesis of pneumonic pasteurellosis and their response to epithelial compromise and microbial invasion will be discussed in this section.

Recent evidence has shown the expression of antibiotic peptides in epithelial cells, and suggests a role in host defence of mucosal surfaces. During a pneumonic episode two antimicrobial peptides belonging to the β-defensin family (Tracheal antimicrobial peptide (TAP) and lingual antimicrobial peptide (LAP) are produced by epithelial cells in the URT. TAP is expressed in the ciliated epithelium of the bovine trachea and was the first member of the β-defensin family to be discovered (Diamond *et al.*, 1991). Upon stimulation of tracheal epithelial cells (TEC) with bacterial LPS, TAP messenger ribonucleic acid (mRNA) levels increase dramatically. LAP has been isolated from squamous epithelial cells of the bovine tongue and its mRNA expression is elevated as a result of tissue injury and inflammation. The mechanism of antimicrobial activity of TAP and LAP is via selective membrane disruption of the pathogen (Russell *et al.*, 1996). Alterations in the expression or biological activity of these defensins can predispose the host to a microbial infection (Russell *et al.*, 1996).

Cathelicidins along with the defensins are members of the antimicrobial peptide family. They are located in epithelial cells and kill bacteria by permeating the LPS membrane (Brogden *et al.*, 2001).

Surfactant proteins are a family of collagenous carbohydrate binding proteins. SP-A-and-D are members of this family and they bind to carbohydrate and lipid moieties of pathogens. This

facilitates the removal of the pathogen from the URT by inhibiting adherence to epithelial lining, enhancing phagocytosis and killing. Lactoferrin is a glycoprotein that sequesters iron from the environment and is secreted from serous cells as well as from polymorphonuclear neutrophils (PMN). It functions by inhibiting bacterial growth by promoting iron-restricted conditions and agglutinating the bacteria (Skerrett, 1994). *P. multocida* has shown to be well equipped for overcoming the effects of lactoferrin and actually utilises this glycoprotein as a source of iron (Sparling, 1983). Lysozyme is present in serous cells of tracheal epithelium and inhibits bacterial survival by digesting the peptidoglycan membrane of the bacteria (Coonrod, 1986).

Immunoglobulins are present in respiratory secretions with IgA confined to the URT while IgG is more prevalent in the alveoli following a *M. haemolytica* infection (McBride *et al.*, 1999). This has not been demonstrated for *P. multocida*. IgA masks inhaled foreign material and protects the mucosa by preventing epithelial adherence, infiltration and by augmenting toxin neutralisation, which is reinforced by non-specific defence mechanisms. Specific IgG acts as an opsonin to promote the uptake of *P. multocida* by alveolar macrophages (Reynolds, 1991).

IgA may be destroyed by enzymatic degradation. Recent findings have reported the production of proteases by *P. multocida* from human pulmonary fluid and these exhibits a high affinity for the cleavage of the heavy chain of human IgA outside the hinge region. Moreover, this protease differs from those that cut at the hinge region, as seen with *Haemophilus influenzae* and *Streptococcus pneumoniae*, and suggests the discovery of a new virulence determinant specific for *P. multocida* (Pouedras *et al.*, 1992). The secretion of these proteases from *P. multocida* into culture medium has been found in isolates from sheep, cattle, pig and chicken (Negrete-Abascal *et al.*, 1999). These proteases exhibit properties identical to that of neutral metalloproteases, in that they show optimal activity at both pH 6 and 7, while their activity is inhibited by EDTA and reactivated by calcium (Negrete-Abascal *et al.*, 1999).

Evolution of proteases appears to be advantageous to pathogens as it enables them to avoid destruction and opsonisation by IgA and IgG by degrading these immunoglobulins. Research has been directed to other functions of proteases other than the cleavage of immunoglobulin molecules, to implicate their involvement in the pathogenesis of respiratory disease (Ohbayashi, 2002). Matrix metalloproteases (MMP) are a group of endopeptidases, dependent on zinc as a cofactor, able to degrade the extracellular matrix of lung tissue. Recent evidence has shown a role for macrophage-derived MMP (MMP-2) in the pathogenesis of pulmonary sarcoidosis (John *et al.*, 2002). Furthermore macrophages were found to secrete MMP-9 in a dose-dependent manner in response to a mycobacterial infection *in vitro* and the immunoregulatory cytokines interferon-gamma (IFN-γ), interleukin (IL) -4 and IL-10 suppressed the bacterial-induced MMP production by reducing the amount of tumour necrosis factor-α (TNF-α) available to the macrophages (Quiding-Jarbrink *et al.*,

2001). The precise mechanisms of lung injury caused by MMP are still unknown but it is believed that MMP facilitate the migration of inflammatory cells and extracellular matrix remodelling (Ohbayashi, 2002). Much attention is being directed towards possible methods of inhibiting such functions of MMP.

1.2.1.2. Host defences of the lower respiratory tract

The LRT consists of the bronchioles and alveoli suspended in connective tissue. Particles less than 5 μm can penetrate to the LRT, as discussed previously. In most cases, invading bacteria are cleared from the alveoli efficiently and quickly, but when the host is immunocompromised the ability of foreign invaders to colonise and proliferate within the URT highlights the importance of the LRT to acquire its own defence mechanisms to contain the spread of such foreign material and minimise the irreversible damage to lung tissue. The extent of pathogenesis is highly dependent on the location and amount of deposited virulent particles. Study of the progression of events within a diseased lung of an experimental animal may lead to a better understanding of host-microbial interaction and improved methods of control (Bienenstock., 1984).

1.2.1.2.1 Cell recruitment following lung injury

Once the bacteria have reached the stage of invasive growth within the lung, the host initiates a cascade of events constituting the inflammatory response. This disrupts the physiological and metabolic equilibrium and causes behavioural and pathological changes (Kushner and MacKiewicz, 1987). Inflammation can be injurious to the host and is subject to multiple levels of control involving a diverse array of cells including vascular endothelial cells, neutrophils, alveolar macrophages, pulmonary intravascular macrophages, airway epithelial cells and soluble mediators such as IL-1, IL-6 and TNF-α.

Within minutes after tissue injury, as may occur during pneumonic pasteurellosis, vasodilation of the microcirculation within the affected region occurs while neutrophils and monocytes attach themselves to the endothelial cells lining the small blood vessels before migrating out of the blood into the tissue spaces. Adhesion molecules expressed by endothelial cells are, to a large extent, responsible for assisting neutrophils to migrate to loci of inflammation. This expression is triggered by products of Gram-negative bacteria, including *P. multocida*, such as LPS, that activates monocytes in the circulation to attract neutrophils by forming a complex with LPS binding protein (LBP). This complex binds to a CD14 membrane receptor on the surface of the phagocyte and initiates a signal for the release of TNF-α through a Toll-like receptor 4 (Tlr4) (Beutler *et al.*, 2001). Other products include molecules produced as a result of damaged tissue, such as thrombin, histamine and IL-1. The cytokines cause endothelial cells to express the adhesion molecule, E-

selectin, while thrombin and histamine are responsible for the enhanced expression of P-selectin (Kerr, 1999).

Mucins, such as L-selectin, are expressed on the surface of circulating neutrophils and aid the transient binding of neutrophils to endothelial cells via attachment to P-selectins (Crockett-Torabi, 1998). This initial adherence is relatively unstable, but it attracts and slows the inflammatory cells so that they begin to roll along the endothelium in the direction of blood flow. A second phase of adhesion then occurs when chemoattractants, such as IL-8, platelet activating factor (PAF) prostaglandins, leukotrienes and other cytokines are released and act upon the neutrophils, releasing a G-protein-mediated transduction signal that enhances the expression of and initiates a conformational change in the integrin CD11a/CD18 on the surface of the leukocyte (Ackermann and Brogden, 2000). This conformational change results in increased affinity for its ligand ICAM-2 expressed on endothelial cells, making the neutrophils stop and bind firmly to the endothelial cells (Coomber *et al.*, 2001). Once tight adherence has been achieved the cells cross the vascular basal lamina and infiltrate the tissue affected, for example the alveoli. This process is called diapedesis and is mediated by platelet endothelial cell adhesion molecule-1 (PECAM-1), which is expressed at intercellular junctions of endothelial cells (Furie and Randolph, 1995). The respiratory epithelial cells lining the bronchioles express ICAM-1 and this mediates the final stage of neutrophil infiltration (Radi *et al.*, 1999).

Paradoxically, neutrophils contribute to tissue damage by releasing elastase, hydrolases, cytokines, oxidative free radicals and chemokines, which are required for the phagocytic killing of pathogens by neutrophils. However, neutrophils can become apoptotic and have shown to play a vital role in the resolution of inflammation induced by *M. haemolytica* by limiting the autotoxic potential of the neutrophil (Chin *et al.*, 2000). To date these is no evidence for the beneficial effects of apoptotic neutrophils in the resolution of inflammation induced by *P. multocida*.

1.2.1.2.2 Phagocytes

The phagocytic cells of mammals belong to two complementary systems: the myeloid system consisting of cells that act rapidly but are unable to sustain their effort, for example PMN and the mononuclear-phagocytic system that includes monocytes/macrophages which acts slowly but is more sustained. Granulocytes of the myeloid system have characteristic lobulated, irregular nuclei and fall into three types; neutrophils, basophils and eosinophils, according to the staining of their cytoplasmic granules, lysosomes which contain an array of lytic enzymes and other proteins. Neutrophils are the major cell of the myeloid system and the granulated cytoplasm takes up neither basic nor acidic dyes. They arise in the bone marrow and are continuously released into the blood at a specific stage of maturity, where they will circulate for 7-10 h. Cattle tend to have a lower percentage of neutrophils, approximately 20 to 30 % in the peripheral blood, compared to 50-70 %

found in other species. They are cells with a short life span of 3 days once they have migrated into tissues, with a multi-lobed nucleus that distinguishes them morphologically from other granulocytes, and possess an array of cytoplasmic granules, which store a variety of chemicals for the degradation of ingested material. The neutrophil granules are of two types: a primary granule contains lysozyme, myeloperoxidase, elastase and cathepsin B and a secondary specific granule contains lactoferrin, lysozyme and collagenase. A unique trait of ruminant neutrophils is the presence of a third type of cytoplasmic granule, larger than the primary granule that is known to contain cationic antibacterial peptides. These peptides possess bactericidal properties for specific bacteria, for example indolicidin has antimicrobial activity for *E. coli* and *S. aureus* (Selsted *et al.*, 1992). However, the selectivity of these peptides for *P. multocida* remains unknown. Furthermore, bovine neutrophils are unusual when compared to other species; for example they have Fc receptors (FcR) for IgM that can act as an effective opsonin. They are chemotactically attracted to certain products of complement activation (C3b). Neutrophils also contain an abundance of glycogen granules, used to supply glucose during glycolysis, enabling the cell to function in anaerobic conditions.

The second type of phagocytic cell, the macrophage belongs to the mononuclear cell lineage, characterised by a single rounded nucleus, and is capable of sustained phagocytic activity. While some remain motile as circulating monocytes in the blood, others migrate into particular tissues and differentiate into tissue specific macrophages. Those located within alveoli are alveolar macrophages and they act as a first line of defence for the removal of foreign material. They process the antigen or inhaled foreign material for presentation to T-cells in association with major histocompatibility complex (MHC II) molecules. In addition they relay signals to other cells in response to foreign agents by the release of an array of cytokines including IL-1, IL-6 and TNF-α. Furthermore, they repair tissue damage by removing dead or damaged tissue and thereby assist the healing process (Tizard, 2000).

1.2.1.2.3 Phagocyte recognition of pathogen

For phagocytosis to occur binding between phagocyte and pathogen must occur and there are two basic mechanisms that are either opsonin-independent or opsonin-dependent. In the absence of opsonins, Ofek *et al.*, (1995) summarised three modes of recognition between the pathogen and the phagocytic cell that would result in phagocytosis.

1.2.1.2.3.1. Opsonin-independent phagocytosis

The first process of phagocytosis occurs by hydrophobic interactions between molecules on the surfaces of the pathogen and phagocytic cells. The second form involves protein-protein interactions through a tripeptide motif, RGD (arginine-glycine-aspartic acid) located in micro-organisms more often associated with *Yersinia* sp. invasin and *Bordetella pertussis* filamentous

hemagglutinin. This RGD motif promotes these bacteria to associate with macrophage adhesion molecules ICAM-1, leading to phagocytosis (Saukkonen *et al.*, 1991). The final form of opsonin-independent mediated phagocytosis is lectinophagocytosis, which involves recognition between bacterial surface lectins and carbohydrates on the host cell (Ofek and Sharon, 1988).

Fimbriae type 4 identified in all capsule types of *P. multocida* is an example of a bacterial protein lectin that facilitates the binding of bacteria to phagocytes by attaching to sugar residues on the host cell surface (Al-Haddawi *et al.*, 2000). A further example of a lectin is the mannose receptor, specific for all tissue macrophages examined, including alveolar and hepatic macrophages. It is a type-I transmembrane glycoprotein consisting of a single subunit, a short cytoplasmic tail and 8 carbohydrate-binding domains. This receptor expresses a high affinity for oligosaccharides located on the surface of microbes, such as *K. pneumoniae* (K21a), that express capsular polysaccharides presenting the following sequence, Man-α2/3-Man. This form of recognition of encapsulated bacteria inevitably results in endocytosis of pathogen and stimulation of respiratory burst (Keisari *et al.*, 1997). Protein lectins are thought to play a major role in the host response against infection and the development of innate immunity. Even though they facilitate the engulfment of pathogens, this may be beneficial for the microbe. For example the fungus *Cryptococcus neoformans* survives and grows readily after phagocytosis by human macrophages but is killed by the chymotrypsin-like cationic proteins and oxygen-dependent system of human polymorphs (Chaturvedi *et al.*, 1996).

Studies on lectinophagocytosis (Ofek and Sharon, 1988) have shown its inefficiency in bacterial recognition when compared to opsonin mediated phagocytosis. This mode of phagocytosis could have evolved as a result of bacteria evading the lectin mediated interactions. Varieties of opsonins exist within serum and lymph fluid and include naturally or acquired immunoglobulins, complement components, acute phase proteins (APP) and pulmonary surfactant proteins.

1.2.1.2.3.2. Opsonin-dependent phagocytosis

When host cell and pathogen are suspended in milieu they tend to have a negative charge due to the presence of surface polymers such as oligosaccharides, lipids and protein, and will tend to repel each other (Patrick and Larkin, 1995). Therefore, for binding to occur it is essential that the negative charge on the bacterium is neutralised by a positively charged protein. Examples of such positively charged proteins include pulmonary surfactant protein-A (SP-A), APP, LBP and mannose-binding protein (MBP), all of which opsonise the polysaccharide surface of bacteria providing it contains mannose in the O-antigenic region of LPS. For example, *Salmonella enterica* group B, D1, D2 and E1 contain an O-antigen with a repeating unit of 1-galactose and 4-mannose residues (Curd *et al.*, 1998).

However, antibodies are by far the major proteins of the immune system and are the most effective opsonins, coating bacteria and linking them to the CD32 (FcγRII) receptor located on the surface of neutrophils and macrophages. Bovine macrophages possess a unique Fc-R called Fcγ2R which binds bacteria coated with IgG2. FcγR I, II, III bind to the Fc portion of IgG and this ligand binding between pathogen and host cell induces receptor cross-linking, stimulating the *src* family kinases to phosphorylate tyrosine residues within the immunoglobulin tyrosine activation motif (Nakamura *et al.*, 2002).

Another opsonin of great importance is C3b, the third component of the complement system. Complement activation is triggered by the presence of antigen-antibody complexes on cell surfaces and leads to the proteolytic cleavage of complement components. The complement receptor CD35 (CR1) is common to both macrophages and neutrophils, but an additional CD11b/CD18 (CR3) complement receptor is expressed by macrophages. These receptors are able to recognise the corresponding complement molecule present on microbes that have reduced surface charge facilitating attachment to the negatively charged phagocytes (Carroll, 1998). However, the binding of opsonised bacteria to receptors CR1 and CR3 fails to trigger the respiratory burst and is a preferred route for the bacteria and are able to thrive within a phagocytic cell. This may explain in part the observation that *M. haemolytica* can survive in alveolar macrophages for a limited period *in vitro* (Hodgson *et al.*, 2002). At present, there is no information available with regard to the ability of *P. multocida* to survive phagocytosis.

1.2.1.2.4 Uptake of the pathogen

Adherence of the pathogen stimulates the phagocyte pseudopodia to fuse and enclose the bacterium within a membrane bound vacuole called a phagosome (Tizard, 2000). This process is thought to occur by the polymerisation of actin and is followed by a signalling cascade initiated by phosphorylation of tyrosine residues and mediated by protein kinase-C (PK-C). A major substrate of PK-C is a membrane-associated protein called myristoylated alanine-rich C kinase (MARCKS) that regulates the structure of the actin filaments at the membrane anchored to a skeleton of microtubules in the cytoplasm by cross-linking with F-actin and Myosin-I (Allen and Aderem, 1995).

Once the pathogen has progressed towards the interior of the phagocyte and fused with the lysosome granules to form a phagolysosome, the lysosome granules release their destructive agents, such as oxygen free radicals, hydrogen peroxide, lysozyme, hydrolytic enzymes and peroxidase into the vacuole. In addition, carbohydrate, phosphate or sulphate groups removed from molecules fuse with the phagosome and the pH becomes more acidic, activating the acid hydrolases. It is fortunate that hydrolases are only active at pH 5 because if they were to leak out into the cytoplasm of the host cell which has a pH of 7.2, they would be likely to damage the cell. A

hydrogen ion ATPase is found in the membrane of the lysosome to maintain the acidic environment. Once the granules release their reactive intermediates the oxygen-dependent and independent killing may commence.

1.2.1.2.5 Oxygen-dependent killing mechanisms

There are two oxidative-generating pathways, one found in macrophages and the other in neutrophils, that facilitate bacterial clearance and which could result in lung injury if uncontrolled. These pathways are slightly different with respect to the toxic agents produced.

1.2.1.2.5.1. The macrophage-associated nitric oxide mechanism

Production of O_2^- allows for its intercalation with the nitric oxide (NO) pathway. LPS, TNF-α, IL-1 and IFN-γ bind to macrophages, triggering transcriptional induction of inducible NO synthase (iNOS) through Tlr. This event occurs only within alveolar macrophages or type II epithelial cells. The enzyme iNOS mediates the conversion of L-arginine into NO and citrulline. At this point O_2^- facilitates the conversion of NO into an array of reactive nitrogen intermediates (RNI) possessing potent antimicrobial activity, and which can lead to acute lung injury. Evidence for this was forthcoming when experimental rats were given an intravenous injection of LPS in order to produce acute endotoxemia. A time-dependent increase in expression of iNOS mRNA was induced in the lung and reached a peak 24h p.c, (Wizemann *et al.*, 1994).

An experiment was carried out to assess *M. haemolytica* LPS induced NO production, its role in BPP and to reduce lung injury by blocking the action of TNF-α, IL-1, IFN-γ and L-NMMA, *in vivo* (Han Sang Yoo *et al.*, 1996). This investigation showed that the antimicrobial activity of NO in alveolar macrophages was better than other RNI. There is evidence that *Mycobacterium avium* can survive attack by NO because it is able to produce superoxide dismutase with manganese as a cofactor. To investigate the bacterium's mode of survival, synthetic manganese containing a superoxide dismutase mimetic (SODm) M40403 has been designed for the specific removal of O_2^- (Salvemini *et al.*, 2001). To date, no information exists with regard to the ability for *P. multocida* to survive attack by host reactive intermediates.

1.2.1.2.5.2. Respiratory burst associated with neutrophils

Once the phagocytes have been exposed to a foreign stimulus they consume a large amount of oxygen as they generate the respiratory burst. This accompanies the capture of the pathogen and is required for killing rather than the process of phagocytosis (Mims, 1982). The oxygen is consumed by the phagocyte in the end reaction of glucose oxidation through the hexose monophosphate shunt, during which the addition of a single electron to oxygen, mediated by the activation of

plasma membrane-bound NADPH oxidase, results in the production of superoxide anions (O_2^-) (Lohmann-Matthes *et al.*, 1994).

$$O_2 + NADPH \rightarrow (NADPH\ oxidase)\ O_2^- + NADP^+ + H^+$$

Oxide radicals may be produced also by other routes not involving NADPH oxidase (Wang *et al.*, 2000).

The catalytic activity of superoxide dismutase generates hydrogen peroxide (H_2O_2), from which potent oxidant species may be produced by either of two methods. The first involves an enzyme myeloperoxidase (MPO), which is released after phagocytosis into the phagolysosme forming a complex with H_2O_2 and which has the potential to react with chloride ions. This oxidation reaction will produce an array of toxic, longer lasting antimicrobial agents such as hypochlorous acid that either attack directly the pathogen or react with nitrogen intermediates to form highly reactive compounds such as chloramines (Shepherd, 1986).

The second method for the conversion of O_2^- into toxic free radicals adopts a non-enzymatic approach and occurs in macrophages, neutrophils and monocytes. O_2^- reduces Fe^{3+} in ferritin compartments to create Fe^{2+}, which is highly reactive and catalyses the conversion of H_2O_2 into a hydroxyl radical, at the same time regenerating Fe^{3+}. Individuals suffering from chronic granulomatous disease have defective reactive oxygen intermediate producing systems and are more prone to bacterial infections. Anti-oxidants such as glutathione, vitamins E and C, catalase and β carotene protect the phagocyte from attack by free hydroxyl radicals by scavenging these radicals or reducing them to water.

The respiratory burst is abolished by the action of macrophage deactivating factor (MDF) or transforming growth factor-β (TGF-β). MDF suppresses the respiratory burst by 50 % and the suppression remains at this level regardless of any attempt to trigger it artificially, for example by the use of phorbol myristate acetate (PMA) (Li *et al.*, 2000) or LPS (Jian *et al.*, 1995). Treatment with factors TGF-β1 and TGF-β2 produces a result similar to that of MDF except that the suppressive effect can be eliminated by IFN-γ or TNF-α (Nathan, 1991). The mechanism of deactivation of the respiratory burst by MDF or TGF-β remains elusive but research has been directed on changes in the levels and translocation of NADPH oxidase components.

Quantification of the respiratory burst in lung phagocytes using luminol-dependent chemiluminescence (LDCL) has been used to examine the effects *in vivo* and *in vitro* of exposure of lung cells to PI-3 or *M. haemolytica*. Ovine alveolar macrophages challenged with either PI-3 or *M. haemolytica* exhibited an increase in light emission in this system indicating an increased

activity in respiratory burst (Hodgson *et al.*, 2002). The highest chemiluminescence (CL) responses were recorded from lambs exposed to *M. haemolytica* 6 days after PI-3 exposure, the group of lambs that had the poorest pulmonary clearance. Overall, CL responses were higher in lambs where bacterial proliferation occurred than in those lambs that controlled the infection (Davies *et al.*, 1986). Similar phagocytic studies are needed with *P. multocida* to determine the efficiency of phagocytosis and the relationship with BPP caused by this pathogen.

1.2.1.2.6 Oxygen-independent killing mechanisms

There are a number of possible mechanisms for the killing and digestion of microbes that do not require the presence of oxygen. Immediately after phagocytosis the pH level within the phagosome decreases to approximately 3, and creating a hostile, unfavourable environment for the pathogen. During the phagolysosome generation pathway a number of granules located within the cytoplasm of neutrophils and macrophages fuse with the phagosome and release their antimicrobial contents into the vacuole (Spitznagel and Shafer, 1985).

Lactoferrin and transferrin are examples of iron chelators found in specific granules of polymorphs and serum, respectively, that restrict the growth of bacteria and facilitate rapid destruction (Jacques *et al.*, 1994). However, some bacteria have developed ways of obtaining iron from such chelating agents. One such microbe is *Listeria monocytogenes* which produces a reductant that reduces Fe^{3+} in lactoferrin and transferrin to Fe^{2+}, thereby releasing the sequestered iron (Patrick and Larkin, 1995).

It has become clear over the past decade that cationic peptides form a significant part of the oxygen-independent antibacterial system in phagocytes. Defensins (α and β) and cathelicidins are two important cysteine-rich peptides that contribute to innate immunity and anti-microbial defence by binding to the bacterial cell membrane and neutralising the virulent effects of the bacteria (Yang *et al.*, 2001). These peptides have been subjected to crystallisation and nuclear magnetic resonance and exhibit distinct hydrophobic and charged regions that allow them to permeate the phospholipid membrane and stimulate bacteriolysis. This property is attributed to 6 cysteines of each peptide that form a circular molecule, stabilised by intramolecular disulphide bonds and creating ion-permeable channels in the membrane (Patrick and Larkin, 1995). Their broad-spectrum bactericidal effects are non-specific and apply to Gram-negative and Gram-positive bacteria as well as some enveloped viruses. These effector molecules have been implicated also in the release of chemokines (IL-8) and degranulation of mast cells, providing evidence for their involvement in the inflammatory response.

Resistance to the antimicrobial effects of defensins has developed in some Gram-negative bacteria, for example *Salmonella typhimurium* is able to survive and replicate within host cells through the

expression of PhoQ, a sensor kinase that responds to changes in pH, ion concentration and osmolarity (Gunn and Miller, 1996).

1.2.1.2.7 Bacterial evasion of phagocytosis and survival

Pathogens such as *Neisseria* spp. have developed strategies of surface antigenic variation to avoid recognition by antibodies and to avoid phagocytosis via the FcγR. Another way that bacteria can avoid phagocytosis is to alter the Fc region of IgG and preventing the protein from interacting with the bacteria. This phenomenon has been identified in *Staphylococcus aureus* (Celli and Finlay, 2002). Although, neither survival mechanism has been demonstrated for *P. multocida*.

Reports have suggested that organisms, including *N. meningitidis* and *S. aureus*, are able to interfere with complement deposition, thus preventing phagocytosis. For example, capsule made from surface polysaccharide may protect against complement-induced bacterial killing by preventing complement from binding to the bacterial membrane. In addition, bacteria expressing smooth LPS can also deter complement-mediated lysis of bacterial cell membrane (Lindahl *et al.*, 2000).

In addition to surviving within phagocytes, some bacteria have developed the ability to escape from the phagocytic vacuole into the cytoplasm or to prevent phagolysosomal fusion. The former has been discussed above with reference to *S. typhimurium* and resistance to cationic antimicrobial peptides. *L monocytogenes, Shigella flexneri* and *Rickettsia conorii*, but not *P. multocida*, are able to polymerise actin into a tail-like structure in order to allow them to migrate out of the phagolysosome and into the cytosol of infected phagocytes (Gouin *et al.*, 1999 and Goebel and Kuhn, 2000). It is assumed that all bacteria that gain access to the cytosol are able to survive and replicate.

Bacteria are able to flourish intracellularly by preventing phagolysosomal fusion; this protects them from reactive oxygen intermediates and some oxygen-independent compounds. *Mycobacterium tuberculosis* is often coated with antibody to entice phagocytosis which prevents phagolysosomal fusion from occurring, allowing bacteria to multiply. It is believed that some mycobacterial products, such as sulphatides, interact with the lysosomal membrane, reducing its mobility within the cytoplasm and thus reducing the chance of phagosome and lysosome combining (Britton *et al.*, 1994). In contrast, molecules present in the wall of *Chlamydia psittaci* interfere with the phagosome membrane and prevent fusion (Moulder, 1991). Although information is lacking on *P. multocida*, *Haemophilus somnus*, an organism with similar attributes to *P. multocida*, is able to survive within bovine macrophages and neutrophils by removing H_2O_2, and inhibiting the respiratory burst. In addition, it is capable of resisting nitric oxide mediated killing (Gomis *et al.*, 1997) although the mechanism for this has not been identified.

1.2.1.2.8 Systemic acute phase response and molecular components of the lower respiratory tract

This phase of the host response following a bacterial infection is associated with pain, fever, endocrine and metabolic changes and alterations in serum protein concentrations, characterised by production of liver derived APP such as haptoglobin (Hp), serum amyloid A (SAA), α_1-acid glycoprotein (AGP). Cytokines, namely IL-1, IL-6 and TNF-α produced by monocytes and macrophages activated by bacterial products, are responsible for mounting the systemic acute phase response (APR).

The APR is a beneficial reaction designed to prevent further damage to the site of injury. It acts to restrict the growth of and aids elimination of the infective agent, and to remove damaged tissue and activate the repair process, thus re-establishing homeostasis. The APR is essentially a non-specific immune response, and is succeeded by a specific immune response (Hirvonen, *et al.*, 1999). Under the influence of IL-1 and TNF-α and in particular IL-6, hepatocytes increase the synthesis of APP, a group of plasma glycoproteins, the concentration of which increases by greater than 25 % in response to tissue damage. The rate of increase depends on the protein and also varies among species. APP can function as either mediators, inhibitors, modulators, scavengers and immunomodulators during inflammation.

Plasma APP profiles differ between animal species. The major bovine APP associated with different pathological conditions include Hp and SAA, while AGP is a moderately responding APP. These proteins have shown to be sensitive markers for inflammation in cattle (Horadagoda *et al.*, 1999). Bremner (1964) was the first to document that the concentration of Hp, a protein that is virtually undetectable in the plasma of healthy calves, can reach approximately 1 mg ml^{-1} during inflammation. He also recognised that serum Hp levels could decrease during haemolysis due to an increased uptake of Hp-Haemoglobin complexes by the hepatic system where they are subsequently recycled, (Tizard, 2000) and that this should be taken into account when interpreting serum Hp levels. Spooner and Miller (1971) reported that Hp-Haemoglobin complexes were detected in only 0.6 % of clinically healthy cattle, but were found in most cows diagnosed with bacterial infection. The main antimicrobial property of Hp is due to this complex formation that renders iron present in haemoglobin unavailable for bacterial proliferation. Hp detection, based on its ability to bind haemoglobin, is a useful diagnostic test for the detection of inflammatory disorders (Eckersall *et al.*, 1999).

Bovine serum amyloid A (b-SAA), a precursor of amyloid protein A, increases 100-fold in cattle inoculated experimentally with *E. coli* LPS (Boosman *et al.*, 1989). Other experimental studies have measured the SAA response in cattle following inoculation with *M. haemolytica* or *P. multocida* (Horadogoda *et al.*, 1993, 1994) and after physical stress in calves (Alsemgeest *et al.*,

1993). From these studies SAA was judged to be a sensitive APP, reacting faster than Hp following a bacterial infection (Gruys *et al.*, 1993; Horadagoda *et al.*, 1994). Serum levels of b-SAA can be determined by ELISA (Boosman *et al.*, 1989; Horadagoda *et al.*, 1993) and, with a commercial assay now available, b-SAA is another useful marker of disease in cattle.

Plasma concentrations of AGP, a constitutive plasma protein, rise slowly following tissue injury in cattle (Conner *et al.*, 1988). Although AGP is an abundant protein, its physiological significance is not fully understood. Inhibition of neutrophil function, transport of molecules through the endothelial layer and platelet aggregation has been reported, though it is not known to possess any immunoregulatory functions (Hochepied *et al.*, 2000). Concentrations of AGP are known to increase in several cattle diseases including pneumonia, mastitis, arthritis and following subcutaneous inoculation of *M. haemolytica* in calves (Walker *et al.*, 1994). Bovine AGP is quantitatively analysed using a single radial immunodiffusion method (Tamura *et al.*, 1989).

Studies focused on antimicrobial peptides, adhesion molecules and metalloproteases have opened prospective avenues for determining the microbicidal activities of the respiratory tract. Further research on host-pathogen interactions is required to enhance our knowledge of the pulmonary host defence system against pathogens and to provide novel treatment and control for such microbicidal activity.

1.2.2. Experimental ruminant models for the induction of bovine pneumonic pasteurellosis

Over the past three decades *M. haemolytica* has been found to be the most frequent *Pasteurella* spp. isolated from the lungs of ruminants affected with BPP. Most investigations on the design of experimental models have therefore been focused on this species of pasteurella.

Experimental reproduction of pneumonic pasteurellosis in ruminants tends to be expensive, partly due to the requirement that animals are free from *Pasteurella* spp. and is a limiting factor. This was shown following a survey into the prevalence of *M. haemolytica* in sheep, when it was found that 95 % of the conventionally reared sheep were found to carry *M. haemolytica* in their tonsils and 64 % carried bacteria in the nasopharynx (Gilmour *et al.*, 1979). Most of these animals had either passive immunity or immunity as a result of natural exposure to the organism, with possible implications for the consistent reproduction of disease for research purposes. As a result of these findings specific-pathogen free animals have been used for the characterisation of *M. haemolytica* infection and these have been invaluable in the analysis of pathophysiological responses and the testing of vaccines specific for this organism in a controlled environment (Sharp *et al.*, 1978; Gilmour *et al.*, 1979). The identification of IRPs, as primary and novel antigens produced by the

bacteria *in vivo* (Gilmour *et al.*, 1991) has proven to be a major advance in the search for a vaccine against *M. haemolytica* infection.

In the past decade *P. multocida* types A and D have become more frequently associated with pneumonic pasteurellosis in ruminants. It is important therefore to develop a suitable, model that will reproduce the disease seen in the field, in order to study the pathogenesis of infection, analyse the efficacy of immunisation and to develop new methods of control. Many of the experimental models developed at the Moredun Research Institute (MRI) for control of *M. haemolytica* infection would assist the development of a calf model for *P. multocida*.

The choice of method and route of inoculation in experimental studies have major consequences for the desired results (Jericho, 1987). Stress factors such as weaning, transportation, dehorning, handling, castration and accompanying predisposing agents such as mycoplasmas, viruses and bacteria play a major role in the production of pneumonic pasteurellosis and will often exacerbate the response.

1.2.2.1. Models using natural routes of infection

Various investigators have induced experimentally pneumonic pasteurellosis in calves using diverse strains of either *P. multocida* or *P. multocida* in conjunction with *M. haemolytica* or with a virus as a predisposing agent.

Pneumonic pasteurellosis in calves aged 2-6 months of age was produced with a combination of SF-4 bovine strain of myxovirus parainfluenza-3 and *P. multocida*. The viral inoculum, $5x10^7$ $TCID_{50}$, was administered intranasally, a natural route of infection, and involved atomisation of the inoculum into the nostrils through a hole in a plastic bag held over the calf's head, causing the forced breathing of the inoculum (Hetrick *et al.*, 1963). Two days later, *P. multocida* at 10^6 cfu ml^{-1} suspended in 5 ml of allantoic fluid was administered intratracheally and by day 2 following exposure to the organism clinical signs, including changes in demeanour, respiratory distress, diarrhoea, anorexia and nasal exudate of varying severity, were found in all calves. These clinical signs associated with respiratory illness were observed 11 days post-infection with *P. multocida* when the calves were exposed to the same agents but in reverse order, suggesting that *P. multocida* was only able to multiply following a virus attack, when the host's immune system was compromised. When calves were separately given either *P. multocida* or SF-4 virus only a mild form of respiratory disease developed, indicating that calves were able to harbour such infectious agents without exhibiting overt clinical signs (Hetrick *et al.*, 1963).

A model for pneumonic pasteurellosis was established in goats using *P. multocida* types A and D harvested from pneumonic lungs of goats and using 4 ml of the bacterial suspension administered

intratracheally (Zamri-Saad *et al.*, 1996). This model established that type D isolates produce more severe lung lesions than do type A strains, a finding in agreement with research that showed that type D toxins have been linked with severity of lesion (van Diemen *et al.*, 1994).

Previous work conducted at the MRI (Donachie *et al.*, unpublished), and elsewhere (Ames *et al.*, 1985; Gourlay *et al.*, 1989), has demonstrated the lower virulence of *P. multocida* relative to *M. haemolytica* when the bacteria were administered in small volumes of PBS. Gourlay *et al.*, (1989) produced pneumonic pasteurellosis in calves aged 2-6 months with *P. multocida* serotype A by infecting them through either the intratracheal or intravenous route. The inoculum ranged from 1.5 x 10^9 to 1.5 x 10^{10} cfu. Clinical illness was mild in all calves, with pyrexia and respiratory signs being only temporary in the worst affected calves.

Ames *et al.*, (1985) produced pneumonic pasteurellosis in 2 day old calves with either *M. haemolytica* or *P. multocida*. A rebreathing bag was used to make the calves hyperpnoeic and, 60 ml of culture was then injected into the trachea. The degree of clinical signs and the extent of the lesions produced were in proportion to the amount of the organism in the inoculum. Moreover he concluded that *P. multocida* alone produced a mild clinical response and that the extent of lung lesions, including pneumonic consolidation, was minimal compared to that caused by *M. haemolytica*. Both experimental approaches however failed to produce overt disease according to the criteria put forth by Houghton, (1995). The standard approach used by Donachie and co-workers when infecting conventional calves with *M. haemolytica* has been to use 60 ml of bacterial suspension. However, more recently, higher volumes (300 ml) of challenge inoculum have resulted in a more reliable and progressive development of the disease, mimicking that seen in the field. A similar approach could be applied to *P. multocida* infection in cattle with the aim of improving our understanding of the interactions between the bacteria and the host immune system.

1.3. Other disease caused by *P. multocida*

P. multocida is the causative agent of other important diseases such as haemorrhagic septicaemia, fowl cholera, atrophic rhinitis and rabbit septicaemia, these are summarised below and listed in Table 1.1.

1.3.1. Haemorrhagic Septicaemia

Haemorrhagic septicaemia (HS) is an acute, often fatal septicaemic disease affecting cattle and buffaloes, with a wide geographical distribution. In most Asian and African countries HS is endemic and is considered to be one of the most economically significant bacterial diseases (Jones

et al., 2002). Following exposure to virulent organisms, clinical signs appear after a short incubation period and affected animals develop fever, respiratory distress and profuse mucosal discharge from the nostrils and mouth. Within a few days, most cases become recumbent and die from septicaemia. Subcutaneous oedema, widespread petechial haemorrhages in submucosal tissues, congested lungs and enlarged lymph nodes are observed at PM (DeAlwis, 1992). In endemically infected areas immunisation using adjuvant vaccines provides the most effective means of controlling HS (Jones *et al.*, 2002).

1.3.2. Fowl cholera

An infectious avian disease of world wide distribution, caused by *P. multocida* serotypes A:1, A:3 or A:4 (Rhoades and Rimler 1987). The disease often manifests as the septicaemic acute form with high morbidity and mortality. The period of acute illness is very short and consists of anorexia, mucosal discharge from the eyes, mouth and nostrils, increased effort for respiration and bloody diarrhoea and death may occur within hours or days. The lesions seen in birds that have died of acute illness include small haemorrhages of internal organs and mucous membranes (Blackall and Miflin, 2000). The disease is treated with chemotherapeutic agents, primarily sulphonamides and antibiotics, and these agents are subjected to *in vitro* sensitivity testing to ensure maximum efficacy against *P. multocida* (Blackall and Miflin, 2000). Vaccination is recommended in regions where the disease is known to occur though, for *P. multocida*, 16 serotypes exist and the commercially available vaccine contains only the most common serotypes A:1, A:3 and A:4.

Table 1.1. Specific serotypes associated with livestock disease caused by *P. multocida*, designated by the Carter:Heddleston method.

Capsular type	Somatic type	Serotype	Disease	Host species
A	1, 3, 4	A:1, A:3, A:4	Fowl cholera	Poultry/ turkeys
	5, 6	A:5, A:6		
	7, 8, 9, 10	A:7, A:8, A:9, A:10		
	12, 13, 14, 15	A:12, A:13, A:14, A:15		
	16	A:16		
	3	A:3	Bovine pneumonic pasteurellosis	Cattle
			Pneumonia	Pigs
B	2	B:2	Haemorrhagic Septicaemia in Asia	Cattle and buffalo
D	11	D:11	Atrophic rhinitis	Pigs
E	2	E:2	Haemorrhagic Septicaemia in Africa	Cattle
F			Fowl cholera	Turkeys

1.3.3. Atrophic rhinitis

Atrophic rhinitis is a common disease of swine that is of worldwide economic importance to the pig rearing industry and is characterised clinically by sneezing, coughing, and nasal discharge. The resulting inflammation and atrophy of the nasal turbinates causes crooked or shortened snouts (Chanter and Rutter, 1989). The disease presents as either a non-progressive mild form caused by *Bordetella bronchiseptica* or a progressive form caused by toxigenic strains of *P. multocida* (types D and A) alone or in combination with *B. bronchiseptica*. The toxigenic strains of *P. multocida* produce potent cytotoxins and *B. bronchiseptica* produces a dermonecrotic toxin (Brockmeier *et al.*, 2002). Both forms inhibit osteoblastic activity and promote osteoclastic reabsorption in nasal bones (Lopez, 2001). Available vaccines use a combination of *B. bronchiseptica* bacterin with toxoids prepared from toxigenic *P. multocida* strains (Sakano *et al.*, 1997).

1.3.4. Snuffles

P. multocida A:12, A:3 and D:1 strains are a major cause of mortality and morbidity in rabbits. The predominant syndrome is "snuffles", which is characterised by conjunctivitis, rhinitis, sinusitis and pleuropneumonia. Antibiotics are often prescribed as the first line of treatment and control, but studies have focused on vaccination as a means of control in rabbitries where pasteurellosis is common (Deeb, 2000). Preliminary vaccination studies in rabbits have demonstrated the ability of *P. multocida* IR-OMP administered intranasally to stimulate immunity against experimental pneumonic pasteurellosis (Confer *et al.*, 2001).

In addition to these diseases caused by *P. multocida* in domestic livestock, the sporadic occurrence of *P. multocida* infection in other species has also been documented in elephants, donkeys and horses (Pavri and Apte, 1967), camels (Hassan and Mustafa, 1985), deer (Aalbaek *et al.*, 1999) and in a snow leopard (Chaudhuri *et al.*, 1992). Humans may become infected by *P. multocida* following an animal bite. Patients tend to exhibit swelling, cellulitis and some bloody drainage at the wound site and infection may move to nearby joints where it can cause swelling and arthritis. However, infections unrelated to animal bites have been frequently reported and include meningitis, and pneumonia (Klein and Cunha, 1997).

1.4. Aims of the study

The aims of the thesis are as follows: Firstly, commensal and virulent *P. multocida* isolates amongst cattle within the Midlothian region will be characterised by molecular typing and LPS chemotyping

and compared with the MRI-*P. multocida* A:3 used for the production of pneumonic pasteurellosis in this study. Secondly, to produce a progressive and realistic model of pneumonic pasteurellosis due to *P. multocida*, in cattle and by characterising the pathophysiological and biochemical responses of the host to either low or high volumes of challenge inocula. It is anticipated that an optimal dose and volume will produce disease comparable to that seen in field cases and will assist the development of treatment strategies. Thirdly, the reproducibility of the model will be assessed and bronchoalveolar lavage fluid (BALF) will be screened for bacterial and host proteins involved in pathogenesis using novel proteomic techniques. Fourthly, the clinical, APP and pathological responses attributable to formalin-killed *P. multocida* will be characterised and compared with those recorded after challenge with live formulations of *P. multocida*. Finally, the mechanisms of host-pathogen interactions will be determined by analysing responses of lavaged lung cells to formalin-killed *P. multocida* challenge.

Chapter 2. General Materials and Methods

2.1. Biochemical characterisation of *P. multocida*

2.1.1. Colony Morphology

Initial characterisation of *P. multocida* strains recovered from nasal swabs and lung were determined by colony morphology. After overnight incubation at 37°C on 5 % (v/v) sheep blood agar (SBA) plates (appendix 1.2.1), one of two colony types was produced by *P. multocida*; smooth, circular greyish colonies 1-2 mm in diameter or greyish mucoid colonies 3-5 mm in diameter (Barrow and Feltham, 1993). Mucoid colonies ranged from discrete, circular slightly mucous-like to large 'watery' mucoid colonies that often coalesced. Growth on SBA produced a characteristic odour. Nasal swab and lung isolates with a colony morphology similar to that seen with *P. multocida* were assessed for their oxidase and catalase activity.

2.1.2. Oxidase activity

This was ascertained by Oxoid touch-sticks (Oxoid Ltd, Basingstoke, Hampshire, UK). The impregnated end of the stick was touched on to the surface of a well separated colony and rotated to remove a mass of cells. The stick was examined after 30 s; if no colour change occurred it was examined 3 min later. A deep purple colour indicated a positive reaction consistent with the colony being *P. multocida*.

2.1.3. Catalase activity

Using sterile loops small amounts of growth were transferred from SBA plates onto microscope slides and each was mixed with a loopful of catalase reagent (equal volumes of 3 % (v/v) hydrogen peroxide and 1 % (w/v) aqueous methylene blue). The enzyme catalase is located within Gram negative bacteria and catalyses the conversion of hydrogen peroxide to oxygen. Coverslips were placed over the reaction mixtures and if bubbles were produced within 10 s, isolates were considered catalase positive. If no bubbles were produced then bacteria were considered catalase negative. Field isolates that tested positive for oxidase and catalase were selected for further characterisation.

2.1.4. Urease activity

For each isolate, several colonies were resuspended in 0.5 ml of sterile distilled water in a bijou, and a urea disk (BD Biosciences-Life Research, Between Towns Rd, Cowley, Oxford, UK) added using sterile forceps and incubated at 37°C for 4 h. A positive reaction was indicated by the disk turning pink in colour, and strongly positive isolates were detectable after 10 min.

2.1.5. Indole production

A single colony of each isolate was inoculated into 10 ml nutrient broth (NB) and incubated for 48 h static at 37°C. Following incubation, 2 ml of the broth cultures were transferred to sterile bijoux containing 0.1 ml of Kovács reagent (bioMerieux, 69280 Marcy l'Etoile, France) and mixed well. Solutions were incubated for 1 min at room temperature (RT) and the production of indole was detected by the development of a deep pink colour at the surface of the broth.

2.1.6. Carbohydrate fermentation tests

Carbohydrate media consisted of 1 % (w/v) solutions of each substrate in peptone water sugar base (BD Biosciences-Life Research) (appendix 1.1.1), dispensed in 3 ml volumes. Sugar-free controls were also prepared. Bacterial suspensions with McFarland values of 3-4 were prepared using a densimat (bioMerieux), from the growth of bacteria on SBA plates resuspended in saline. The McFarland values were multiplied by a conversion factor, 3×10^8 and a correction factor of 1.5 applied, to provide an estimated viable count of 1.4 to 1.9×10^9 cfu ml^{-1}. The carbohydrate media were inoculated with 0.2 ml of these bacterial suspensions, and incubation was carried out for up to 14 d at 37°C. Every 2 d the broths were examined for a change of colour from purple to yellowish-brown, indicating the production of acid from sugar substrates (Bisgaard *et al.*, 1991).

2.1.7. Detection of β-galactosidase activity (ONPG test)

Bacterial suspensions, prepared as described under section 2.1.6, were inoculated into *O*-nitro-phenyl-D-galactopyranoside (ONPG) broth (appendix 1.1.2) and incubated for 48 h at 37°C. β-galactosidase activity was detected by the development of a yellow colour.

2.1.8. Ornithine decarboxylation

Moeller decarboxylase broth (BD Biosciences-Life Research) was prepared, with and without L-ornithine (Sigma, Co. Ltd, Poole, Dorset, UK), according to the manufacturer's instructions, and dispensed aseptically in 3 ml volumes into bijous. Bacterial suspensions, prepared as described under section 2.1.6., were inoculated in 0.4 ml volumes into the decarboxylase broths. The cultures were then overlaid with 1 ml of sterile mineral oil, and incubated for 7 d at 37°C. Ornithine decarboxylation was detected when media changed purple.

2.1.9. Capsule characterisation

Determination of *P. multocida* capsular types was performed using hyaluronidase (capsular type A) (Carter and Rundell, 1975) and acriflavine tests (capsular type D) (Carter *et al*, 1973; Rimler 1994). The former test identified type A strains by growth restriction due to the depolymerisation of capsular hyaluronic acid by hyaluronidase. Tests for enzyme decapsulation activity were carried out using a disc diffusion technique, whereby a culture was streaked on to the surface of a dextrose starch agar plate, and a sterile filter paper disc placed over the streaked area. A 20 µl volume of filter-sterilised hyaluronidase solution (3 units (U) hyaluronidase and 0.02M PBS pH 7) was added to the disc and cultures incubated for 18-24 h at 37°C. Decapsulation was shown by a clear circular zone of colony forms that surrounded the disc (Rimler 1994).The latter test identified type D strains by the production of a heavy flocculent precipitate following the addition of a 0.1 % (w/v) aqueous solution of acriflavine to a concentrated broth culture (Carter *et al*, 1973).

2.2. Molecular characterisation of *P. multocida*

2.2.1. Pulsed-field gel electrophoresis

An initial study was performed to determine the most appropriate restriction endonuclease for *Pasteurella* spp. *Apa*I proved to be more suitable than *Dra*I, producing well-defined profiles of bacterial DNA, in agreement with a previously reported observation (Lainson *et al.*, 2002).

Suspensions of pure cultures were diluted in 1.5 ml phosphate buffered saline (PBS), to a culture density of McFarland 2.5 (approximately equivalent to 7.5×10^8 cfu ml^{-1}). Conditions for the digestion of chromosomal DNA using *Apa*I were those used by Lainson *et al.*, (2002), with slight modifications. Briefly, cells were harvested by centrifugation at 11, 340 x *g* for 10 min at RT. Supernatant fractions were discarded, and bacterial pellets were washed four times with Pett IV

buffer (appendix 1.3.1); subsequently, bacteria were resuspended in 0.5 ml of Pett IV buffer. Cells were warmed for 30 min at 40°C and suspensions were mixed with equal volumes of 2 % (w/v) low gelling temperature agarose (Promega, Madison WI, USA) (appendix 1.3.10) and dispensed into plug moulds (Bio-Rad Laboratories, Hemel Hempstead, UK); 3 plugs were made per strain and digested in 5 ml of EC lysis buffer (appendix 1.3.2) containing lysozyme to lyse the bacterial cell wall, at 37°C for 24 h. Following proteinase K (100 μg ml^{-1} Sigma) digestion of bacterial proteins in 3 ml ESP buffer (appendix 1.3.3) at 50°C for 60 h, plugs were incubated at RT for 30 min in 5 ml of TE buffer (appendix 1.3.4) containing 0.1 M PMSF (Sigma) (appendix 1.3.5). The plugs were then washed with 8 x 5 ml Tris-EDTA (appendix 1.3.8 and 1.3.9) on a shaker at RT for 15 min each. Four mm sections (inserts) were cut from the plugs and equilibrated in distilled water (DW) containing 1 x *Apa*I digestion buffer (Promega) and 10 mg ml^{-1} BSA (Promega) final concentrations for 1 h at RT. Subsequently the inserts were digested in equilibration buffer containing 20U of *Apa*I (Promega), at 30°C for 48 h. Plugs were then washed in 1 ml of TE buffer (appendix 1.3.4) for 15 min, then loaded onto 1 % (w/v) agarose gels (Promega) (appendix 1.3.11). Lambda DNA 340 ladder PFGE markers (New England BioLabs Inc, USA) were included as molecular weight standards.

Electrophoresis was carried out in TBE buffer (appendix 1.3.7) on the BioRad CHEF DRII system at 14°C and 6v cm^{-1}, with an initial switch time of 1 s increasing to a final switch time of 40 s for a duration of 23 h. Gels were then stained at RT using 100 μl of 0.25g ml^{-1} ethidium bromide (Promega) mixed with 250 ml DW for 45 min and were washed in DW for 20 min at RT with gentle shaking. Banding patterns were recorded using an Image Master Video Display System (Pharmacia, Amersham Biotech, Buckinghamshire, England).

2.2.2. Identification of *P. multocida* by PCR

P. multocida in bovine nasal swabs was identified by PCR, using primers specific for the *kmt1* gene that encodes a protein involved in the synthesis of capsular polysaccharide. Conditions for the detection of *P. multocida* were those used by Townsend *et al.*, (2001).

2.2.2.1. Bacterial DNA extraction for PCR

Loopfuls of bacterial growth taken from SBA plates, were resuspended in 250 μl of nuclease-free water, and incubated at 99°C for 10 min to lyse the bacterial cells. The bacterial lysates were cooled at -20°C for 5 min and centrifuged at 3, 000 x *g* for 10 min at RT. A distinct layer was then detected containing bacterial genomic DNA which was removed and stored at -20°C or analysed directly by PCR.

2.2.2.2. *PCR conditions*

The *P. multocida* specific PCR primers, *kmt1t7* (5'-ATCCGCTATTTACCCAGTGG-3') and *kmt1sp6* (5'-GCTGTAAACGAACTCGCCAC-3'), were designed to amplify a 460bp fragment of the *kmt1* gene (Townsend *et al.*, 2001). 2.5 µl aliquots of extracted *P. multocida* DNA were resuspended in 25 µl of PCR amplification mixture, consisting of *P. multocida* specific primer at a concentration of 1 µM (Invitrogen Ltd, Inchinnan Business Park, Paisley, UK), with each deoxynucleoside triphosphate at a concentration of 200 µM (Invitrogen), 1 x PCR buffer (Promega), 1.5 mM $MgCl_2$ (Promega), and 1 U of *Taq* DNA polymerase (Promega). In addition, all modifying enzymes (including restriction endonucleases and DNA polymerases) were purchased from Promega, unless stated otherwise. All thermal cycling was performed in a Thermo Hybaid PCR system Px2 (Thermo Life Sciences, Edison Rd, Hampshire, UK) under the following conditions: initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for 5 min.

2.3. Characterisation of *P. multocida* lipopolysaccharide

2.3.1. Phenol-hot water micro-extraction of bacterial LPS

Single *P. multocida* colonies were inoculated into 10 ml of NB that were incubated statically overnight at 37°C. Cells were harvested by centrifugation at 2, 000 x *g* for 30 min at 20°C. Subsequently, cells were washed twice in 20 ml of PBS and resuspended in 500 µl pyrogen-free water. Five hundred µl of 90 % (w/v) aqueous phenol was added to each sample. The mixture was vortexed for 10 s, heated in a water bath at 70°C for 10 min, vortexed once, cooled on ice for 2 min, and then centrifuged at 3, 000 x *g* for 4 min at RT (Westphal and Jann, 1965). The clear upper aqueous layer, containing LPS, was either stored at -20°C or analysed directly by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot.

2.3.2. SDS-Polyacrylamide LPS Gels

LPS species of different molecular weights were separated by discontinuous SDS-PAGE using 1.0 mm thick pre-cast 12% (w/v) acrylamide / bis-acrylamide gels (NuPAGE gel system, Invitrogen). Smooth LPS extracted from *E. coli* serotype O55:B5 (Sigma) was used as a positive control alongside molecular weight standards of 3-188 kDa (SeeBluePlus2; Invitrogen), which covered the expected range of LPS masses. The pre-stained markers also provided immediate visual confirmation of successful immunoblot transfer. Equal volumes of LPS extract and NuPAGE

sample buffer were mixed, boiled for 5 min and loaded at 3 µl per well. Electrophoresis took place at 200V (70-80mA) for approximately 35 min, at RT, until the dye front reached the bottom of the gel.

2.3.3. Characterisation of LPS by Silver stain

LPS bands were silver stained (SilverQuest™ silver staining kit, Invitrogen). The methods applied for the running and silver staining of LPS polyacrylamide gels are more rapid, reliable and reproducible than those described by Uchida and Mizushima, (1987) and Tsai and Frasch, (1982). All incubations were performed on a rotary shaker (1 revolution s^{-1}) at RT. Volumes were 100 ml unless otherwise stated. Gels were submerged in fixative solution (proprietary formulation) for 20 min, then washed in 30 % (v/v) ethanol for 10 min. Sensitising solution 30 % (v/v) ethanol, 10 % (v/v) sensitiser; (proprietary formulation) was added for 10 min, before gels were washed again in 30 % (v/v) ethanol followed by DW for a further 10 min. Staining solution 1 % (v/v) (proprietary formulation) was added to gels for 15 min, and, subsequently, gels were washed with ultra pure water for 20-60 s. Gels were then incubated in developing solution 10 % (v/v) (proprietary formulation), with the duration of incubation being determined by the differential staining intensity of the background and LPS in the gel. Once the desired stain was obtained 10 ml of stopper solution (proprietary formulation) was added. LPS bands were designated upwards from 1, equivalent to the lowest molecular weight species.

2.3.4. Immunoblotting

Electrophoretically separated LPS species were transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using a Trans blot semi-dry transfer cell system (Bio-Rad Laboratories) at 70 mA per gel for 2 h at RT in Tris-glycine buffer (Sigma) (transfer buffer, appendix 1.4.1). Conditions for the detection of LPS bands were modified from the method of Lacroix *et al.*, 1993. Transfer membranes were immersed in a high salt blocking buffer (0.5 M NaCl-PBS pH 7.0, 0.5 % (v/v) Tween-80 [PBS-Tween]) (appendix 1.4.2) for 1 h at RT (Huntley *et al.*, 2001). Membrane-bound LPS was probed for 1 h at RT with the following polyclonal antisera diluted in blocking buffer: Bovine anti-*P. multocida* immune serum (1:200), and rabbit anti-*P. multocida* immune serum (1:40). After four washes in a high salt blocking buffer, membranes were incubated for 1 h at RT with the appropriate horseradish peroxidase (HRP)-conjugated sheep anti-bovine IgG or anti-rabbit IgG (Serotec, Kidlington, Oxford, UK) at a 1/1000 dilution in blocking buffer, depending upon the species from which the primary antiserum was derived. Immunoblots were then washed four times in blocking buffer and freshly prepared DAB HRP substrate (Sigma)

(appendix 1.4.5) was added. Once the desired staining was obtained DW was added to stop the colourimetric reaction.

2.4. Preparation of *Pasteurella multocida* antigens for *in vitro* studies

An isolate of *P. multocida* (MRI reference 619/90) was retrieved in 1990 from the lung of a calf challenged with a mixture of five *P. multocida* isolates recovered from field cases of BPP. The isolate was passaged through another calf to confirm its virulence and stored in NB at -70°C. This freshly passaged isolate (MRI reference 671/90), confirmed by somatic typing (VLA, Edinburgh) as *P. multocida* A:3, was used as the challenge isolate for this study. PFGE analysis (Kodjo *et al.*, 1999) was used to compare the DNA content of the challenge isolate with that of bacteria recovered from infected tissue.

A 10 ml starter culture of *P. multocida* MRI-671/90, was prepared by resuscitating a stored culture and isolating pure colonies for inoculation in 10 ml of NB. This 10 ml starter culture was incubated at 37°C overnight for 16 h and then inoculated into 1, 200 ml of NB. This culture was placed on a rotary shaker at 100 rpm for 4 h 30 min at 37°C to obtain a log phase culture that was then centrifuged at 3976 x *g*, for 30 min at 4°C. Most of the supernatant fraction was poured off and the remainder was used to resuspend the cell pellet. The pellet was spun down at 2040 x *g*, for 30 min at 4°C, washed and resuspended twice in PBS. The final cell pellet was resuspended in 20 ml of a 10 mM Tris solution at pH 7.6 (appendix 1.3.9) containing 200 µl 10 mg ml^{-1} PMSF (in ethanol), as a protease inhibitor. The pellet and diluent were stored overnight at 4°C.

The whole cell suspension was sonicated in a glass universal for 5 x 30 s bursts, cooling on ice between each burst. This was repeated until the sonicate was clear, whereupon unbroken cells were then removed by centrifugation at 2040 x *g* at 4°C for 30 min. The pellet was discarded and the supernatant fraction transferred to 4 x 5 ml 70-Ti ultracentrifuge tubes (Beckman Coulter Life Sciences, Fullerton, CA, USA). The supernatant fraction was centrifuged at 82600 x *g* at 4°C for 1 h 30 min. The pellets were separated from the supernatant fractions, pooled, washed and resuspended in 6 ml of a 10 mM Tris solution. The resuspended preparation was centrifuged at 191940 x *g* for 45 min, at 4°C. The supernatant fraction was discarded and the final pellet comprising cell envelopes was resuspended in 2 ml of a 10 mM Tris solution at pH 7.6 and stored at -20°C.

2.5. Antiserum production for *P. multocida* LPS characterisation

2.5.1. Generation of rabbit anti-*P. multocida* hyperimmune sera

Rabbits of either sex, between the ages of 4-6 months, bred at MRI, were used to raise hyperimmune serum against formalin-killed *P. multocida* (serotype A:3 isolate 671/90). A stored (-70°C) vial of *P. multocida* (671/90) was plated out onto SBA plates and incubated overnight at 37°C in a moist chamber. Growth from the plate was harvested with 10 ml of saline using a plastic loop and cells centrifuged and washed once in 10 ml of saline. Washed cells were resuspended in saline containing 0.3 % (v/v) formalin, and adjusted to give a final concentration of 10^6 cfu ml^{-1} and stored at 4°C until time of challenge.

Pre-immunisation blood samples were taken from a marginal ear vein of each rabbit, and tested for the presence of pre-existing antibodies by immunoblotting and enzyme-linked immunosorbent assay (ELISA). Immunisations were carried out according to the schedule in Table 2.1. Three days after the last immunisation, rabbits were euthanased with 3 ml (0.6 ml kg^{-1}) of Pentobarbitone Sodium B.P. (200 mg ml^{-1}; Animal Care, Lancaster, UK) intravenously (i.v.), and exsanguinated by cardiac puncture. Blood was allowed to clot at RT overnight. Serum was separated by centrifugation and stored in 5 ml aliquots at -20°C.

Table 2.1. Immunisation schedule for the production of hyperimmune serum.

Day	Treatment
1	Pre-immunisation blood sample
2	0.5 ml subcutaneously at one site
4	1.0 ml subcutaneously at one site
7	2.0 ml subcutaneously at one site
10, 13, 16, 19, 22, 25	2.0 ml intravenously
32	Trial bleed
35	Exsanguination

2.5.2. Generation of bovine anti-*P. multocida* hyperimmune sera

Bull calves at 8 weeks of age were used to raise hyperimmune serum against live *P. multocida* (serotype A:3 isolate MRI-671/90 challenge strain, Chapter 4). Carriage of, or prior exposure to, *P. multocida* was checked by the culture of nasal swabs on vancomycin (1 mg ml^{-1}, Sigma) SBA plates (appendix 1.2.2) and analysis of antibody to *P. multocida* by ELISA, respectively. Calves were challenged intratracheally via a bronchoscope with 300 ml of PBS containing 10^9 cfu ml^{-1} of *P. multocida* (section 2.8.4).

Six to fourteen hours after challenge, calves were given 5 ml long-acting tetracycline intramuscularly (1 ml 10 kg^{-1}: 200 mg ml^{-1}) (Intervet UK Ltd, Cambridge, UK) along with 2.5 ml of a non-steroidal anti-inflammatory drug, flunixin meglumine (2.2 mg kg^{-1}: 2 ml 45 kg^{-1}) (Schering-Plough Animal Health, Uxbridge, Middlesex, UK) administered i.v., after antibiotic to prevent overt disease and undue suffering to the animals. Blood samples were taken 21 days after challenge and ELISA and immunoblot analyses were used to assess titres. Calves were re-challenged because of low titres and for ethical reasons a lower volume (10^9 cfu *P. multocida* in 60 ml PBS) was used. At 28 d post re-challenge, the calves were euthanased with 25 ml of Pentobarbitone Sodium B.P. (200 mg ml^{-1}; Animal Care), and antisera were collected.

2.6. Serology

2.6.1. Blood sampling

Jugular vein blood samples (9 ml) were collected into chilled vacutainers (Becton Dickinson, Cowley, Oxford, UK) containing 1 ml ethylenediaminetetraacetic acid (EDTA) (appendix 1.9.1) from all calves on day of arrival, the day of challenge (day 0), 4 h p.c. and daily thereafter until the day of necropsy. Plasma was separated and stored at - 40°C until analysis for APP (Hp, AGP and SAA), IgG, and plasma antibody to parainfluenza virus type-3 (PI-3), respiratory syncitial virus (RSV), infectious bovine rhinotracheitis virus (IBRV) and bovine viral diarrhea virus (BVDV).

2.6.2. Detection of acute phase proteins

2.6.2.1. Haptoglobin

Plasma Hp concentrations were determined using the Phase ^{TM} haptoglobin-haemoglobin binding assay (Tridelta Ltd, Greystones, Ireland) (Eckersall *et al.*, 1999). This is a non-species specific colorimetric assay that uses a peroxidase substrate to prevent non-specific binding between serum proteins and haemoglobin (Hb), without altering the binding affinity between Hb and Hp. Unbound Hb exhibits peroxidase activity, which is inhibited at a low pH. Hp present in the sample will form a complex readily with haemoglobin under these pH conditions. As the amount of Hp rises, amount of Hb-Hp complex is enhanced with a simultaneous increase in peroxidase activity. Hp levels in either plasma or serum can therefore be established directly when compared against standards prepared from a range of known concentrations of Hp. In duplicate, 7.5 µl of each prepared calibrator (0, 0.5 and 2 mg ml^{-1}) along with test specimen was transferred to a fresh microplate. 100 µl of Reagent 1 (equal volumes of Haemoglobin (v/v) and Haemoglobin diluent (v/v)) was added to each well and the plate tapped to ensure that calibrators/specimens and haemoglobin were mixed. Shortly after 140 µl of Reagent 2 (Chromagen and substrate mixed in a ratio of 9:5) was added to each well and the plate incubated for 5 m at RT and read immediately at 630 nm. A calibration curve was generated by plotting absorbance (630 nm) versus Hp concentration (mg ml^{-1}) to facilitate the calculation of the haptoglobin concentration in test samples. Test values were read from the curve and multiplied by an appropriate factor if dilution had been required. The normal range of haptoglobin in the bovine is between 0 and 0.5 mg ml^{-1} and the acute range is between 0.5 and 3 mg ml^{-1}.

2.6.2.2. Serum Amyloid-A

Plasma concentrations of SAA were determined using the Phase ™ range ELISA SAA kit (Tridelta Ltd Greystones, Ireland) that employs a monoclonal antibody specific for SAA coated onto microtitre well strips. Diluted test samples or standards of known SAA content (300, 150, 75, 37.5, 18.8, 0 ng ml^{-1}) were added to the wells and a second biotinylated anti-SAA monoclonal antibody added. The immobilised antibody bound SAA present in the well, where it was detected with the conjugate antibody. After washing, to remove unbound material, Streptavidin-HRP conjugate was added, the plate incubated for 30 min at RT, in the dark and 3,3,5,5-tetramethylbenzidine solution (TMB) substrate solution added. After stopping the reaction with H_2SO_4 the absorbance at 450 nm was measured and SAA concentration derived by comparison to the standards.

2.6.2.3. α_1 Acid glycoprotein

The AGP concentrations in plasma were measured using a radial immunodiffusion kit (Saikin Kagaku Institute, Japan). Each test sample (5 µl) was placed in an individual test well. As the sample diffused from the well into the agar gel containing specific antiserum to bovine α_1AG, a precipitin reaction occurred and a ring was produced, with the radius proportional directly to the concentration of α_1AG in the test sample. The concentration was computed by reference to two known standard solutions of α_1AG at concentrations of 250 µg ml^{-1} and 1000 µg ml^{-1}.

2.6.3. Detection of serum IgG

ELISA plates were coated with 50 µl per well of *P. multocida* A:3 envelope at 1/100 dilution in carbonate-bicarbonate buffer pH 9.6 (appendix 1.5.1) and left at 4°C overnight. The plate was then washed 3 times with ELISA wash buffer (appendix 1.5.2) to remove any unbound envelope protein. The plate was blocked with 200 µl per well of a high salt blocking buffer (0.5 M NaCl-PBS, pH 7.0, in 0.5% (v/v) Tween-80 {Sigma}) (appendix 1.4.2) for 1 h at 37°C (Huntley *et al.*, 2001). This stage was to prevent subsequent non-specific binding of uniformly diluted antisera to the well surface. After washing a further three times with ELISA wash buffer, test and standard sera were added to 100 µl volumes, in duplicate to antigen coated wells, and to one uncoated well to estimate the extent of any non-specific antibody binding. The standard positive convalescent and negative sera were used at a dilution of 1/100, while during a preliminary study, the test sera were titrated from 1/50 to 1/6400, much variation amongst animals was noted. Therefore an arbitrary dilution of 1/100 was implemented for the detection of test serum IgG raised to *P. multocida* A:3. Plates were washed with ELISA wash buffer and sheep anti-bovine IgG-HRP conjugate (Serotec, Oxford, UK) added (1/1000) to each well before incubation for 1 h at 37°C. After washing, 50 µl of the substrate

(*O*-phenylenediamine (OPD) and hydrogen peroxide (H_2O_2), appendix 1.5.5) was added to each well and the plate incubated for 5 min at RT. The reaction was stopped by the addition of 2.5 M H_2SO_4 (appendix 1.5.6) and optical density (O.D) in the wells read at 492 nm.

2.6.4. Detection of respiratory viral antibodies

The measurement of virus specific IgG levels in bovine plasma used an ELISA combining the following viral antigens: PI-3, RSV, BVDV and IBRV in a single test microplate format. All viral antigens except for RSV which was isolated from the Rispoval vaccine (Pfizer, UK) originated from local Scottish bovine isolates. Bound antibody was detected subsequently by the reaction of an anti-bovine HRP-conjugate followed by the addition of a substrate, either OPD or TMB.

The ELISA method used to test the presence of viral antibodies in experimental samples was adopted from Caldow *et al.*, (1988) with slight modifications. The appropriate viral antigens were diluted in 0.05 M carbonate buffer (pH 9.6) and 100 µl was added per well to the microtitre plate (Greiner bio-one Ltd, Stonehouse, Gloucester, UK) which was incubated overnight at 4°C in a humidified chamber. Coated plates stored at 4°C were used up to 5 d after coating with antigen. Plates were washed with PBS-Tween (0.01M PBS and 0.1 M EDTA in 0.05 % Tween-20). Plasma samples were diluted 1/50 in ELISA diluent (PBST-0.5 % (v/v) ovalbumin (appendix 1.6.1) and mixed thoroughly. The control sera were also diluted 1/50 in PBST- 0.5 % (v/v) ovalbumin ELISA diluent. Control and experimental samples were loaded onto the ELISA plate, 4 wells per sample at 100 µl per well and incubated for 1 h at 37°C in a humidified chamber. After washing, bound antibody was detected by the addition of rabbit anti-bovine IgG HRP-conjugate at 1/3000, 1/6000, 1/2000, 1/4000 dilution for BVDV, IBRV, RSV and PI-3, respectively (Sigma) for 1 h at 37°C in a humidified chamber, followed by washing and the addition of the substrate OPD (Sigma) (0.8 mg ml^{-1}) in substrate buffer (appendix 1.6.2) with 30 % (v/v) H_2O_2. The colour reaction was measured on a Dynex MRX microplate reader (Dynex technologies, Ashford, Middlesex, UK) at a wavelength of 492 nm. The results were expressed in OD units as mentioned earlier and adjusted in accordance with a standard curve determined for each test. The ELISA was calibrated so that an OD reading ≥ 0.1 was regarded as specific antibody positive.

2.7. Proteomics-2D-Electrophoresis

All reagents within this section were purchased from Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, England, unless stated otherwise.

2.7.1. BAL sample preparation

A 10 ml volume of supernatant fraction recovered by bronchoalveolar lavage (BAL) (section 2.9.1), was desalted by placing the sample in 10 cm of snakeskin dialysis tubing (Pierce Biotechnology Inc, Rockford, IL, USA) and dialysing against 3 changes of DW for 24 h at 4°C. The desalted solution was concentrated down to approximately 1 ml using Spectra/Gel absorbent (Spectrum Labs, Medicell, UK). The 1 ml of solution was dried using a freeze dryer (Edwards 12K Supermodulyo, Alpha Laboratories Ltd, Hampshire, UK). The lyophilised powder was dissolved in 200 µl of rehydration buffer (appendix 1.7.1) and protein content was determined using a 2-D Quant protein assay kit.

2.7.2. Quantification of protein in BAL fluid samples

The PlusOne 2-D Quant Protein assay kit was used to determine the concentration of protein present in the sample. The assay was performed according to manufacturer's instructions. The 2-D Quant kit uses a combination of precipitant and co-precipitant to precipitate quantitatively the sample protein. The protein is sedimented by centrifugation and resuspended in a solution of cupric ions. These ions bind to the polypeptide backbone of protein and any unbound cupric ions (Cu^{2+}) then react with the colourimetric agent (proprietary formulation). The colour density is inversely related to the concentration of protein in the sample (PlusOne 2-D Quant Kit protocol). All of the solutions (precipitant, co-precipitant, copper solution, colour reagents A and B) described in this procedure were included in the 2-D Quant Kit and all reactions were performed at RT unless stated otherwise.

10 µl of protein solution, in rehydration buffer was assayed using the protocol described. The absorbance of each sample was read at 490 nm using a microplate reader (Dynex MRX II Endosafe Ltd, Kent, UK) and the concentration of protein in each sample was determined from the standard curve. From the results, samples were diluted with rehydration buffer to a concentration of 100 µg of protein in 200 µl. Preliminary results suggested that 100 µg of BAL proteins were well differentiated by 2-DE when staining with colloidal Coomassie blue stain (Genomics Solutions, Ann Arbor, MI, USA).

2.7.3. First dimension electrophoresis: Isoelectric focusing of sample

Prior to isoelectric focusing (IEF), 40 mM dithiothreitol (DTT) and immobilised pH gradient (IPG) buffer (3-10) (propriety formulation) were added to the samples to give final concentrations of 0.28 % (w/v) and 0.5 % (v/v), respectively. IEF was used as the first dimension to separate proteins

according to their isoelectric points (pI) and was performed on an IPGphor IEF system. The procedure was as follows: 100 µg of protein in 200 µl of rehydration buffer was placed in an 11 cm ceramic strip holder and an 11 cm IPG gel strip, pH range 3-10, was placed gel-side down on top of the solution. PlusOne dry strip cover fluid was placed over the IPG strip and used to fill the rest of the strip holder to prevent the strip from drying out during IEF. The ceramic holders were then loaded onto the IPG phor and the strips rehydrated for 12 h at 20°C. They were then subjected to IEF by means of a series of increasing voltage steps; 100 V for 2 h, 500 V for 1 h, 1000 V for 1 h, 2500 V for 1 h and 8000 V up to 38.8 kVh. According to Strahler *et al.*, (1988) the increasing voltage gradient allows the separation of samples independent of varying salt concentrations. Once the strips were electrofocused they were stored either at -80°C or prepared directly for 2-dimensional electrophoresis (2-DE).

2.7.4. Second-dimension electrophoresis: SDS-Polyacrylamide gel electrophoresis of the isoelectrically separated sample

The 11 cm strips were equilibrated in 7 ml of equilibration buffer (appendix 1.7.2) containing 70 mg of DTT for 15 min at RT on a shaker. The solution was decanted and the strips were equilibrated for a further 15 min in 7 ml of equilibration buffer containing 280 mg of iodoacetamide (Sigma). During equilibration of the IPG strip, the Multiphor II flatbed system was prepared for 2-DE. The temperature was set to 15°C on the MultiTemp III Thermostatic Circulator and 2 ml of IPG cover fluid was pipetted onto the Multiphor II cooling plate prior to addition of the 2-D Gel. The strips were left on a paper towel to remove as much of the equilibration solution as possible without over-drying. A 12.5 % (w/v) homogeneous 2D-ExcelGel was used to separate the proteins according to their molecular weights with gel buffer strips placed on either side of the gel as contacts between the gel and the electrodes. Two 11 cm IPG strips were placed on each Excel Gel and a piece of application paper (0.5 x 0.5 cm) containing 15 µl of molecular weight standards (Mark 12 MW: Invitrogen) was placed between the two IPG strips. Electrophoresis was performed at 20 mA / 600 V until the bromophenol blue had moved out of the IPG strips after which the IPG strips were discarded and the cathode buffer strip moved over to the position previously occupied by the IPG strips, and the electrode moved to the new location of the cathode buffer strip. The current was increased to 40 mA and the run was continued until the dye front reached the anode buffer strip.

2.7.5. Detection of protein by colloidal Coomassie blue

After electrophoresis, gels were removed from the Multiphor and fixed in a solution of 40 % (v/v) methanol, 10 % (v/v) acetic acid, 50 % DW for 2 h at RT on a shaker. A staining solution was prepared from 320 ml of colloidal Coomassie blue stain (Genomics Solutions) mixed with 80 ml of methanol. The fixing solution was drained off and the staining solution was added overnight. Coomassie blue binds to the proteins stoichiometrically. A disadvantage of Coomassie staining is its relatively insensitive detection limit. This limitation was catered for by ensuring that an adequate amount of protein was present during sample preparation. Following colloidal Coomassie blue staining, gels were placed in 25 % (v/v) methanol to decrease background staining, and destaining was performed twice for 30 m on each occasion or until excess stain had leached out from the gel. Subsequently, gel images were captured using an Image scanner and analysed using the ImageMaster 2D Elite software (Amersham Pharmacia). This program provides essential tools for automatic protein spot detection, quantification of spot density and allows equivalent spots to be matched between different gels.

2.7.6. Protein identification by matrix assisted laser desorption ionisation time of flight (MALDI-TOF) mass spectrometry

Following analysis of 2-D gels, spots were excised for further characterisation by MALDI-TOF. This is a highly sensitive (femtamole level) technique, performed on a Voyager-DE PRO mass spectrometer (Applied Biosystems, CA, USA). It can detect and characterise biomolecules such as proteins, peptides, oligosaccharides and oligonucleotides with molecular masses between 400 and 300,000 Da. Protein identification by this technique is rapid and requires only a small amount of sample (less than 1 p mol). The biomolecule is fixed in an UV-light absorbing matrix, α-cyano-4-hydroxycinnamic acid (CHCA), onto a target plate that is placed in the mass spectrometer. A nanosecond laser pulse irradiates the sample spot. Most of the laser energy is absorbed by the matrix, preventing unwanted fragmentation of the biomolecule. An electrical field confers the same kinetic energy on the ionised peptides so that they pass down a field-free flight tube under vacuum according to their mass to charge ratio and reach the detector plate at the end of the flight tube at proportionately different times. In this way each molecule yields a distinct signal and the percentage incidence of each ion will be unique for each trypsin digested protein analysed by MALDI mass spectrometry (MS). The masses of the peptides can be determined by reference to calibration peptides.

2.7.6.1. Trypsin digestion of proteins in 2DE gels for MALDI-TOF

Prior to MALDI-TOF it was necessary to fragment proteins with trypsin (a proteolytic enzyme that cleaves proteins after the amino acid lysine to produce peptides) digestion, so that the mass of each fragment could then be determined by mass spectrometry. The Moredun Functional Genomics Unit provided the protocol for in-gel trypsin digestion of proteins, and all steps were performed at RT unless stated otherwise. The spots of interest from 2-D gels were excised manually using a spot picking pen (The Gel Company, San Fransisco, CA) which ensured that no excess gel around the spot was extracted. Meticulous care was taken to avoid keratin contamination (from skin) by cleaning the spot picking pen (The Gel Company) with methanol between each excision and gloves were worn throughout the procedure. The excised spots were placed into sterile 1.5 ml siliconised microfuge tubes (Anachem Ltd, Luton, UK), covered with 100 mM ammonium bicarbonate / 50 % (v/v) acetonitrile, and incubated for 15 min. These destaining and dehydrating reagents respectively, were discarded and this step was repeated until the colloidal Coomassie blue stain (Genomics Solutions) had been removed from the gel spots. Gel pieces were dehydrated with 100 % acetonitrile (Sigma) for 10 min and then dried by centrifugal evaporation using a Speed Vac (Savant Instruments Inc, Hicksville, NY) for 20 min at RT.

Sequencing grade trypsin (Promega) was dissolved in 25 mM ammonium bicarbonate to a concentration of 10 ng μl^{-1}. Just enough trypsin solution was added to cover and rehydrate gel pieces, which were left to stand for 15 min. Following rehydration, 25 mM ammonium bicarbonate was added to the microfuge tubes in order to cover the gel pieces. The tubes were incubated at 37°C for at least 16 h. Following protein digestion supernatants containing peptides of interest were either stored at –20°C or 0.5 µl aliquots were taken for analysis by MALDI-TOF MS.

2.7.6.2. MALDI-TOF MS

Equal volumes (0.5 µl) of peptide digest and 10 mg ml^{-1} of CHCA matrix (Ciphergen Biosystems, Inc, Fremont, CA) suspended in 50 % (v/v) acetonitrile / 0.1 % (v/v) trifluoroacetic acid (TFA) were applied to the designated circle on the target plate and left to dry. Standards of a known molecular mass were spotted in 0.25 µl volumes on the top right hand side of the designated circle for calibration. The standards were left to dry and then automated MS was performed.

2.7.7. Database search

An online database constructed by a Swiss proteomics company (Swiss-Prot) contains "fingerprints" comprising the peptide masses generated by trypsin digestion of known proteins. Comparison of the fingerprints obtained from MALDI-TOF MS with the fingerprints on the Swiss-

Prot database was used to identify proteins of interest detected in the BAL samples. An equivalent database with which to identify *P. multocida* proteins in lung fluid was available following recent sequencing of the entire genome of a turkey capsular serotype A strain (Pm70) of *P. multocida* (May *et al.*, 2001).

2.8. Animal experiments

2.8.1. Animals

Calves used in experiments were Holstein x Friesian of both sexes and were bred at farms within the Midlothian region. Calves were 8 weeks old at the start of the experiments.

2.8.2. Culture conditions of live *P. multocida*

Stored cultures, thawed and plated onto 5 % (v/v) SBA (appendix 1.2.1), were incubated at 37°C overnight to check purity and to observe growth of bacteria. Single colonies were picked from plates and inoculated into 10 ml of NB. After static incubation at 37°C for 16 h, 0.5 ml aliquots of culture were inoculated into further 10 ml aliquots of NB and the subcultures were transferred to a rotary shaker (Forma Scientific Inc, Ohio, USA) for 3.5 h.

Counts of viable bacteria were estimated by inoculating 4 x 20 µl volumes of ten-fold dilutions of the original bacterial suspension onto SBA and incubated overnight. The colonies from the sample dilution giving discernible colonies were counted and the mean count was multiplied by 50 and the dilution factor to obtain the viable counts ml^{-1}.

2.8.3. Production of formalin-killed *P. multocida*

0.5 ml of static overnight culture, in NB, at 37°C of the MRI-671/90 challenge strain was inoculated into 50 ml NB and placed on a rotary shaker for 4-6 h at 37°C. The total dose of 10^9 cfu cells was centrifuged at 2,000 x *g* for 30 min at 20°C and killed by re-suspending in 0.3 % (v/v) formalin overnight at 4°C. The dead bacteria were washed in PBS to remove the formalin, centrifuged at 2,000 x *g* for 20 min at 20°C and resuspended in 300 ml PBS. The formalin-killed *P. multocida* suspension was streaked out onto 5 % (v/v) SBA at weekly intervals throughout the experiment to check for bacterial contaminants.

2.8.4. Procedure for intratracheal challenge with *P. multocida*

For challenge, calves were restrained whilst a fibre optic bronchoscope was inserted via a nostril into the trachea and the challenge instilled at the bronchial bifurcation. The two types of formulations, live and formalin-killed were delivered into the same calf, on different occasions via a sterile syringe attached to the bronchoscope and into the airway during which time calves were observed for any adverse effects of the challenge procedure itself. The bronchoscope was sterilised with weak chlorhexidine solution (Novartis Pharmaceuticals UK Ltd, Camberley, Surrey, UK) and rinsed with saline prior to re-use.

2.8.5. Clinical monitoring

Following challenge, calves were monitored at 4 h intervals for clinical signs of disease, using a scoring system similar to that established for sheep (Hodgson *et al.*, 1995) in which 0 represented a normal state and higher scores represented increasing disease. Signs were scored as follows: demeanour (normal, dull, depressed, recumbent) 0-3; respiratory rate (<60, 60-80, 80-100 and >100) 0-3; nature of respiration (normal, increased effort, laboured, dyspnoea) 0-3; rectal temperature (38-39.5, 39.51-40.5 and >40.5 or <38) 0-2; nasal discharge (absent, mild, moderate, profuse) 0-3; appetite (normal, depressed, anorexic) 0-2. The maximum score possible was 16 but intervention was set at half this limit and any calf that developed acute respiratory problems and dullness with a score between 8 and 10 was killed to minimise the degree and duration of any suffering.

2.8.6. Necropsy

Calves were killed by intravenous injection with 25 ml of Pentobarbitone Sodium B.P. (200 mg ml^{-1}; Animal Care) on day 4 p.c. The trachea was exposed and clamped to avoid intratracheal contamination with blood, lungs were removed and the presence, extent and nature of superficial lesions were determined using a method developed for sheep (Gilmour et al, 1983). Briefly, the percentage of affected whole lung surface area was calculated from diagrams produced during the PM examination, whereby various regions of the lung comprised a particular percentage according to their area (Fig. 2.1). In addition, the severity and extent of pleurisy associated with each lung was assigned a score between 0 and 3, three being the most severe.

Representative samples from lung, heart, liver, kidney, bronchiolar and mediastinal lymph node were placed in 10 % (v/v) formol-saline and prepared for histopathological examination by

embedding in paraffin wax and cutting into 5 micron sections, followed by staining with haematoxylin and eosin.

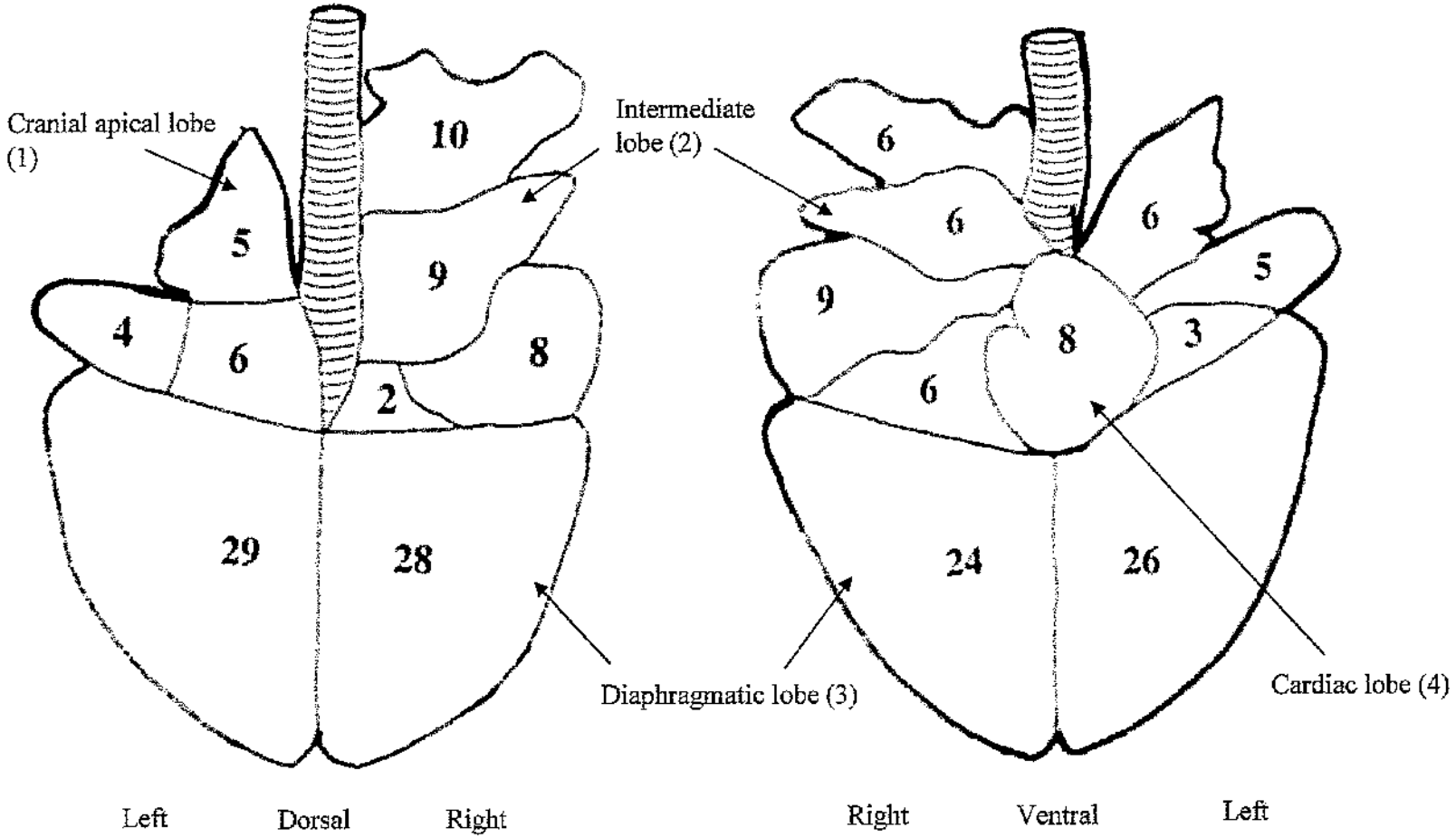


Fig. 2.1. A schematic diagram of calf lungs to illustrate the areas of lungs sampled at post-mortem for bacteriology examination, denoted by (1, 2, 3, 4). The numbers add up to 200 and represent the relative area of different lung lobes and were used to assess the extent of consolidation. This diagram was kindly produced by Mr Malcolm Quirie, Bacteriology Department, Moredun Research Institute.

2.8.7. Bacteriological examination

Small samples of tissue (approximately 1 cm cubes, about 1 g) taken from four pre-selected lung sites (apical, intermediate, diaphragmatic and cardiac lobes) (Fig. 2.1), bronchial lymph nodes, heart, spleen, liver and kidney were homogenised in 9 ml peptone water and diluted in 10-fold steps to 10^{-6}. Aliquots (10 µl) of each dilution from 10^{-6} to 10^{-2} were applied to SBA plates and incubated at 37°C for 16-20 h. Viable counts were determined and expressed as cfu g^{-1} of tissue.

2.9. Phagocytic activity of host defence cells

2.9.1. Bronchoalveolar lavage

Prior to PM examination, calves were restrained while a flexible fibrescope was inserted via a nostril into the trachea, past the bronchial bifurcation and into the lower airways until resistance was felt. Two successive 60 ml aliquots of Ca^{2+}, Mg^{2+} indicator-free hanks balanced salt solution (HBSS) (Sigma) at RT were infused into the alveolar space via a syringe attached to the bronchoscope. Dislodged cells were recovered by withdrawing the fluid from the lungs into the syringe. The mean recovery of BAL fluid was approximately 60 % of the 120 ml instilled. Calves were monitored throughout and showed only moderate discomfort during the procedure.

2.9.2. Separation of alveolar macrophages and neutrophils from lung wash

The recovered fluid was separated into lung phagocyte cells and supernatant fractions by centrifugation at 470 x *g* for 10 min, at 5°C. The supernatant fractions were placed in a 75 cm^2 culture flask, (Costar, USA), weighed and stored at -40°C prior to subsequent protein analysis. The pelleted fractions were washed once in 10 ml of Ca^{2+}, Mg^{2+} indicator-free HBSS (Sigma), then resuspended in 5 ml of isotonic Percoll (Amersham Pharmacia, Biotech, Ltd), diluted to a density of 1.075 g $ml^{-1}$with HBSS and 10 ml of Percoll (density 1.055 g ml^{-1}) (appendix 1.8.2) layered on top. The gradients were centrifuged at 1,200 x *g* for 15 m at 4°C to produce two bands; the pellicle (Band 1) contained approximately 90 % alveolar macrophages and the pellet (Band 2) comprised 95 % neutrophils with some alveolar macrophages. Both cell types were harvested and washed twice in Ca^{2+}, Mg^{2+} indicator-free HBSS at 4°C or until the supernatant was clear.

2.9.3. Viability of lung phagocytes

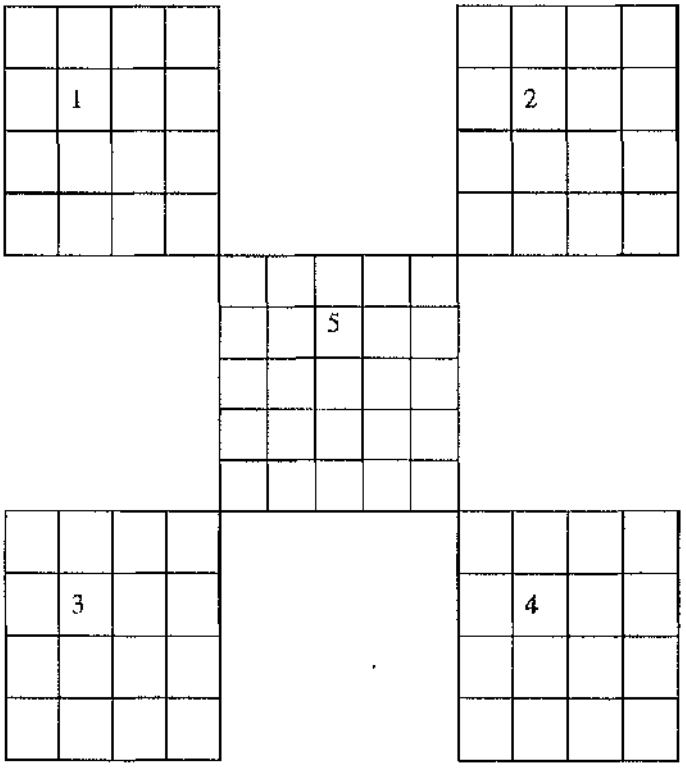
After the final wash cell pellets were resuspended in a weighed universal with 5 ml of Ca^{2+}, Mg^{2+} indicator-free HBSS and concentration and viability of the cells determined using a haemocytometer (ORME technologies, Whitbrook Way, Stakehill Industrial Park, Middleton, Manchester, UK) and a light microscope (Olympus Optical Co. Ltd, 2-8 Honduras street, London, UK) at x 40 magnification by counting the cells over 25 large squares from 10 µl of a 1/10 dilution of the macrophages or neutrophils in 0.1 % (w/v) nigrosin (appendix 1.8.1) (Fig. 2.2). The nigrosin enters dead cells through perforated cell membranes. Viable cells and percentage viability were determined as follows:-

$$\text{Number of live or dead cells per ml} = (\text{cells} \times 10 \times 10^4)$$

$$\text{Viability} = (\text{Number of live cells/ total cell count}) \times 100$$

The total volume was obtained and the suspensions were diluted in HBSS to give a count of 2×10^6 live cells ml^{-1}

Fig. 2.2. Counting grid present on haemocytometer for the accurate determination of live and dead cells within a given area.



Count 5 large squares

2.9.4. Measurement of respiratory burst

Luminol-dependent chemiluminescence (LDCL) is a sensitive method for measuring changes in the oxidative metabolism of phagocytes. The chemiluminescence (CL) response of bovine alveolar macrophages and neutrophils undergoing phagocytosis was determined at ambient RT using a luminometer (Model TR717, PE Biosystems). Potential stimulants of the respiratory burst included a.) McCoys 5A medium, b.) *P. multocida* opsonised with bovine antiserum (section 2.9.5), c.) *P. multocida* A:3, non-opsonised and d.) PMA with Ca^{2+} and Mg^{2+} which generates a high LDCL response as a standard for comparison within and between assays.

2.9.4.1. Reagents for the luminometer

A stock solution of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, Sigma) was prepared at a concentration of 2 mg ml^{-1} in PBS and stored in 20 µl volumes in foil-wrapped eppendorf tubes at -20°C. Stock luminol was thawed and diluted 1/500 in Ca^{2+}, Mg^{2+}, indicator-free HBSS and warmed to 37°C immediately before use. A stock solution of PMA (Sigma) was prepared at a concentration of 10 mg ml^{-1} and stored in 5 µl aliquots in eppendorfs at -20°C. The working reagent was prepared by diluting the stock PMA solution 1/2000 in Ca^{2+}, Mg^{2+}, indicator-free HBSS and also warmed to 37°C immediately before use.

2.9.4.2. Luminol-dependent chemiluminescence assay (LDCL)

An *in vitro* assay based on LDCL measures the photons of light generated by the reaction between luminol and reactive oxygen intermediates (ROI) produced by neutrophils or macrophages during and after phagocytic stimulation (Richards and Renshaw, 1986). The light emitted is a product of the phagocytically induced respiratory burst associated with oxygen-dependent activation of the hexose monophosphate shunt (Chang *et al.*, 1985). The respiratory burst uses molecular oxygen to produce ROI, used by the phagocyte for microbial killing (Briheim *et al.*, 1984).

LDCL assays were performed using 100 µl of cells at 2 x 10^6 cells ml^{-1} in triplicate in Dynex Microlite white opaque 96-well microplates (Dynex Technologies Ltd). All wells contained phagocytes and luminol and the incubation temperature in the luminometer was maintained at 37°C. Microplates were incubated for 7 m before adding the pre-warmed stimuli to appropriate wells. Plates were placed in the luminometer and the phagocytic respiratory burst measured. Measurements were obtained over a 32 min period, at 37°C with a sample time of 1 s repeated every 30 s. The mean area under the CL curve, of relative light units (RLU) against time

represented the amount of reactive intermediates produced, and the rate of the initial CL peak was measured when the phagocytes were stimulated.

2.9.5. Optimisation of the opsonisation procedure

This method was performed as described by Lagergard *et al.*, (1995) with slight modifications. *P. multocida* were grown overnight and suspended at 1 x 10^8 cfu ml^{-1} in McCoys 5A medium, with concentration determined using a MacFarland standard number and diluting with PBS if required. Bacteria in a 100 µl suspension at 1 x 10^6 cfu were incubated with different volumes of heat inactivated hyperimmune bovine antiserum raised against *P. multocida* A:3 (20 µl, 40 µl, and 60 µl), at 37°C for 15, 30, 45 and 60 min and harvested by centrifugation at 12,000 x *g* for 10 min. This preparation was dispensed into duplicate wells (100 µl) of a 96-well microplate containing 100 µl of HRP-anti-bovine IgG conjugate at 1/1000 and incubated at 37°C for 30 min. After washing three times with PBS, OPD substrate was added (appendix 1.5.5) and incubated for 5 min at RT. The reaction with OPD was stopped by the addition of 50 µl 2.5M H_2SO_4 and absorbance was read at 490 nm. The absorbance in wells containing bacteria incubated with McCoys 5A medium served as background and was subtracted from those of the test samples.

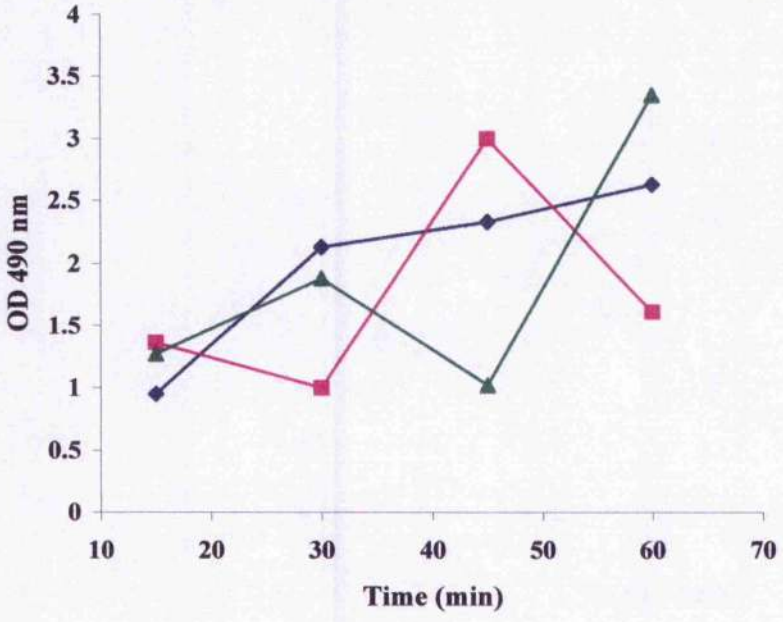


Fig. 2.3. A graph to determine the optimum volume of bovine antiserum and duration of incubation with *P. multocida* to achieve efficient opsonisation; 20 µl bovine antisera (◆), 40 µl bovine antisera (■) and 60 µl bovine antisera (▲).

The graph (Fig. 2.3) indicated that 20 µl of bovine antiserum raised to a live challenge of *P. multocida* incubated for 30 min with *P. multocida* was sufficient for optimal opsonisation. The shape of the 20 µl curve was more consistent than the others, but the problem with this assessment is with the time and volume so variable, the opsonisation may vary widely also.

2.9.6. Phagocytic capacity determined by the uptake and killing of *P. multocida in vitro*

Phagocytic uptake of *P. multocida* by bronchoalveolar macrophages harvested from calves challenged with either live or dead *P. multocida* A:3, was studied using the acridine orange /crystal violet (AO/CV) fluorescent technique (Goldner *et al.*, 1983) (appendix 1.8.3) and adhesion slides (Paul Marienfeld GMBH & Co, Lauda-Konigshofen, Germany). This method can be used to differentiate between ingested and extracellular adherent bacteria by the addition of CV dye to quench any bacterium that has not been internalised by the phagocyte (Zanetti *et al.*, 1987).

Phagocytes, extracted on the day of a lung lavage, at a concentration of 2×10^6 cells ml^{-1} (10 µl = 2×10^4 cells) were added to each reaction field and the slide placed in a 5 % (v/v) CO_2 incubator at 37°C for 15 min.

Reagents warmed to 37°C were RPMI 1640 (appendix 1.8.4) (Life Technologies Ltd, Paisley, UK) modified by the addition of 20 mM HEPES, 1 % (v/v), 1 % (v/v) L-glutamine, and lacking bicarbonate and antibiotics, while supplemented with 10 % (v/v) heat-inactivated (1 h at 56°C) foetal calf serum (FCS); (Life Technologies Ltd); McCoy's 5A medium (Life Technologies Ltd); Ca^{2+}, Mg^{2+}, indicator-free HBSS; acridine orange (AO) (10 mg ml^{-1}) (Molecular Probes Europe BV, Rijnsburgerweg 10, AA Leiden, The Netherlands) and crystal violet (CV) (1% aqueous solution (v/v) (ORME technologies) (appendix 1.8.3).

After incubation, non-adherent cells were removed from the reaction fields by washing with Ca^{2+}, Mg^{2+} indicator-free HBSS into a weighed container and the cells were counted using a haemocytometer (ORME technologies). The number of adherent cells (approximately 10^5) were determined by difference. RPMI at 37°C was applied to the adherent cells instead of Ca^{2+}, Mg^{2+} indicator-free HBSS (to provide nutrient) and the reaction fields were incubated for 1 h in a 5 % (v/v) CO_2 incubator at 37°C.

Suspensions of *P. multocida* type A:3 (MRI strain 671/90) in mid-log phase of growth in NB from a 3.5 h culture were prepared, whereby the estimated viable count of bacteria were 10^8 cfu ml^{-1} using a McFarland standard reading of 2 and a correction factor of 1.5, which takes into account variable colony mass and growth kinetics of different bacterial strains (Miles *et al.*, 1938). The culture

suspension was adjusted by dilution in McCoys 5A medium (Life Technologies Ltd) so that 200 µl contained 2 x 10^6 live organisms (approximately 10 times the number of adherent cells). *P. multocida* was opsonised directly prior to use by incubating 500 µl of bacterial suspension with 100 µl of *P. multocida* A:3 hyperimmune serum, heated previously at 56°C for 30 min to remove complement factors (20 µl per 10^6 bacteria; Hodgson *et al.*, 1996), at RT for 30 min. Triplicate reaction fields were incubated with 20 µl of opsonised bacterial suspension, 20 µl of sterile McCoys 5A solution (control) or 20 µl of non-opsonised bacterial suspension, for 1 h (Hodgson *et al.*, 1996). All reaction fields were then washed three times with Ca^{2+}, Mg^{2+} indicator-free HBSS to remove non-adherent organisms, 20 µl of pre-warmed RPMI was added and the reaction fields incubated for a further 30 min. After a final wash with Ca^{2+}, Mg^{2+} indicator-free HBSS each preparation was stained with 20 µl of AO (14.4 mg 100 ml^{-1} GBSS) for 45 s and using Ca^{2+}, Mg^{2+} indicator-free HBSS, washed twice, stained with 20 µl of 1 % (v/v) CV (50 mg 100 ml^{-1} PBS) for 45 s and washed twice before cell-side of the adhesion slide was covered by a cover slip (Chance Propper Ltd, West Midlands, England) and sealed with clear nail varnish. Slides were viewed under oil immersion (Leitz antifluorescent oil) at a magnification of 1,250 x using an Olympus BX50 fluorescent microscope (Olympus Optical Co. Ltd). The following measurements of phagocytic activity were recorded: the mean number of ingested bacteria per macrophage, the mean percentage of macrophages that were phagocytic, *in vitro* per 100 BAM and the mean number of bacteria killed *in vitro* per BAM. CV quenches any extracellular fluorescence generated by AO, ensuring that only ingested bacteria are counted. Viable AO-stained organisms fluoresce green (orthochromasy) under ultraviolet excitation; whereas killed bacteria fluoresce dark orange (metachromasy) indicating a loss of viability of ingested organisms (Williams *et al.*, 1991).

2.9.7. Storage of cells

The remaining cell suspension was centrifuged at 470 x *g* for 10 min, at 5°C and the pellet of cells was re-suspended in freeze mixture (appendix 1.8.5) to 1 x 10^7 cells ml^{-1}. The suspension of freeze mixture and cells was transferred to cryovials for overnight storage at -70°C followed by storage in liquid nitrogen prior to *in vitro* analysis.

2.9.8. Differential immune cell counting

BAL fluid samples comprising 5 x 10^5 cells ml^{-1} were centrifuged at 70 x *g* for 5 min using a cytocentrifuge (Life Sciences International, Cheshire, England) and forced through a filter assembly onto a slide partly covered with filter paper but with an uncovered target staining area. The cells were fixed immediately and stained with Diff-Quik® (Dade Behring, Switzerland). In this

procedure, the air-dried cytospin was immersed initially in a fixative solution (fast green in methanol, 0.002 g l^{-1}) for 5 s, then dipped into the first staining solution (Eosin G 1.22 g l^{-1} in phosphate buffer pH 6.6), for a further 5 s and finally coloured with Thiazin-Farbsstoff dye (1.1g l^{-1} in phosphate buffer pH 6.6) for 10 s. The duration of cytospin immersion in the Diff-Quik® solutions was optimised to achieve the appropriate staining intensity for the detection of lung immune cells. Finally the cytospin was rinsed with DW and air-dried for viewing under a microscope. Total and differential cell counts were determined for the BAL fluid samples recovered at live lavage.

2.10. Statistical analysis

A variety of statistical methods were used in the analysis of the data. Data were examined for symmetry, and where necessary were transformed using a square-root or logarithmic function to remove skewness. Where comparisons were being made between a pair of groups, t-tests were performed and means and standard errors of the mean (SEM) were calculated using Minitab version 13. More complex statistical designs were analysed using analysis of variance (ANOVA) or related statistical methods. The experiment described in Chapter 4 was designed using a factorial formulation to increase the statistical power. Such a statistical design must be analysed in terms of estimating effects and interactions. However, the experiment described in Chapter 4 gave rise to unbalanced data, making simple ANOVA analysis difficult. In this case, Residual Maximum Likelihood (REML) was used to estimate differences between group data over time. REML is commonly regarded as the most appropriate statistical methodology for unbalanced data sets such as those of Chapter 4. By contrast, data from the experiment described in Chapter 6 were analysed using ANOVA, a simpler methodology which could be applied to this data set. It should be noted that the small sizes of the groups of calves and the high variability between these animals made it likely that only large effects would be identified as statistically significant.

Chapter 3. MOLECULAR CHARACTERISATION AND LPS CHEMOTYPING OF COMMENSAL AND VIRULENT *PASTEURELLA MULTOCIDA* ISOLATED FROM CONVENTIONAL CALVES.

3.1. INTRODUCTION

P. multocida isolates are grouped into five serogroups (A, B, D, E and F) based on capsular antigens, or into 16 somatic serotypes based on variations of LPS structure, designated according to the Carter-Heddleston system (Rimler and Rhoades, 1987). The organism may be isolated from the respiratory tract of both healthy and diseased cattle (Frank, 1989; Biberstein, 1990) indicating that pathogenic and commensal types may exist, but the role of possible commensal strains in the development of disease has not been determined. There are several potential mechanisms for commensal involvement; firstly, disease may result from a simple overgrowth of bacteria resident in the nasopharynx resulting from immunosuppression associated with certain stressful conditions such as weaning, dehorning, viral infection or transport (Frank, 1989). Subsequent shedding and inhalation of bacteria in numbers sufficient to overcome defences in the URT (Gonzalez and Maheswaran, 1993) may occur and cause pneumonic pasteurellosis, an acute disease associated with welfare problems and significant economic losses to the livestock industry (Dalgleish, 1989). In such cases, the bacteria involved are genetically and phenotypically identical to the organism causing disease within the lung, as was demonstrated in a proportion of acutely ill calves described by DeRosa *et al* (2000); however, this latter work gave no information on strains present in clinically healthy calves and, therefore, no distinction as to whether a commensal or new virulent strain was involved in the pathogenesis of disease.

This forms a second hypothesis where the disease-causing organism is entirely disparate from the commensal and not analogous with any strains recovered from clinically healthy animals.

A third hypothesis is that commensal bacteria may undergo genetic or phenotypic modifications as a result of some stimulus in healthy animals, conferring virulent attributes and enabling them to locate to, and subsequently infect, the lung. For example, research on serotype predominance in the URT in a closely related organism to *P. multocida*, *M. haemolytica*, has provided evidence for the rapid switch from commensal to pathogen. It has been shown that microfloral population shifts in favour of a more virulent *M. haemolytica* strain, serotype 1 (ST1) revealed by its smooth LPS, from

serotype 2 (ST2) possessing the less virulent rough LPS (Gonzalez and Maheswaran, 1993). Overall however, there is still insufficient information regarding prevalence, genetic and antigenic variation of *P. multocida* thus limiting our understanding of its virulence and pathogenesis (Davies and Donachie, 1996).

Recent evidence suggests that antigenic variation of cell surface structures, including LPS, could be a key factor impeding the development of effective vaccines (Preston and Maskell, 2002). Variation can be assessed by characterising genetic and phenotypic differences, including key surface structures, such as LPS. PFGE remains a useful tool for assessing the degree of genetic relatedness amongst a broad array of bacterial species (Tenover *et al.*, 1995), and has been shown to be more discriminatory than ribotyping, random amplified polymorphic DNA and REP PCR (Hunt *et al.*, 2000) in the differentiation of *P. multocida and M. haemolytica* strains. DNA:DNA hybridisation techniques and extensive phenotypic testing led to the reclassification of the *Pasteurella* genus (Mutters *et al.*, 1985) when *P. multocida* was divided into the 3 subspecies, *P. multocida* subsp. *multocida*, subsp. *septica* and subsp. *gallicida*. The recent development of the multilocus sequence typing method has been of value for differentiating isolates to the subspecies level in addition to identifying outbreak isolates and the clonal relationships between strains (Maiden *et al.*, 1998). This technique has been developed for *Streptococcus suis*, *Enterococcus faecalis* and *Listeria monocytogenes* (Nallapareddy *et al.*, 2002; King *et al.*, 2002; Salcedo *et al.*, 2003) to address these issues and would be of value for *P. multocida*.

Prior to this investigation allocation of biotype was based upon characteristics typical of *P. multocida* on conventional bacteriological media: lack of growth on MacConkey agar; fermentative reaction patterns observed with acid production from certain pentoses, disaccharides and polyhydric alcohols; negative reaction with urease and positive reactions for oxidase, catalase and indole (Bisgaard *et al.*, 1991).

Some key surface structures that interact powerfully with the host immune system, such as LPS, may be altered through antigenic variation, so that a single bacterium can express more than one form of the antigenic component, which is then recognised by the host immune system (Maskell *et al.*, 1993). Although there is no evidence for this phenomenon in *Pasteurella* spp., whereas a related organism, *Haemophilus influenzae*, displays an array of highly immunogenic LPS antigens. Such variation presents problems for effective vaccination, and a better understanding of the natural diversity and the host immune responses to these antigens will aid strategies for pathogen control.

The common and unique LPS structures associated with *Pasteurella* spp. enable this species to be classified, however the variation of antigenicity shown by LPS provides scope for diversity and thus further classification. The intra-specific diversity of LPS has been assessed previously by

analysing variations in the LPS profile of *M. haemolytica* and *P. trehalosi* between and within capsular polysaccharide serotypes (Davies and Quirie, 1996; Davies and Donachie, 1996), but *P. multocida* LPS has not been characterised.

The present work assessed the phenotypic, genotypic and LPS chemotype relatedness of *Pasteurella* spp. isolates recovered from both clinically healthy and diseased animals during a spontaneous outbreak of pneumonic disease at the MRI.

3.2. EXPERIMENTAL DESIGN AND METHODS

3.2.1. Bacterial isolates

A total of 30 clinical isolates were recovered from 28 conventional calves at 8 weeks of age, by nasal swabbing from all animals on arrival at MRI and taking lung tissue samples from clinical cases at post-mortem. These samples were examined and compared with 19 reference strains representative of the currently recognised *P. multocida* serotypes; an MRI pneumonic A:3 strain and 3 type strains of *P. multocida* (subsp. *multocida*, subsp. *septica* and subsp. *gallicida*) obtained from culture collections (Table 3.1). All samples were cultured on 5 % (v/v) SBA plates, and incubated aerobically for 16 h at 37°C; single colonies were picked and sub-cultured to ensure purity, and stored either in NB at −70°C or in a lyophilised state at RT until the time of characterisation.

3.2.2. Molecular strain characterisation

Isolates with colony morphology similar to *P. multocida* and which were oxidase and catalase positive were selected for phenotypic characterisation. Tests used were: production of urease and indole, β-galactosidase activity, production of acid from arabinose, maltose, mannose, sorbitol, mannitol, trehalose and xylose, and decarboxylation of ornithine (Bisgaard *et al.*, 1991).

Capsular types D or A of *P. multocida* were determined using acriflavine or hyaluronidase tests, respectively (Carter *et al.*, 1973 and 1975, Rimler 1994). The former test identified type D strains by the production of a heavy flocculent precipitate on the addition of an aqueous solution of acriflavine to a concentrated broth culture. The latter test identified type A strains by growth restriction due to the depolymerisation of capsular hyaluronic acid by hyaluronidase. Bacterial culture and PFGE conditions used were those described by Lainson *et al.*, (2002) (section 2.2.1).

LPS characterisation of *P. multocida* A:3 and essential reagents to assist this are described in detail in (section 2.3) and (section 2.5) respectively.

P. multocida in bovine nasal swabs and lung tissue was identified by PCR, using primers specific for the *kmt1* gene encoding a protein involved in the synthesis of capsular polysaccharide. Conditions for the detection of *P. multocida* were those used by Townsend *et al.*, (2001) with slight modifications.

Table 3.1. *Pasteurella* spp. isolates from a natural outbreak of bovine pneumonic pasteurellosis in Scotland and reference strains.

Source	Host / bacterial species	MRI and Rimler reference number	Total no. of isolates / strains
Natural outbreaks			
Nasopharynx	Bovine	14/00R, S, B, C, G, Q, 15/00F, 14/00A, D, E, H, J, L, P, N, K, T, F, I, M, U, 15/00A, B, C[a], D[b], E	26
Lung	Bovine	12/00, 20/00, 21/00[b], 23/00[a]	4
Reference strains			
Lung	Bovine	MRI-671/90[1]	1
Rimler reference strains	Chicken	116/95A	
	Bison	116/95B	
	Turkey	116/95C, D, E, F, I, J, O, P, S	19
	Herring gull	116/95G	
	Pine siskin	116/95H	
	Porcine	116/95K	
	Human	116/95L, M	
	Bovine	116/95N, Q, R	
NCTC[2] 10322	subsp. *multocida*	34/01A	
CIP[3] A125	subsp. *septica*	34/01B	3
NCTC[2] 10204	subsp. *gallicida*	34/01C	

[1]671/90 is an MRI pneumonic strain, somatically typed as A:3. [2]NCTC; National Collection of Type Cultures (NCTC), London, UK. [3]CIP Collection de l'Institut Pasteur, Paris, France. 15/00C[a]/ 23/00[a] and 15/00D[b]/ 21/00[b] are the paired nasal and lung isolates.

3.3. RESULTS

3.3.1. Molecular characterisation of test isolates

3.3.1.1. Phenotypic characterisation

All 30 test isolates were catalase and oxidase positive and urease negative. PFGE profiles, capsular types and sugar fermentation characteristics are presented in Table 3.2. Using the *P. multocida* MRI-671/90 challenge strain (Chapter 4) as a positive control, 20 out of the 30 bovine isolates were found to be *P. multocida* subsp. *multocida*, while the remainder were assigned to other taxa within the *Pasteurellaceae* family, primarily *Mannheimia* sp. (Table 3.2). Data presented in Table 3.2 show that phenotypic analysis of these isolates revealed the presence of 11 biovars (I to XI). Of the test isolates identified as *P. multocida* subsp. *multocida* the majority fell into biovar I and the remainder were distributed between biovars II-VI. 19 of the 20 *P. multocida* subsp. *multocida* isolates were mucoid confirming production of capsule type A, whereas the remaining isolate was capsular type D. Similar data for the 19 reference isolates and the type strains are presented in Table 3.3. Sixteen of these reference isolates were identified as *P. multocida* subsp. *multocida*, 2 as *P. multocida* subsp. *gallicida* and 1 as *P. multocida* subsp. *septica*, and all fell within 6 biovars (I, XI-XV). The majority (12) of the reference strains and the MRI (671/90) strain shared the same biovar (XI).

Table 3.2. Phenotypic characters and PFGE profiles of field isolates[a]

Biovar and isolate	Profile	Mucoid	Capsule type	Bacteria	I	[b]ODC	[c]ONPG	A	M	Ma	S	T	X	Man
I 12/00	P10	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
14/00I	P9	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
14/00M	P4	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
14/00U	P11	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
15/00A	P2	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
15/00B	P2	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
15/00C	P2	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
15/00D	P2	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
15/00E	P2	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
20/00	P2	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
21/00	P2	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
23/00	P2	Y	A	*multocida*	+	+	-	-	-	+	+	+	+	+
II 14/00T	P4	Y	A	*multocida*	+	+	-	-	-	-	+	+	+	+
III 14/00P	P7	N	D	*multocida*	+	+	-	-	-	+	+	+	-	+
IV 15/00F	P3	Y	A	*multocida*	-	-	+	-	-	+	+	+	+	+
V 14/00B	P5	Y	A	*multocida*	-	-	-	-	-	+	+	+	+	+
14/00C	P6	Y	A	*multocida*	-	-	-	-	-	+	+	+	+	+
14/00G	P5	Y	A	*multocida*	-	-	-	-	-	+	+	+	+	+
VI 14/00R	P5	Y	A	*multocida*	-	-	-	-	-	-	+	+	+	+
14/00S	P5	Y	A	*multocida*	-	-	-	-	-	-	+	+	+	+
VII 14/00A	P1	N		*haemolytica*	-	-	+	-	+	+	+	-	+	-
14/00D	P1	N		*haemolytica*	-	-	+	-	+	+	+	-	+	-
14/00E	P1	N		*haemolytica*	-	-	+	-	+	+	+	-	+	-
14/00H	P1	N		*haemolytica*	-	-	+	-	+	+	+	-	+	-
14/00J	P1	N		*haemolytica*	-	-	+	-	+	+	+	-	+	-
14/00L	P1	N		*haemolytica*	-	-	+	-	+	+	+	-	+	-
14/00P	P1	N		*haemolytica*	-	-	+	-	+	+	+	-	+	-
VIII 14/00K	P8	N		*haemolytica*	-	+	+	+	+	+	-	-	+	-
IX- 14/00Q	P1	N		*haemolytica*	-	-	-	-	+	+	+	-	+	-
X- 14/00N	P1	N		Pasteurella *spp.*	-	-	+	-	+	+	+	+	+	-
XI- 671/90	P12	Y	A	*multocida*	+	+	-	-	-	+	+	-	+	+

Bovine test isolates grouped according to biovar (denoted by Roman numerals) as determined from biochemical fermentation patterns, showing PFGE profile, capsule characteristics and the class of sub species to which each belonged. [a]All bovine field isolates were catalase/oxidase positive, urease negative and resided within biovars I to X. The MRI-challenge strain 671/90 had a unique biovar pattern (XI). [b]ODC = Ornithine decarboxylase activity; Detection of β-galactosidase activity: [c]ONPG = (O-nitro-phenyl-D-galactopyranoside); A = Arabinose; I = Indole; M = Maltose; Ma = Mannitol; S = Sorbitol; T = Trehalose; X = Xylose; Man = Mannose; + positive reaction; – negative reaction.

Table 3.3. Phenotypic characters and PFGE profiles of Rimler reference *P. multocida* isolates and type strains[a].

Biovar and isolate	Mucoid	Capsule type	Bacteria	I	[b]ODC	[c]ONPG	A	M	Ma	S	T	X	Man
I 116/95H	N	D	*multocida*	+	+	-	-	-	+	+	+	+	+
XI 116/95B	N	B	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95C	Y	A	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95D	Y	A	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95E	Y	A	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95I	Y	A	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95J	Y	A	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95K	N	D	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95L	Y	A	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95P	Y	A	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95Q	N	E	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95R	Y	D	*multocida*	+	+	-	-	-	+	+	-	+	+
116/95S	Y	F	*multocida*	+	+	-	-	-	+	+	-	+	+
XII 116/95F	N	D	*multocida*	+	+	-	-	-	+	+	-	-	+
116/95G	N	D	*multocida*	+	+	-	-	-	+	+	-	-	+
XIII 116/95O	N	D	*multocida*	+	+	+	-	-	+	+	-	+	+
XIV 116/95M	N	D	*septica*	+	+	-	-	-	+	-	+	+	-
XV 116/95A	Y	A	*gallicida*	+	+	-	+	-	+	+	-	-	ND
116/95N	N	A	*gallicida*	+	+	-	+	-	+	+	-	-	ND
Type strains													
34/0A		A	*multocida*	+	+	-	-	-	+	+	+	+	
34/0B			*septica*	+	+	-	-	-	+	-	+	+	
34/01C			*gallicida*	+	+	-	+	-	+	+	-	-	

[a]All reference isolates and type strains were grouped according to biovar (denoted by Roman numerals) as determined from biochemical fermentation patterns and all isolates were catalase/ oxidase positive and urease negative. I, Indole; [b]ODC, Ornithine decarboxylase activity; [c]ONPG, *O*-nitro-phenyl-D-galactopyranoside (β-galactosidase activity); A, Arabinose; M, Maltose; Ma, Mannitol; S, Sorbitol; T, Trehalose; X, Xylose; Man, Mannose; +, positive reaction; –, negative reaction. ND = not determined.

3.3.1.2. Genetic characteristics

Of the test isolates examined, including the MRI-671/90 pneumonic strain, 12 pulsotypes (Table 3.2) were identified by PFGE (Fig. 3.1and Fig. 3.2) showing between 5 and 12 bands, ranging in size from below 48.5 to 485 kb. A dendrogram was produced of best-fit analysis using the Dice coefficient function of the Image Master Database 3D Elite software to show the percentage similarity between representative isolates of the 12 pulsotypes (Fig. 3.3). Within this study, 8 of the 9 *Mannheimia* sp. isolates were uniquely associated with pulsotype P1 profile and were assigned to 3 biovars (VII, IX and X); the remaining isolate (14/00K) was assigned to pulsotype P8, biovar (VIII). Pulsotype P2 comprised 8 of the 20 *P. multocida* subsp. *multocida* isolates biovar (I) whereas pulsotypes P3, P6, P7, P9, P10 and P11 contained only single isolates and were distributed amongst 4 biovars (I, III, IV and V). Four of the test isolates were typed as P5, biovar (V and VI) and two as P4, biovar (I and II). The dendrogram demonstrated a correlation between genetic relatedness and biochemical features among test isolates and the MRI-671/90 pneumonic strain, such that isolates 14/00C (P6), 14/00F (P7) and 12/00 (P10) expressed a genetic similarity of >70% but belonged to different biovars, whereas isolates 14/00M (P4), 14/00U (P11), 14/00I (P9) and 20/00 (P2) were 70% similar and biochemically identical. Test isolate 14/00F showed a distinct PFGE profile (pulsotype P7) and was the only profile that matched any of the type strains, showing >95% similarity to subsp. *multocida*.

3.3.2. LPS gel profiles

Analyses of silver stained SDS-PAGE gels of phenol-extracted LPS from test isolates identified 6 different banding patterns within the core oligosaccharide region, each comprising between 1 and 7 bands (Fig. 3.4;Table 3.4). Despite using a standard methodology, silver staining of LPS from *M. haemolytica* produced clearer bands than with *P. multocida*, the reasons for which are unknown. There was no staining in the high molecular weight region of the gels, indicating the absence of O-antigen side-chains in all isolates. The LPS profiles of *P. multocida* in lung samples obtained at PM and in nasal samples taken a day earlier from the 3 clinical cases were identical (profile B, bands 3, 4, 5, 6), and distinct from the lung isolate (MRI challenge strain 671/90; bands 5, 6, 7, 8) used in previous work. The core banding pattern of the reference strains was highly variable (results not shown), and bovine strain 116/95Q was the only one to exhibit an O-polysaccharide side chain.

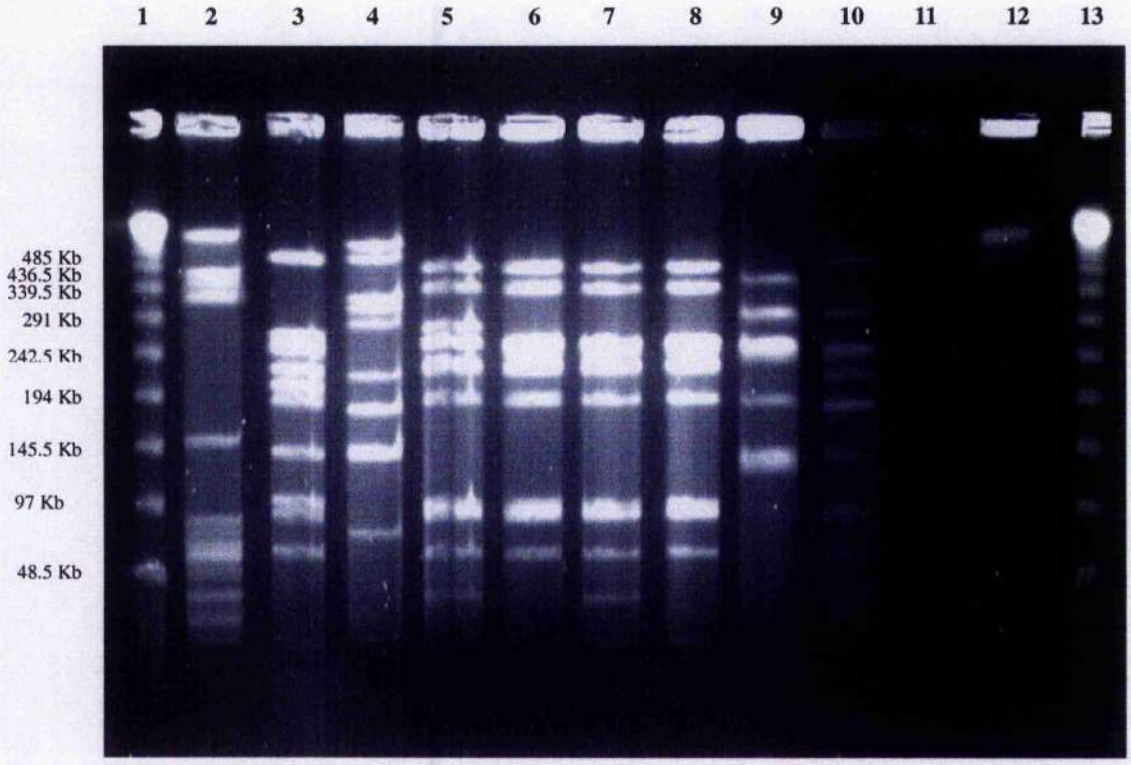


Fig. 3.1. Seven *Apa*I PFGE profiles of Scottish bovine *Pasteurella* spp. test isolates. Lanes 1 and 13 contain molecular mass standards 48.5-kb concatemer (lambda DNA ladder, New England Biolabs). Lane 2, pulsotype 1 (isolate 14/00D, *M. haemolytica*); lane 3, pulsotype 2 (isolate 20/00, *P. multocida*); lane 4, pulsotype 3 (isolate 15/00F, *P. multocida*); lane 5, pulsotype 9 (isolate 14/00I, *P. multocida*); lane 6 and 8, pulsotype 4 (isolates 14/00M and 14/00T, *P. multocida*); lane 7, pulsotype 11 (isolate 14/00U, *P. multocida*); lane 9, pulsotype 5 (isolate 14/00B, *P. multocida*); lanes 10, 11 and 12 are the type strains subsp. *multocida*, subsp. *septica* and subsp. *gallicida*.

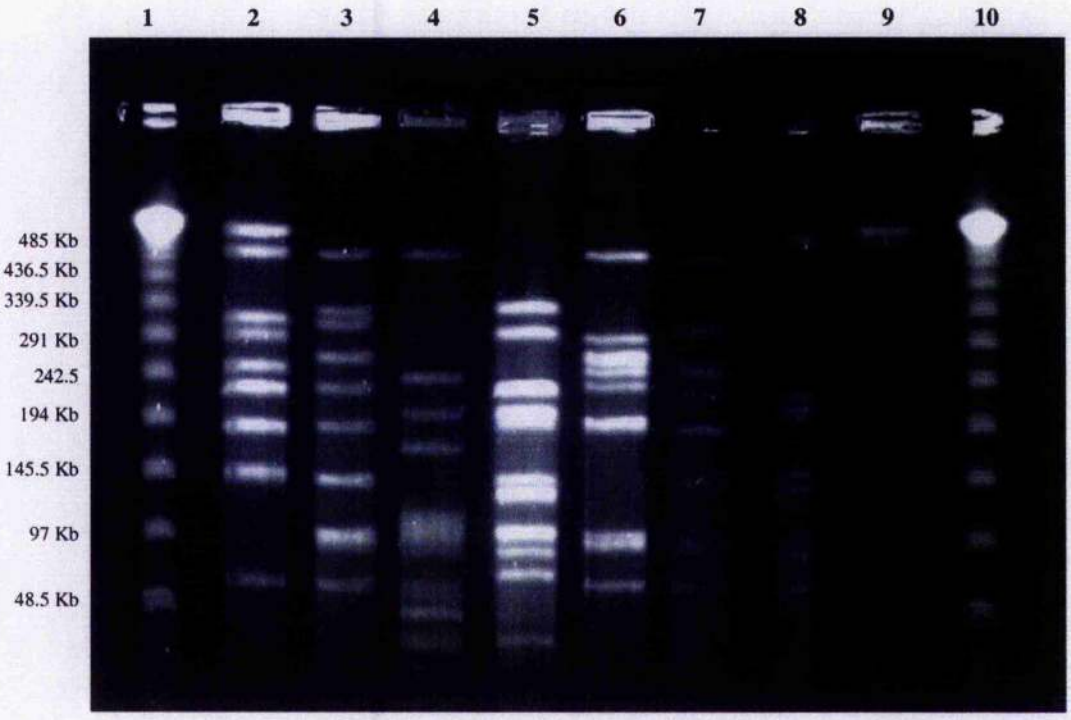


Fig. 3.2. Five *Apa*I restriction PFGE profiles of Scottish bovine *Pasteurella* spp. test isolates. Lanes 1 and 10 contain molecular mass standards 48.5-kb concatemer (lambda DNA ladder, New England Biolabs). Lane 2, pulsotype 6 (isolate 14/00C, *P. multocida*); lane 3, pulsotype 7 (isolate 14/00F, *P. multocida* type D); lane 4, pulsotype 8 (isolate 14/00K, *M. haemolytica*); lane 5, pulsotype 12 (isolate MRI-671/90, *P. multocida*); lane 6, pulsotype 10 (isolate 12/00, *P. multocida*), lanes 7, 8 and 9 are the type strains *P. multocida* subsp. *multocida*, subsp. *septica* and subsp. *gallicida*.

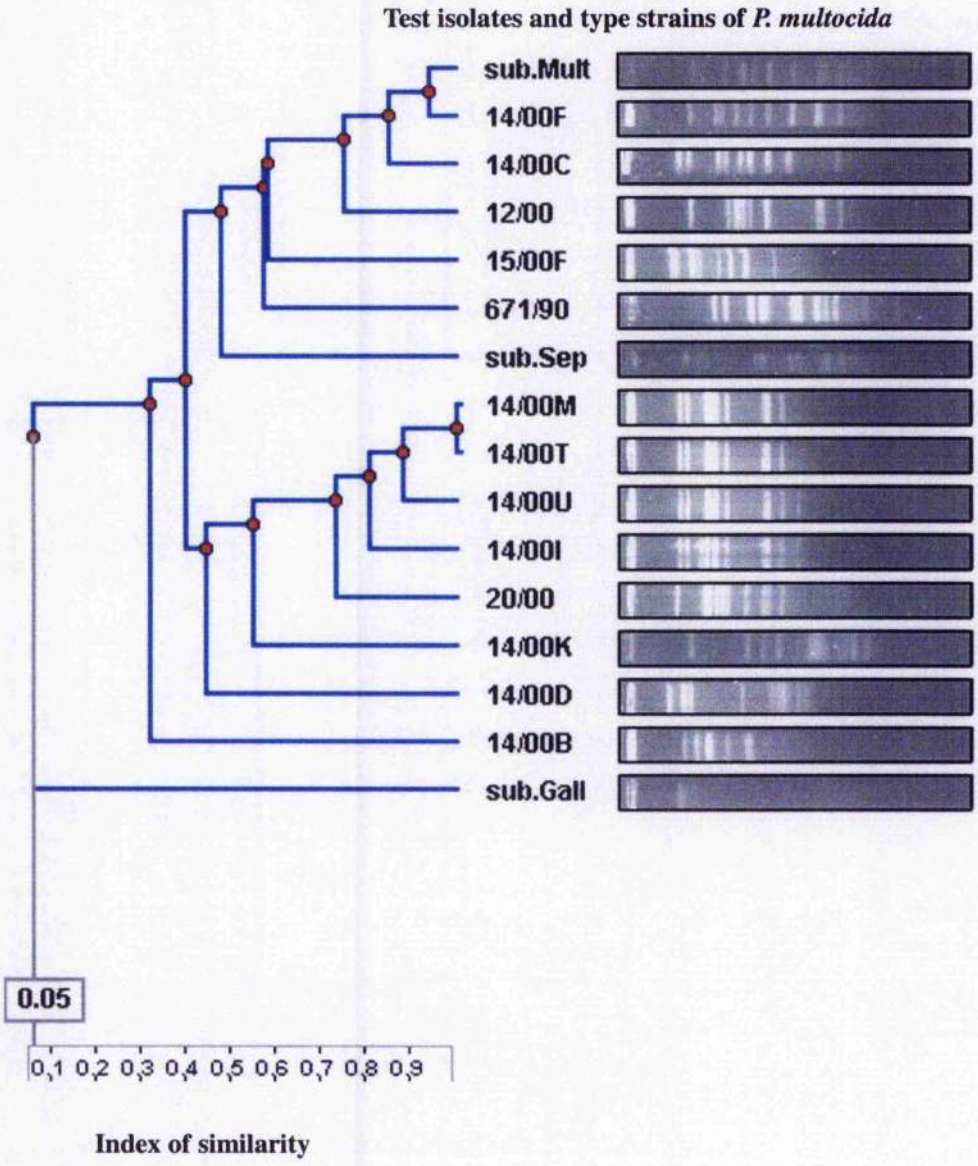


Fig. 3.3. Dendrogram illustration of the genetic relatedness amongst *Pasteurella* spp. isolates, type strains and the MRI-671/90 challenge strain.

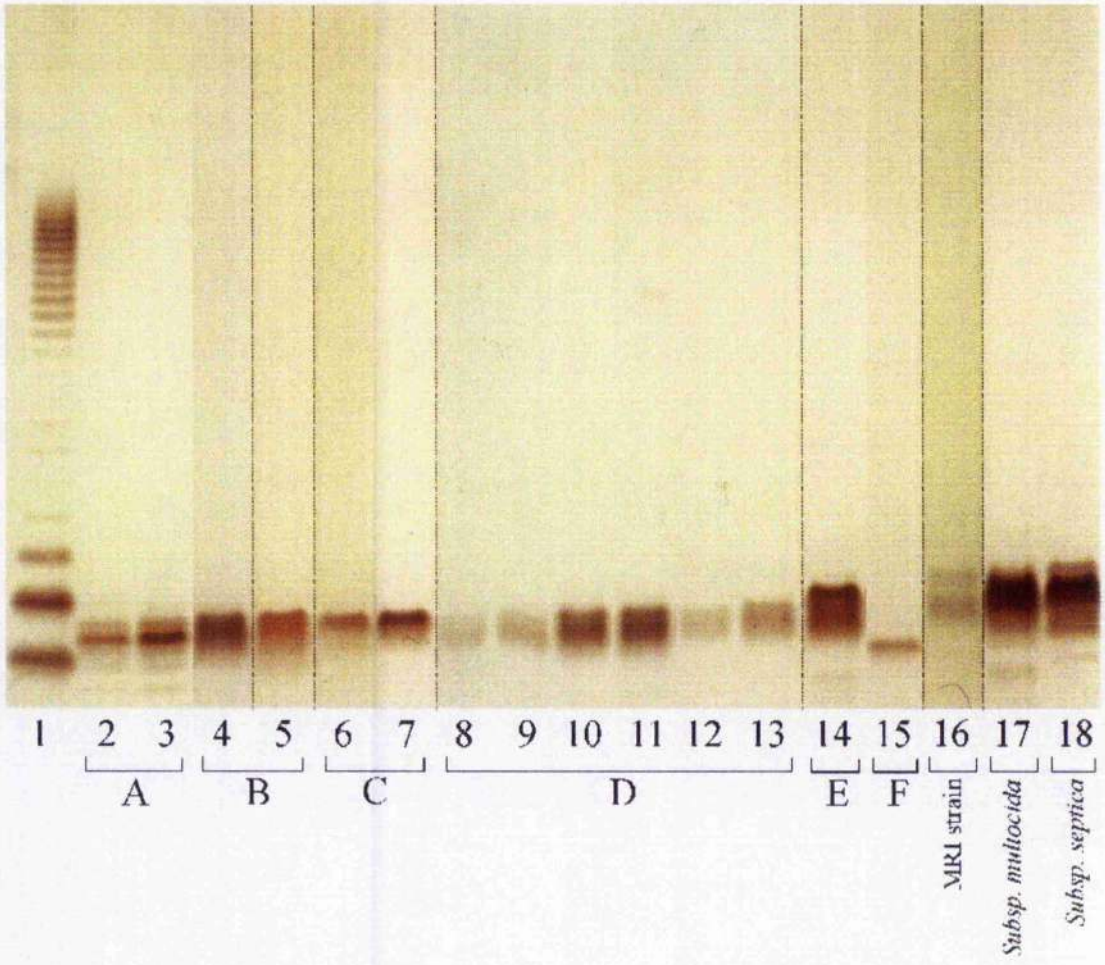


Fig. 3.4. Silver stained SDS-PAGE gels of phenol-extracted LPS from Scottish bovine *Pasteurella* and *Mannheimia* isolates showing 6 profiles (A-F) comprising between 1 and 7 core bands with no evidence of O-antigen. Lane 1: control profile from *E. coli* serotype O55:B5: Lanes 2, 3; profile A (14/00A, D, *M. haemolytica*): Lanes 4, 5; profile B (15/00A, 12/00, *P. multocida*): Lanes 6, 7; profile C (15/00F, 14/00C, *P. multocida*): Lanes 8, 9, 10, 11, 12, 13; profile D (14/00I, M, U, T, B, G, *P. multocida*): Lane 14; profile E (14/00F, *P. multocida*): Lane 15; profile F (14/00K, *M. haemolytica*): Lane 16; MRI strain (671/90, *P. multocida*) bands 5, 6, 7, 8: Lane 17, 18; subsp *multocida* and subsp *septica*.

Table 3.4. Correlation of LPS core profile types A – F with pulsotypes P1 – P11 of bovine field isolates.

Bacterial species	LPS core profile†	LPS core banding pattern	Pulsotype (n*)	Isolate reference numbers
Mannheimia	A	1, 3, 4, 5, 6	P1 (9)	14/00A, D, E, H, J, L, N, P, Q
P. multocida	B	3, 4, 5, 6	P2 (8), P10 (1)	15/00A, B, C, D, E, 20/00, 21/00, 23/00, 12/00
P. multocida	C	4, 6	P3 (1), P6 (1)	15/00F, 14/00C
Mannheimia	D	4, 5, 6	P4 (2)	14/00M, T
P. multocida	D	4, 5, 6	P9 (1); P11 (1); P5 (4)	14/00I, 14/00U, 14/00B, G, R, S
P. multocida	E	2, 4, 5, 7	P7 (1)	14/00F
Mannheimia	F	4	P8 (1)	14/00K

*Number of isolates with indicated LPS core profile

†Isolates confirmed as *M. haemolytica* or *P. multocida*

+ Indicates a semi band.

3.3.3. LPS Immunoblot profiles

The immune reactivity of the 6 LPS core bands were studied by immunoblotting using two sources of polyclonal antiserum, raised either to a live challenge of *P. multocida* A:3 in cattle or to formalin-killed *P. multocida* A:3 in rabbits (Table 3.5). All LPS core bands reacted with bovine antiserum and were immunogenic except for band 3 in profile F (Fig. 3.5). Band 3 (profile F) was also not detected by the rabbit antiserum. The bovine antiserum raised against a rough form of *P. multocida* A:3 was able only to recognise the O-antigen of phenotypically characterised *M. haemolytica* strains (P1, LPS type A); immunoblots were still unable to reveal possible immunogenic O-antigen moieties for the rough *P. multocida* test isolates. Rabbit antiserum raised against formalin killed rough *P. multocida* A:3 was unable to detect any O-antigen moieties, and was able only to identify LPS core banding profiles of *P. multocida* (Fig. 3.6). The immunogenic components of the MR1 challenge strain (P12) for both target species displayed entirely disparate

immunoblot profiles. The bovine antisera (Fig. 3.5; lane 14) detected only two reactive core bands (4, 5) compared to the rabbit antiserum (Fig. 3.6; lane 10) that detected an additional three reactive core bands (4, 5, 6, 7).

3.3.4. Correlations between pulsotype, biovar and LPS profile

Test isolates 14/00M (P4), 14/00U (P11), 14/00I (P9) and 20/00 (P2) showed that they were genetically similar (70%) and biochemically identical, with the exception of 14/00T (P4) which was identical in terms of genetic make-up (>95%) to 14/00M but distinct biochemically in its inability to ferment mannitol. On all occasions it seemed that the 6 LPS banding types of the individual test isolates correlated well with pulsotype (Table 3.4).

3.3.5. PCR analysis of bovine test isolates

A single band on a DNA gel at a fragment size of 460 bp confirmed the morphological and biochemical characterisations of the following *P. multocida* test isolates; 14/00B, C, F, G, I, M, R, S, T, U, 15/00A, B, C, D, E, F, 12/00, 20/00, 21/00, 23/00. The MRI-671/90 challenge strain was incorporated into the assay as the positive control (Fig. 3.7). The following test isolates 14/00A, D, E, H, J, K, L, N, P, Q identified phenotypically as *M. haemolytica* were unable to produce the desired PCR product, and a band was missing at the 460 bp point. Although, this PCR typing method was unable to reproducibly, differentiate between capsular types, the desired outcome was achieved using biochemical tests.

Table 3.5. Variation in immune reactivity between the 6 LPS types shown by immunoblot using bovine or rabbit polyclonal antiserum raised to live or formalin-killed *P. multocida* A:3, respectively (numbers in brackets refer to reactive core bands).

Anti-serum	O-antigen	A *Mannheimia*	B *P. multocida*	C *P. multocida*	D *Mannheimia*	D *P. multocida*	E *P. multocida*	F *Mannheimia*
Bovine	P1	P1 (4, 6)	P2 (4, 5, 6) P10 (5)	P3 (6) P6 (5)	P4 (6)	P11 (6), P5 (6)	P7 (5, 6)	* NR
Rabbit	* NR	* NR	P2 (4, 6) P10 (6)	* NR	P4. 14/00T (6), *NR with 14/00M	P11 (4), P5. 14/00G, S (6) *NR with 14/00B, R	P7 (6)	* NR

*NR = No reaction

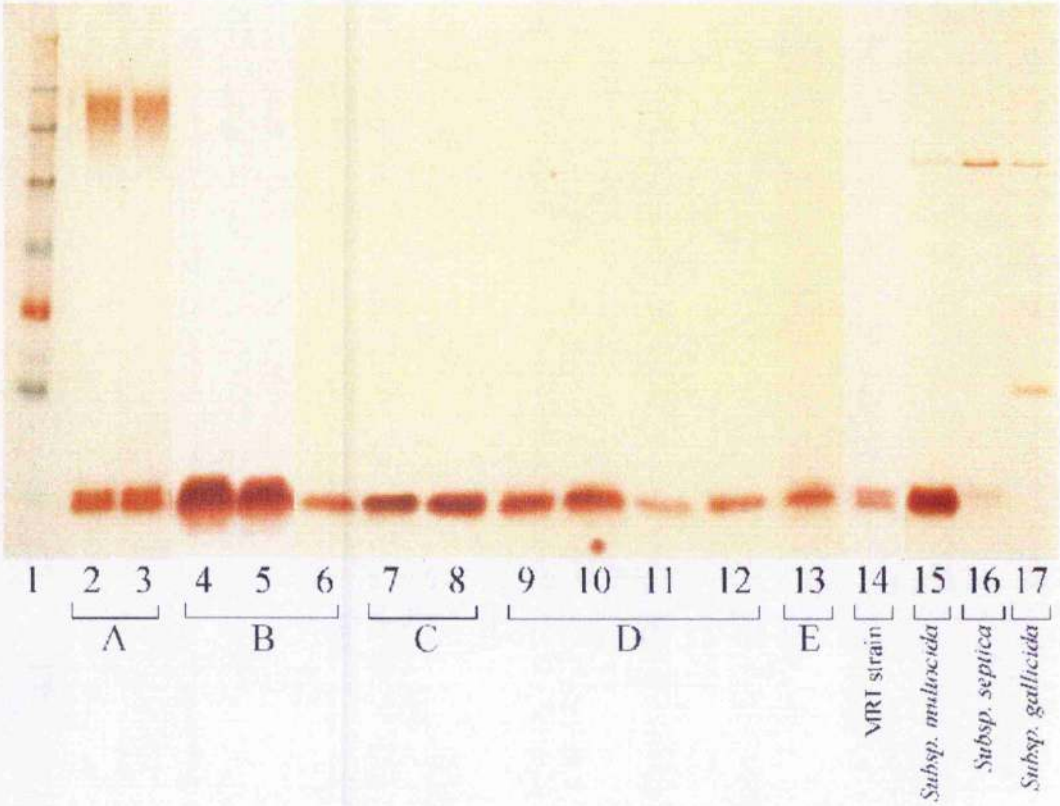


Fig. 3.5. Immunoblots of the 6 LPS profiles (A-F) from Scottish bovine *Pasteurella* spp. isolates showing the immune reactive core bands when exposed to polyclonal antiserum raised to a live challenge of *P. multocida* A:3 in cattle. Lane 1: molecular weight standard of 3-188 kDa (See Blue Plus 2; Invitrogen); Lanes 2, 3: profile A (14/00A, D, *M. haemolytica*); Lanes 4, 5, 6: profile B (15/00A, B, 12/00, *P. multocida*); Lanes 7, 8: profile C (15/00F, 14/00C, *P. multocida*); Lanes 9, 10, 11, 12: profile D (14/00I, M, U, T, *P. multocida*); Lane 13: profile E (14/00F, *P. multocida* type D); Lane 14: MRI challenge strain (671/90, *P. multocida*); Lane 15, 16, 17: *P. multocida* subsp *multocida*, subsp. *septica*, subsp. *gallicida*.

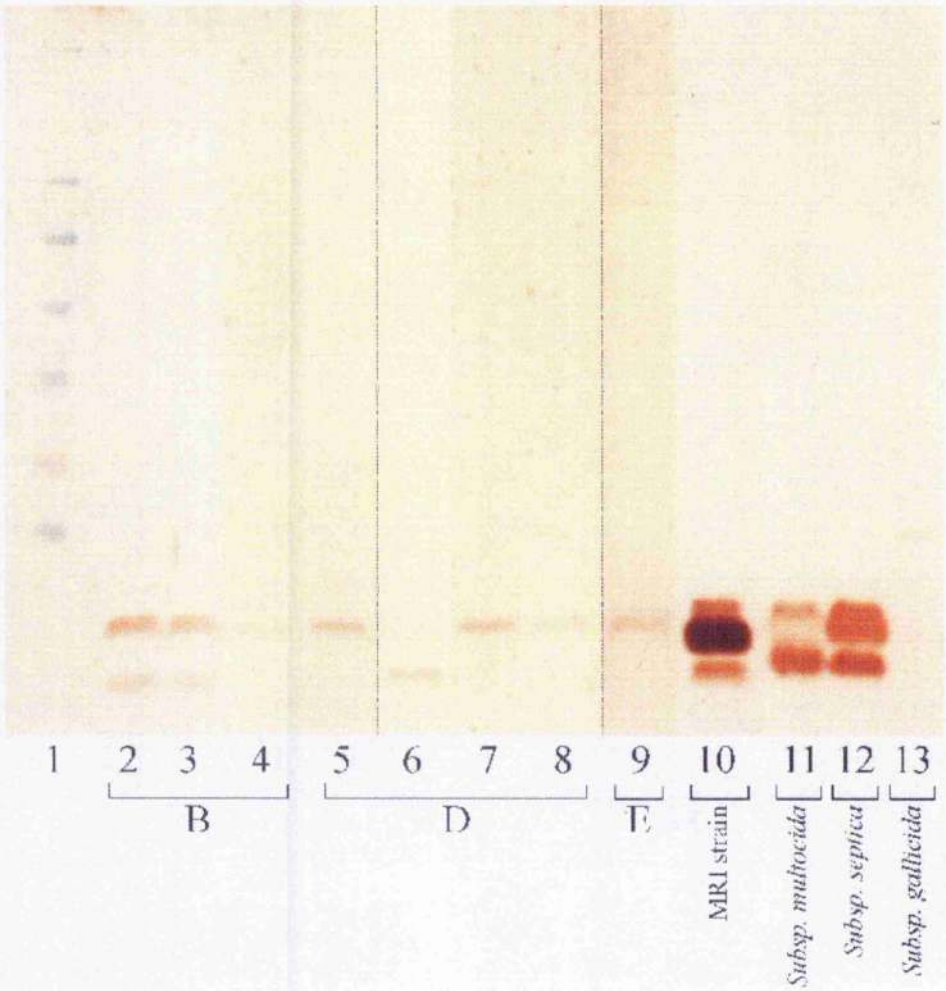


Fig. 3.6. Immunoblots of the 6 LPS profiles (A-F) from Scottish bovine *Pasteurella* spp. isolates showing the immune reactive core bands when exposed to polyclonal antiserum raised to formalin-killed *P. multocida* A:3 in rabbits. Lane 1: molecular weight standard of 3-188 kDa (See Blue Plus 2; Invitrogen); Lanes 2, 3, 4 :profile B (15/00A, B, 12/00, *P. multocida*); Lanes 5, 6, 7, 8: profile D (14/00T, U, G, S, *P. multocida*); Lanes 9: profile E (14/00F, *P. multocida* type D); Lane 10: MRI strain (671/90, *P. multocida*); Lane 11, 12, 13: *P. multocida* subsp *multocida*, subsp. *septica*, subsp. *gallicida*.

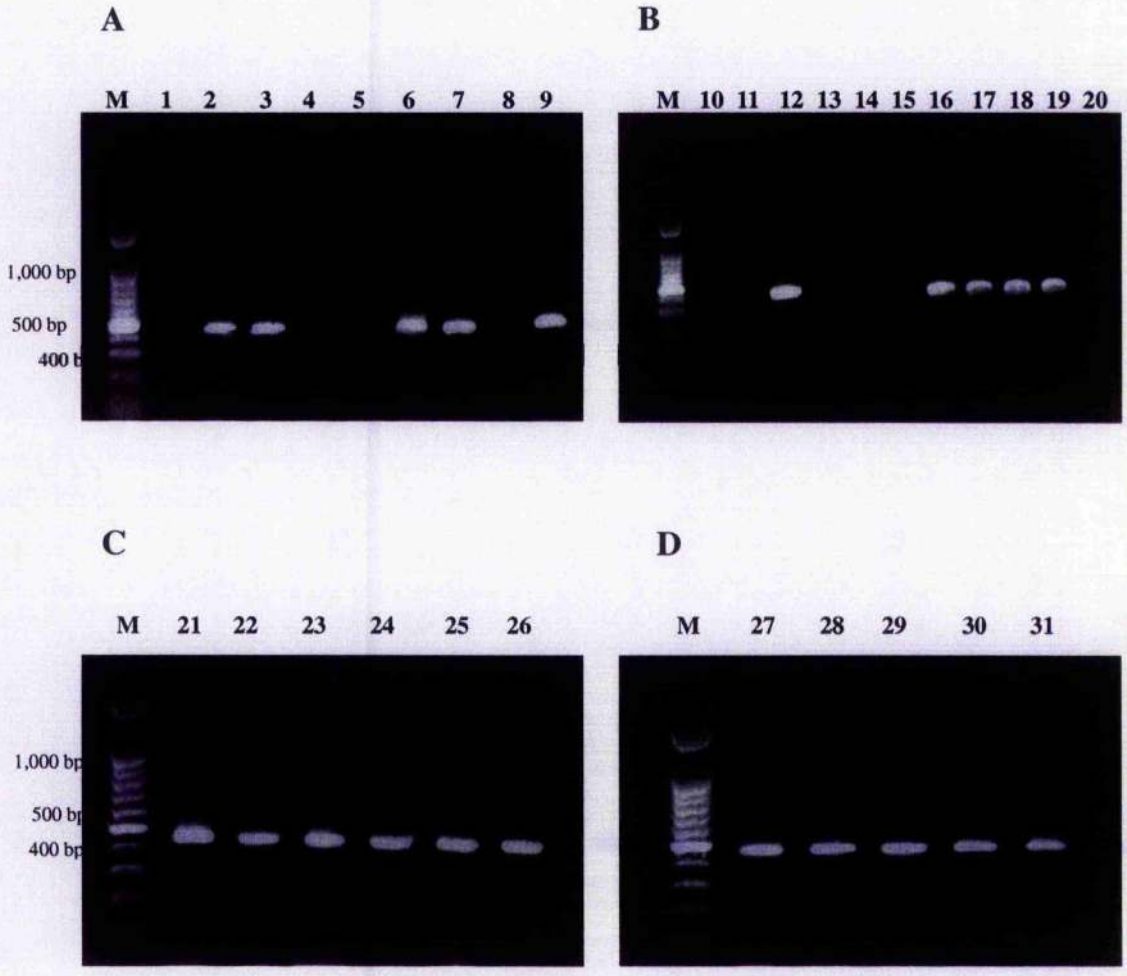


Fig. 3.7. PCR analysis of bovine *Pasteurella* spp. test isolates. The presence of a single 460bp DNA fragment confirmed the test isolate to be *P. multocida*. Lanes M contain molecular mass standards from 100-1500 bp (100bp DNA ladder).

A: Lanes 1, 4, 5, 8, *M. haemolytica* isolates 14/00A, D, E, H; lanes 2, 3, 6, 7, 9, *P. multocida* isolates 14/00B, C, F, G, I. **B:** Lanes 10, 11, 13, 14, 20 *M. haemolytica* isolates 14/00L, P, K, Q, J and *Pasteurella* sp, 14/00N; lanes 12, 16, 17, 18, 19, *P. multocida* isolates 14/00M, R, S, T, U. **C:** Lanes 21-26, *P. multocida* isolates 15/00A, B, C, D, E, F: Lanes 27-31, *P. multocida* isolates 671/90, 12/00, 20/00, 21/00, 23/00.

3.4. DISCUSSION

Limited research has been conducted on the characteristics and diversity of a commensal bacterial population residing in the upper respiratory tract within a herd or between herds of cattle. It is believed that the flora of the nasopharynx is the potential source of infection, responsible for weakening the innate immune status and stimulating acquired immunity through antigenic factors that *P. multocida* possesses. The foremost *Pasteurella* spp. recovered from clinically healthy and pneumonic cases are considered to be *M. haemolytica* and *P. multocida* (Bisgaard *et al.*, 1991). For this study we analysed the potential molecular and LPS diversity of the MRI *P. multocida* strain, 26 nasal and 2 matched nasal and lung *Pasteurella* spp. field isolates from 28 calves. Phenotypic analyses of these field strains revealed that 20 were *P. multocida* subsp. *multocida* and the remainder were assigned to other taxa within the genus *Pasteurella* spp. *P. multocida* subsp. *multocida* could be differentiated by their fermentative patterns and their mucoid properties, grouping them into 7 out of the 11 biovars designated for field isolates. These results alone indicate the scope for prevalence of *P. multocida*; however, a more detailed study involving the genetic profile of these isolates is required to further discriminate *P. multocida* samples of remarkable likeness and epidemiological significance.

The generation of a single DNA band of 460 bp indicated the use of this PCR assay for differentiating *P. multocida* test isolates from other morphologically and biochemically similar bacteria (Townsend *et al.*, 2001).

PFGE was used as the method for genotyping isolates, and digestion of genomic DNA with the restriction endonuclease, *Apa*I, generated clear banding patterns. This allowed discrimination between different species of *Pasteurella* spp., suggesting that PFGE, in combination with *Apa*I digestion, is a powerful epidemiological tool. Since there is no commonly agreed directive for designating pulsotypes to the fragment patterns produced by PFGE, guidelines devised by Tenover *et al.*, (1995) were employed in this study. According to Tenover *et al.* (1995) isolates of the same pulsotype are impossible to differentiate. Twelve pulsotypes were identified amongst the 26 nasal, 2 paired nasal and lung isolates and the MRI challenge strain (671/90). These PFGE findings indicate that a high frequency of genetic diversity exists among a small pool of *Pasteurella* spp. test isolates confirming the heterogeneity of *P. multocida*. A similar observation has been made by others (Lainson *et al.*, 2002; Buttenschon and Rosendal, 1990), and should be taken into account when devising control measures.

The presence or absence of 1 or 2 bands differentiated the PFGE patterns generated from *Apa*I digestion. These observations, following passage through different hosts, can be caused by random

genetic events such as insertions and deletions of DNA (Connor *et al.*, 2000). Equivalent alterations in genomic DNA restriction patterns have been observed in bacterial species such as *Yersinia pestis* and *Pseudomonas aeruginosa* after *in vitro* passage or passage through different animal hosts *in vivo* (Kodjo *et al.*, 1999). However, no variations were observed between the PFGE profile of the challenge material of an MRI pneumonic strain 671/90 type A:3 and the strain re-isolated after *in vivo* passage. *In vitro* stability of *M. haemolytica* strains has been reported (Kodjo *et al.*, 1999).

Correlations were observed amongst biovar and pulsotype, however, there were instances where PFGE required phenotypic analysis to discriminate strains of the same pulsotype, indicating the fallibility of relying on one epidemiological technique to differentiate strains.

One of the molecules responsible for the virulence of pasteurellosis is LPS (Hodgson *et al.*, 2003), however, the range and role of different LPS chemotypes in healthy and diseased animals has not been defined. The LPS profiles from all of the field isolates contained only a core banding pattern, indicating that strains were rough. Such a characteristic would indicate the loss of diversity, variation and virulence. These attributes are associated with many Gram-negative agents including *Salmonella typhimurium*, *Haemophilus*, *E. coli* and *Pasteurella* spp. all possessing O-antigen.

Amongst our small pool of field isolates and the MRI-671/90 challenge strain, 6 different LPS banding patterns were identified, indicating a considerable amount of diversity within the core region. Chemotype profiling distinguished efficiently the core banding patterns of *P. multocida* strains from *M. haemolytica* strains, and would appear, therefore, to be a useful tool for epidemiological studies. Evidence for the variation in immune reactivity amongst the 6 LPS core banding patterns was illustrated by immunoblotting of phenol-extracted LPS from test isolates probed with the appropriate antisera and this can be compared with early work by others (Brogden and Rebers, 1978).

Bovine antiserum raised against the MRI challenge strain containing rough LPS was able to recognise the O-antigen of *M. haemolytica* strains on immunoblot. A possible explanation is that the antiserum obtained was from 8 week old conventional calves that had been exposed previously to *M. haemolytica*. Neither bovine nor rabbit antisera were able to detect LPS core profile F. Similar observations have been made previously (Hodgson *et al.*, unpublished data), indicating that low molecular weight core bands may be concealed from the host immune system or that the host is unable to differentiate the pathogen from self. In this regard it is interesting to note the ability of some LPS outer core structures to mimic human gangliosides (Harvey *et al.*, 2001; Moran & Prendergast, 2001; Guerry *et al.*, 2002).

In the case of the rabbit antiserum, a unique profile was observed, in that 5 additional LPS core bands were recognised in the MRI-671/90 challenge strain LPS that were not detected by bovine

antiserum. The rabbit antiserum was unable also to detect O-antigen or core bands from LPS types A and C because in the case of type A, strains were *Mannheimia* sp. A similar phenomenon has been reported for *M. haemolytica* (Ali *et al.*, 1992), but this observation involved only 1 core LPS band, and this has not been investigated with respect to *P. multocida* where the phenomenon seems more evident. The difference may indicate simply that formalin-killed preparations present otherwise hidden antigens to the host's immune surveillance system; however, bacteria will also be killed and lysed following live challenge, and the observation may indicate that some core LPS structures are similar in composition to bovine but not to rabbit structures and therefore not recognised as foreign by bovine immune cells. If so, this has important implications for our understanding of immune evasion and perhaps also for colonisation and existence as a commensal by *P. multocida*.

Research using bovine antiserum raised against killed bacteria is required to allow a resolution of this matter by direct comparison with equivalent rabbit antiserum. The antiserum should provide an explanation of the underlying mechanisms of virulence, which will be of value when investigating possible antigenic variation between commensal and virulent states in *P. multocida*. Commonality among epitopes to both host and pathogen is vital for adhesion and recognition.

LPS chemotyping has signalled the importance of this antigenic molecule for host pathogen interaction and for inducing a specific host immune reaction, making this antigen a prospective vaccine candidate. The methods employed by Davies and Donachie, (1996) to assess diversity have established their effectiveness as an epidemiological tool, capable of identifying surface components with a capacity for virulence. Comparison of isolates from 2 of the 4 calves that developed clinical disease from the nasopharynx pre-infection and from the lung post-infection showed no phenotypic or genotypic differences, indicating that in some cases the same strain was able to operate both as a commensal and pathogen. These findings are in agreement with DeRosa *et al.*, (2000) who showed that *Pasteurella* spp., isolated from the URT and lungs of acutely ill animals were identical; however, it is questionable whether this discovery applies in the case of a clinically healthy animal.

Isolates obtained from diseased lung tissue were identical in phenotype and genotype to a previously obtained paired nasal isolate. This result supports the finding that a commensal of *P. multocida* resident in the nasopharynx can proliferate unchanged in animal's immunosuppressed, leading to shedding and inhalation of *P. multocida* in numbers sufficient to cause disease in the LRT. What remains unknown is that at the time of nasal swabbing the calf could have become infected with a virulent strain of *P. multocida*, the clinical development of disease would have been observed at a later period. Clearly further research is required to determine the mechanism by which a commensal may become a pathogen. The same LPS rough chemotype could be present

with or without disease. No evidence of a specific link between LPS structure and disease virulence was found.

Chapter 4. EXPERIMENTAL INDUCTION OF PNEUMONIC PASTEURELLOSIS IN CALVES BY INTRATRACHEAL INFECTION WITH *PASTEURELLA MULTOCIDA* BIOTYPE A:3

4.1. INTRODUCTION

P. multocida is an opportunistic pathogen of wild and domesticated animals, as well as humans (Adler *et al.*, 1999) and, similarly to *M. haemolytica* (Donachie, 1995), is an important cause of respiratory disease in cattle. However, the pathogenesis of bovine pneumonic disease caused by *P. multocida* is not well characterised (Confer, 1993) and the lack of a reproducible experimental model in calves has limited research into the pathogenic determinants of this organism and the development of effective and safe vaccines. Previous attempts to induce BPP experimentally included challenge of calves intratracheally with either 10 or 60 ml of culture media containing between 10^9 to 10^{10} cfu of *P. multocida* (Ames *et al.*, 1985; Gourlay *et al.*, 1989); however, lesions and clinical signs of disease were mild in all calves, with only transient signs of pyrexia and respiratory distress in the most affected calves. Thus, neither of these experimental approaches met criteria suggested (Houghton, 1995) for experimental induction of overt disease at an incidence and degree equal to or exceeding that observed for the natural disease. Conversely, in our laboratory, use of *M. haemolytica* serotype A:1 showed that a high volume (300 ml) dose containing 2×10^8 cfu given intratracheally resulted in a reliable and progressive development of overt pneumonic disease, similar to that seen in the field (Donachie, unpublished observations).

The aim of the present work was to investigate whether a similar approach could be used to generate a realistic, progressive and reproducible model of pneumonic pasteurellosis in cattle caused by *P. multocida* by characterising the clinical, APP changes and pathological responses of the host to either low or high volumes of challenge inocula. It was anticipated that future exploitation of such a model might assist the development of novel strategies and products for treatment and prevention of infection by *P. multocida*.

4.2. MATERIALS AND METHODS

4.2.1. Calves and experimental design

Holstein cross Friesian male calves (n=16) of approximately two weeks of age were purchased from a local farm and housed in individual calf pens. Calves were assessed for prior exposure to *P. multocida* by nasal swabbing and plasma antibody analysis (Fig. 4.8) by ELISA (section 2.6.3); all calves seemed free from *Pasteurella* spp., in the nasopharynx.

Calves were weaned at 7 weeks of age from a liquid milk diet on to hay and mixed pellets and held in open pens for the duration of the experiment. At 8 weeks of age (designated experimental day 0) the 16 calves were allocated randomly to groups of four and challenged according to the 2 x 2 factorial experimental design summarised below.

Group 1: 10^9 cfu *P. multocida* A3 in 300 ml of PBS

Group 2: 10^9 cfu *P. multocida* A3 in 60 ml of PBS

Group 3: 10^{10} cfu *P. multocida* A3 in 300 ml of PBS

Group 4: 10^{10} cfu *P. multocida* A3 in 60 ml of PBS.

It seemed unlikely that instillation of sterile saline alone into the lung would cause significant clinical or biochemical changes, as other studies of experimental *P. multocida* pneumonia in calves found no effect of intratracheal instillation of sterile brain heart infusion broth (Gourlay *et al.*, 1989). Therefore, an uninfected negative control group was not included, in order to restrict the number of experimental animals required. Instead, pre-infection data from each animal were used to determine negative control baselines. All experimental protocols were approved by the MRI Animal Experiments Committee, authorised under the Animals (Scientific Procedures) Act 1986. Access to veterinary care was available at all times.

4.2.2. Preparation of *P. multocida* for challenge

Live cultures of *P. multocida* A:3 MRI challenge strain 671/90 were pooled and McFarland readings of culture density were obtained using a Densimat (bioMerieux) and converted to estimated viable counts using a correction factor determined previously using standard techniques (Miles *et al.*, 1938). Cultures were diluted subsequently with pre-warmed (37°C) PBS to produce challenge inocula containing an estimated 10^9 and 10^{10} cfu in 60 and 300 ml volumes. Actual

bacterial doses, estimated retrospectively by plating out serial dilutions of each dose onto blood agar, were 1.5 x 10^9 cfu and 2.2 x 10^{10} cfu.

4.2.3. Clinical monitoring

A total clinical score (TCS) for each 24 h observational period was calculated for each calf from which a mean TCS over the duration of the experiment was derived (section 2.8.5).

4.2.4. Statistical analyses

Mean TCS, pleurisy scores and viable lung counts were distributed in a skewed manner, and were transformed using a square root or logarithmic transformation, as appropriate, to produce a more symmetrical distribution of data (Crowder and Hand, 1990). Differences between factor levels for these measurements were analysed for statistical significance using the residual maximum likelihood (REML) application of the Genstat software (Release 4.2) (Payne, 2000). The use of REML was indicated by the non-orthogonality of the data after humane removal of two animals that developed endotoxic shock. Respiratory rates, rectal temperatures and APP concentrations against time were summarised using the area under the curve (AUC) adjusted to individual time-zero baseline values and analysed using REML. The choice of AUC was chosen to provide a robust method of analysing repeated measures, from a relatively small number of animals in which the responses were not linear with time.

4.2.5. Plasma assays

Jugular vein blood samples were collected into vacutainers (Becton Dickinson) containing 1 ml of EDTA (Sigma) as anticoagulant from all calves on day of arrival, the day of challenge (day 0), 4 h p.c., and daily thereafter until the day of necropsy (day 4). Plasma was separated and stored at -40°C until analysis for viral antibodies; BVDV, PI-3, RSV and IBRV, APP; AGP, SAA , Hp and IgG levels.

4.3. RESULTS

4.3.1. Clinical observations

All calves were healthy and alert prior to and immediately after infection. The challenge procedure took approximately 10 min and was well tolerated by all animals, causing only mild coughing. Within 4-6 h p.c. 12 of the 16 calves became depressed and recumbent, with signs of laboured breathing, mild nasal discharge and reduced appetite, and were unresponsive to mild noise or visual stimuli. Rectal temperature increased rapidly in all groups after infection, peaking at 5 h p.c. (Group 2) and at 23 h p.c. for all other groups (Fig. 4.1a). Mean temperatures fell thereafter in all groups but did not return to normal during the course of the experiment. The AUC for rectal temperature was significantly higher in calves given the high volume challenge ($P < 0.05$). The volume by dose relationship was not statistically significant; however, calves in the high dose high volume group did show the highest temperature rises. Rapid and marked rises in respiratory rates (Fig. 4.1b) were observed in all four treatment groups after infection. In calves given the high dose, low volume challenge mean respiratory rates varied between 35 and 59 min^{-1} (clinical score of 0) prior to infection, increasing to an average of 81 min^{-1} (clinical score of 1) 5 h p.c,. Differences in mean AUC of respiration rate against time were not statistically significantly affected by dose, volume or dose by volume interactions; however, high dose, high volume challenge tended to be associated with high respiration rates.

Within 5 h p.c. one animal in each of the high dose groups (Groups 3 and 4) showed signs of endotoxic shock, including depression, dyspnoea, a rise followed by a drop in rectal temperature, salivation and recumbency (Nagaraja *et al.*, 1979; Lohuis *et al.*, 1988). To minimise suffering, both were killed humanely.

Overall clinical signs peaked at 5-23 h p.c, thereafter signs began to resolve such that there was an obvious improvement in the health status of the calves towards the latter stages of the experiment. Calves given the high dose challenge exhibited a secondary clinical response during which respiratory rates increased at 48 h p.c. and remained elevated until the scheduled PM date. Both high dose challenges caused greater disease, as measured by mean TCS, but group differences were not statistically significant ($P>0.05$). The mean TCS in Group 2 (low dose, low volume) was consistently low and associated with mild disease only (Table 4.1).

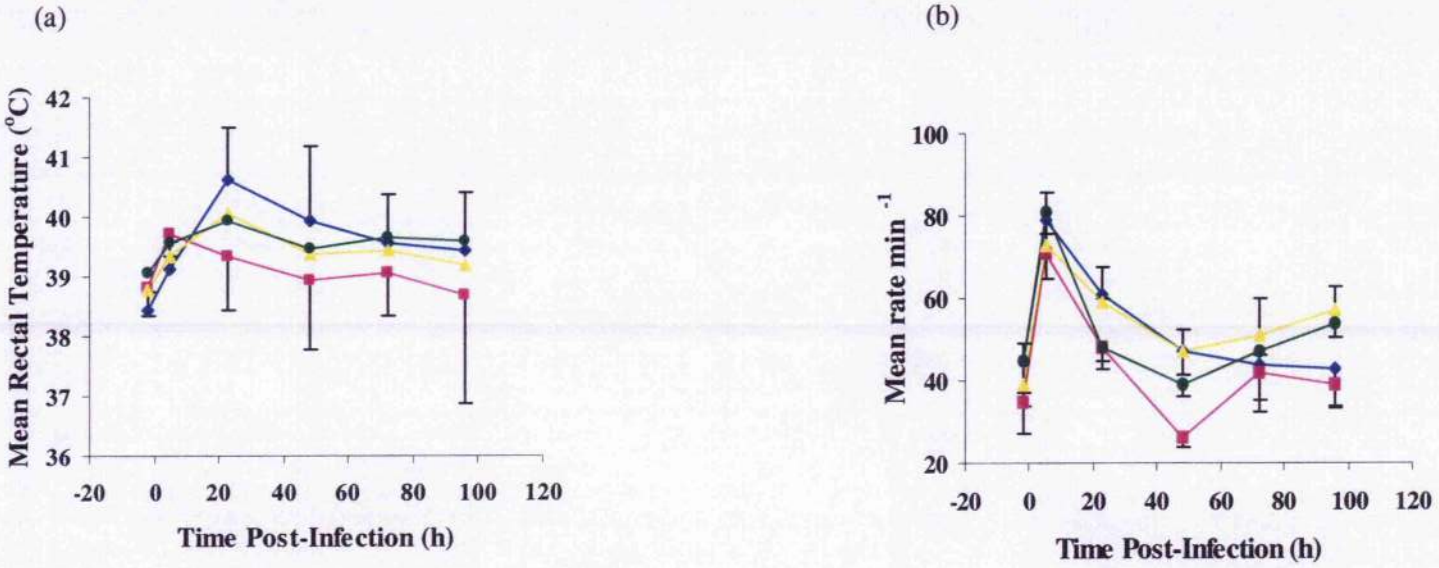


Fig. 4.1. Mean (± S.E) clinical measurements from calves inoculated with *P. multocida* A:3 at either 10^9 or 10^{10} in 60 or 300ml PBS. (a) Mean rectal temperatures (°C) and (b) Mean respiratory rates (rate min^{-1}). Data presented correspond to challenge groups. Group 1 (▲); 10^9 cfu *P. multocida* in 300ml PBS, Group 2 (■); 10^9 cfu *P. multocida* in 60ml PBS, Group 3 (◆); 10^{10} cfu *P. multocida* in 300ml PBS, Group 4 (●); 10^{10} cfu *P. multocida* in 60ml PBS.

4.3.2. Gross pathology and bacteriology

Lung lesions varying from pale grey to purple, often associated with acute congestion, abscessation and thickened gelatinous pleura, were present in all calves (Fig. 4.2D), and with the apical lobes being most affected (Fig. 4.2A). On sectioning, the lesions were solid and the colour varied between red to brown. White small nodules were frequently observed (Fig. 4.2C). Pleurisy and pleural adhesions were observed in all groups (Fig. 4.2B), but there was no correlation between degree of pleurisy and dose or volume by dose of challenge. Surface lesion scores (Table 4.1) tended to be low in Group 2 calves, high in calves given the higher challenge dose (Groups 3 and 4), but overall there was no statistically significant effect of dose, volume, or dose by volume interaction on the area of lung affected. *P. multocida* was recovered from the lungs of 9 pneumonic calves and from the 2 animals that developed signs of endotoxic shock, primarily in lobules exhibiting extensive consolidated lesions. The viable counts of individual calves varied from 3 x 10^4 cfu g^{-1} to 1.4 x 10^9 cfu g^{-1}, and analysis of the recovered bacteria by PFGE confirmed that isolates were identical to the inoculum strain. Recovery of bacteria from lung tissue was lowest in Group 1 animals (Table 4.1). Bacteria were not recovered from heart, heart blood, spleen, kidney, liver and lymph node tissues.

Table 4.1. Mean TCS, surface lesion scores, pleurisy scores and bacterial recoveries from lung tissue (cfu g^{-1}) of all calves challenged intratracheally with *P. multocida*.

Group	Calf	Dose (cfu)	Volume (ml)	Mean TCS	Surface lesion score	Pleurisy	Viable lung count (cfu g^{-1})
	1105			4	10	0	1.4×10^9
1	1115	10^9	300	2	0	0	No bacteria
	1380			3	20	1	No bacteria
	1388			4	10	3	No bacteria
	1111			2	10	2	No bacteria
2	1384	10^9	60	2	10	2	No bacteria
	1386			2	5	0	3×10^4
	1401			1	5	1	1×10^6
	1109			5	20	2	3×10^8
	1112	10^{10}	300	2	10	0	1.2×10^9
3	1377*			11	5	0	2.4×10^7
	1379			7	20	3	1.3×10^8
	1106			4	10	1	1×10^6
	1113	10^{10}	60	2	20	1	4×10^8
4	1389*			12	5	0	6.5×10^7
	1390			3	10	1	4×10^6

Calves 1377* and 1389* developed endotoxic shock and were euthanased prior to the scheduled PM date.

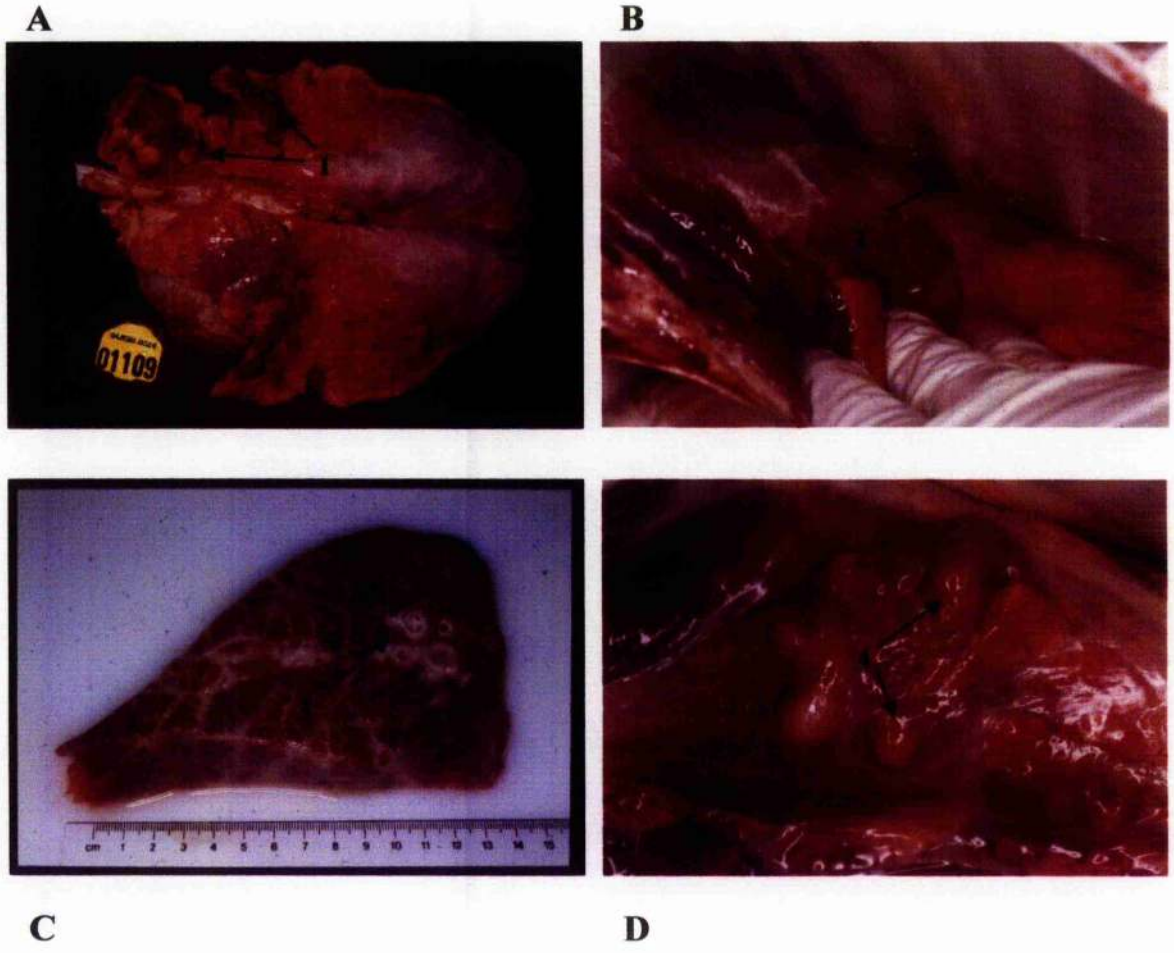


Fig. 4.2. Gross pathological changes observed in calf 1109 given a high volume, low dose challenge of *P. multocida* A:3, 4 d p.c. **A:** Whole excised lungs to illustrate the bilateral cranioventral area (1) as being most affected. The lesions appear typical of a bacterial induced purulent pneumonia similar to those seen in field cases of *M. haemolytica* infections. **B:** Ventral surface of the lung lying within the thoracic cavity, note the excess pleural fluid (2) between the rib cage and the lung. **C:** Cross section through a right apical lobe of a pneumonic lung to demonstrate consolidation and the presence of fibrin and oedema obstructing the bronchial airways (3). **D:** Multiple abscesses on the cranioventral lung surface (4).

4.3.3. Histopathology

Lesions observed in lung samples varied from mild to severe multifocal fibrinopurulent bronchopneumonia with a tendency to abscessation (Fig. 4.3A), but there was no apparent association between the degree of effect and the administered treatment. The predominant findings (9 of the 16 cases) were foci of central coagulative necrosis, surrounded by a zone of densely packed neutrophils, mononuclear cells and spindle-shaped, degenerative inflammatory cells (Fig. 4.3A), commonly known as oat cells (Dungworth, 1993). On occasion, large numbers of *P. multocida* could be observed in these foci. In addition, in areas less affected lesions were characterised by the accumulation of loosely packed neutrophils and macrophages (Fig. 4.3B). In 10 of the 16 calves, there was severe oedema as well as fibrin clots within the interlobular septa (Fig. 4.3C). In 3 animals, encapsulated abscesses were observed in the lung parenchyma (Fig. 4.3D).

Activation of the mediastinal lymph nodes was evident from the presence of a large number of secondary follicles. No significant histopathological changes were observed in any of the samples taken from spleen, heart, liver or kidney tissues.

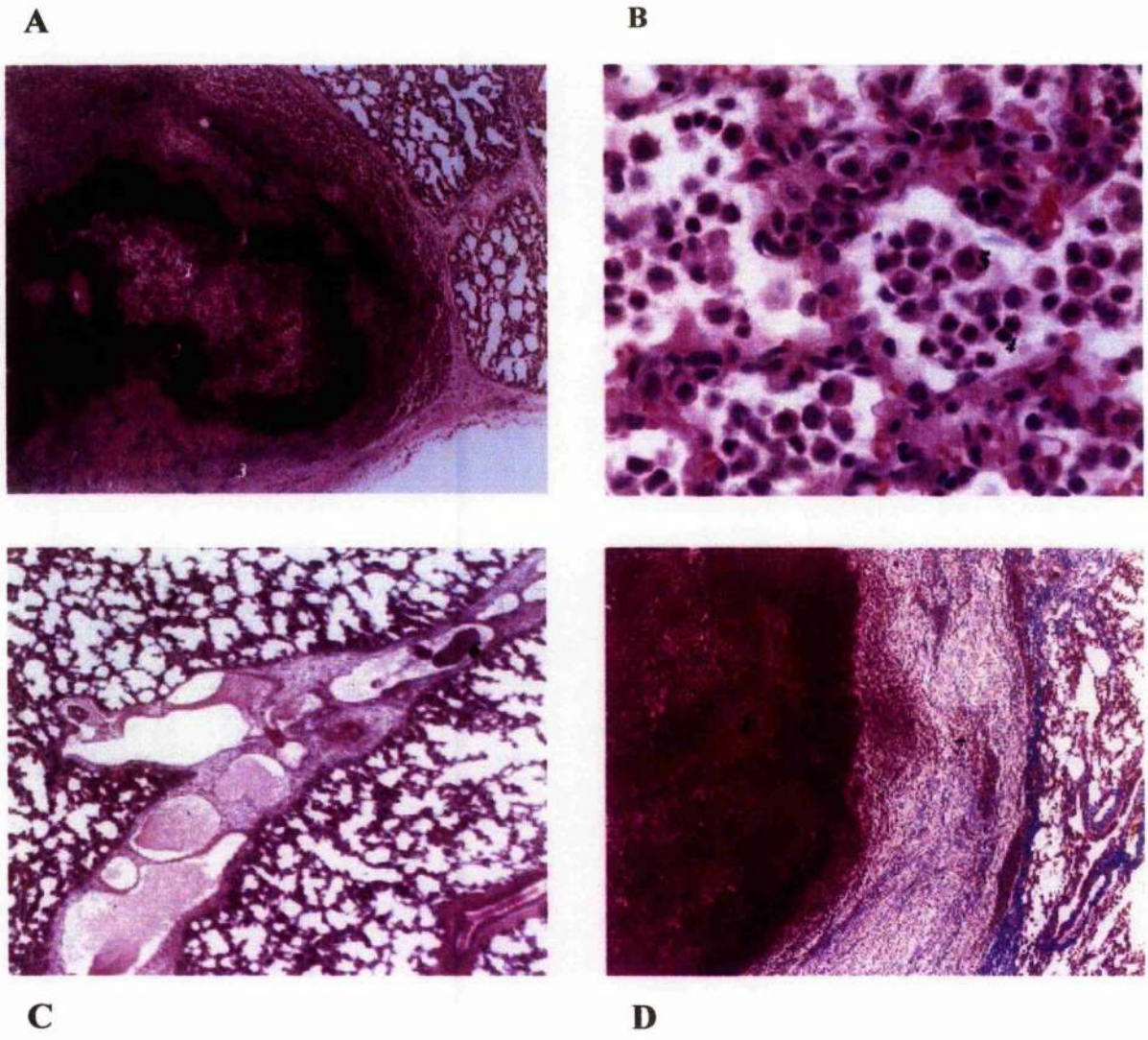


Fig. 4.3. Histopathological changes observed in calves infected with *P. multocida*. **A:** calf 1380 given high volume, low dose challenge, 4 d p.c. (H&E, original magnification x 40); necrotic tissue (1), surrounded by a zone of densely packed inflammatory cells, sometimes referred to as 'oat cells' (2), thickening of the pleura (3); **B:** calf 1112 given high volume, high dose challenge 4 d p.c. (H&E, original magnification x 400) accumulation of neutrophils (4) and macrophages (5) in alveoli; **C:** calf 1384 given low volume, low dose challenge, 4 d p.c. (H&E, original magnification x 100); oedema and dilation of lymphatics in the interlobular septa (6); **D:** calf 1111 given low volume, low dose challenge, 4 d p.c. (Masson's trichrome, original magnification x 40) abscess with necrosis (1) and prominent capsule (7).

4.3.4. Plasma assays

4.3.4.1. Viral antibody analysis

Standard ELISA for detection of antibodies against viral antigens showed that all pre-infection plasma samples were negative for IBRV with the exception of calf 1112 that gave a reading of 0.286 units, but most samples contained antibodies to BVDV (Fig. 4.4) PI-3 (Fig. 4.5), RSV (Fig. 4.6). For most calves, comparison of titres in samples taken before infection and immediately prior to post-mortem showed a decrease in titre; however, in six calves, BVDV antibody titres increased by 30 % during the experiment, although no viraemia was detected. Evidence for seroconversion was observed for PI-3 (1388, 1389) and RSV (1388, 1389, 1401).

4.3.4.2. Analysis of acute phase proteins

In all calves, mean plasma Hp concentrations increased linearly to values significantly greater ($P<0.05$) than those of the mean pre-infection samples, falling gradually thereafter (Fig. 4.7 a). A statistically significant effect ($P<0.05$) of high volume on the Hp AUC was identified. Increases in the concentration α_1AGP were more gradual than those observed for SAA or Hp and maintained for longer (Fig. 4.7b). All calves exhibited a steady increase in the α_1AGP response over the course of the experiment, but for Group 2 calves this rise was less sustained and reached a peak mean concentration of 435 mg l^{-1} at 48 h p.c., declining thereafter with a slight recovery between 72-96 h p.c. The mean AUC for α_1AGP was higher after high volume challenge but the effect was not statistically significant, and there was no evidence of a dose effect.

Plasma concentrations of SAA increased rapidly between 5 and 23 h p.c. (Groups 1, 3 and 4) or between 5 and 48 h p.c. (Group 2) (Fig. 4.7c). Thereafter concentrations in Groups 1 and 4 remained elevated (approximately 550 - 600 mg l^{-1}) until falling dramatically to approximately 5 mg l^{-1} between 72 and 96 h p.c. Concentrations in Groups 2 and 3 fell progressively from peak values (approximately 415 - 465 mg l^{-1}) to between 70 and 120 mg l^{-1} at 96 h p.c; however, there was no statistically significant evidence of any effect that could be attributed to either dose or volume.

4.3.4.3. Analysis of antibody to P. multocida

Plasma concentrations of IgG prior to challenge with *P. multocida* appeared to exhibit a positive response which varied from an OD of 0.3 to 1.55, in addition a similar range was observed post-infection, 0.33 to 1.65 (Fig. 4.8). Calves that tended to have a high IgG concentration prior to

challenge remained close to that OD value post-infection, this was particularly observed with Group 1 calves.

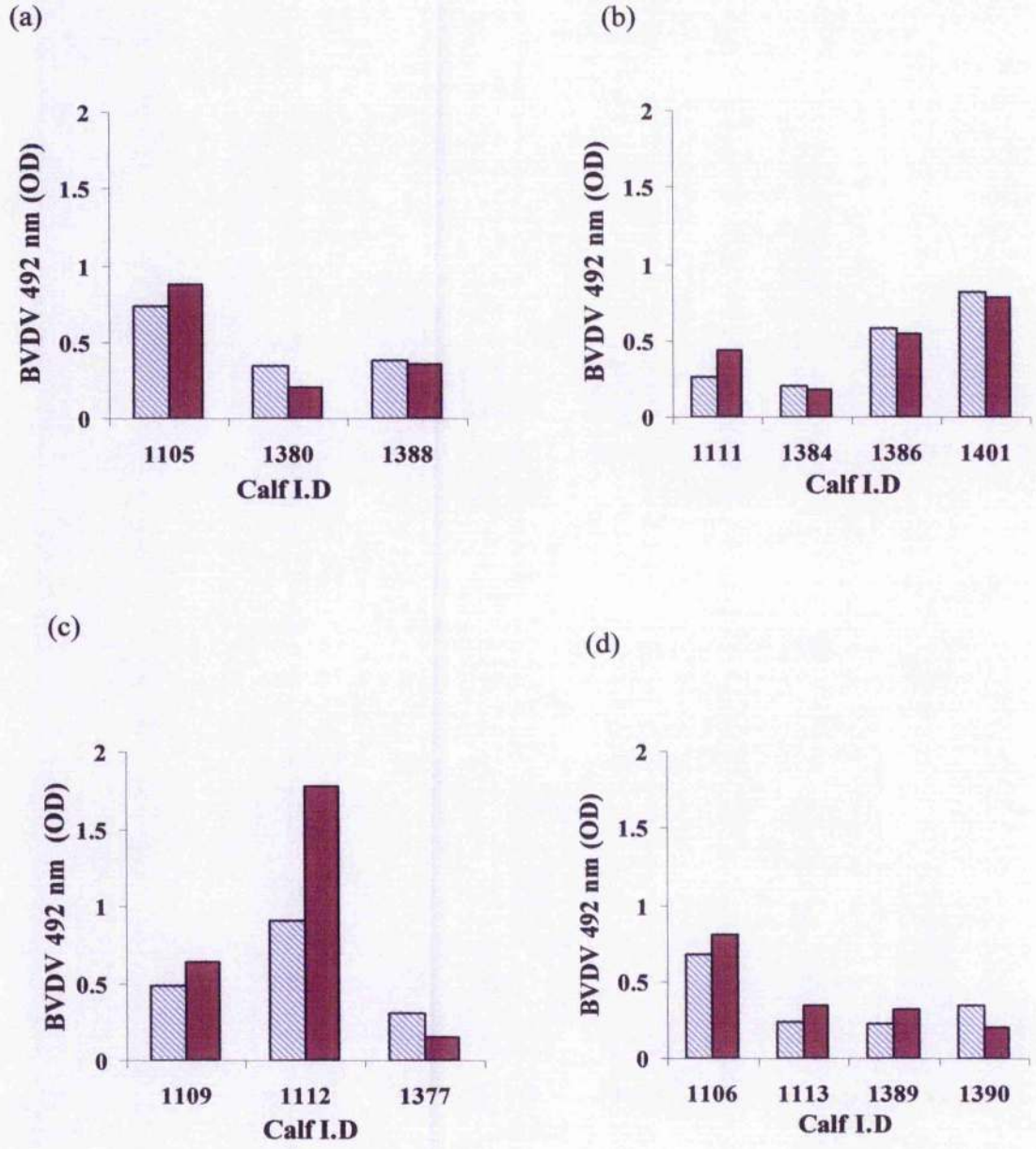


Fig. 4.4. BVDV antibody titres in pre-infection (▨) and post-mortem (■) bleeds from calves inoculated with *P. multocida* A:3 at either 10^9 or 10^{10} in 60 or 300 ml PBS. Data presented corresponds to challenge groups; (a) Group 1 10^9 cfu *P. multocida* in 300ml PBS: calf 1115 no response; (b) Group 2 10^9 cfu *P. multocida* in 60ml PBS; (c) Group 3 10^{10} cfu *P. multocida* in 300ml PBS: calf 1379 no response; (d) Group 4 10^{10} cfu *P. multocida* in 60ml PBS.

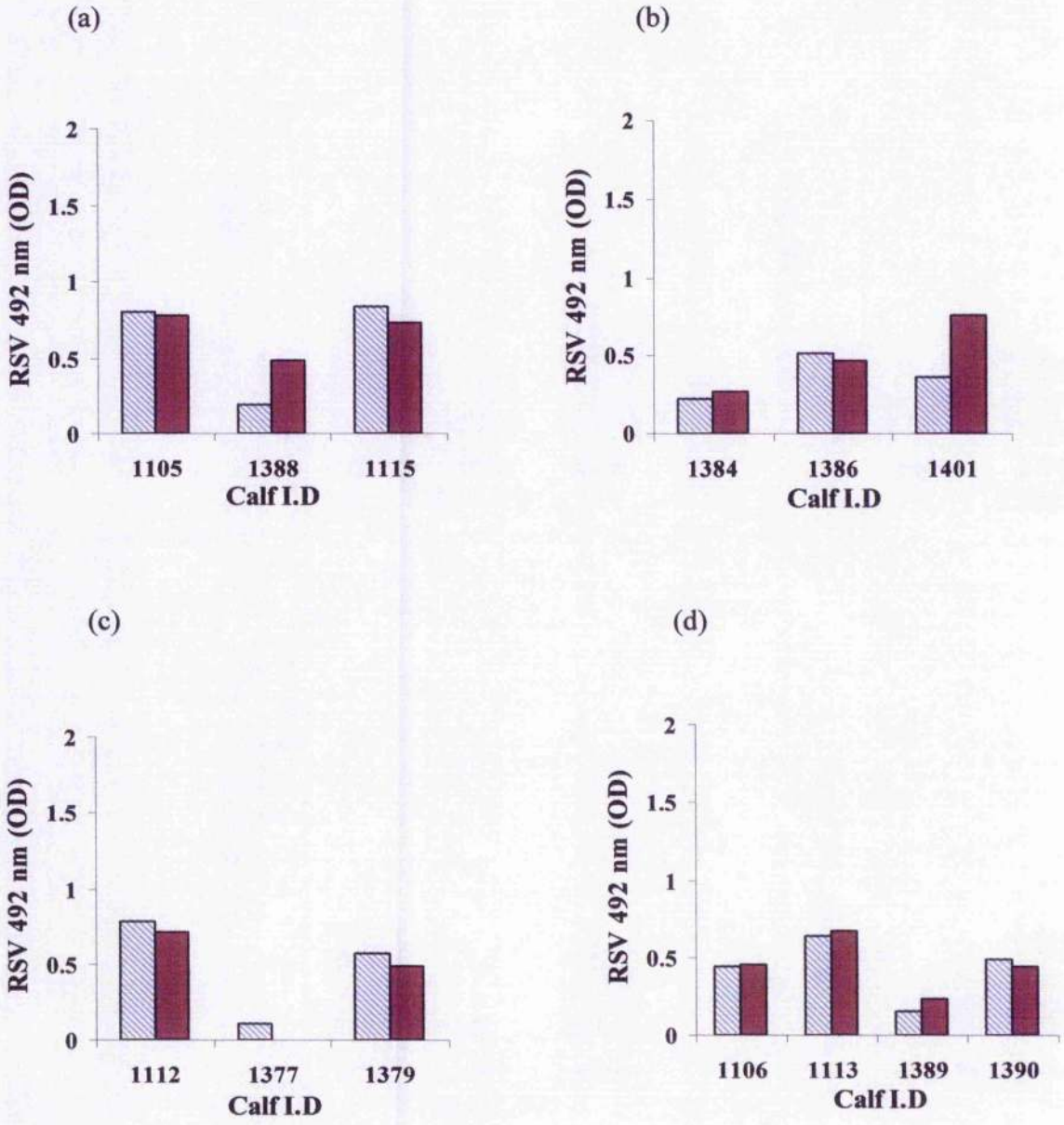


Fig. 4.5. RSV antibody titres in pre-infection (▨) and post-mortem (■) bleeds from calves inoculated with *P. multocida* A:3 at either 10^9 or 10^{10} in 60 or 300 ml PBS. Data presented corresponds to challenge groups; (a) Group 1 10^9 cfu *P. multocida* in 300ml PBS: calf 1380 no response; (b) Group 2 10^9 cfu *P. multocida* in 60ml PBS: calf 1111 no response; (c) Group 3 10^{10} cfu *P. multocida* in 300ml PBS: calf 1109 no response; (d) Group 4 10^{10} cfu *P. multocida* in 60ml PBS.

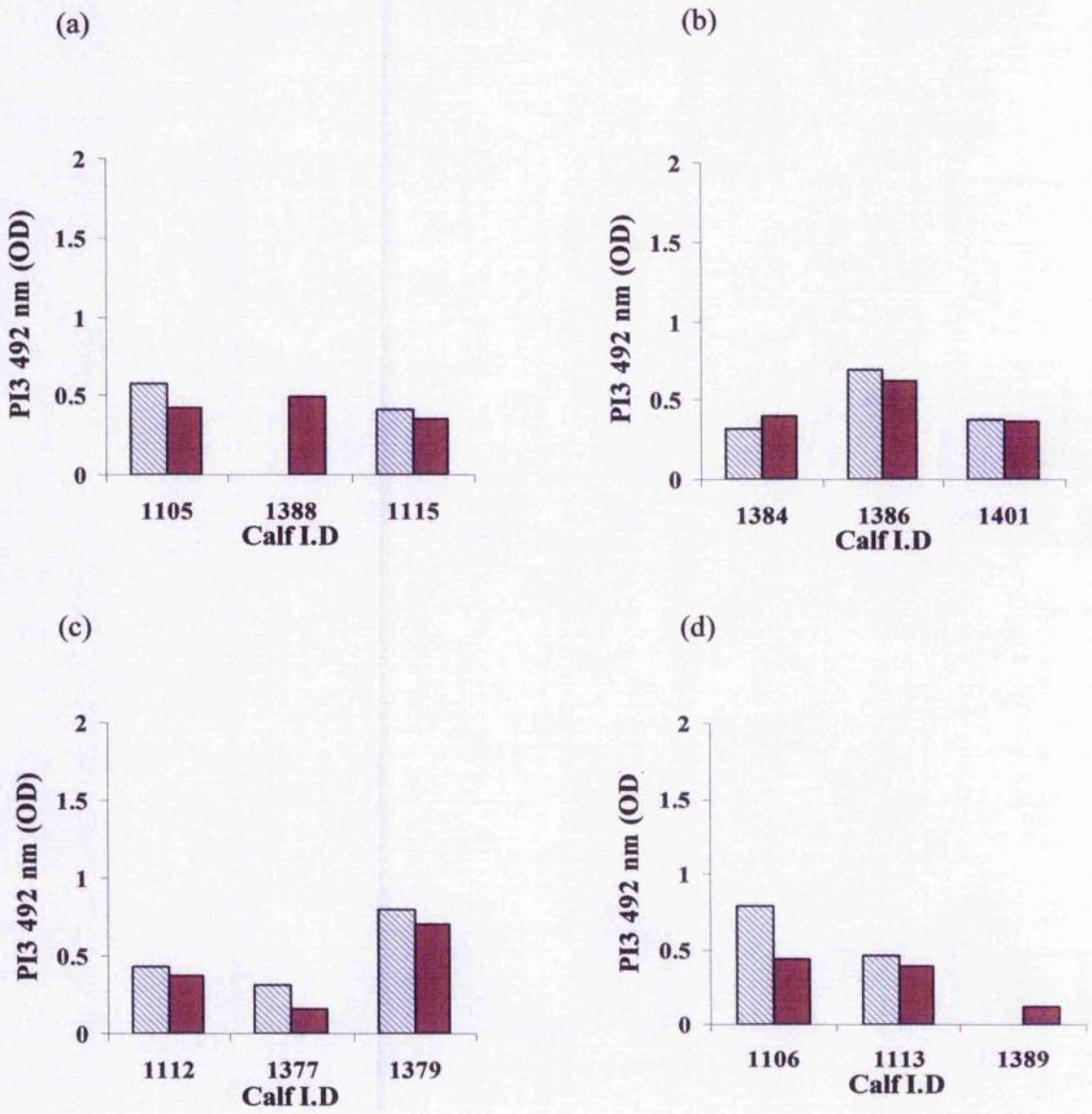


Fig. 4.6. PI-3 virus antibody titres in pre-infection (▨) and post-mortem (■) bleeds from calves inoculated with *P. multocida* A:3 at either 10^9 or 10^{10} in 60 or 300 ml PBS. Data presented corresponds to challenge groups; (a) Group 1 10^9 cfu *P. multocida* in 300ml PBS: calf 1380 no response; (b) Group 2 10^9 cfu *P. multocida* in 60ml PBS: calf 1111 no response; (c) Group 3 10^{10} cfu *P. multocida* in 300ml PBS: calf 1109 no response; (d) Group 4 10^{10} cfu *P. multocida* in 60ml PBS: calf 1390 no response.

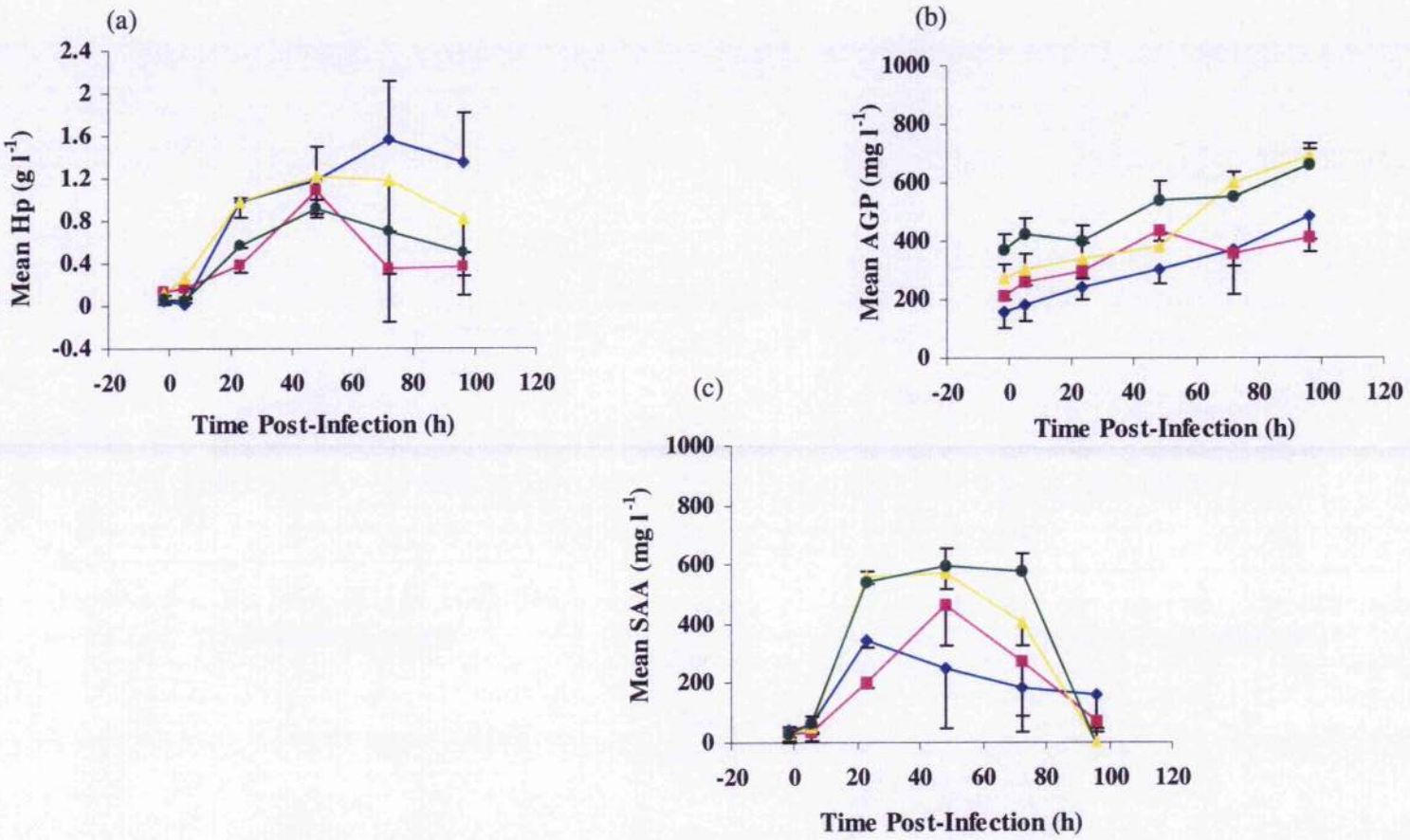


Fig. 4.7. Mean (± S.E) plasma concentrations of Hp, AGP and SAA in calves inoculated with *P. multocida* at either 10^9 or 10^{10} in 60 or 300ml PBS. (a) Mean concentrations of plasma Hp (g l^{-1}); (b) Mean concentrations of plasma AGP (mg l^{-1}); (c) Mean concentrations of SAA (mg.l^{-1}). Data presented corresponds to challenge groups. Group 1 (▲); 10^9 cfu *P. multocida* in 300ml PBS, Group 2 (■); 10^9 cfu *P. multocida* in 60ml PBS, Group 3 (◆); 10^{10} cfu *P. multocida* in 300ml PBS, Group 4 (●); 10^{10} cfu *P. multocida* in 60ml PBS.

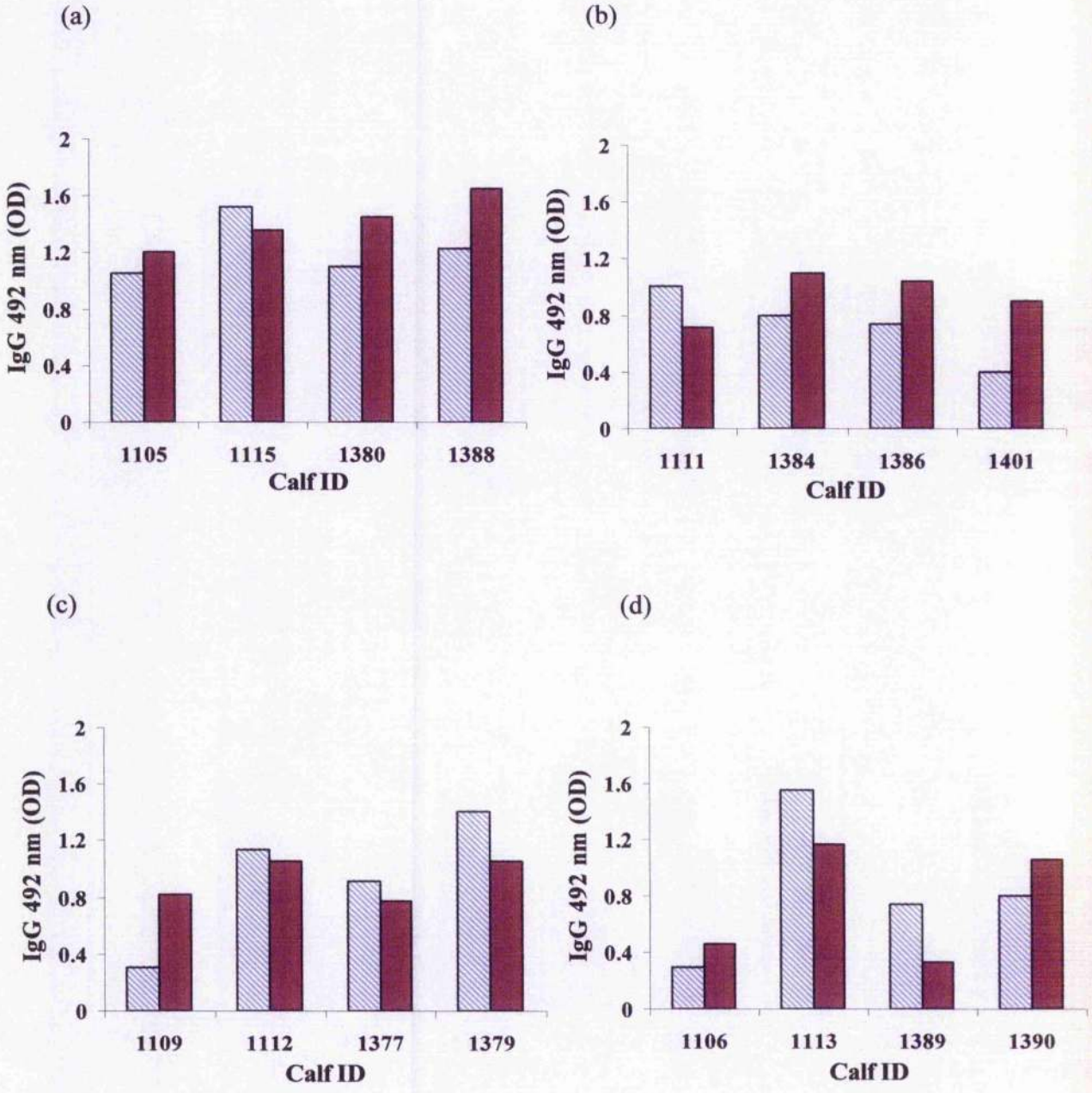


Fig. 4.8. IgG plasma antibody titres in pre-infection (▨) and post-mortem (■) bleeds from calves inoculated with *P. multocida* A:3 at either 10^9 or 10^{10} in 60 or 300 ml PBS. Data presented corresponds to challenge groups; (a) Group 1 10^9 cfu *P. multocida* in 300ml; (b) Group 2 10^9 cfu *P. multocida* in 60ml PBS; (c) Group 3 10^{10} cfu *P. multocida* in 300ml PBS; (d) Group 4 10^{10} cfu *P. multocida* in 60ml PBS.

4.4. DISCUSSION

The investigation established for the first time an experimental model for BPP caused by *P. multocida* and provided an opportunity to evaluate the effect of infectious lung challenge with the bacterium on pathological and biochemical changes in calves. It should be noted that the small number of animals available for analysis reduced the effective power of the REML procedure, making it likely that only large effects would be identified as statistically significant by this study. This applies particularly to the investigation of interaction effects. Nevertheless, various aspects of biological interest to this study were highlighted. Such as, the effect of treatment on respiration rates, area of lungs affected and pleurisy scores.

All treatments elicited a moderate to severe response, and induced clinical signs of bovine pneumonic pasteurellosis consistent with those characteristically observed in natural cases of disease, including moderate depression, pyrexia, laboured breathing, mild nasal discharge and anorexia. In addition, the gross pathological and microscopic changes observed were similar to those reported both for experimental induction (Furrow *et al.*, 1986) and field cases (Blood *et al.*, 1979) of bovine pneumonic pasteurellosis associated with *P. multocida*. The APP results indicated that, of the two treatment variables used (dose and volume), volume was the more influential factor inducing pneumonic disease, and this, combined with the observation of two cases of endotoxic shock in calves given high dose challenge, mitigated against using the higher dose in any model formulation. These considerations, supported by the observation that the low dose, high volume treatment produced significant effects on rectal temperature and plasma Hp concentrations, identified this approach as the one to adopt in future experimental studies of the pathogenesis and prevention of *P. multocida* respiratory disease.

Plasma concentrations of APP change by at least 25 % during inflammation (Godson *et al.*, 1995) and are useful markers with which to determine the progression of disease with time and to characterise initial host responses. Hp is particularly useful as a sensitive marker of bacterial infection (Conner *et al.*, 1988; Heegaard *et al.*, 2000), increasing between 10 to 100-fold after challenge (Godson *et al.*, 1996; Skinner *et al.*, 1991). It is interesting that animals given the high volume challenge exhibited higher ($P<0.05$) peak responses in Hp. The high volume challenge probably affected greater lung areas, especially if an initially slow response in Hp production allowed more time for the bacteria to proliferate. Examination of α_1AGP concentrations for all treatments indicated that the high volume challenge gave rise to a greater increase from resting level than the low volume challenge. Similar correlation of α_1AGP with severity of disease has been reported for other disorders aside from pneumonic pasteurellosis (Horadagoda *et al.*, 2001),

and in previous work examining natural acute versus chronic inflammation in cattle (Horadagoda *et al.*, 1999).

P. multocida challenge dose used was deliberately 1 or 2 logs higher (10^9 or 10^{10} cfu) than that routinely used in our laboratory for *M. haemolytica*, because of the expected lower virulence of the former strain (Ames *et al.*, 1985). Indeed, even at the dose administered, bacteria were not recovered from lung tissue in 3 out of 4 (Group 1) and 2 out of 4 (Group 2) animals. This may have been due to the sampling technique involving small samples taken from a restricted selection of set sites that did not include infected tissue. Alternatively, it may reflect a faster lung clearance of bacteria over the 4 d between challenge and PM by animals receiving the lower challenge dose, possibly linked with an effect of APP responses helping restrict bacterial numbers, as suggested by others (Horadagoda *et al.*, 2001). However, in the present work there was no correlation between APP concentrations and lung bacterial clearance.

All calves were reared conventionally with access to maternal colostrum and it was not surprising that we detected antibody to a range of viruses. In most samples the antibody titre decreased with time indicating that viral antibodies resulted from passive colostrum transfer and were not an active response to infection; however, the rise in antibody to BVDV in some calves indicated an active infection. Recent work has shown an enhanced risk of bovine respiratory disease in association with BVDV (Martin *et al.*, 1999) but the present results suggested that severity of disease was associated with dose and volume of challenge and not with BVDV antibody status.

It was hardly surprising that calves prior to challenge exhibited rather high IgG levels, which was attributable to the passive transfer of IgG through maternal colostrum. Following challenge with *P. multocida* some calves showed a decrease in IgG concentration because at 8 weeks of age the level of maternal antibody is decreasing and the naïve calf is developing its innate immunity, also when the animal grows the plasma blood volume expands and becomes diluted, thus lowering the concentration of IgG. The reasons for an increase in IgG could also be due to analytical variation within the assay, as it is highly improbable that an immune response could develop by 5 days following a bacterial infection.

To conclude, the model produced a range of detectable clinical and pathological responses consistent with the natural disease, and showed the validity of APP as indicators of bovine pneumonic pasteurellosis. Future work will use the model to improve our understanding of host-pathogen interactions after infection with *P. multocida*, and in the longer term use this knowledge in an attempt to develop a multivalent vaccine, effective against agents responsible for bovine respiratory disease.

Chapter 5. A PRELIMINARY STUDY TO IDENTIFY DISEASE MARKERS IN LAVAGE FLUID FROM CALVES INFECTED EXPERIMENTALLY WITH *PASTEURELLA MULTOCIDA* USING 2D-ELECTROPHORESIS AND MALDI-TOF MASS SPECTROMETRY.

5.1. INTRODUCTION

Disease mechanisms attributable to *P. multocida* in BPP have not been fully defined. Recent evidence in calves suggests that the presence of *P. multocida* either alone, or in association with a virus, attenuates the animal's natural resistance, promoting bacterial colonisation of the mucociliary apparatus, invasion of the innate mucosal defence system and subsequent infection of the lung (Ackermann and Brogden 2000).

There are numerous proteins present in bronchial secretions, released from secretory cells such as goblet cells and mucus glands that can both assist mucociliary clearance and act as antibacterial factors during host-bacterial immune interaction. These proteins comprise the film of mucus on the alveolar surface and which aids gaseous exchange, and others that encompass surfactant which prevent the lung from collapsing (Wu *et al.*, 2003). Evidence has been presented of the importance of epithelial lining fluid in the diagnosis of human lung diseases such as idiopathic pulmonary fibrosis, sarcoidosis and adult respiratory distress syndrome (Lenz *et al.*, 1993). It is believed, from the analysis of human BALF that the overall protein composition of the epithelial lining fluid of the alveolar and distal airways of the lung may change during the initiation and developmental stages of pneumonic pasteurellosis (Chang He *et al.*, 2003).

Innate defences of the lung against *P. multocida* are mediated by an array of resident inflammatory and immune effector cells. In the healthy animal these are mainly macrophages lying within the alveolar epithelial lining fluid (Babiuk *et al.*, 1995). Upon stimulation by bacteria, alveolar macrophages generate a chemotactic gradient through the production of proinflammatory cytokines and chemokines (IL-8, IL-1, TNF-α) to recruit neutrophils into the alveolar lumen (Sibille and Reynolds, 1990). These cells act in conjunction with a variety of soluble factors, in particular chemokines and cytokines that either up or down-regulate the activity of specific leukocytes (Babiuk *et al.*, 1995). Secretory products released by both macrophages and PMN can alter the protein composition of pulmonary fluid in such a manner as to deter bacteria from proliferating and

colonising the lung, by inhibiting adherence to epithelial lining and enhancing subsequent phagocytosis and killing. These secretions include proteases, oxidants, lactoferrin and cytokines. Other proteins that are located on mucosal surfaces and secreted from epithelial cells located in the URT and LRT include defensins (TAP and LAP), cathelicidins, lysozyme, lactoperoxidase, Ig-A and G and surfactant proteins (SP-A and SP-D) (Ganz and Weiss, 1997). It may be postulated that expression of the genes encoding these proteins will vary according to their requirement during the disease process. Collectively they alter the composition of pulmonary fluid

Recent studies have shown the protective effects of these proteins and their possible role in controlling respiratory infection. For example, an ovine cathelicidin SMAP29 decreased the concentration of bacteria in both BALF and necrotic lung tissues of pneumonic lambs (Brogden *et al.*, 2001). The roles for other proteins in the lung have been discovered more recently; for example, Hp is expressed at a high level in lung cells, and may help reduce oxidative damage associated with haemolysis (Yang *et al.*, 2003).

Stressors, such as viral or bacterial overload, may compromise the functional integrity of the epithelial cell surface, and allow infection to spread out of control, with the result that pulmonary fluid becomes altered in such a manner as to promote bacterial survival. Low levels of antimicrobial proteins, and high levels of proteins that make the alveolar mucus more viscous, may contribute towards reducing bacterial clearance from the lung by cilia and promoting bacterial proliferation (Babiuk *et al.*, 1995).

Recently, a technique of sequential live bronchoalveolar lavage has been developed to provide samples of lung fluid prior to infection and at different stages of disease (Caldow, 2001). This technique, combined with recently available cytokine probes, will provide valuable information on the cellular and secretory components of the lung, particularly those that may influence the initiation of pneumonic pasteurellosis. Such evidence may provide useful markers of clinical disease. Changes in inflammatory cytokine profiles in BALF following inoculation with *M. haemolytica* have been reported (Morsey *et al.*, 1999; Malazdrewich *et al.*, 2001), but changes in the overall protein composition of bovine lung fluid following bacterial infection have not been assessed. This is now possible through advances in the field of proteomics, using state of the art technology that allows the identification of proteins responsible for virulence, pathogenesis, and protective immune responses that may be increased or decreased specifically in the diseased lung. Characterising protein changes during pneumonic pasteurellosis may provide insight into the underlying mechanisms of pathogenesis and lead to new and improved methods of disease prevention and control. In addition, the characterisation of this disease process may shed light on a related human disease, pulmonary sarcoidosis that is also associated with the accumulation and stimulation of inflammatory cells within the lung (Sabounchi-Schutt *et al.*, 2003).

Aims of the study

The proposed work will extend the established experimental model that successfully indicated the susceptibility of calves to an intratracheal challenge of *P. multocida* at 10^9 cfu in 300 ml of PBS and the extent of disease. The model will be used to provide a detailed description of the clinical development of pneumonic pasteurellosis and preliminary information on changes in pulmonary fluid content during lung infection with *P. multocida* A:3. Pre-infection lung fluid samples will be included in the study to assess the protein composition of a normal healthy lung. Results from the former will be compared with post-infection samples and, using 2-DE, MS and databases, changes in protein composition following infection will be tracked and any novel bovine and or bacterial proteins identified.

5.2. EXPERIMENTAL DESIGN AND METHODS

5.2.1. Animals and challenge procedure

Male Holstein cross Friesian calves (n=16) of approximately two weeks of age were purchased from farms within Midlothian and housed in individual calf pens. Calves were assessed for prior exposure to *P. multocida* by nasal swabbing and plasma antibody analysis, performed by an ELISA specific to *P. multocida* cell envelope; all tests were negative.

Calves were weaned at 7 weeks of age from a liquid milk diet to hay and mixed pellets, and held in open pens for the duration of the experiment.

At 8 weeks of age (designated experimental day 0) the 16 calves were allocated randomly to two groups. A control group (Group 1; n=6) were given 300 ml PBS to allow comparison of clinical responses to those of experimentally infected animals, and to see how protein production in the lung was affected by the live lavage procedure. The remaining 10 animals (Group 2) were challenged with 10^9 cfu of exponentially growing (log phase) *P. multocida* A:3 in 300ml of PBS. Calves in Groups 1 or 2 were challenged intratracheally via a bronchoscope at 8 weeks of age (day 0) with either PBS or *P. multocida*, respectively (Chapter 4).

5.2.2. Live lung lavage procedure

Lung lavage samples were taken from all calves 2 d before challenge (day -2) to determine negative control baselines and on day 1 and day 4 after challenge and at PM (Table 5.1). All experimental protocols were approved by the MRI Animal Experiments Committee, authorised

under the Animals (Scientific Procedures) Act 1986, and the intervals used complied with the Home Office (HO) project licence. Access to veterinary care was available at all times.

5.2.3. Lung fluid protein identification

Unless stated otherwise all reagents and protocols used for the analysis of proteins in lung fluid were purchased from Amersham Pharmacia Biotech UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire, England. Lung fluid isolated from only 3 of the 6 Group 1 calves (20, 35 and 41) and 3 of the 10 Group 2 calves (11, 42 and 43) were randomly selected for protein analysis.

2-DE is a technique in which proteins are separated according to their pI (first dimension) and then subsequently on the basis of size (second dimension). As a result, proteins present in a sample are displayed as spots on a gel. Protein positions can be mapped and differences in composition or level of expressed protein compared in samples obtained at different stages of disease and from other sources (O'Farrell, 1975).

Full details of lung fluid protein analysis, separation by electrophoresis and detection by mass spectrometry are given in section 2.7.

A PlusOne 2-D Quant Protein assay kit was used according to the manufacturer's instructions (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, England) to determine accurately the concentration of protein present in samples prior to 2-D gel analysis. It was found that 100 µg of protein resuspended in 200 µl of rehydration buffer was optimum for the procedure. Samples were loaded on 11 cm IPG strips, pH range 3-10 and run on an IPGphor at 100 V for 2 h, 500 V for 1 h, 1000 V for 1 h, 2500 V for 1 h and 8000 V up to 38.8 kVh. (section 2.7.3).

Table 5.1. Live lung lavage and bacterial challenge schedule for both groups of animals, control and infected.

	Treatment								
Day	**-2**	**0**	**1**	**4**	**7**				
Calf I.D									
Control Group 1	LV	Challenge	LV	LV	PM				
9, 14, 20,	1		2	3					
Infected Group 2	LV	Challenge	LV	LV	PM				
10, 11, 12, 24, 25,	1		2	3					
			-5			**0**	**1**	**4**	**7**
Control Group 1			LV			Challenge	LV	LV	PM
35, 41, 38			1				2	3	
Infected Group 2			LV			Challenge	LV	LV	PM
39, 42, 43, 45, 48			I				2	3	

Challenge = 300ml PBS, 10^9 CFU *P. multocida* A3 MRI-671/90 challenge strain.

LV = Lavage

Following first dimension electrophoresis, second dimension was carried out by placing each 11 cm IPG strip on a 12.5 % (w/v) homogeneous 2D-ExcelGel on a Multiphor II (Amersham Biosciences) that separated proteins according to their molecular weight (section 2.7.4). This size of gel showed that 100 μg of protein in 200 μl of rehydration buffer was an optimum concentration for clear separation of proteins of interest within lung fluid samples and for comparative studies to be conducted between gels.

Following electrophoresis, protein in the gel was detected by Colloidal Coomasie blue stain (Genomics Solutions, Ann Arbor, Michigan) (section 2.7.5), scanned using Imagemaster (Amersham Bioscience) and processed using Imagemaster 2D software.

Following the analysis of 2-D gels, spots were excised for further analysis by MALDI-TOF (section 2.7.6). Prior to MALDI-TOF it was necessary to fragment the 155 spots of interest by digestion with trypsin (a proteolytic enzyme that cleaves proteins after the amino acid lysine to produce peptides), so that the mass of each fragment could be determined by mass spectrometry. Proteins were identified from Swiss-Prot, Mass Spectrometry Database (MSDB) and *P. multocida* databases.

5.2.4. Statistical analyses

The mean rectal temperature and respiratory rate were analysed by subtracting the baseline value, to adjust for intrinsic animal variability and a two sample t-test was performed on the data set to determine statistical significance between the two animal groups.

5.3. Results

5.3.1. Clinical observations

Clinical signs such as demeanour, laboured breathing, and reduced appetite were observed 2-6 h p.c. in all Group 2 animals. Mean rectal temperatures for Group 2 calves increased significantly ($P = 0.001$) from 38.9 (± 0.09) °C before inoculation to a peak of 40.2 (± 0.1) °C 30 h p.c. (This peak occurred at 23 h p.c. in work reported in Chapter 4). Mean rectal temperatures in Group 1 calves did not differ significantly for the first 8 h p.c., increasing from 38.9 (± 0.1) °C 2 h before challenge to 39.4 (± 0.2) °C 8 h p.c. (Fig. 5.1a) Following this sampling point and until 68 h p.c. mean rectal temperatures were erratic, fluctuating between 38.8 (± 0.1) °C and 39.4 (± 0.05) °C. Mean respiratory rates for Group 2 calves increased significantly ($P = 0.001$) from 33 (± 2.3) min^{-1} prior

to infection to 103 (± 2.9) min^{-1} 8 h p.c. and remained higher than Group 1 calves for the duration of the experiment (Fig. 5.1b). Group 1 calves demonstrated a slight increase in respiratory rate from a baseline value of 29.5 (± 2.6) min^{-1} to 47.8 (± 3.5) min^{-1} by 8 h p.c., values that were within the normal respiratory range and remained so for the duration of the sampling period.

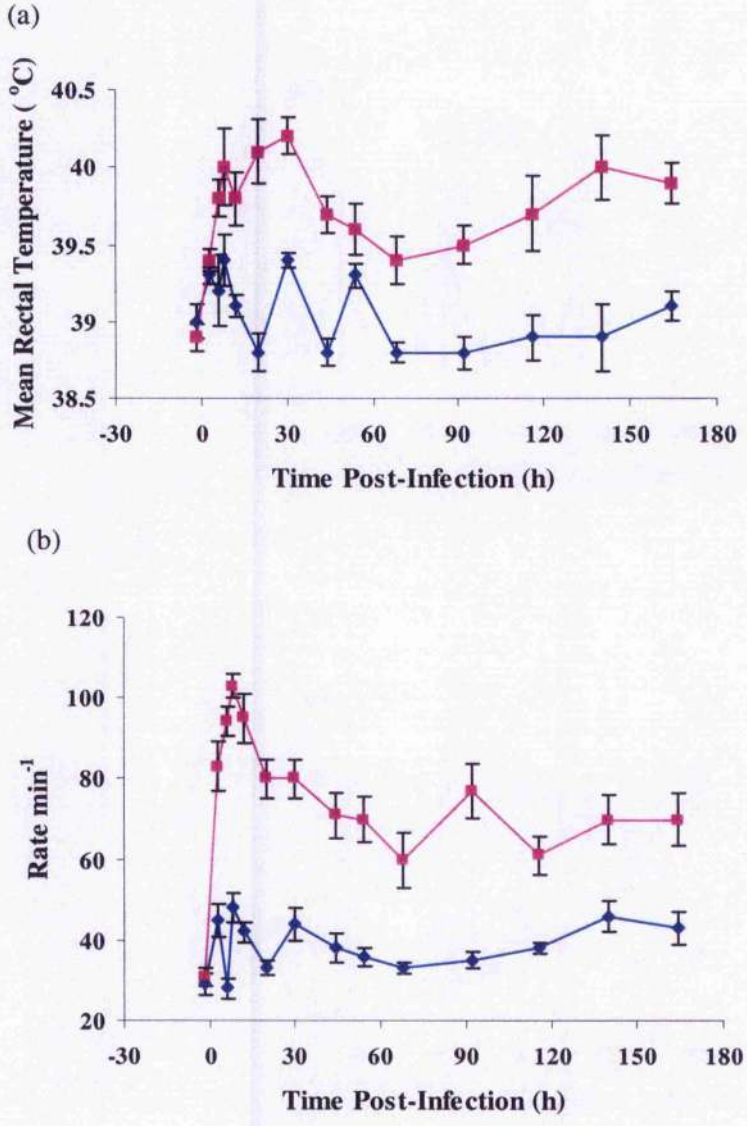


Fig. 5.1. Mean (± S.E) clinical measurements from calves inoculated with *P. multocida* A:3 at 10^9 cfu in 300 ml PBS (a) Mean rectal temperature (°C); (b) Mean respiratory rate (rate min^{-1}). Data presented corresponds to challenge groups 1 (control 300ml PBS◆) and 2 (Infected 10^9 cfu *P. multocida* A:3 in 300 ml PBS ■).

5.3.2. Gross pathology and bacteriology

Lung surface lesions from Group 2 calves were characterised by severe congestion and haemorrhage, giving the affected area an intense red to purple discolouration (Fig 5.2A) due to the active hyperaemic response during early stages of inflammation; the apical lobes were most affected, as described in Chapter 4. Plaques of fibrinous exudate were located on the lung surface and were often responsible for the cranioventral lobes adhering to the thoracic cavity and to each other (Fig 5.2B). On sectioning, the lesions seemed nodular and often associated with abscessation. A high level of fibrinous exudate was present in the bronchial airways. All calves in Group 1 had healthy lungs; in some cases (calves 14, 38 and 41) there was evidence of old lesions that were not consolidated and some flecks of necrotic tissue that were not acute.

Pleurisy and pleural adhesions were observed in 40 % of calves in Group 2, with no such evidence for Group 1 calves. *P. multocida* was recovered from the lungs of 8 pneumonic calves (Group 2), largely in areas exhibiting congestion and abscessation. The viable counts from lungs of individual calves varied from 2.5×10^6 cfu g^{-1} to 1.9×10^9 cfu g^{-1}. A high bacterial count and high percentage of surface lesions were observed in this study, similar to that seen in Chapter 4 for this specific formulation (10^9 cfu in 300 ml PBS).

There was no bacterial recovery from heart, heart blood, pericardial or pleural fluid, spleen, kidney, liver and lymph node tissues taken from Group 1 animals (Table 5.2).

Table 5.2. Percentage of surface lesions, pleurisy scores and bacterial recoveries from lung tissue (cfu g^{-1}) of all calves challenged intratracheally with *P. multocida* A:3 at 10^9 cfu ml^{-1} in 300 ml PBS.

Group	Calf	% lung surface affected	Pleurisy	Viable mean lung count (cfu g^{-1})
Control	9	0	0	0
	14	5	0	0
	20	0	0	0
	35	0	0	0
	38	6.5	0	0
	41	2	0	0
Infected	10	36	0	4×10^7
	11	52	2	1.2×10^9
	12	26	0	1.9×10^9
	24	4	0	0
	25	47	0	1×10^9
	39	20	1	4.2×10^7
	42	47	2	2.8×10^7
	43	25.5	0	0
	45	19	1	2.1×10^8
	48	10.5	0	2.5×10^6

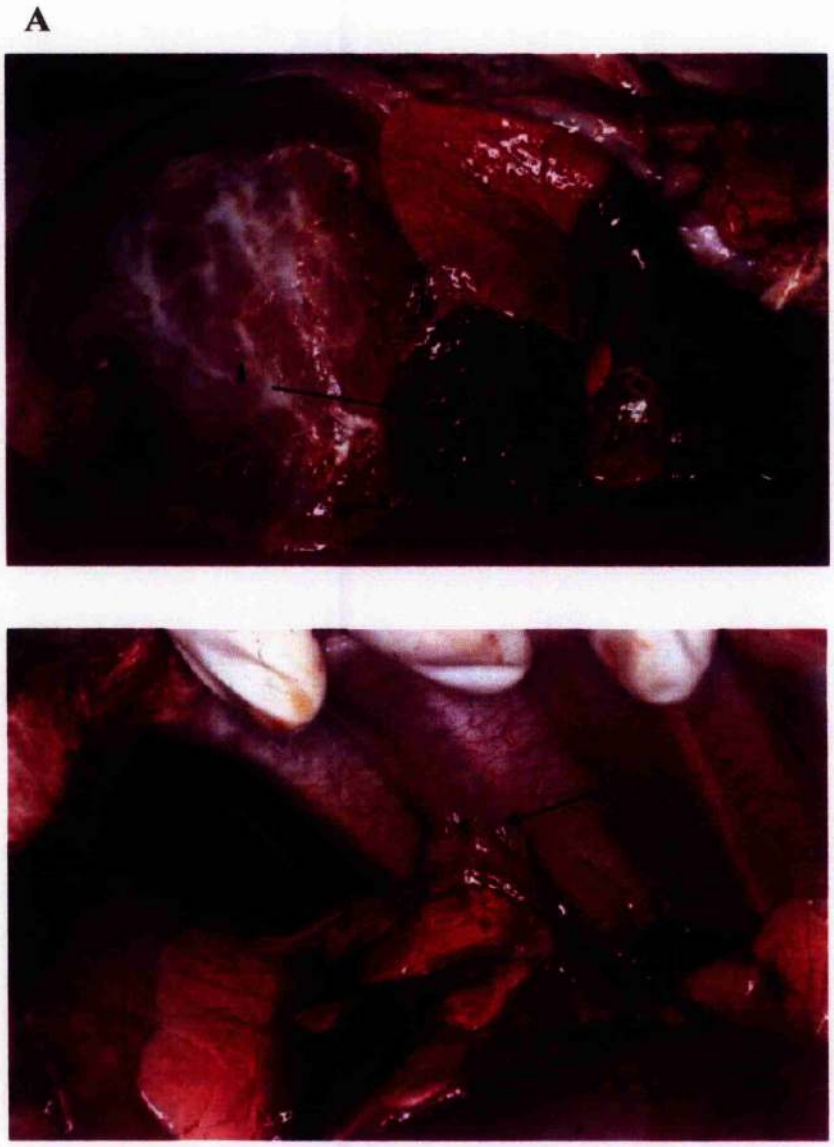


Fig. 5.2. Gross pathological changes observed in calf 11 given 10^9cfu of *P. multocida* A:3 in 300 ml PBS, 4 d p.c; **A:** whole excised lungs to illustrate the cranioventral area as being most affected, characterised by consolidation and red to purple discolouration (1) of the lung surface; **B:** ventral surface of the same lungs lying within the thoracic cavity, note fibrinous plaques on the cranioventral lobe adhering to the thoracic cavity (2).

5.3.3. Histopathology

Microscopic examination of the selected lesions in Group 2 calves showed that they were typical of a moderate to severe fibrinopurulent bronchopneumonia. In all Group 2 calves the interlobular septa appeared oedematous as indicated by their marbled appearance and they were filled with fibrinocellular exudate, neutrophils and macrophages. As a result, the air supply and muco-ciliary apparatus for that part of the lung became disabled, resulting in tissue necrosis. Focal areas of coagulative necrosis contained densely packed inflammatory cells and were present in pulmonary parenchyma of all calves in Group 2 except for calf 24. In contrast, the lung tissue of all Group 1 calves appeared healthy, containing large alveolar air spaces and only the occasional activation of bronchial associated lymphoid tissue (BALT) in confined areas around the bronchi. No significant histopathological changes were observed in any of the samples taken from spleen, heart, liver or kidney tissues.

5.3.4. 2-Dimensional Electrophoresis; Protein spot patterns

The protein content of lavaged lung fluid detected by the 2D-Quant kit ranged from 154 – 5200 μg ml^{-1} and each sample was adjusted so that 100 μg of protein (section 2.7.2) was loaded on each gel.

2D-gels of lung fluid from both groups of calves resolved up to 155 protein spots of interest, with numbers and staining intensity on most occasions being greatest 1 d p.c. The spots were located within the region corresponding to molecular masses of 6-75 KDa and within a pI range of 3-10. All 2D-gels seemed to present a similar spot pattern whether the animal was in a healthy or diseased state. A large dense area between 80 and 66 KDa, identified as albumin, was observed on all 2D-gels and comprised up to 50 % of the total protein. Colloidal Coomasie staining detected a dense area of protein (90 to 75 KDa), above albumin that was present throughout the experiment but at varying levels of intensity, illustrated by spots 89, 90 and 91 (Fig 5.3.b and Fig 5.3.c, calf 11) and spots 51, 52 and 53 (Fig. 5.6, calf 20), on all gels except for those shown in Fig 5.3.a and Fig. 5.4.c.

Below the albumin area, between 66 and 31 KDa the degree of protein expression varied and there was evidence of spots appearing and disappearing at different stages of sampling. A line of 4 spots (93, 94, 95 and 96) are absent in Fig 5.3.a. but appear faintly in Fig 5.3.b and with increased expression in Fig 5.3.c. These spots were selected for identification by MS as it seemed that they might represent suitable markers of disease, although these spots were observed also at all sampling stages in a control calf (Fig. 5.6, calf 20).

Varying levels of protein expression were observed in an area directly below albumin (Fig. 5.6, calf 20). This area showed a high level of protein expression in all animals at the sampling phase prior to challenge and appeared to decrease markedly 1 d p.c., only to increase 4 d p.c (spots 44 to 47). This spot pattern was also observed in gels shown in Fig. 5.8, calf 41, Fig. 5.7, calf 35 and Fig. 5.5, calf 43. MALDI-TOF analysis and a database search showed that spots excised from this area of the gel were albumin fragments in multiple forms.

The central region of the gel, between 36 and 28 KDa exhibited a consistent overall pattern of spots, although levels of intensity and expression of spots were altered throughout the experiment. For example, 6 spots (26, 27, 28, 29, 30 and 31) were present only prior to challenge, Fig. 5.5.a indicating their association with normal, healthy bovine BALF. Spot 40 was selected for MS due to its appearance 1 d p.c. (Fig. 5.5.b, calf 43). Spots 119 and 120 were taken from the gel shown in Fig. 5.4.a because they were absent from samples collected on day 1 and 4 p.c., and thus corresponded to healthy BALF. Additional spots (113, 114, 115, 116, 117 and 118) were detected 1 d p.c., and may play a role in infection by *P. multocida*, although BALF taken on day 4 p.c., from calf 42, did not show this protein spot pattern, despite re-analysis.

Differences in levels of protein expression were observed in the lower molecular weight region of the gel (14.4 to 6 KDa), illustrated in Fig. 5.6.a., Fig. 5.6.b., Fig. 5.6.c. Spot 56 showed decreasing intensity throughout the sampling period, whereas spots 57 and 58 exhibited maximum intensity 1 d p.c; spots were only excised from this region if they were stained intensely, indicating a high concentration of protein.

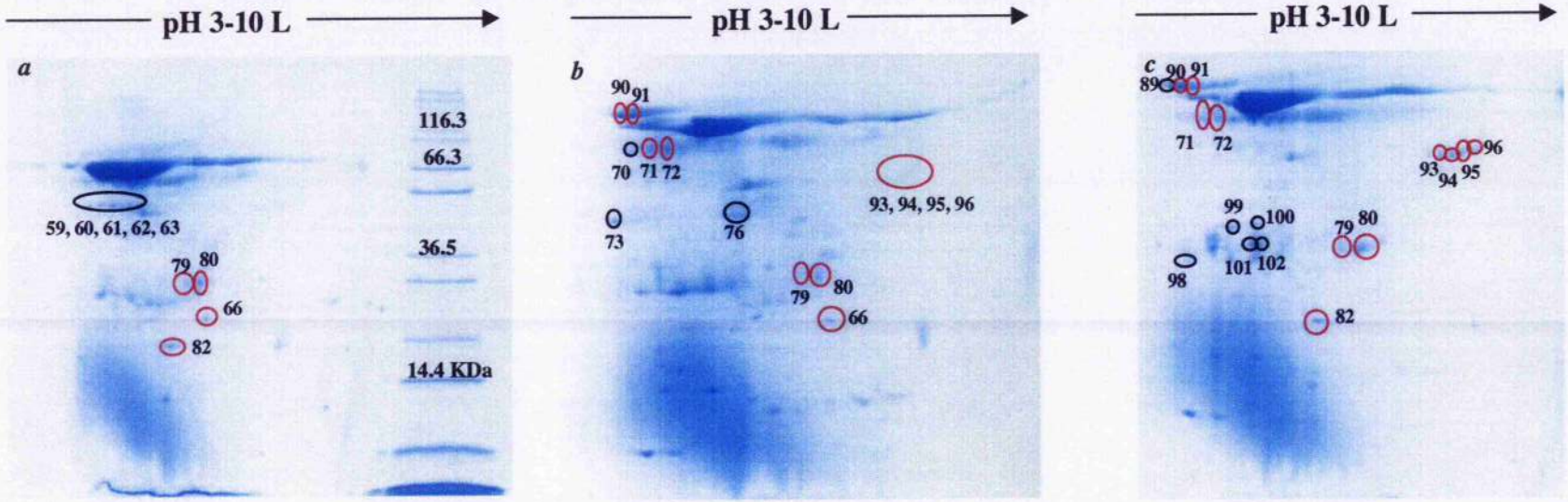


Fig 5.3. Protein spot patterns of bronchoalveolar fluid extracted from calf 11; (a) 2 d prior to infection, (b) day 1 and (c) day 4 following challenge with *P. multocida* at 10^9 cfu in 300 ml PBS. Black circles indicate different spots and red circles indicate same spots with variable levels of expression.

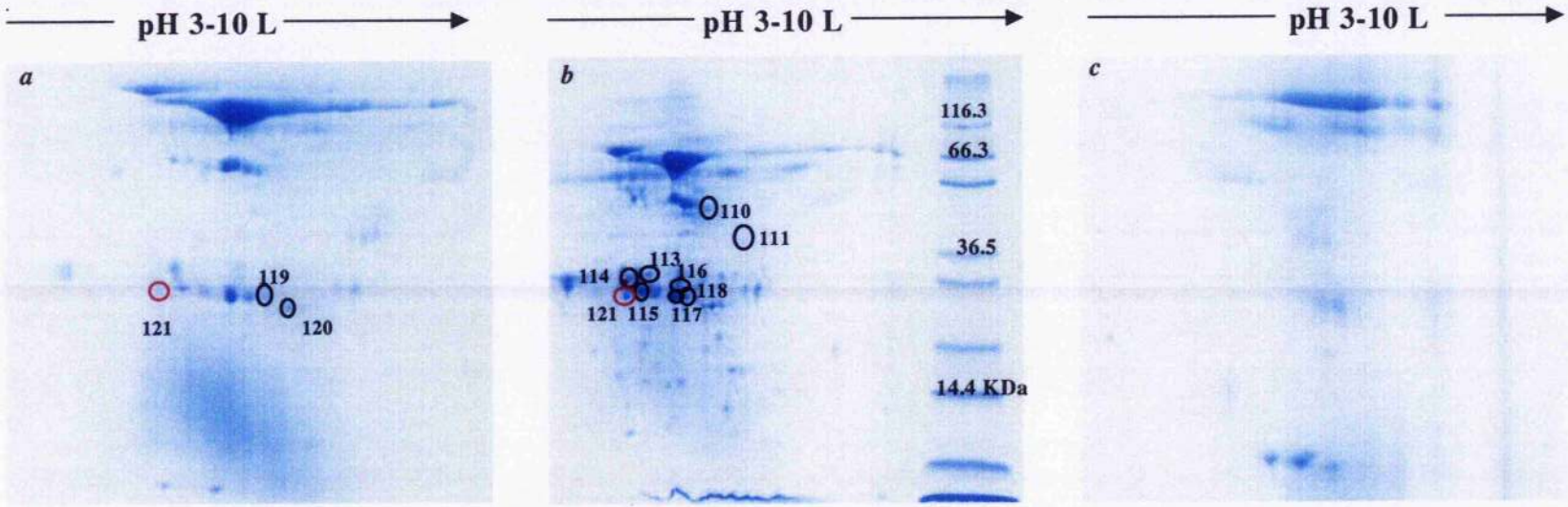


Fig. 5.4. Protein spot patterns of bronchoalveolar fluid extracted from calf 42; (a) 2 d prior to infection, (b) day 1 and (c) day 4 following challenge with *P. multocida* at 10^9 cfu in 300 ml PBS. Black circles indicate different spots and red circles indicate same spots with variable levels of expression.

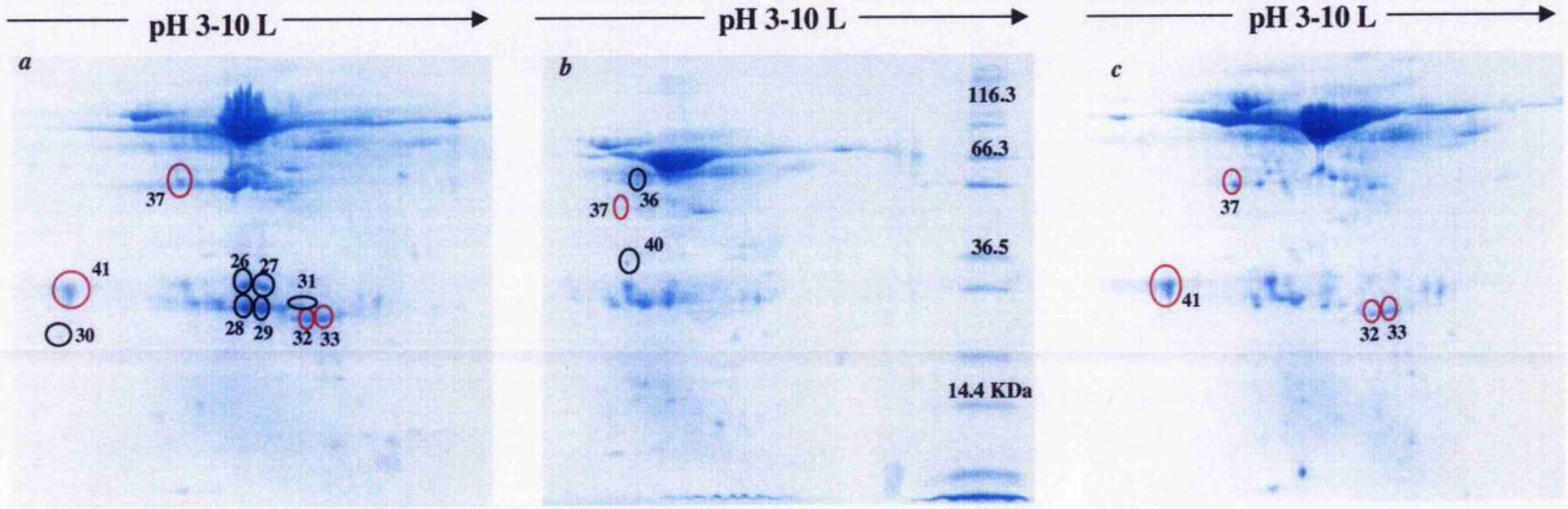


Fig. 5.5. Protein spot patterns of bronchoalveolar fluid extracted from calf 43; (a) 2 d prior to infection, (b) day 1 and (c) day 4 following challenge with *P. multocida* at 10^9 cfu in 300 ml PBS. Black circles represent different spots and red circles represent same spots with variable levels of expression.

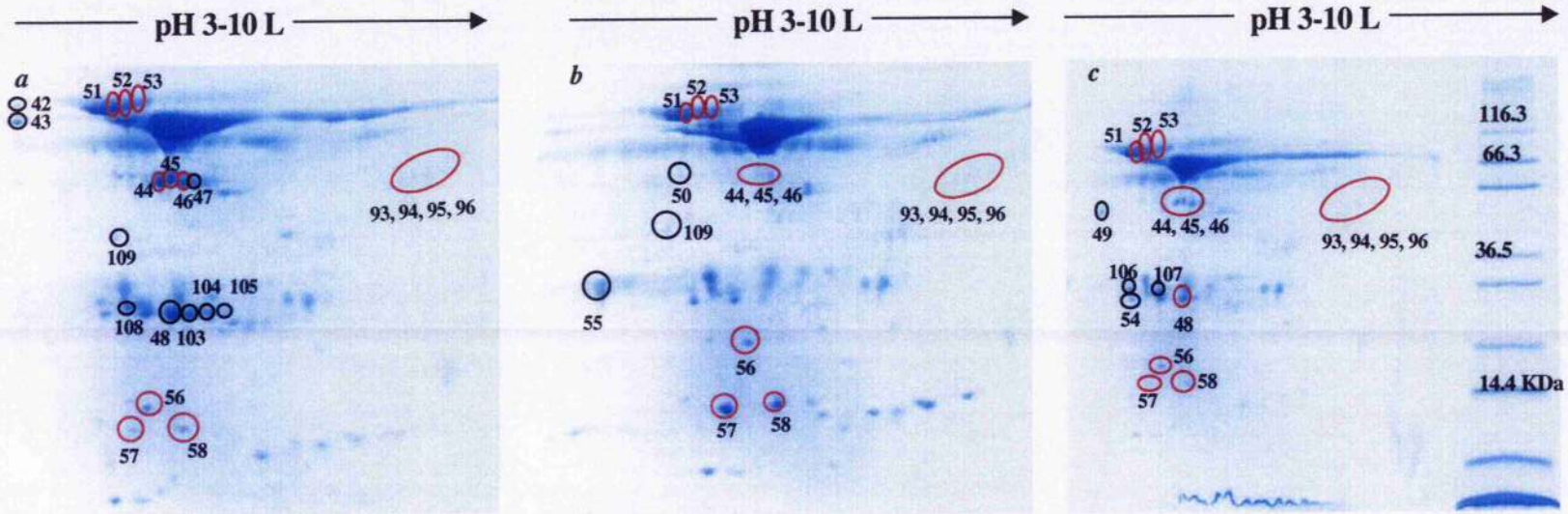


Fig. 5.6. Protein spot patterns of bronchoalveolar fluid extracted from calf 20; (a) 2 d prior to infection, (b) day 1 and (c) day 4 following challenge with 300 ml of PBS. Black circles represent different spots and red circles represent same spots with variable levels of expression.

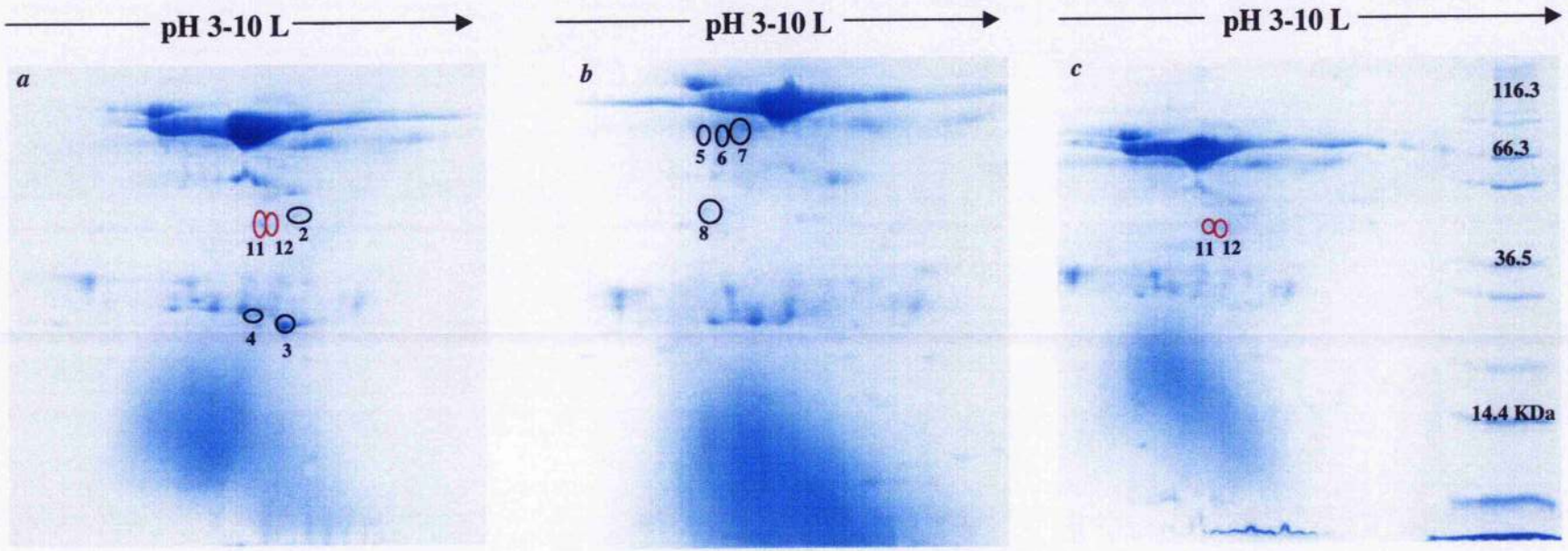


Fig. 5.7. Protein spot patterns of bronchoalveolar fluid extracted from calf 35; (a) 2 d prior to infection, (b) day 1 and (c) day 4 following challenge with 300 ml of PBS. Black circles represent different spots and red circles represent same spots with variable levels of expression.

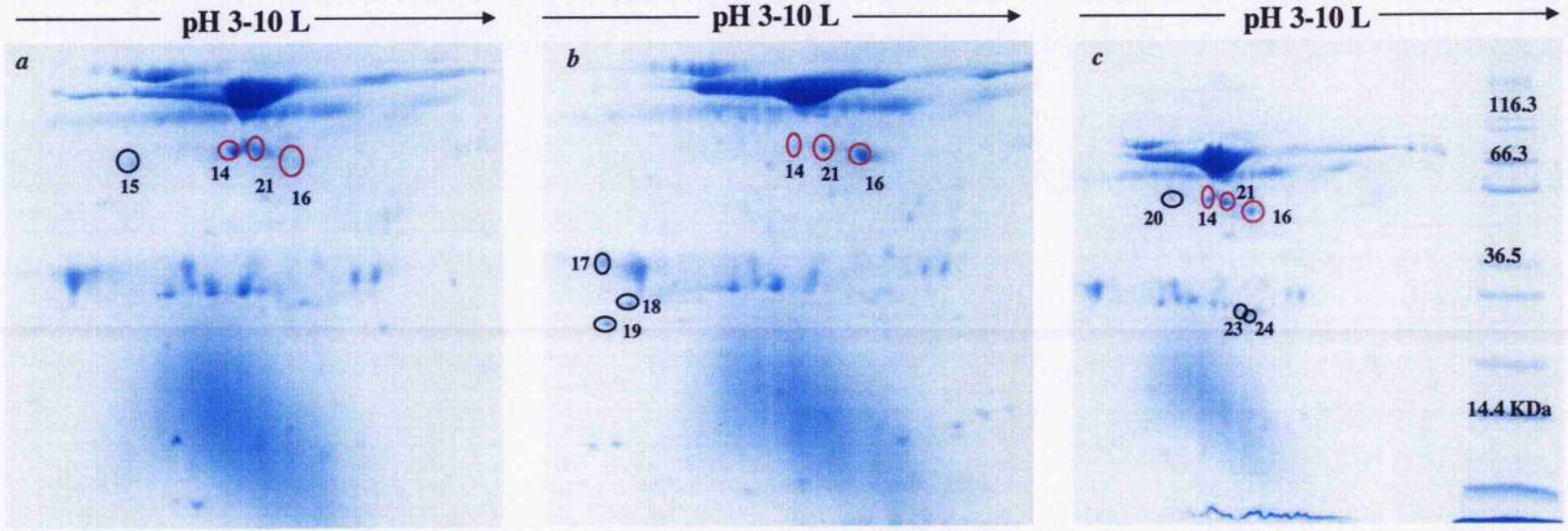


Fig. 5.8. Protein spot patterns of bronchoalveolar fluid extracted from calf 41; (a) 2 d prior to infection, (b) day 1 and (c) day 4 following challenge with 300 ml of PBS. Black circles represent different spots whereas red circles represent the same spots with variable levels of expression.

5.3.5. Mass spectrometry results

In order to identify the proteins that were either absent or present at designated sampling periods or those with varying levels of expression, in 6 experimental calves, all 155 protein spots were subjected to MALD-TOF MS. All spots circled on the gel Fig 5.3 to Fig. 5.8 yielded results by MS and keratin contamination was avoided. The fingerprints from each sample exhibited three sets of peaks, one set corresponding to the CHCA matrix, a second corresponding to autodigestion products of trypsin and a third for the protein excised from the 2-DE gel. The fingerprints generated by the MS for the standards and the proteins with significant hits are located in Appendix 2. MALDI-TOF results are in the form of molecular masses of each fragment from the trypsin digest. These masses were placed into three databases; Swissprot, MSDB (http://ca.expasy.org/), (http://linux1/mascot/cgi/index.pl?page=../home.html) and *P. multocida* (http://lenti.med.umn.edu/pub/PM70/) to identify proteins of interest.

5.3.6. Protein identification in bronchoalveolar lavage fluid

Protein identification from excised spots of interest was successful only using the Swissprot database. The *P. multocida* database was unable to identify any bacterial proteins from the 155 spots excised. The results from the Swissprot database search identified 12 proteins that were different among Group 1 calves (Table 5.3) and Group 2 calves (Table 5.4) from the 155 spots excised. Many of these proteins were present in multiple forms (isoforms and fragments). Among proteins present in multiple forms were apolipoprotein A-1 precursor, serotransferrin precursor, bactenecin (antimicrobial peptide precursor), hypothetical protein precursor (a novel protein that has yet to be annotated), and bovine serum albumin precursor. Additional proteins that were not fragments included aldehyde dehydrogenase-1, annexin-I, tropomyosin-2, antioxidant protein-2, actin, annexin V and tropomysoin α-4 chain. It was found that fragments of bovine serum albumin were the most predominant protein isolated from all calves. Annexin V was considered the only protein with association to the disease state as this was detected 1 d following challenge with *P. multocida* in calf 42 (Table 5.4) and was not identified in BALF of control animals. Annexin I from the same family as Annexin V was primarily associated with control animals 1 d p.c. All other proteins mentioned above were found to some degree in samples from both control and infected animals.

Table 5.3. The detection of significant lung fluid proteins isolated at 3 sampling periods (LV1, 2, 3) from control calves (20, 41 and 35) using a Swissprot database.

Calf I.D	Spot Numbers	Estimated mass kDa	Actual mass kDa	pI	% peptide masses matched	% sequence coverage	Protein Identity Accession number
20 LV 1 Gel Fig 5.6.a	44, 46, 48, 108, 103, 105	46, 46, 28, 29 28, 28	71.2 30.3	5.82 5.71	32, 66, 46, 54 28, 29	16, 14, 6, 17 29, 38	BSA precursor; ALBU_BOVIN (Appendix 2.6) Apolipoprotein A-I precursor; APA1_BOVIN (Appendix 2.5)
20 LV 2 Gel Fig 5.6.b	50	55	71.2	5.82	68	17	BSA precursor; ALBU_BOVIN
	51, 52, 53	80	80	6.75	83, 35, 60	23, 21, 29	Serotransferrin precursor; TRFE_BOVIN (Appendix 2.7)
	56	25	32.6	4.58	33	19	Tropomyosin 2; TPM2_BIOGL (Appendix 2.10)
	57, 58	17, 18	17.9	7.57	33	40	Cyclic dodecapeptide precursor; BCT1_BOVIN (Appendix 2.4)
20 LV 3 Gel Fig 5.6.c	54	28	24.9	6.02	18	29	Antioxidant protein 2; AOP2_BOVIN (Appendix 2.2)
41 LV 1 Gel Fig 5.8.a	14	50	71.2	5.82	47	24	BSA precursor; ALBU_BOVIN
41 LV 2 Gel Fig 5.8.b	16	47	42.2	5.3	39	21	Actin, ACT_THELA (Appendix 2.3)
41 LV 3 Gel Fig 5.8.c	20	54	71.2	5.82	35	19	BSA precursor; ALBU_BOVIN
35 LV1 Gel Fig 5.7.a	2	46	71.2	5.82	26	18	BSA precursor; ALBU_BOVIN
	3	29.5	30.3	5.71	60	42	Apolipoprotein. A-I precursor; APA1_BOVIN
	4	30.5	24.9	6.02	24	34	Antioxidant protein 2; AOP2_BOVIN
35 LV 2 Gel Fig 5.7.b	5, 6	55.3	55.3	6.23	23, 25	15, 14	Aldehyde dehydrogensae 1, A1; DHA1_BOVIN (Appendix 2.9)
	8	39	39.2	6.44	38	14	Annexin I; ANX1_BOVIN (Appendix 2.1)

Table 5.4. The detection of significant lung fluid proteins isolated at 3 sampling periods (LV1, 2, 3) from calves (42, 43 and 11) infected with *P. multocida*, using a Swissprot database.

Calf I.D	Spot Numbers	Estimated mass kDa	Actual mass kDa	pI	% peptide masses matched	% sequence coverage	Protein Identity Accession number
42 LV 1 Gel Fig 5.4.a	120	28	56.3	9.67	27	13	Hypothetical protein precursor Y7J3_ANASP (Appendix 2.11)
42 LV 2 Gel Fig 5.4.b	110	47	42.2	5.3	33	22	Actin; ACT_THELA (Appendix 2.3)
	111	37	36	4.94	28	27	Annexin V; ANX5_BOVIN (Appendix 2.8)
	112, 117, 118	66.3, 29, 29	71.2	5.82	47, 50, 39	12, 18, 12	Bovine serum albumin precursor; ALBU_BOVIN (Appendix 2.6)
	113, 115, 116	29, 29, 30	25	6.02	24, 18, 18	43, 39, 33	Antioxidant protein 2; AOP2_BOVIN (Appendix 2.2)
43 LV 1 Gel Fig 5.5.a	26, 29	31, 28	71.2	5.82	43, 34	11, 14	Bovine serum albumin precursor; ALBU_BOVIN
	32, 33	25, 25	30.3	5.71	27, 28	21, 23	Apolipoprotein. A-I precursor; APA1_BOVIN (Appendix 2.5)
11 LV 1 Gel Fig 5.3.a	61, 62, 63	49.5, 48.5, 48	71.2	5.82	16, 31, 42	21, 15, 12	Bovine serum albumin precursor; ALBU_BOVIN
11 LV 2 Gel Fig 5.3.b	70	55	55.3	6.23	32	21	Aldehyde dehydrogenase 1A1; DHA1_BOVIN (Appendix 2.9)
	79	32	28.5	4.65	23	29	Tropomyosin alpha 4 chain; TPM4_HORSE (Appendix 2.12)

5.4. Discussion

The investigation demonstrated clinical and pathological responses consistent with pneumonic pasteurellosis and these responses were reproduced with the challenge formulation 300ml 10^9 cfu ml^{-1} used in the work and described in Chapter 4. Furthermore, these marked clinical effects were not induced by PBS in this experiment, as confirmed by normal clinical signs following administration of PBS in Group 1 animals.

The 2-DE patterns of bovine BALF identified 12 different proteins amongst animal groups 1 and 2, from over 155 colloidal Coomasie-stained protein spots (intact proteins and fragments). Colloidal Coomasie blue was the stain of choice because uptake by different proteins is relatively linear, allowing assessment of differential levels of protein expression, which the stain itself is MS compatible. Silver stain is more sensitive but faint spots probably would not contain sufficient protein for analysis using our MS setup and also uptake of stain is non-linear.

Proteins which could not be identified by MS were generally those with low intensity, small diameter and often within the low M_r region. These proteins could be analysed further by digestion with another protease and the peptide sequence elucidated using automated Edman degradation. All the proteins identified were host proteins, secreted by pulmonary epithelial cells in response to *P. multocida* infection and to PBS. There was no evidence of bacterial protein, with no hits on the *P. multocida* database search.

Pneumonic pasteurellosis is known to be associated with the accumulation and activation of inflammatory mediators and immune cells within the lung (Babiuk *et al.*, 1995). However, secretory products such as IL-8 and IL-1β (chemoattractants for neutrophils secreted by macrophages), surfactant (reduces bacterial growth), Hp (antimicrobial agent), granule contents of neutrophils such as lysosomal enzymes, MPO and collagenases were not identified. Other constituents of neutrophil granules, antimicrobial peptides, were detected in BALF but they were located primarily in the low *Mr* region, making detection by MS difficult. Products from neutrophil granules, for example, may compromise the integrity of the alveolar-capillary barrier, leading to an increased passage of solutes and plasma proteins and a loss of the gas-exchange capacity during pneumonic pasteurellosis (Maheswaran *et al.*, 1992).

In this study the majority of protein spots identified in BALF 2D-E related to either serum albumin or albumin precursors, which were indicated by a large spot with an undefined perimeter at approximately 70 to 66 KDa, and present in all lavage samples irrespective of treatment. A similar albumin pattern and content has been observed for BALF isolated at various stages of disease from sheep infected with *M. haemolytica* (unpublished observations).

This result is in agreement with the composition of BALF which is reported to contain approximately 50 % albumin and 30 % IgA/IgG (Noel-Georis *et al.*, 2002). Surprisingly immunoglobulins did not produce significant results with the Mascot search in this study. The abundance of albumin may mask the detection of high molecular weight proteins, such as those implicated in the pathogenesis of pneumonic pasteurellosis. To date there is no efficient method available for the removal of these major proteins without affecting the composition of the minor BAL fluid proteins (Lenz *et al.*, 1993). Preliminary studies however have been conducted at MRI to address this problem and to improve the identification of possible disease markers in BALF that would otherwise be masked by albumin and immunoglobulins. It is essential that an additional step is included to remove the albumin, and this could be achieved by running the samples through an affinity column with antibodies to bovine albumin (Lopez *et al.*, 2002) or a column (affinity-blue) with immobilin dye, known to bind albumin, and allowing other proteins of a similar mass and pI to appear on the 2D-gel.

Another reason for the inability of 2-DE to detect proteins in low abundance and at high molecular weight may be due to the dilution of lung fluid during bronchoalveolar lavage. Mammalian lungs contain only a thin layer of epithelial lining fluid that surrounds the airways and the alveoli (Wattiez *et al.*, 2000); any more fluid would affect the surface area for gaseous exchange and reduce lung efficiency. Host proteins are confined to this layer of epithelial lining fluid, though some may originate from the blood due to pulmonary tissue damage.

The value of using bronchoalveolar lavage as a means to analyse the cellular components of lung fluid has been established as a research tool (Caldow, 2001). It maybe that technical limitations in this study may affect the recovery of low abundance proteins. For instance the infusion of 2 x 60 ml Ca^{2+}, Mg^{2+} free HBSS solution at the alveolar epithelial lining would dilute any low abundance proteins present at the time of lavage and they may even not appear as only about 60 % of the fluid can be aspirated from the lung. Therefore protein such as albumin that constitutes a large amount of total protein within BALF has a greater probability of being recovered.

Annexins are proteins that bind phospholipids in a Ca^{2+}-dependent manner. Pulmonary tissues, macrophages and neutrophils are rich in these proteins and Annexins I, II, IV and VI have been purified from bovine pulmonary tissue. These proteins may be involved in the suppression of inflammation, suggesting a role for them in the process of pulmonary inflammatory disease (Katoh *et al.*, 1999). Annexins I and IV have been detected in BALF from calves inoculated experimentally with *M. haemolytica*. Annexin IV was detected more specifically in response to bacterial challenge (Katoh *et al.*, 1999). In the present study Annexin I was primarily associated with control calves and its detection has been associated with glucocorticoids, which are increased through stress or underlying bacterial infection (Breazile, 1988; Peers *et al.*, 1993). The insertion of

a bronchoscope for bronchoalveolar lavage or bacterial challenge could be a cause of stress. In the study of formalin-killed *P. multocida*-induced pneumonic pasteurellosis (Chapter 6), concentrations of SAA, Hp and AGP (indicators of increased stress) were elevated in all calves following bronchoalveolar lavage prior to challenge with either *P. multocida* or PBS.

It would be of interest to measure the serum glucocorticoid levels of all calves in this study to strengthen the explanation that stress was the causative agent of the indirect release of Annexin I in Group 1 calves, as no bacteria were recovered from these animals at post-mortem and their serum viral antibody titers were decreasing.

By comparison with Annexin I, the detection of Annexin V was associated essentially with infected calves. This is the first isolation of Annexin V from bovine BALF, this calcium and phospholipid binding protein possesses potent anticoagulant activity (Andree *et al.*, 1990) and has been shown to inhibit phospholipase A2 activity. This latter property appears to determine the functional role of Annexin V as an anti-inflammatory mediator by preventing the release of arachidonic acid by phospholipase A2 (Ahn *et al.*, 1988). One may expect the secretion of Annexin V in response to *P. multocida* infection considering its anti-inflammatory function. However, Annexin V was isolated only from one inoculated animal and for this protein to become a potential marker for inflammatory disease caused by *P. multocida* it is essential that it is isolated from a majority of infected calves to allow definitive conclusions to be made. Further research would be required to elucidate the role of Annexin V in the pathogenesis of pneumonia in naturally and experimentally infected animals.

Other host proteins of interest include antioxidant protein 2, apolipoprotein A-1 precursor, aldehyde dehydrogenase A-1, serotransferrin precursor, antimicrobial peptide, actin and tropomyosin. None of these proteins were specifically present in lung fluid because of a *P. multocida* challenge because some of these proteins were also present in the lung fluid of animals given only PBS.

Future studies should focus on the removal of albumin from the sample by pre-fractionation leading to the reduced overlapping of spots with a similar pI and mass to albumin, so that proteins in low abundance could be detected. In addition, the production of a clearer gel with the spots well separated and easy to see can be achieved by use of the longer 18 cm IPG strips; but this was outwith the scope of this preliminary investigation. Attention should be focused on methods to improve the efficiency of the aspiration technique to ensure the recovery of proteins in low abundance which may have possible roles in the pathogenesis of disease. Very recently, the use of a series of narrow range IPG strips covering the pH interval 4.5 - 6.7 enabled the detection of low abundance proteins (Sabounchi-Schutt *et al.*, 2003). Pre-fractionation can be compared to the use of narrow range gradient gels, as more protein extract can be loaded onto a narrow range gradient

and thus the probability of detecting the scarcer proteins will be greater (Sabounchi-Schutt *et al.*, 2003).

It would be of interest to isolate bacterial proteins from lung fluid which may provide clues to the pathogenesis of disease; this could be addressed by probing bovine lung fluid sample with specific immune serum. 2-DE proved to be a useful research tool in the analysis of relative changes in lung fluid protein composition as the appearance, disappearance and alterations in level of protein expression could be observed. However, these relative changes were not associated specifically with pneumonic disease and were observed in control animals as well. It is possible that the removal of major proteins such as albumin will reveal proteins concerned primarily with disease.

Chapter 6. PATHOPHYSIOLOGICAL AND PHAGOCYTIC RESPONSES PRIOR TO AND FOLLOWING LUNG CHALLENGE WITH FORMALIN-KILLED *PASTEURELLA MULTOCIDA* BIOTYPE A:3 IN CALVES.

6.1. INTRODUCTION

Chapter 4 summarised novel information on the interaction of an active infection of *P. multocida* with the host in an experimental model of pneumonic pasteurellosis, in which significant clinical and systemic responses were recorded.

There is little if any information on how *P. multocida* bacterin may interact with, or stimulate, the immune mechanisms within the lung. Components of bacterin include LPS and, following the interaction of bacterial LPS with macrophages resident in the lung alveoli, it is understood that recruitment of neutrophils occurs by local production of pro-inflammatory mediators (IL-8, IL-6, IL-1 and TNF-α) (Soethout *et al.*, 2002). The migration of neutrophils to the site of inflammation occurs soon after infection and assists resident macrophages in the phagocytic killing of pathogens and prevents bacterial dissemination to the systemic system. Sustained neutrophil activity often contributes to tissue necrosis and levels of these phagocytes tend to be highest in areas of lung affected by lesions and high numbers of *P. multocida* (Davies and Penwarden, 1981).

It is believed that bacterin introduced *in vivo* would be unable to produce virulence factors and antigens such as IRP due to their inability to multiply compared with live bacteria that have demonstrated the production of IRP-OMP. Antibodies against these iron sequestering proteins will starve the bacteria of iron, rendering them non-viable (Ruffolo *et al.*, 1998). However IRP are only likely to exist if the bacteria were grown initially in iron-restricted conditions.

The systemic response to infectious challenge of the lung is variable and will depend on the bacterial agent. For example, intratracheal inoculation of *M. haemolytica* in calves caused an increase in serum TNF-α (Horadagoda *et al.*, 1994) but research in buffalo challenged intranasally with a haemorrhagic form of *P. multocida* reported a lack of response in serum TNF-α (Horadagoda *et al.*, 2001). Additionally, APP such as Hp was measured in both experiments and exhibited a more rapid response following challenge with *P. multocida* than with *M. haemolytica*. SAA was also measured in the study of Horadogoda *et al.*, (1994) and showed a marked early rise following challenge with *M. haemolytica*. These APP tend to have longer half lives than TNF-α and may

prove better indicators of systemic disease than pro-inflammatory mediators. There is growing evidence that APP are expressed, and presumably secreted, by tissues other than the liver. The lung in particular produces mRNA for Hp (Yang *et al.*, 1995), even in response to a systemic stimulation. Presumably, non-hepatic production would be even more responsive if the stimulus was local, as in the form of a Gram-negative bacterial infection. In earlier work (Chapter 4) Hp and AGP were produced in response to an intratracheal challenge of live *P. multocida* and AGP may have a direct effect on increasing resistance to bacterial infection (Crestani *et al.*, 1998).

Many researchers have looked at the stimulatory or inhibitory effects of bacterial components, such as exotoxin on resident macrophages. Results from these studies have highlighted the detrimental effects of *M. haemolytica* leukotoxin, on phagocytes and their ability to phagocytose pathogens (Markham and Wilkie, 1980). No such exotoxin exists for *P. multocida*, but this bacterium plays a significant role in the pathogenesis of pneumonic pasteurellosis (Ashfaq and Campbell, 1986). The LDCL assay has been used to measure production of oxygen radicals during the interaction between phagocyte and pathogen, and by applying this technique it is possible to determine the up or down regulatory effects of bacterial components on lung macrophages.

Aims of the study

The study was designed to evaluate the clinical, pathological, lung phagocytic cell and systemic responses to an inactivated challenge in which the *in vivo* expression of virulence factors and virulence associated with the ability of bacteria to grow and disseminate in host tissue were disabled. The host responses during the early onset and development of disease will be compared with equivalent responses following challenge with live organism (Chapter 4). In order to obtain information on the effect of exposure to bacterial antigens *in vivo* on the efficiency of uptake and killing of live *P. multocida* by alveolar macrophages *in vitro*, lung cells were harvested at intervals by a technique of sequential bronchoalveolar lavage.

6.2. EXPERIMENTAL DESIGN AND METHODS

6.2.1. Animals

Prior exposure to *P. multocida* was determined by screening serum samples for the presence of antibody using a specific ELISA and by culturing nasal swab samples on vancomycin SBA, a selective medium that excludes Gram-positive bacteria such as *Streptococcus* spp, which often

mask underlying *P. multocida* colonies. Only calves that showed no growth of *P. multocida* were selected for the experiment.

Twenty clinically healthy calves aged 1 week old and of either sex were selected from farms within the Midlothian region and housed as ten pairs, in individual calf pens. Nasal swabs and blood samples were collected weekly and all nasal swabs were negative for *P. multocida* prior to challenge, though the ELISA detected high antibody titre to *P. multocida* in a majority of calves prior to challenge. Calves were weaned at 7 weeks of age on to hay and mixed pellets, and remained housed in individual pens until day 21 (Chapter 7).

6.2.2. Experimental procedure

At 8 weeks of age (designated experimental day 0) the 20 calves were paired according to age and allocated randomly to 2 treatment groups of 10 animals each: Group 1 (300ml PBS) and Group 2 (formalin-killed *P. multocida* in 300 ml PBS). Intratracheal challenge with either PBS or formalin-killed *P. multocida* was given using a fibre optic bronchoscope, in accordance with the experimental design summarised in Table 6.1.

Calves were monitored throughout the experiment for clinical signs of disease, using a scoring system established previously that provides a quantitative measure of disease for control and infected animals (section 2.8.5) and allows corrective action to be taken if necessary. Access to veterinary advice and treatment was available at all times.

BALF was recovered from all calves, using a fibre optic bronchoscope, 2 d before challenge, and on day 1 and day 4 after challenge, for the measurement of phagocytic function *in vitro* and for 2-DE in future studies (Chapter 5 and section 2.9). Due to the amount of work scheduled for each batch of freshly lavaged cells, the maximum number of lavages that could be processed on any one day and still allow time to monitor and, where necessary, treat challenged animals was four. Thus, as far as possible, calves were procured over a period of 10 days so that no more than 4 calves were scheduled for either lavage and or challenge procedures on any one day.

Alveolar macrophages were separated from PMN by a Percoll gradient centrifugation method (section 2.9.2). The efficiency of separation was evaluated by differential count of Diff-Quik stained cytospin preparations and cell viability was measured by nigrosin exclusion (sections 2.9.8 and 2.9.3). The capacity of macrophages and neutrophils to generate a respiratory burst was measured by an LDCL assay (section 2.9.4). The effect of bacterial exposure *in vivo* on the phagocytic efficiency of macrophages was determined *in vitro* by fluorescence microscopy (section 2.9.6). The first 100 cells in view were counted and examined for intracellular bacteria. The

number of organisms in each cell and the fluorescence (red or green) of each were recorded and the total number of bacteria ingested per reaction field, and the percentage killed, were calculated.

On day 5, at the end of the sampling phase, Group 1 was divided into groups 1a, 1b and Group 2 into groups 2a and 2b. The subscript a refers to the animals given either PBS (Group 1a) or formalin-killed *P. multocida* A:3 (Group 2a). Four animals were assigned to each of these groups to determine the effect of dead challenge and PBS on lung pathology (Table 6.1). The remaining calves of Group 1 (n=6) and Group 2 (n=6) were now placed into Group 1b and Group 2b (Table 6.1). The 6 calves in Group 2b (with Group 1b as controls) were used to evaluate the protective effect of lung exposure to formalin-killed *P. multocida* on subsequent immunity (at day 21) to lung challenge with live *P. multocida* (Chapter 7).

6.2.3. Serology

Blood samples were collected from a jugular vein into heparinised vacutainers from all calves prior to purchase, on day of arrival, day 0, 4 h p.c, and daily thereafter until day 5 p.c. Plasma was separated and stored at - 40°C until analysis for APP. Plasma concentrations of Hp were measured (Eckersall *et al.*, 1999) with purified bovine Hp as a standard. A radial immunodiffusion kit and an ELISA kit were used to measure plasma levels of AGP and SAA respectively, (section 2.6.2).

6.2.4. Statistical analysis

Differences between treatments for AUC (-48 h to 120 h) for Hp, SAA, AGP, respiratory rate and mean rectal temperature were tested for statistical significance by ANOVA with treatment groups fitted as a factor and the value of the variable on day 0 fitted as a covariate. This was preferred to simply looking at the AUC above the day 0 level because on some occasions, and for some variables, this method over-adjusted. The choice of AUC was made to provide a robust method for the analysis of repeated measurements from a relatively small number of animals and where the responses were not linear with time.

Table 6.1. Experimental design illustrating the bronchoalveolar lavage schedule at days -2, 1 and 4 and the intratracheal challenge schedule of calves at day 0 with either PBS or formalin-killed *P. multocida* A:3 (dead challenge).

	Day	**-2**	**0**	**1**	**4**
Group	**Calf ID**				
1a	457, 459, 530, 534	Lavage 1	Intratracheal challenge with 300ml PBS	Lavage 2	Lavage 3
1b	435, 441, 443, 704, 706, 709,				
2a	458, 529, 531, 532,	Lavage 1	Intratracheal challenge with 300ml 10^9 cfu formalin-killed *P. multocida*	Lavage 2	Lavage 3
2b	172, 437, 440, 705, 707, 708				

6.3. RESULTS

Pathophysiological and cellular responses to formalin-killed *P. multocida* and PBS were determined as changes in clinical, acute phase and phagocytic responses.

6.3.1. Clinical observations

All calves appeared healthy and alert prior to and immediately after challenge with either PBS or formalin-killed *P. multocida*. The challenge procedure took about 10 min and was well tolerated by all animals, causing only mild coughing and a slight increase in respiratory rate. Within 3-5 h p.c. clinical signs were observed in Group 2, including dullness, signs of increased respiratory effort, mild nasal discharge and reduced appetite.

Mean rectal temperatures for Group 2 calves increased rapidly from a baseline value of 38.9 (± 0.2) °C to a peak of 40 (± 0.2) °C, at 5 h p.c., returning to within the normal range (38 to 39.5°C; clinical score 0) by 24 h p.c. Mean temperatures for Group 1 calves remained within the normal range throughout (Fig. 6.1.a). A secondary temperature response was observed in Group 2 calves at 48 h p.c. when the mean increased by 0.3°C. Rectal temperatures higher than the mean at the beginning of the experiment tended to stay higher throughout the 5 day measurement period. There was strong evidence ($p = 0.012$) of initial differences (0 to 5 h p.c.) in the rate of temperature rise between Group 1 and Group 2 calves. However, apart from initial differences, there was no statistically significant difference between the two treatment groups for the remainder of the experiment.

Respiratory rates were similar for both groups during monitoring prior to challenge (-48 to 0 h p.c.) (Fig. 6.1. b) and the data were pooled. Rapid and marked rises in respiration rates were observed in both treatment groups after challenge (0 to 5 h p.c.), doubling from 44.5 min^{-1} (± 0.7; clinical score 0) prior to challenge (-48 h to 0 h) to 87.6 min^{-1} (± 6.7; clinical score 2) 5 h p.c. Thereafter, respiratory rates in control animals fell rapidly to within normal levels (<60) by 24 h p.c. However, the decline in Group 2 animals was not as dramatic and from 12 h p.c. a secondary response was observed in which respiratory rates increased from 63.7 min^{-1} (± 2.45; clinical score 1) to a peak of 77 min^{-1} (± 4.89; clinical score 1) at 48 h p.c., remaining higher than Group 1 animals throughout. The mean AUC of respiratory rate against time over the 5 d clinical monitoring period was consistently higher for Group 2 than for Group 1 animals ($p = 0.043$).

Clinical signs for Group 1 animals showed a similar trend to that of Group 2 animals but mean total clinical scores were consistently higher for Group 2 animals, increasing rapidly to 4 within 5 h p.c. and showing two further peaks, although lower than the initial peak, at 48 h p.c and 96 h p.c. Overall, it was evident that adverse responses to challenge were consistently higher for Group 2 than for Group 1 animals, although none of these differences was statistically significant, apart from the accumulated score over the 5 d initial monitoring period for respiratory rate.

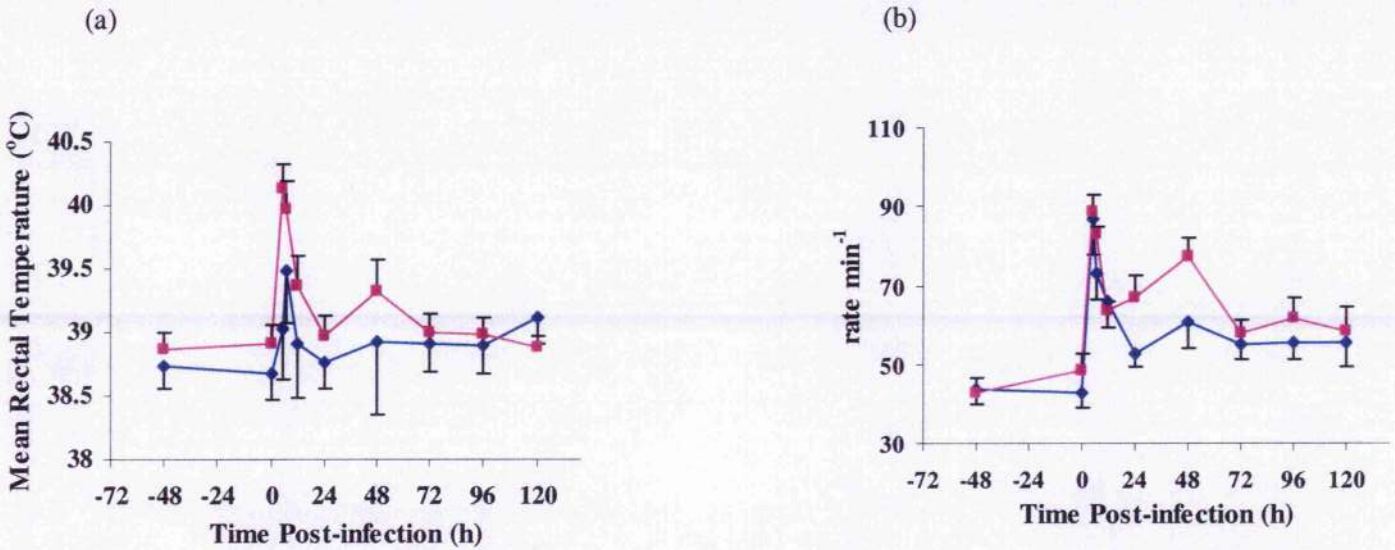


Fig. 6.1. Mean (± SE) over time for clinical measurements from calves given either 300ml PBS (♦) or 10^9 cfu formalin-killed *P. multocida* A:3 in 300ml PBS (■). (a) Mean rectal temperature (°C); (b) Mean respiratory rate (rate min^{-1}).

6.3.2. Serology

Viral antibody and IgG titres for all 20 calves in this experiment are reported in Chapter 7, where the final blood sample was taken at PM on day 28. Plasma levels of APP were determined for the first 5 days following challenge.

6.3.2.1. Acute phase response

Mean plasma Hp concentrations prior to infection were 189 (± 81.7) and 253 (± 114.7) μg ml^{-1} for Group 1 and Group 2 animals, respectively, and increased to 362 (± 111.6) μg ml^{-1} by 24 h p.c. in Group 2 (Fig. 6.2.a.). Changes in mean plasma SAA concentrations were rapid for Group 2 calves between 5 and 24 h p.c., increasing from 29 (± 2.5) to 81 (± 10.3) μg ml^{-1}. The rate of increase during this period was slower for Group 1 calves, increasing from 21 (± 4.0) to 67 (± 22.5) μg ml^{-1}. Thereafter concentrations in Group 2 fell dramatically to 36 (± 4.9) μg ml^{-1} between 24 and 48 h p.c. SAA concentrations in Group 1 fell gradually to 20 (± 3.5) μg ml^{-1} at 120 h p.c. (Fig. 6.2.c) Mean plasma AGP concentrations prior to infection were 369 (± 34.2) and 345 (± 30.2) μg ml^{-1} for Group 1 and Group 2 animals, respectively. After challenge, increases in AGP were more gradual than those observed for Hp and SAA, reaching 438 (± 64.3) μg ml^{-1} at 24 h p.c. and 450 (± 51.9) μg ml^{-1} at 48 h p.c. for Group 1 and Group 2 animals, respectively (Fig. 6.2.b). The AGP concentrations in both treatment groups was sustained above values measured on day 0 for the duration of the experiment. However, none of the differences between the two groups of calves in Hp, SAA or AGP concentrations were statistically significant.

6.3.3. Reactivity and function of BALF cellular components

The recruitment, function and reactivity of phagocytes residing in the lungs of cattle challenged with formalin-killed *P. multocida* and PBS was determined.

6.3.3.1. Measurement of resident and recruited cell populations within the lung

The proportions of lung phagocytes in BALF were assessed on day 2 prior to challenge and on day 1 and day 4 following challenge with either formalin-killed *P. multocida* A:3 or PBS by differential counts of cytospin preparations (Fig. 6.3). On day – 2, macrophages were the dominant cell type, comprising 74 (± 5.5) % and 75 (± 4.7) % of the resident cell population for Group 1 and Group 2 calves, respectively; the remaining cells were PMN (Fig. 6.4.). By day 1 p.c. neutrophils recruited to the lung BALF were the principal cell type comprising 68 (± 4.8) % and 57 ± (6.3) % of the cell

population in samples from Group 2 and Group 1 animals, respectively. There was little change in the proportion of immune cell counts for either treatment groups on day 4 p.c., when 67 (± 4.8) % and 64 (± 5.0) % of the lung fluid cells were neutrophils in Group 1 and Group 2 animals, respectively (Fig. 6.4.).

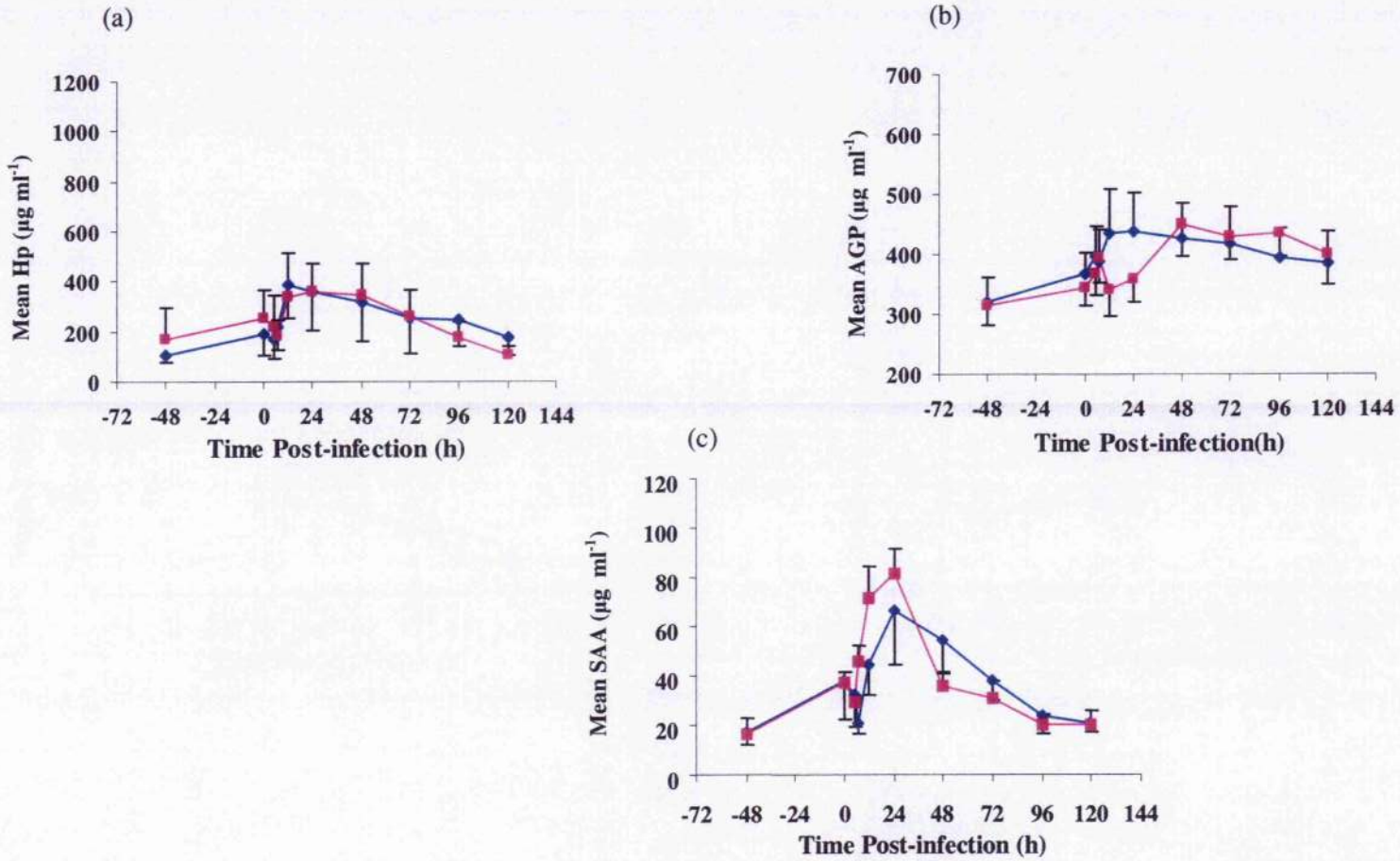
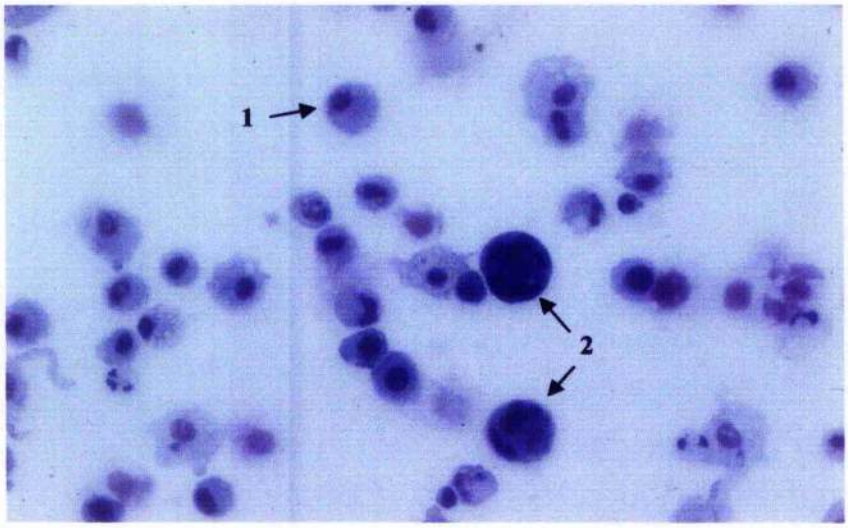


Fig. 6.2. Mean (±SE) over time for plasma concentrations of haptoglobin (Hp), α_1 acid glycoprotein (AGP), and amyloid A (SAA) in calves challenged with either 300ml PBS or 10^9 cfu formalin-killed *P. multocida* A:3 in 300ml PBS. (a) Mean concentrations of Hp (µg ml^{-1}); (b) mean concentrations of AGP (µg ml^{-1}); (c) mean concentrations of SAA (µg ml^{-1}). (◆) Control calves, (■) Infected calves.

A



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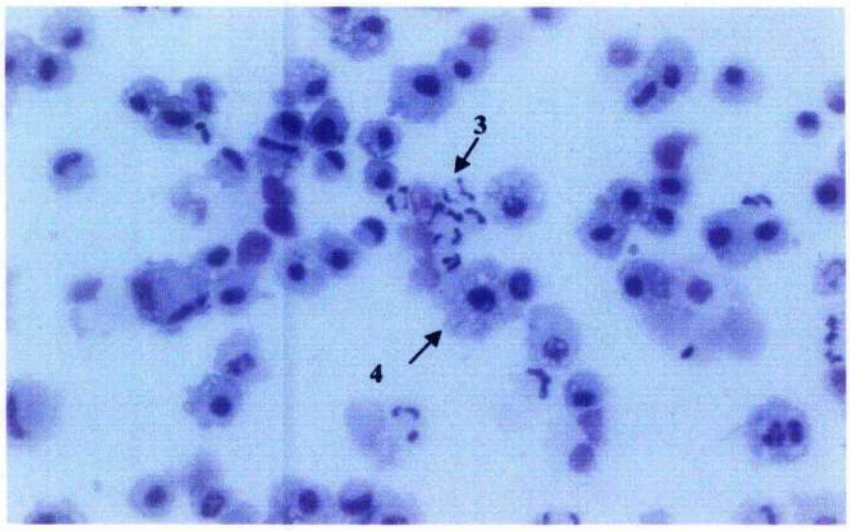


Fig. 6.3. Cytological preparations from bronchoalveolar samples to illustrate the cell types recorded as a percentage of the total cells counted at various stages of disease. **A:** Predominately macrophages (1) with some syncytial cells (2) prior to challenge with formalin-killed *P. multocida* and **B:** the presence of neutrophils (3) and vacuolated macrophages (4), 1 day p.c. with formalin-killed *P. multocida*. Diff Quik stain x 40.

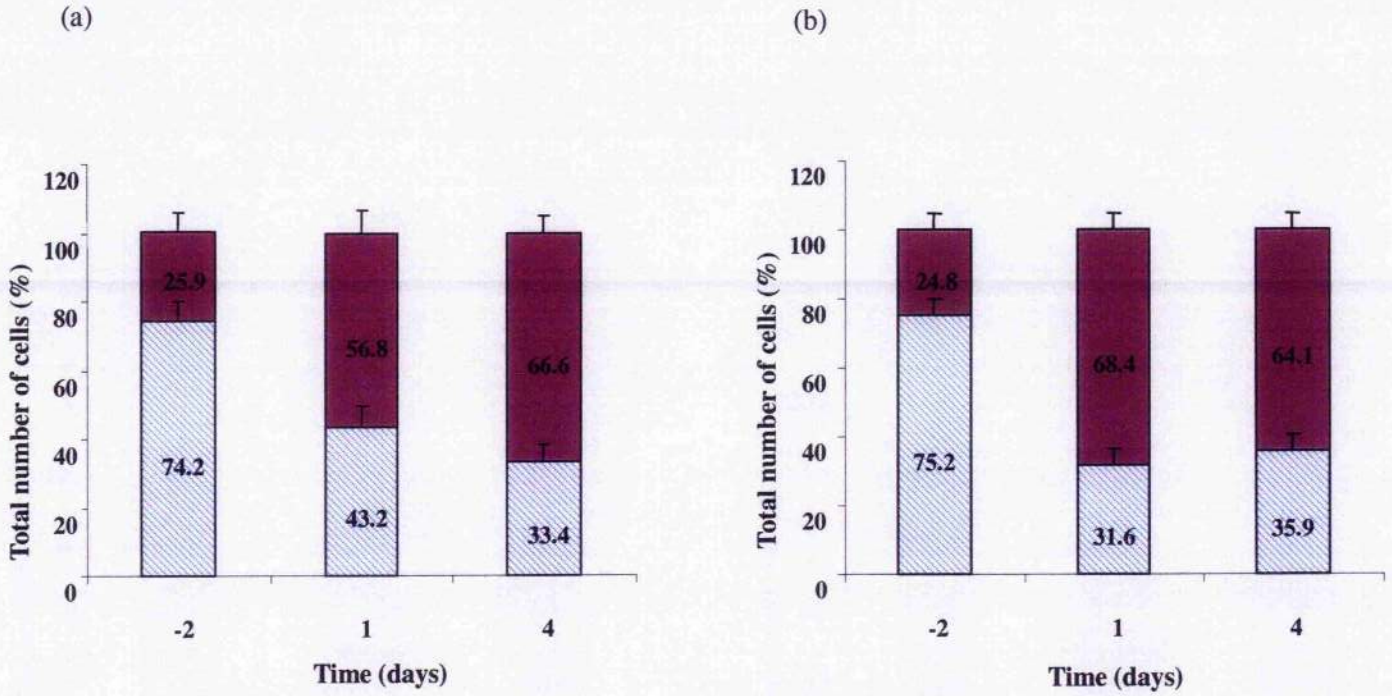


Fig. 6.4. Mean (± SE) differential immune cell counts of lung fluid cells obtained before and after infection. (a.) Calves given 300ml PBS; (b.) Calves given 10^9 cfu formalin-killed *P. multocida* A:3 in 300ml PBS. (▨) alveolar macrophages, (■) neutrophils.

6.3.3.2. Viability of phagocyte populations in lung lavage samples

The effect of treatment on the viability of macrophages and neutrophils were compared on day 1 and day 4 following challenge (Fig. 6.5). Formalin-killed *P. multocida* did not appear to affect the viability of either cell type as similar phagocyte viability was recorded between the two treatment groups. The viability of neutrophils was 89 % in lung wash samples and was consistently higher than that for macrophages by an overall mean of 7 %, irrespective of treatment (Fig. 6.5). There were minor changes in viability between day 1 and day 4 for each cell type and treatment group.

6.3.3.3. In vitro measurement of the uptake and killing of opsonised and non-opsonised bacteria by alveolar macrophages

A summary of results for bronchoalveolar macrophages (BAM) from calves on days -2, 1 and 4 p.c. with PBS and formalin-killed *P. multocida* is presented in Table 6.2. The mean number of ingested bacteria killed during incubation with BAM was not affected by the intratracheal instillation of either PBS or formalin-killed *P. multocida* (Fig. 6.6, Fig. 6.7; Table 6.2.). The number of ingested bacteria per BAM ranged from 2.2 (± 0.2) to 4.8 (± 2.5) for phagocytes incubated with non-opsonised *P. multocida* and from 2.1 (± 0.2) to 2.6 (± 0.1) for phagocytes incubated with opsonised *P. multocida* (Table 6.2; Fig. 6.8 and Fig. 6.9).

The mean phagocytic efficiency of BAM was determined by calculating the number of macrophages that had ingested bacteria. There was a 10 % increase in phagocytic efficiency from day -2 to day 4 for cells from Group 2 animals incubated with opsonised bacteria and a 4 % decrease in phagocytic efficiency for cells from Group 1 animals incubated with non-opsonised bacteria (Fig. 6.10; Table 6.2.). However, this parameter failed to show any statistical significance throughout the sequential lavage or between challenge groups.

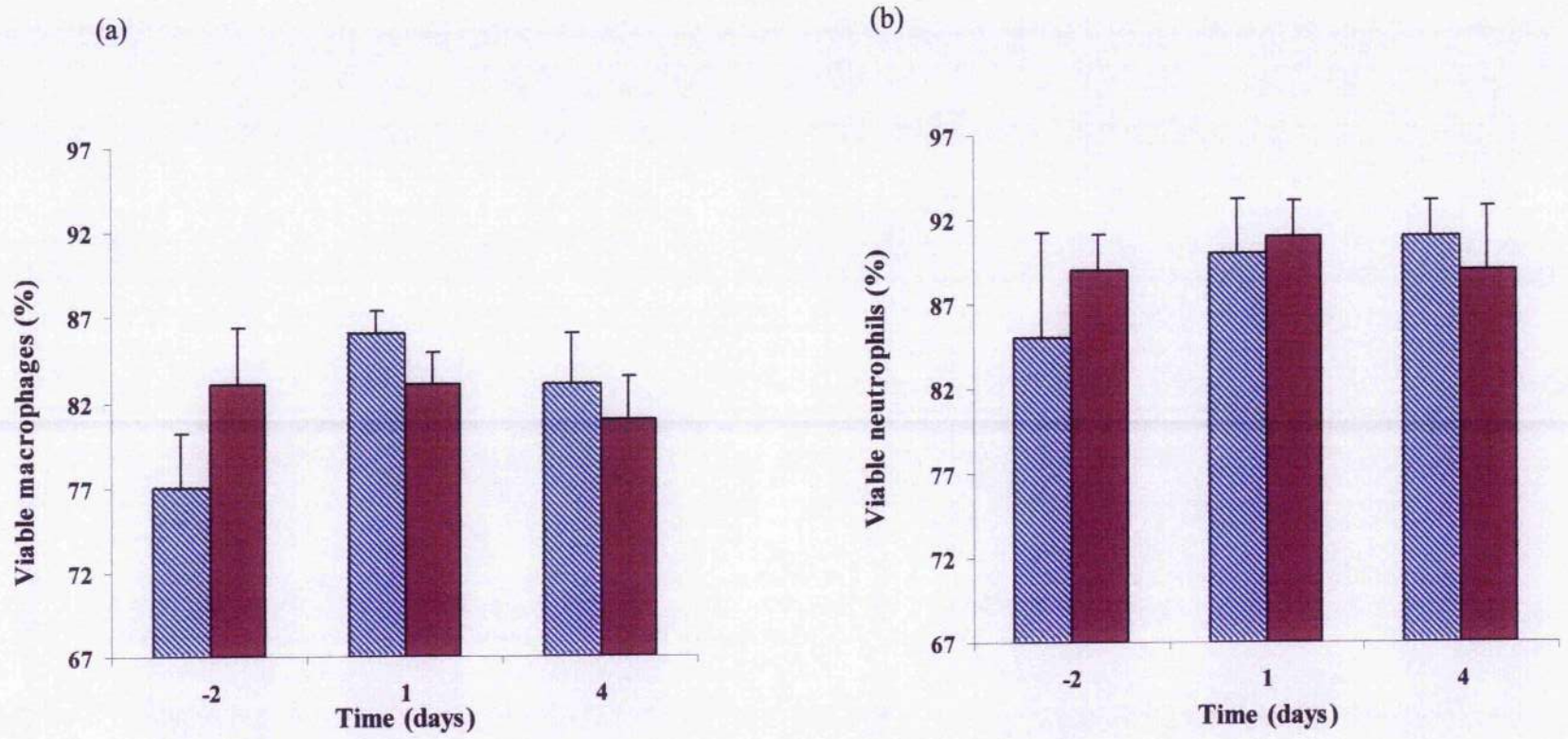


Fig. 6.5. The effects of 300ml PBS (■) and 10^9 cfu formalin-killed *P. multocida* A:3 in 300ml PBS (▨) on the mean (± SE) viability of (a) alveolar macrophages and (b) neutrophils.

Table 6.2. Mean (± SE) characteristics of lung macrophage phagocytic efficiency, assessed prior to challenge and up to 4 days following challenge. Includes mean bacteria ingested per macrophage, mean bacteria killed *in vitro* per BAM and the mean percentage of phagocytosing macrophages.

		Characteristics of lung macrophage phagocytic efficiency								
Treatment	Group	Number of mean bacteria ingested per macrophage			Number of mean bacteria killed *in vitro* per BAM			Mean % of phagocytosing macrophages *in vitro* per 100 BAM		
		-2 d	**1 d**	**4 d**	**-2 d**	**1 d**	**4 d**	**-2 d**	**1 d**	**4 d**
Non-Opsonised	1	2.2 (0.2)	4.8 (2.5)	2.5 (0.1)	0.002 (0.002)	0.02 (0.005)	0.03 (0.02)	27 (4.6)	27 (3.9)	24 (2.2)
	2	2.5 (0.1)	3 (0.2)	3 (0.1)	0.02 (0.01)	0.05 (0.05)	0.02 (0.02)	20 (2.3)	32 (4.0)	27 (2.9)
Opsonised	1	2.6 (0.1)	2.4 (0.1)	2.3 (0.2)	0.005 (0.003)	0.009 (0.006)	0.03 (0.02)	35 (4.1)	24 (2.4)	25 (3.4)
	2	2.1 (0.1)	2.4 (0.2)	2.5 (0.1)	0.02 (0.01)	0.02 (0.02)	0.05 (0.03)	20 (3.2)	27 (3.9)	30 (2.9)

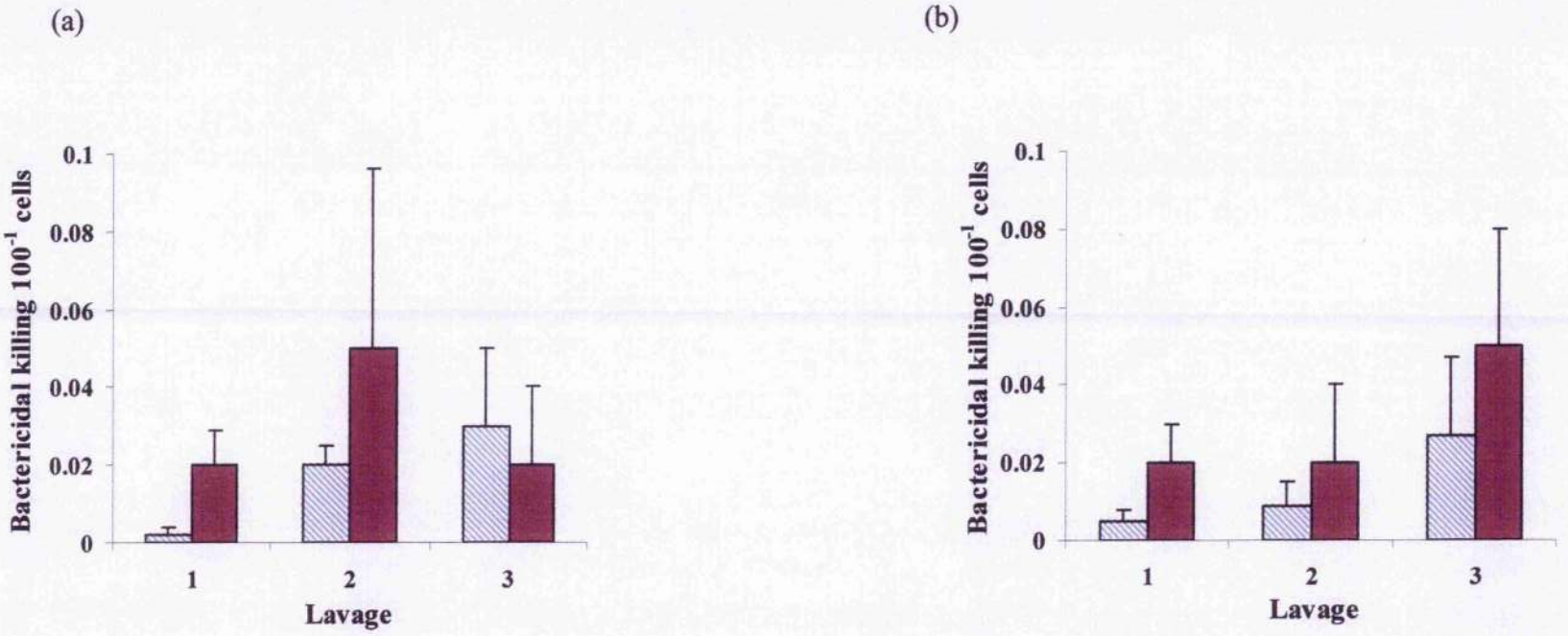


Fig. 6.6. Bactericidal activity of macrophages *in vitro* when incubated with non-opsonised *P. multocida* or opsonised *P. multocida*. (a) Mean (± SE) number of killed non-opsonised *P. multocida* per 100 macrophages; (b) Mean (± SE) number of killed opsonised *P. multocida* per 100 macrophages. Group 1: 300ml PBS (▨) and Group 2: 10^9 cfu formalin-killed *P. multocida* A:3 300ml PBS (■).

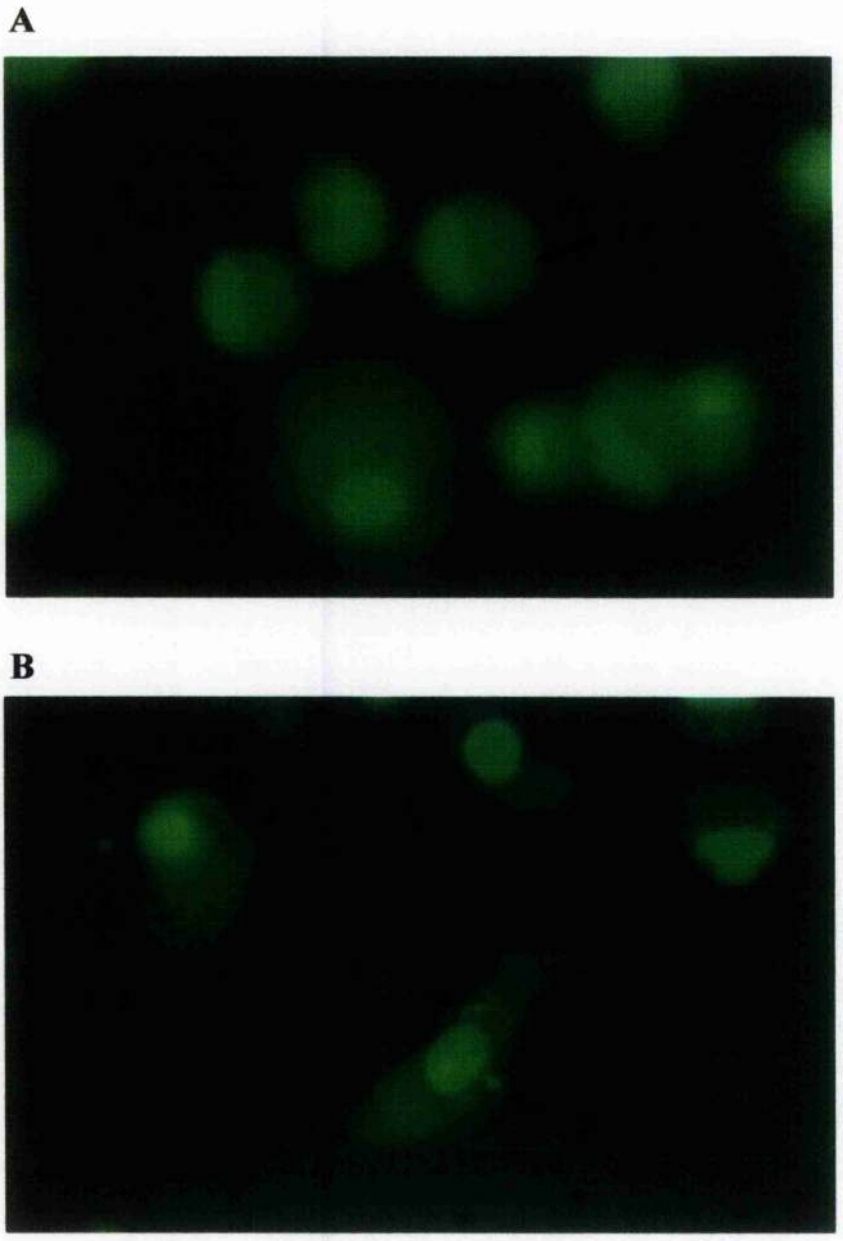


Fig. 6.7. Bronchoalveolar cells isolated from calf 534 1 d p.c. with PBS (Group 1a). **A:** Macrophages (1) devoid of bacteria as they have been placed on a control reaction field of the BioRad slide. **B:** Macrophages incubated with *P. multocida* on another reaction field. The central macrophage contains 6 bacteria (2) within the cytoplasm along the same focal plane as the nucleus (AO and CV fluorescent stain x 1250).

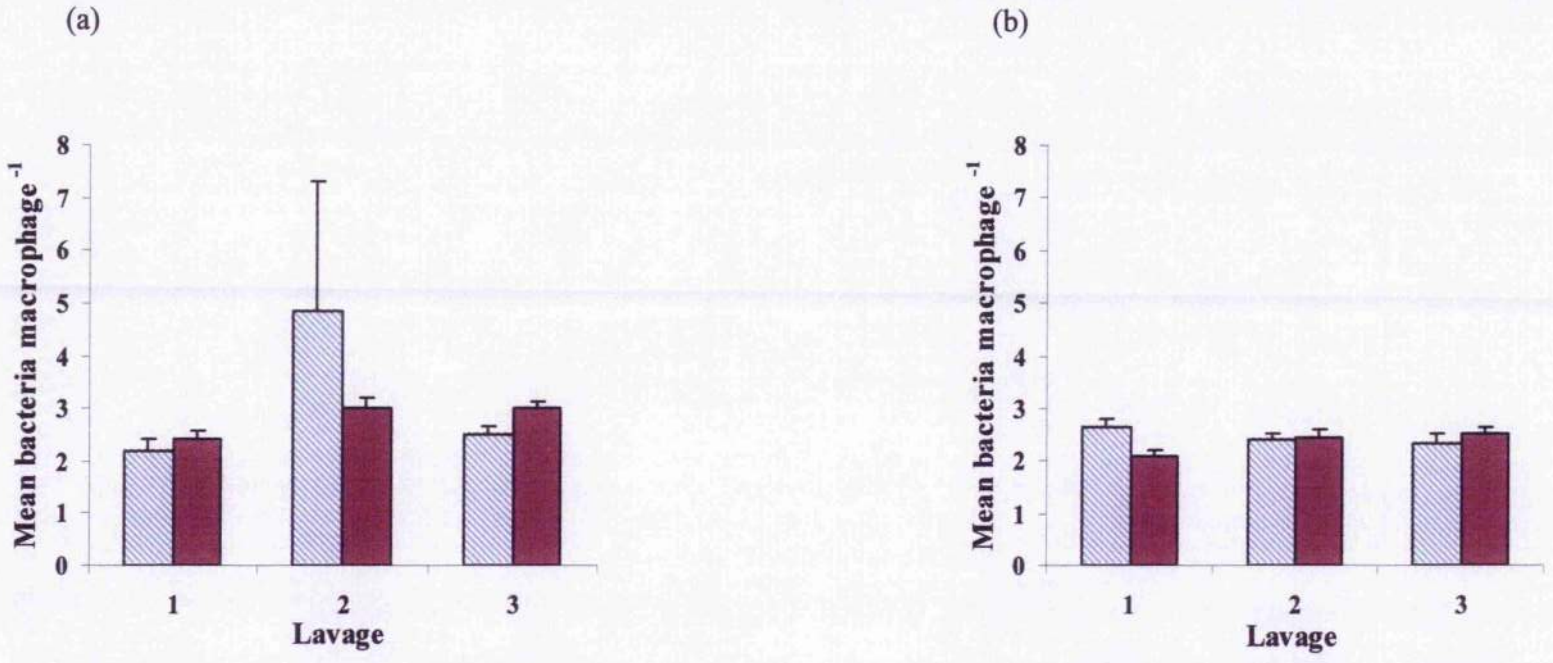


Fig. 6.8. Phagocytic efficiency of macrophages *in vitro* when incubated with non-opsonised *P. multocida* and opsonised *P. multocida* (a) Mean (± SE) number of bacteria in macrophages when incubated with non-opsonised *P. multocida*; (b) Mean (± SE) number of bacteria in macrophages when incubated with opsonised *P. multocida*. Group 1: 300ml PBS (▨) and Group 2: 10^9 cfu formalin-killed *P. multocida* A:3 300ml PBS (■).

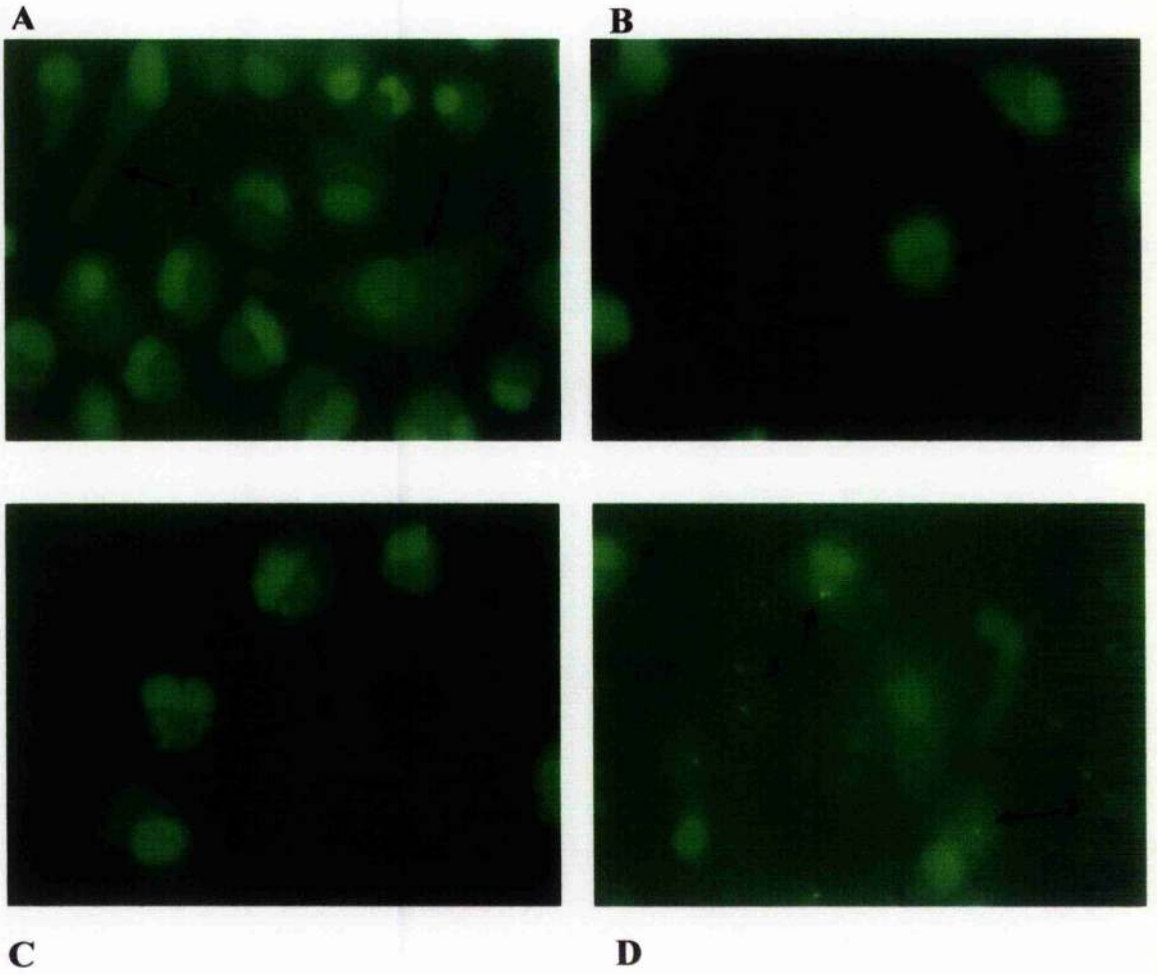


Fig. 6.9. Bronchoalveolar cells isolated from calf 529 1 d p.c. with formalin-killed *P. multocida* (Group 2a). **A:** Macrophages devoid of bacteria as they are from a control reaction field of the BioRad slide, note the activated macrophages (1) possibly due to formalin-killed *P. multocida* exposure *in vivo*. **B:** Two macrophages containing a total of 10 live phagocytosed bacteria (2) within the cytoplasm. **C:** Two macrophages containing a total of 15 live phagocytosed bacteria (2) within the cytoplasm. **D:** Macrophages were incubated with opsonised *P. multocida*, note the presence of 3 dead bacteria (3) within the cytoplasm of the two macrophages.

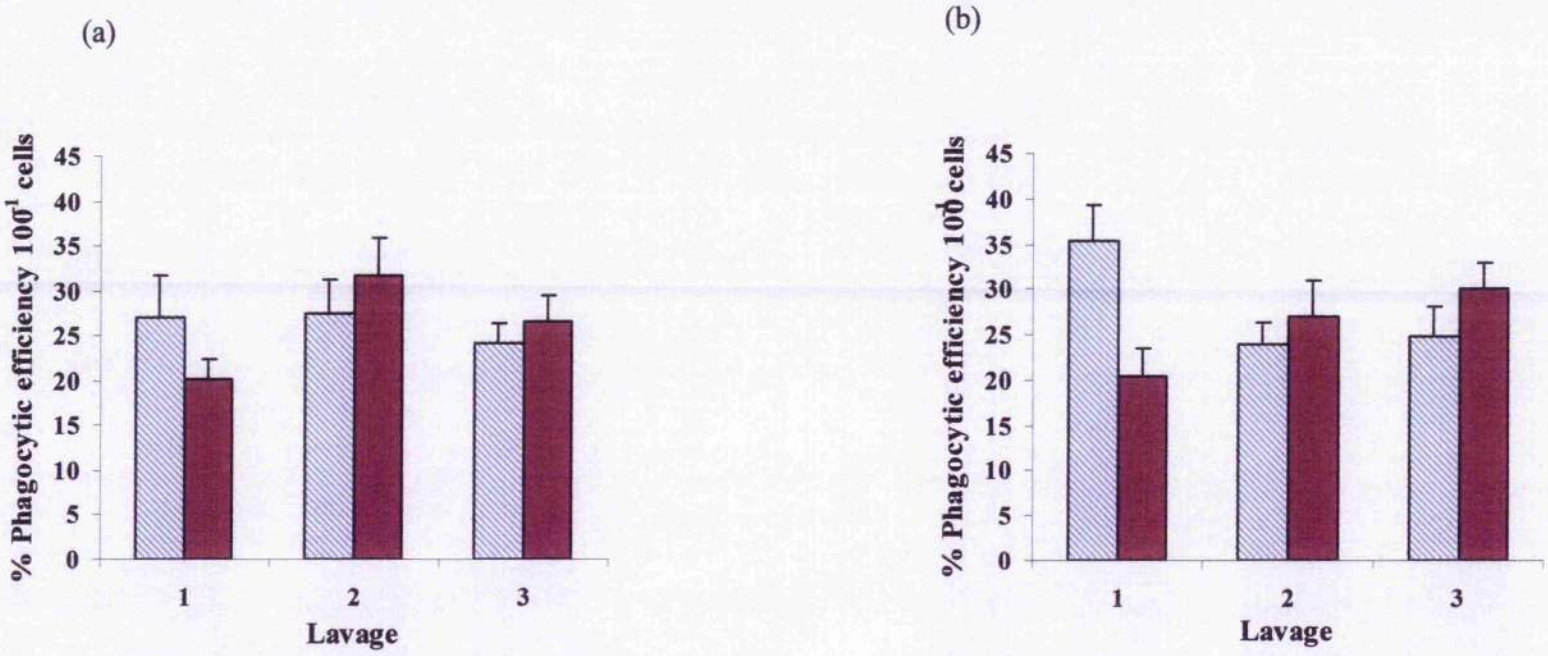


Fig. 6.10. Mean (± SE) phagocytic efficiency (%) of macrophages *in vitro* per 100 cells when incubated with (a) non-opsonised or (b) opsonised *P. multocida*. Group 1: 300ml PBS (▨) and Group 2: 10^9 cfu formalin-killed *P. multocida* A:3 300ml PBS (■).

6.3.3.4. *In vitro respiratory burst of BAM and PMN after exposure to either formalin-killed P. multocida or PBS*

Purified BAM and PMN adjusted to equivalent concentrations (2 x 10^6 cells ml^{-1}) were incubated with a PK-C agonist PMA, and live opsonised or non-opsonised *P. multocida*, and their respiratory burst activity was compared with control treatment (McCoys 5A medium) or antiserum. PMN were incubated with antiserum, opsonised *P. multocida*, PMA and McCoys as a comparison.

Marked increases in LDCL, upon exposure to PMA were observed from both neutrophils and macrophages recovered from all animals. The respiratory burst of macrophages occurred at a rate of 5 541 RLU s^{-1} (Group 2 day 1 p.c.) over 473 s (Fig. 6.11). In contrast, the respiratory burst of neutrophils occurred 213 s earlier than that of macrophages and a maximal respiratory response was 3.5 fold greater for neutrophils at 19 519 RLU s^{-1} (Group 2 day 4 p.c.) (Fig. 6.12). For macrophages the LDCL value decreased gradually thereafter, on average by 42 % regardless of treatment *in vivo* (Fig. 6.11; Table 6.3). The decline in response observed for neutrophils prior to challenge was only 18 % and increased to 33 % p.c. There was no statistical significant difference in the respiratory burst for BAL cells obtained prior to or following challenge *in vivo* or between treatment groups 1 and 2.

Macrophages from day -2 incubated *in vitro* with opsonised *P. multocida* produced a gradual increase in LDCL that reached a plateau at 816 s for both Group 1 and Group 2 (Fig. 6.13).

A different response was observed in cells incubated with opsonised *P. multocida*, obtained at days 1 and 4 p.c., in which the LDCL response increased by 50 % to a peak at 900 s (Group 1, day 1 p.c.) and by 100 % to a peak at 1030 s (Group 2 day 1 p.c.), after which values were lower on day 4 p.c., by 30 % and 14 %, for groups 1 and 2, respectively. A similar pattern for the response curve was observed on stimulation of neutrophils with opsonised *P. multocida* (Fig. 6.14), except that the overall RLU value was 5 fold higher than that generated by macrophages.

The stimulation of macrophages with antiserum produced a LDCL kinetic pattern comparable to that of the opsonised bacteria for Groups 1 and 2 animals prior to and post challenge, except that on days 1 and 4 p.c. the antiserum response was slightly depressed (by 9 % and 21 % respectively, Fig. 6.13). A different relationship between opsonised bacteria and antiserum was observed for neutrophils (Fig. 6.14). A high LDCL response was observed for neutrophils isolated from both treatment groups prior to challenge, reaching accumulated values of 3 700 RLU s^{-1} over 343 s (Fig. 6.14). For both treatment groups this value increased substantially by day 1 p.c., particularly for Group 1 calves. In contrast, on day 4 p.c., the LDCL response for opsonised bacteria and antiserum decreased markedly to the levels of the control, McCoys, with both treatment groups showing a remarkably identical response.

The stimulation of macrophages with non-opsonised *P. multocida* produced only low levels of light emission and exhibited a response curve comparable to that of the response to McCoys. On addition of McCoys to neutrophils only a gradual stimulation was observed and a peak LDCL value was not reached during the assay for either treatment groups (Fig. 6.14)

Total integrated LDCL values expressed as mean RLU sec^{-1} x 10^6 were calculated for the area under the profile curve and shows the overall reactive oxygen intermediate production for the 32 min sampling period. The respiratory burst was 5 fold higher on exposure of PMN (Table 6.4) to PMA than the response from macrophages (Table 6.3). The addition of opsonised *P. multocida* to both cell types produced a measurable respiratory burst, higher than that observed with McCoys and live *P. multocida*. There was no significant difference in the mean integrated LDCL responses for BAL cells obtained prior to or following challenge *in vivo* or between treatment groups 1 and 2 or to any of the stimulants.

6.3.3.5. Initial linear rate of respiratory burst

The rate of increase of the respiratory burst was rapid and more pronounced for neutrophils (Table 6.4) than for macrophages (Table 6.3).

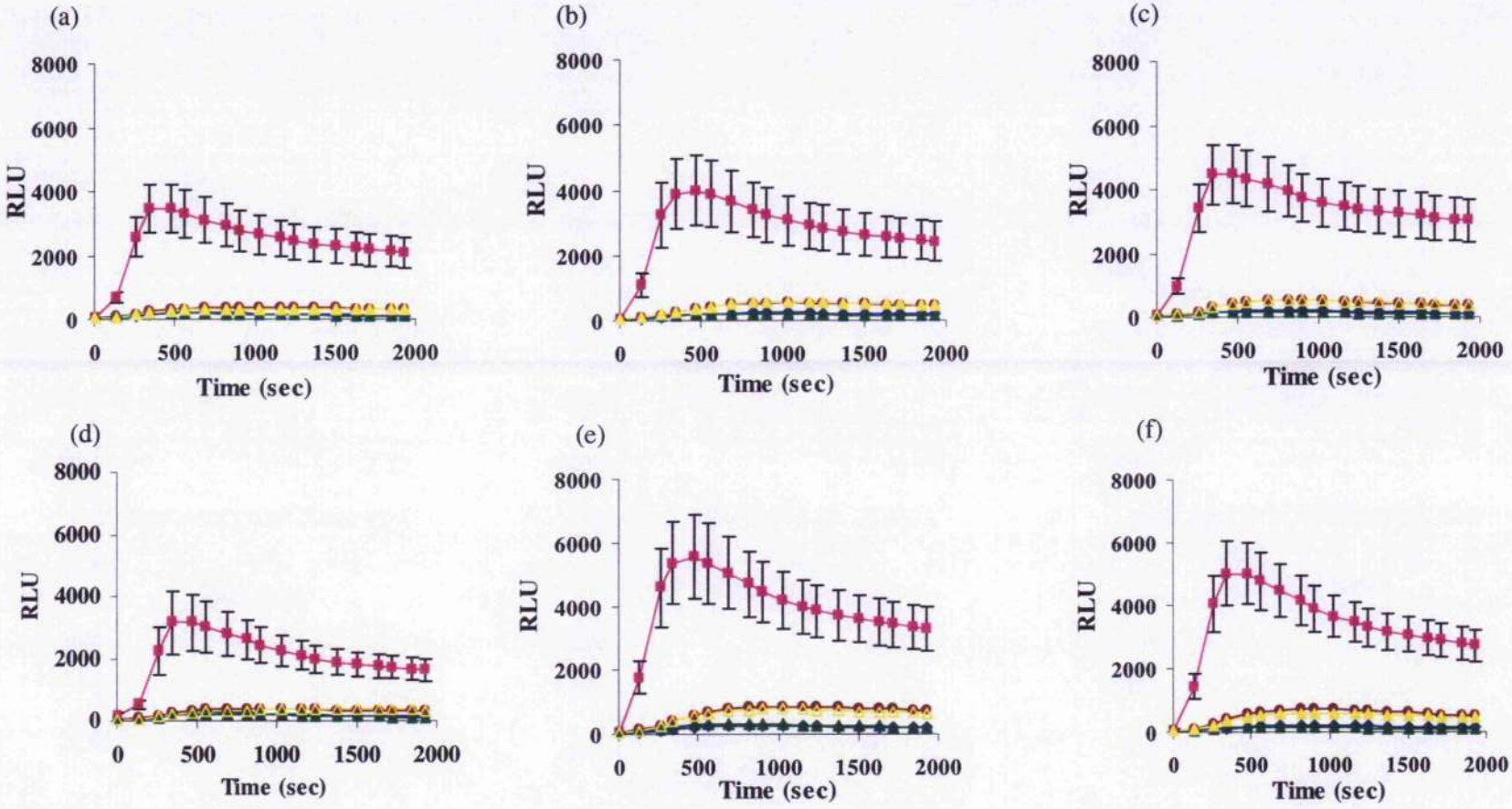


Fig. 6.11. Mean (± SE) LDCL response of macrophages on exposure to a standardised chemiluminescent stimulant, PMA. (a) Group 1 (300ml PBS) calves day -2 prior to challenge; (b) Group 1 calves day 1 post-challenge; (c) Group 1 calves day 4 post-challenge; (d) Group 2 (10^9 cfu formalin-killed *P. multocida* A:3) calves day -2 prior to challenge; (e) Group 2 calves day 1 post-challenge; (f) Group 2 calves day 4 post-challenge. PMA (■), opsonised *P. multocida* (●), opsonin (▲), non-opsonised *P. multocida* (▲) and McCoys (◆).

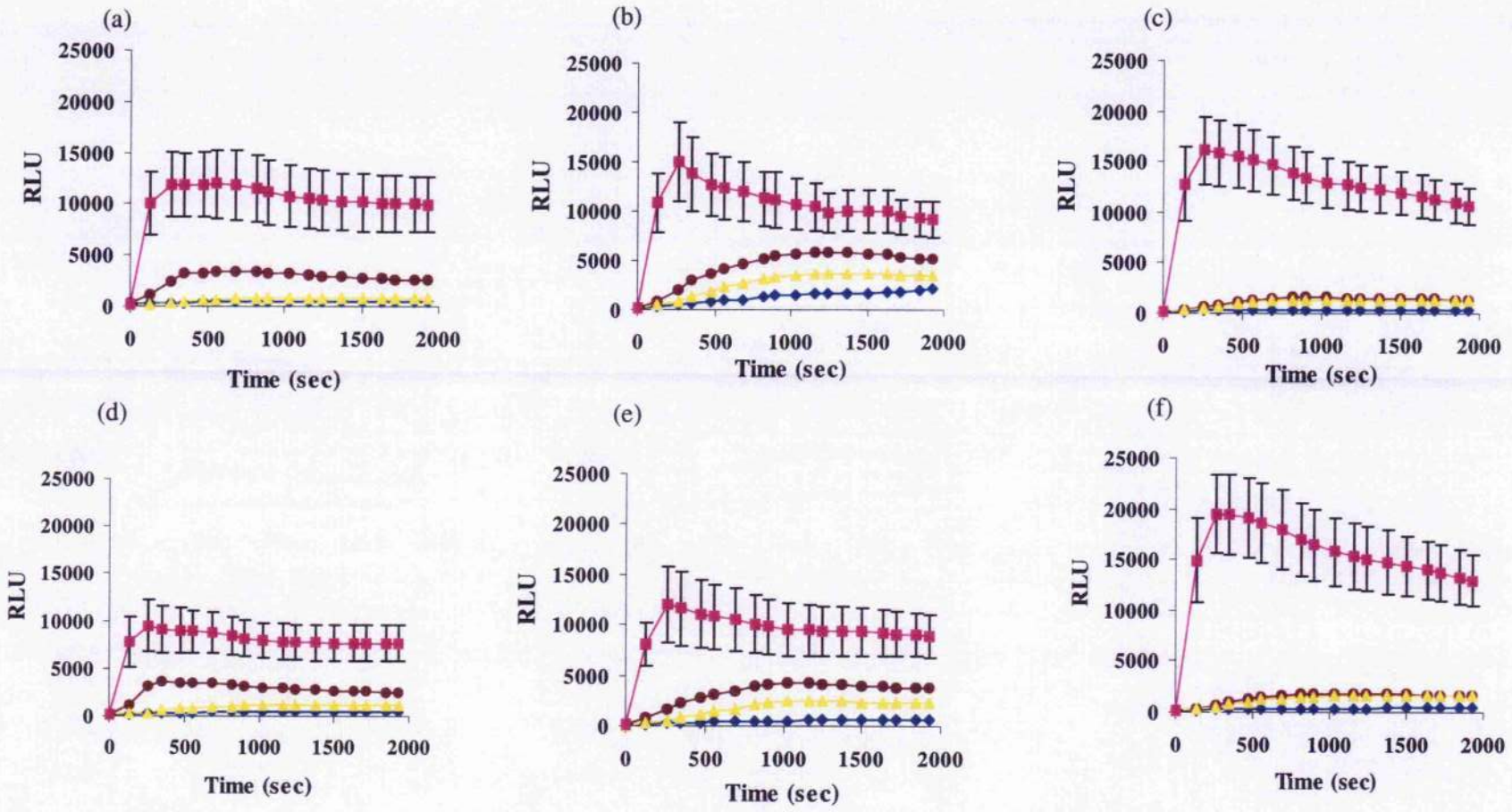


Fig. 6.12. Mean (± SE) LDCL response of neutrophils on exposure to a standardised chemiluminescent stimulant, PMA. (a) Group 1 (300ml PBS) calves day -2 prior to challenge; (b) Group 1 calves day 1 post-challenge; (c) Group 1 calves day 4 post-challenge; (d) Group 2 (10^9 cfu formalin-killed *P. multocida* A:3) calves day -2 prior to challenge; (e) Group 2 calves day 1 post-challenge; (f) Group 2 calves day 4 post-challenge. PMA (■), opsonised *P. multocida* (●), opsonin (▲), and McCoys (◆).

Table 6.3. Mean (± SE) LDCL responses, mean (± SE) rate and time to peak of macrophages to PMA, live *P. multocida*, opsonised and non-opsonised *P. multocida*, antiserum compared to control.

Treatment	Challenge Group	PRODUCTION OF CHEMILUMINESCENCE								
		Mean integrated RLU value / sec (x 10^6)			Mean rate to peak (x 10^3 RLU/sec)			Time to peak (min)		
		-2 d	1 d	4 d	-2 d	1 d	4 d	-2 d	1 d	4 d
McCoys (control)	1	0.3 (0.04)	0.4 (0.1)	0.3 (0.04)	0.2 (0.03)	0.2 (0.07)	0.2 (0.03)	9	13.6	11
	2	0.4 (0.09)	0.4 (0.1)	0.3 (0.08)	0.4 (0.16)	0.3 (0.1)	0.2 (0.05)	8	13.6	13.6
PMA	1	4.8 (1.1)	5.6 (1.4)	6.5 (1.4)	3 (0.9)	4 (1)	4.6 (0.9)	6	8	8
	2	4.0 (1.0)	7.7 (1.7)	6.7 (1.3)	3.3 (1)	9 (4.6)	5.3 (1)	6	8	8
Opsonised	1	0.6 (0.1)	1.0 (0.4)	0.8 (0.15)	0.4 (0.08)	0.7 (0.2)	0.5 (0.1)	19	17	13.6
P. multocida	2	0.6 (0.1)	1.3 (0.4)	1.1 (0.25)	0.4 (0.08)	0.9 (0.3)	0.7 (0.2)	13.6	17	15
Non-opsonised	1	0.3 (0.04)	0.4 (0.1)	0.25 (0.05)	0.2 (0.035)	0.25 (0.07)	0.2 (0.05)	8	11	9
P. multocida	2	0.3 (0.06)	0.5 (0.2)	0.3 (0.08)	0.26 (0.04)	0.3 (0.1)	0.26 (0.07)	9	11	9
Antiserum	1	0.5 (0.12)	0.9 (0.3)	0.8 (0.2)	0.37 (0.08)	0.6 (0.2)	0.6 (0.1)	17	17	15
	2	0.6 (0.09)	1.1 (0.4)	1.0 (0.2)	0.4 (0.06)	0.8 (0.25)	0.6 (0.1)	17	17	15

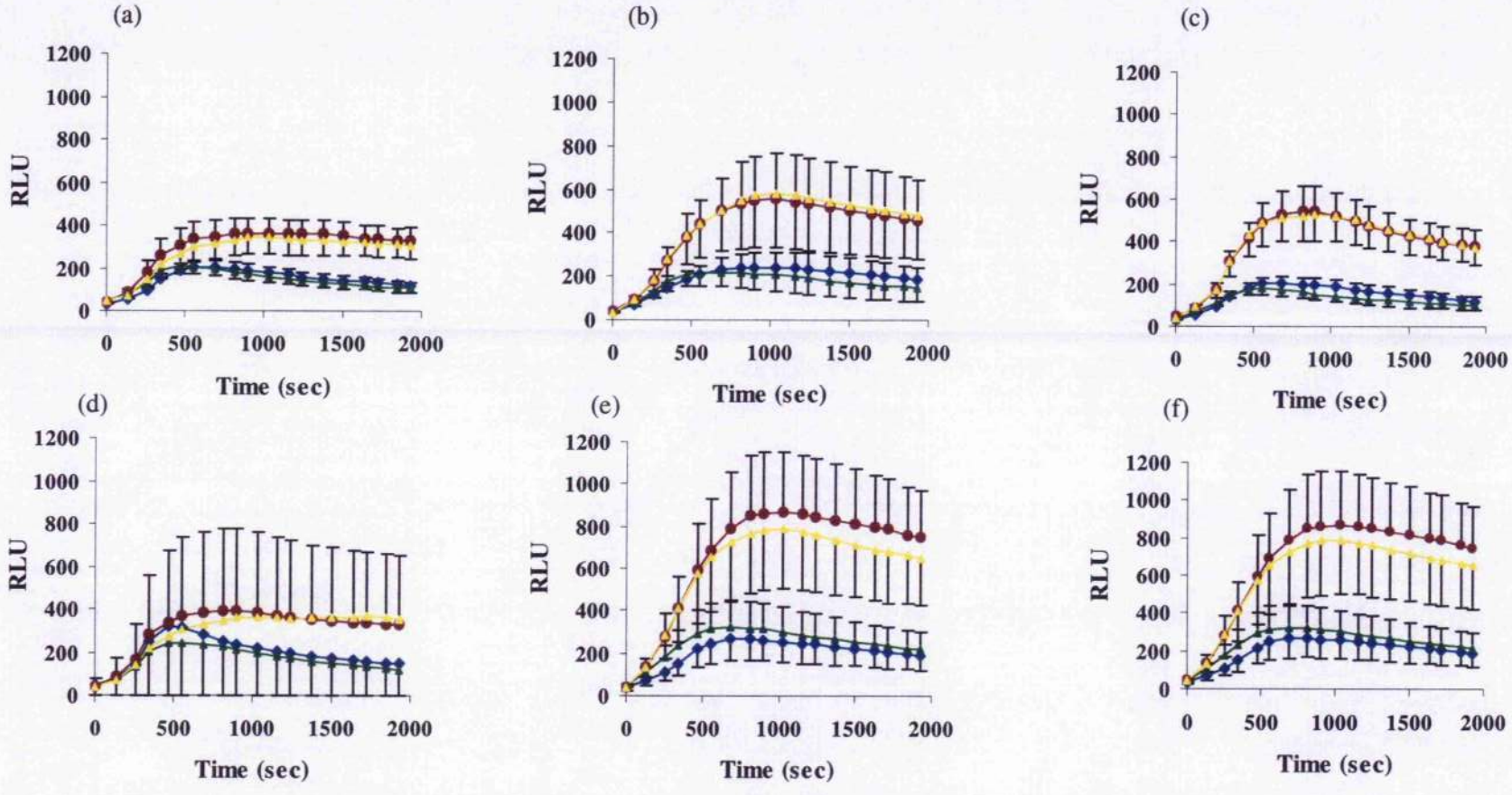


Fig. 6.13. Mean (± SE) LDCL response of macrophages on exposure to; opsonised *P. multocida* (●), opsonin (▲), non-opsonised *P. multocida* (▲) and McCoys (◆). (a) Group 1 (300ml PBS) calves day -2 prior to challenge; (b) Group 1 calves day 1 post-challenge; (c) Group 1 calves day 4 post-challenge; (d) Group 2 (10^9 cfu formalin-killed *P. multocida* A:3) calves day -2 prior to challenge; (e) Group 2 calves day 1 post-challenge; (f) Group 2 calves day 4 post-challenge.

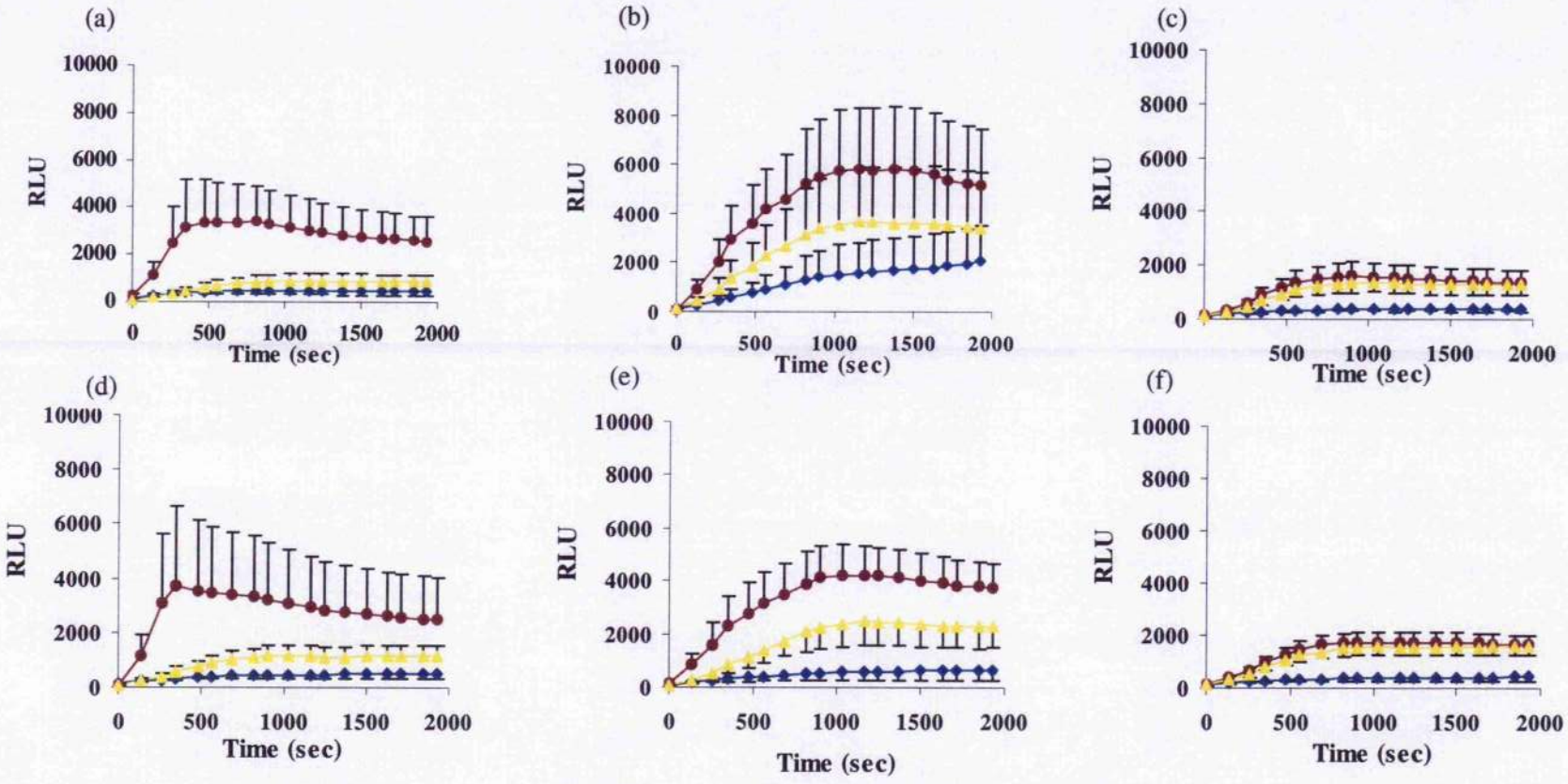


Fig. 6.14. Mean (± SE) LDCL response of neutrophils on exposure to; opsonised *P. multocida* (●), opsonin (▲) and McCoys (◆). (a) Group 1 (300ml PBS) calves day -2 prior to challenge; (b) Group 1 calves day 1 post-challenge; (c) Group 1 calves day 4 post-challenge; (d) Group 2 (10^9 cfu formalin-killed *P. multocida* A:3) calves day -2 prior to challenge; (e) Group 2 calves day 1 post-challenge; (f) Group 2 calves day 4 post-challenge.

Table 6.4. Mean (± SE) LDCL responses, mean (± SE) rate and time to peak of bovine neutrophils to PMA, opsonised *P. multocida*, antiserum compared to control. Live *P. multocida* was not included in the LDCL assay due to low number of fresh PMN cells and time constraint.

		PRODUCTION OF CHEMILUMINESCENCE								
Treatment	**Challenge Group**	**Mean integrated RLU value (x 10^6)**			**Mean rate to peak (x 10^3 RLU/sec)**			**Time to peak (min)**		
		-2 d	**1 d**	**4 d**	**-2 d**	**1 d**	**4 d**	**-2 d**	**1 d**	**4 d**
McCoys (control)	1	0.8 (0.26)	2.5 (1.8)	0.66 (0.13)	0.6 (0.2)	1.8 (0.4)	0.5 (0.1)	21	32	32
	2	0.8 (0.17)	1 (0.5)	0.7 (0.2)	0.6 (0.16)	1.5 (0.8)	0.6 (0.2)	32	32	32
PMA	1	20.4 (5.6)	20.8 (4.9)	25 (4.7)	15 (2.5)	19 (4.5)	17.3 (3.7)	9	4	4
	2	15.4 (3.9)	18.4 (5)	30 (6.3)	12 (2.2)	13.9 (3.2)	20.8 (3.9)	4	4	4
Opsonised *P. multocida*	1	5.4 (1.9)	8.8 (3.6)	2.4 (0.8)	2.1 (0.9)	6.5 (2.7)	1.6 (0.5)	13.6	19	15
	2	5.4 (3.7)	6.5 (1.9)	2.8 (0.6)	1.6 (0.5)	4.5 (1.2)	2.1 (0.5)	6	19	17
Antiserum	1	1.4 (0.6)	5.3 (3.2)	2.1 (0.5)	1.1 (0.4)	3.8 (2.3)	1.6 (0.4)	17	21	17
	2	1.8 (0.6)	3.5 (1.2)	2.5 (0.4)	1.4 (0.5)	2.6 (0.9)	1.9 (3.3)	17	19	17

6.3.4. Gross pathology and bacteriology

Results for gross pathology, bacteriology and histopathology refer only to 8 calves that were challenged either with formalin-killed *P. multocida* (Group 2a, n= 4) or with PBS (Group 1a, n= 4).

Surface lung lesions of all calves in Group 2a were identical to those of calves given live challenge (Chapter 4) except that on sectioning there was little evidence of exudate within the bronchi. The lung lesions were confined to the right and left apical lobes, affecting on average 10 % of the lung surface area (Table 6.5) (Fig. 6.15). Two animals (Calf 457 and 530) in Group 1a had 9 % and 16 % of their lung surface area affected respectively. Calf 457 showed scattered lesions in all four lung lobes. For Calf 530 both apical lobes were consolidated and contained microabscesses and collapsed alveolar tissue, but little evidence of exudate within the bronchi. The remaining control animals, 534 and 459 (Group 1a), had no discernable gross lung pathology and appeared normal.

P. multocida was recovered from the lungs of 6 calves 2 of these animals were given only PBS, and the bacteria were located in lung tissue exhibiting extensive consolidation. The viable counts of individual calves varied from 0 cfu g^{-1} (Group 1a) to 1.2 x 10^7 cfu g^{-1} (Group 2a). On average, recovery of bacteria from lung tissue was lowest in Group 1a animals and highest in Group 2a animals (Table 6.5). There was no significant association of bacterial count with treatment group. There was no evidence of bacterial count being associated with respiratory rates, or any of the acute phase proteins (Table 6.5). Bacteria were not recovered from pericardial, or pleural fluid or heart blood.

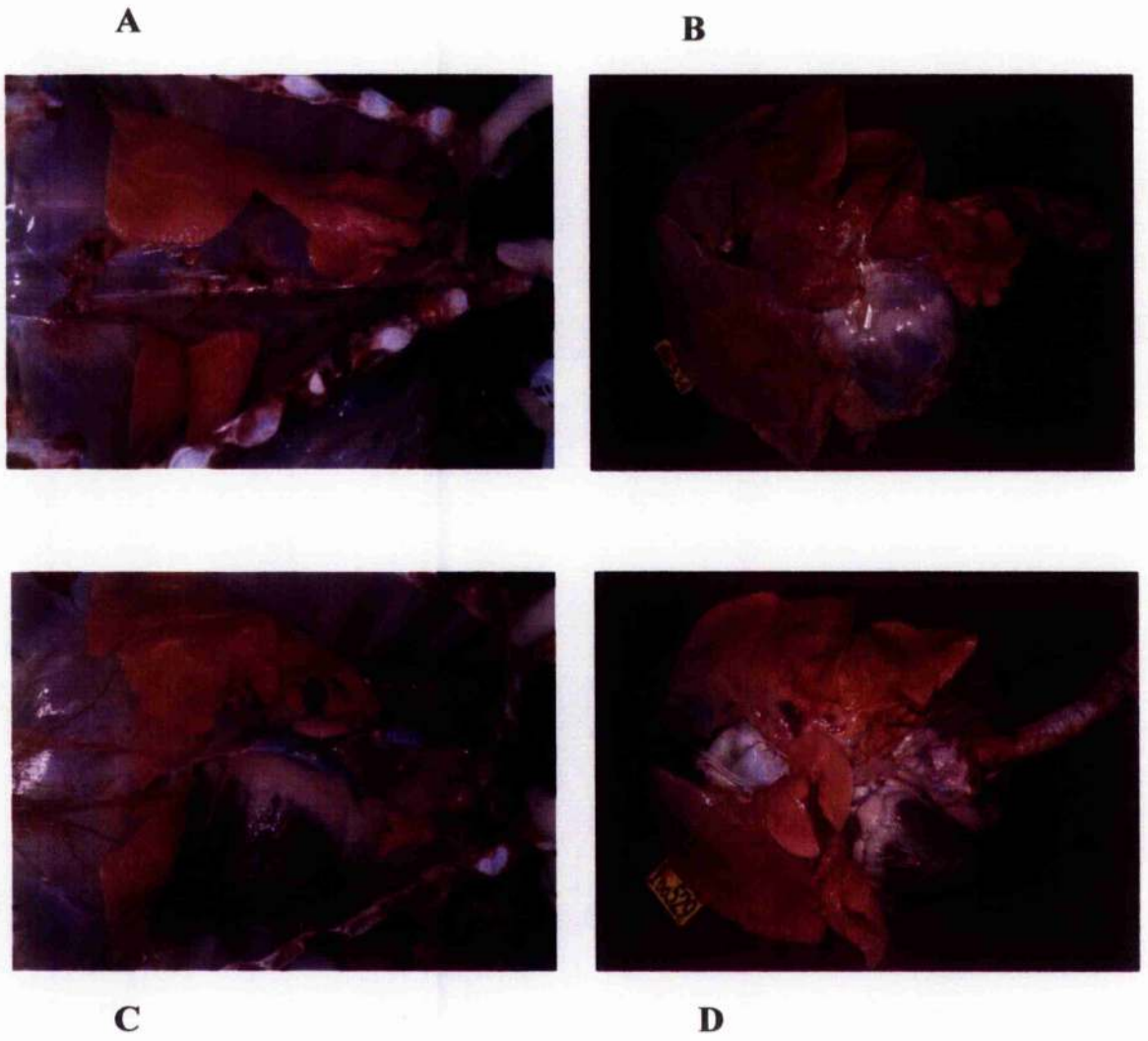


Fig. 6.15. Gross pathological changes observed for calves given 300ml PBS (Group 1a) and 10^9 cfu formalin-killed *P. multocida* A:3 (Group 2a); **A:** healthy lungs in situ from calf 534 challenged with PBS (Group 1a) and; **B:** view of the ventral surface of the same lungs when removed from the thoracic cavity, no evidence of lesions; **C:** lungs in situ from calf 529 challenged with formalin-killed *P. multocida* (Group 2a) and; **D:** view of the ventral surface of the same lungs when removed from the thoracic cavity. Consolidation of the right apical lobe is evident in both photographs.

Table 6.5. Percentage of surface lesions, pleurisy scores and bacterial recoveries from lung tissue (cfu g^{-1}) of calves challenged intratracheally with PBS or formalin-killed *P. multocida* A:3.

Group	Calf	% lung surface affected	Pleurisy	Viable lung count (cfu g^{-1})
1a	457	9.2	0	8.0×10^6
	534	1.3	0	No bacteria
	459	6	1	No bacteria
	530*	16	1	1.3×10^5
2a	458	10.5	0	1.3×10^3
	529	12	0	1.2×10^7
	531	7.2	0	6.5×10^4
	532	9.2	1	1.0×10^6

*Calf 530. Unfortunately this calf was housed with *P. multocida* challenged animals.

6.3.5. Histopathology

All four calves given only formalin-killed *P. multocida* (Group 2a) showed evidence of moderate-severe broncho-interstitial pneumonia in the right apical lobe, and 3 of the 4 calves contained alveolar oedema (Fig. 6.16.). All calves showed evidence of mild-moderate depletion of BALT and in calves 458, 531 and 529 the BALT appeared active. Neutrophils were migrating through the sub-mucosa of the respiratory epithelium and were present in airways.

Two animals assigned to Group 1a (457 and 530) exhibited histopathological findings typical of bacterial purulent pneumonia (Fig. 6.17.). Calf 457 showed evidence of mild, acute broncho-pneumonia in all four lobes and calf 530 showed signs of acute, severe broncho-interstitial pneumonia solely in the right apical lung lobe. The lungs of calves 534 and 459 showed no evidence of bacterial infection. Occasional foci of PMN were located in the respiratory epithelium and airways of all four calves, with greater concentrations found in calves 457 and 530. No significant histopathological changes were observed in any of the samples taken from the spleen, heart, liver or kidney.

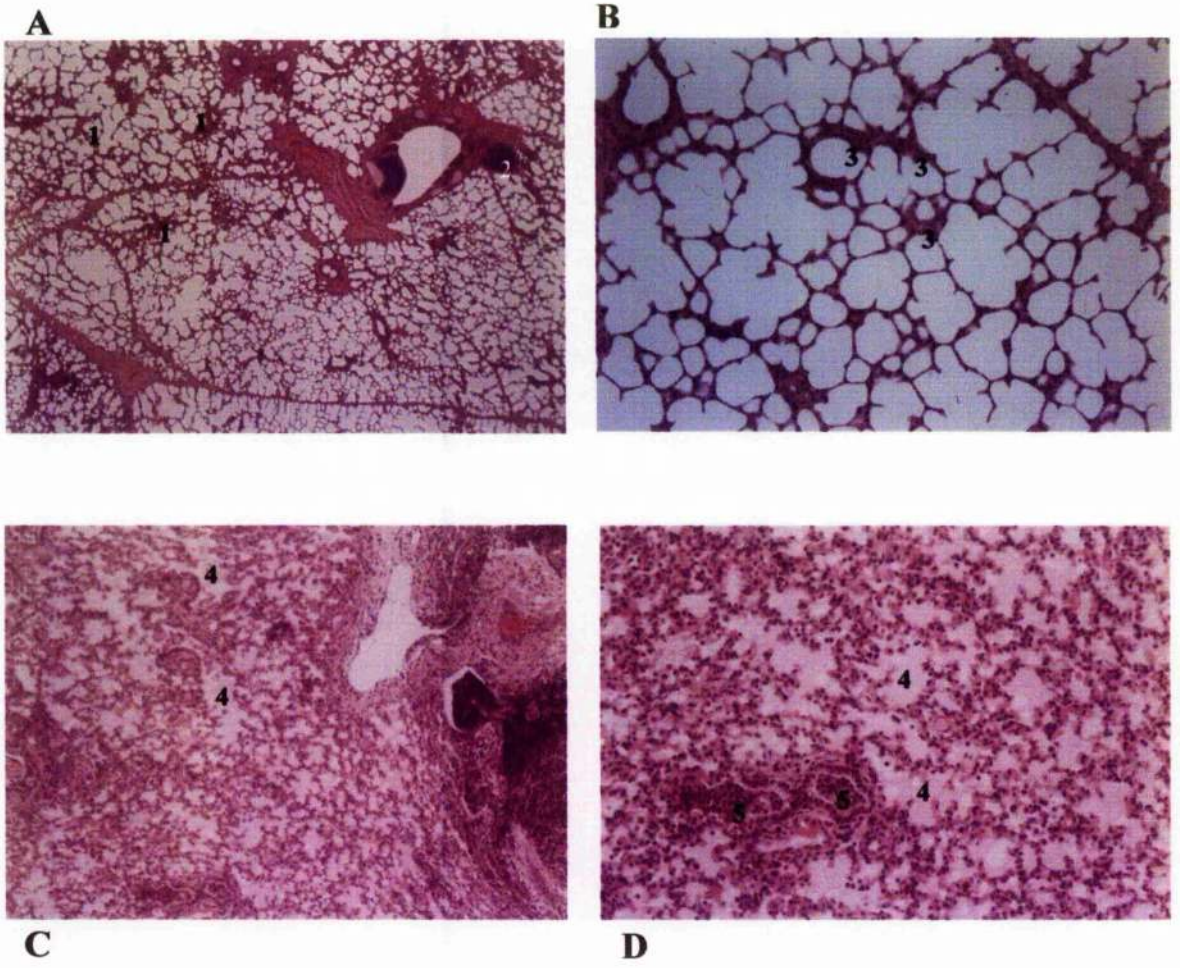


Fig. 6.16. Histopathological changes observed in Group 2a calves challenged with 10^9 cfu formalin-killed *P. multocida* A:3 ; **A:** calf 458 28 d p.c. (H&E, original magnification x 20); mostly normal lung tissue from the left apical lobe with occasional areas of thickening of alveolar septa (1): note the presence of BALT which appears contained (2); **B:** calf 458 28 d p.c., left apical lobe (H&E, original magnification x 100); thickening of the alveolar septa due to the presence of cells, possibly interstitial macrophages, within the septa (3); **C:** calf 529 28 d p.c., (H&E, original magnification x 40): fibrinous broncho-pneumonia of the right apical lobe as shown by the presence of alveolar oedema and fibrin in the alveolar spaces (4), which are in various stages of collapse and consolidation: all bronchioles contain a mixed, predominately PMN, inflammatory cell infiltrate; **D:** calf 529 28 d p.c., (H&E, original magnification x 100): fibrinous broncho-pneumonia of the right apical lobe as denoted by extensive alveolar oedema along with fibrin formation in alveolar air spaces (4): bronchiolar airways are obliterated due to the presence of mixed inflammatory cell infiltrate (5).

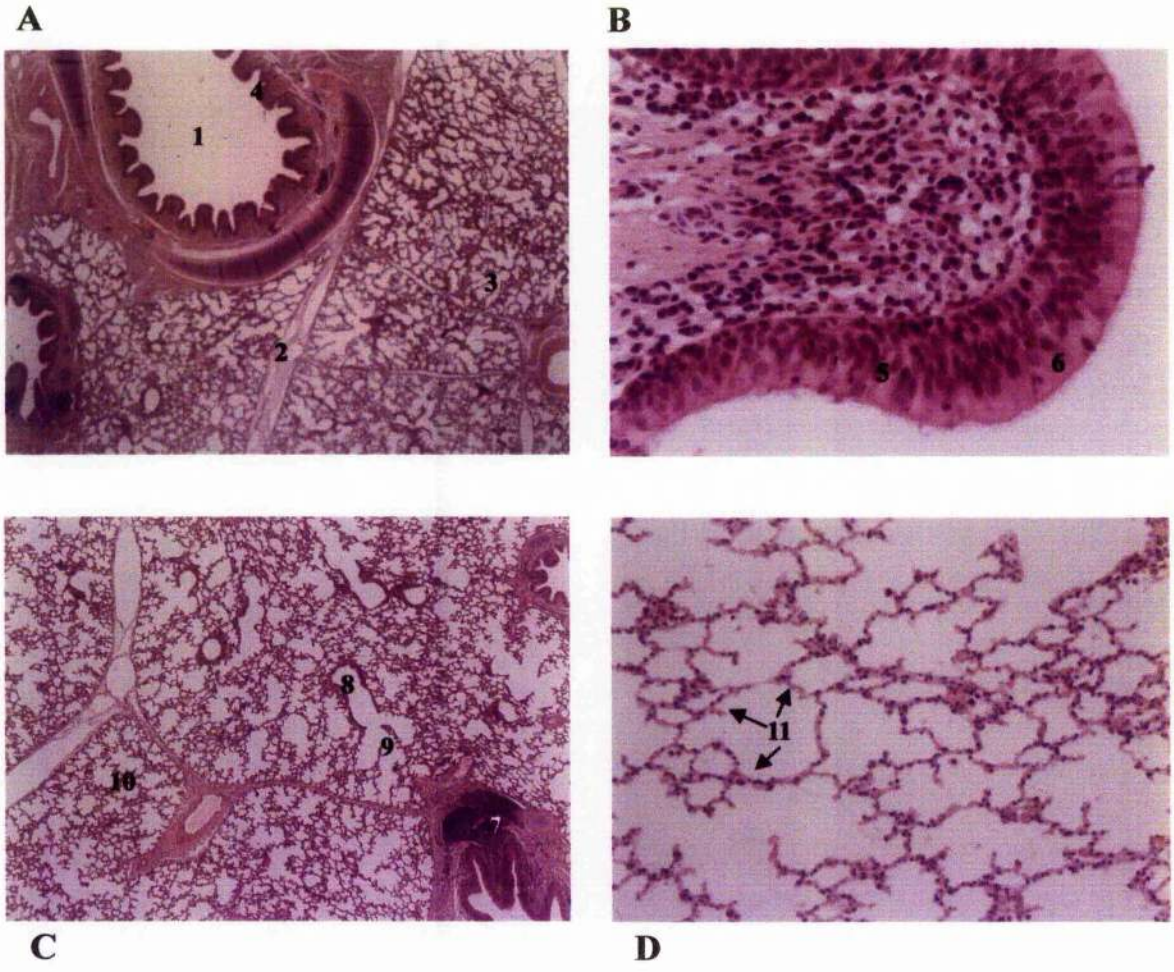


Fig. 6.17. Histopathological changes observed in Group 1a calves challenged with 300 ml PBS; A: calf 534 28 d p.c. (H&E, original magnification x 20); left apical lobe illustrating normal bronchi (1), inter-lobular septa (2) and alveoli (3). The corrugations present in bronchial epithelium are a post-mortem/fixation artifact as is slight collapse of alveoli (4); B: calf 534 28 d p.c., left apical lobe (H&E, original magnification x 400); bronchial respiratory epithelium (5), with occasional PMN migrating through the epithelium (6) in normal lung tissue; C: calf 534 28 d p.c., left apical lobe (H&E, original magnification x 40); normal lung, note BALT (7) and lack of cells within terminal (8) and respiratory (9) bronchioles and alveolar spaces (10); D: calf 534 28 d p.c., left apical lobe (H&E, original magnification x 200); normal expanded alveoli devoid of any inflammatory cell infiltration (11).

6.3.6. Nasal swab analysis

It was found that 2 of the 4 animals by 3 weeks p.c., from each group (457, 534, 459, 530; Group 1a and 458, 532, 531, 529; Group 2a) not given live challenge, tested positive for *P. multocida* (Table 6.6). At PM, bacteria were also recovered from tracheal swabs and lung tissue of all calves except 534 and 459 (Group 1a). These 2 animals remained negative throughout the experiment.

Table 6.6. *P. multocida* detection from nasal swabs taken periodically in all calves prior to and following inoculation with PBS and formalin-killed *P. multocida* A:3 at 10^9 cfu in 300 ml PBS. Data in the table until week 4 (PM) corresponds to the respiratory tract status of *P. multocida* for those calves that were not exposed to live *P. multocida*.

Group	Calf I.D	Prior to challenge	Week 1	Week 2	Week 3: Prior to live *P. multocida*	Week 4 PM
1a	457	-	+	-	+	+
	534	-	-	-	-	-
	459	-	-	-	-	-
	530	-	-	-	+	+
2a	458	-	-	-	+	+
	532	-	+	-	-	+
	531	-	-	-	-	+
	529	-	-	-	+	+

6.4. Discussion

The investigation was designed to compare and contrast the effects of formalin-killed *P. multocida* or PBS on lung and systemic pathophysiology on animals given a live challenge (Chapter 4). In addition, the effects of formalin-killed *P. multocida* on lung cell-mediated changes were assessed.

6.4.1. Clinical responses to challenge

Adverse responses such as pyrexia, increased respiratory rate and laboured respiration were observed for calves given formalin-killed *P. multocida*, but, surprisingly, similar responses were observed also for animals challenged with PBS. It is unlikely that these clinical responses found in control animals were due to the volume of fluid and or the physical insult of the challenge procedure, because a clinically normal response to PBS alone was observed in work described in Chapter 5, suggesting that these unexpected responses may be attributable to environmental conditions. The question to address, which leads us to Chapter 7 is whether an adaptive response against a homologous challenge had been generated.

6.4.2. Acute phase protein responses to challenge

To my knowledge the increase in SAA shown here is the first description of an APR to a killed pathogen, but along with Hp and AGP was not significantly greater in the treated than in control animals. Hp and especially SAA are sensitive indicators of inflammatory disease in cattle and their levels in blood during acute events are usually 10 to 100 fold (Conner *et al.*, 1988) and 100 to 1000 fold greater, respectively, than the normal physiological levels, which are often near the limit of detection (Eckersall and Conner, 1988). Although the calves appeared healthy at the beginning of the study, elevated plasma concentrations of these acute phase proteins were observed in approximately 30 % of the calves prior to challenge and indicated the presence of a subclinical infection.

Any changes in mean Hp and AGP concentration were small and statistically non-significant for either group throughout the study. The slight rise in Hp and AGP was more than likely due to the challenge procedure and not the effect of formalin-killed *P. multocida*. The responses for Hp and AGP were similar in Group 1 calves, although in Group 2 animals the progressive rise in AGP concentration was delayed until 12 h p.c. The Hp and AGP responses to formalin-killed challenge were markedly different from those seen following a live challenge (Chapter 4 and Chapter 7). The Hp response following live *P. multocida* challenge (Chapter 4) increased linearly and peaked at 1 g

l^{-1} as opposed to peaking at 400 μg ml^{-1} by 5 h p.c., observed in Group 2 calves. Whereas the AGP response following live *P. multocida* challenge (Chapter 4) was more gradual and sustained reaching concentrations of 620 mg l^{-1}, a delayed AGP response was observed for Group 2 calves in this study.

The rise in plasma SAA level was rapid for Group 2 calves, occurring after challenge more quickly than Hp. A similar observation has been reported for SAA response to *M. haemolytica* in cattle where it was suggested that SAA could have been released from local cells such as alveolar macrophages instead of hepatocytes (Horadogoda *et al.*, 1994). The differences in SAA concentration between infected and control animals in the current study were not significant, although the degree of increase in SAA for Group 2 calves was higher. This finding may be explained by the presence of *P. multocida* antigens in the blood triggering the synthesis of SAA for the purpose of scavenging foreign material. A similar but greater SAA response was observed following live *P. multocida* challenge (Chapter 4) peaking at 600 mg l^{-1} between 5 and 12 h p.c., although SAA levels fell at the end of the sampling period which was not the case in this study.

6.4.3. Cellular responses to challenge

Resident alveolar macrophages constitute a primary line of innate defence against foreign invaders and keep microbes at bay before neutrophils arrive in substantial numbers from the blood (Celli and Finlay, 2002). The presence of neutrophils in BALF may be used as an indicator of inflammatory disease (Walker *et al.*, 1985; Caldow, 2001) and in this study formed part of the cellular response of calves to formalin-killed *P. multocida*. In normal, healthy calves alveolar macrophages constitute 90 % of the cells present in BAL fluid, the remaining cells being neutrophils, epithelial cells and lymphocytes (Allen *et al.*, 1992a). However, with the onset of bovine respiratory disease, the proportion of macrophages decreases as neutrophils are recruited into the lung tissue and become the dominant cell type (Allen *et al.*, 1992b).

In this study the unusually high proportion (25 %) of neutrophils present in the pre-challenge lavage was surprising as neutrophils are usually recruited to the site of infection as a result of a foreign invader. This may indicate an underlying infection of a bacterial or viral nature, prior to challenge, as indicated also by the high initial concentrations of APP.

Additional neutrophil recruitment was observed in both groups following challenge but formalin-killed *P. multocida* produced a greater effect on the recruitment of neutrophils on day 1 p.c. compared with the PBS challenge by 11.6 %, though the difference between the two groups was not statistically significant. These findings are supported by a study on differential cell counts in feedlot calves treated for respiratory diseases (Allen *et al.*, 1992b) while similar findings have been

reported in cases where the resident alveolar macrophages have been overloaded with bacteria (Davies and Penwarden, 1981). To my knowledge there is no evidence within the public domain showing recruitment of cells in the presence of dead bacteria. However, it is unlikely, with a challenge of formalin-killed bacteria for overloading to occur, as no viable bacteria were present. It seems more credible that the physical presence of the bronchoscope and the volume of fluid that entered the lung is responsible for the similar percentage of neutrophil recruitment in both challenge groups on day 4 p.c., which would be supported by the demonstration in dogs and monkeys that a pulmonary lavage alone can stimulate neutrophil movement from the blood into the lung (Cohen and Batra, 1980).

Moreover, subclinical disease unrelated to experimental treatment may have been affecting the lung on any of the sampling days (Allen *et al.*, 1992b) as indicated by the recovery of *P. multocida* by nasal swabbing of the animals. Gram positive bacteria could also have been present in the URT though this cannot be confirmed as the swabs were plated onto SBA, suppressing any growth of Gram-positive organisms so that the presence of Gram-negative organisms would be easily visible if present.

The *in vitro* viability of recovered neutrophils was consistently higher than that of macrophages by a mean of 10 % for all calves throughout the study. The LDCL response was also higher for neutrophils than macrophages, even though the same number of viable cells was used in either test. It is possible that the resident alveolar macrophages were disabled by the unlooked for presence of a pathogen, whereas the neutrophils had been recruited fresh to the site of infection from the blood, ready for phagocytic killing as reflected by their large LDCL response in this study.

6.4.4. Phagocytic responses to stimulants following *in vivo* challenge with bacterin

The interactions between bovine mononuclear cells and *P. multocida* are poorly understood and have not been characterised to the extent that other respiratory pathogens, such as *M. haemolytica* and *Haemophilus somnus* (Czuprynski and Hamilton, 1985 and Czuprynski *et al.*, 1987) have been. In this study of *in vitro* interactions between alveolar macrophages and *P. multocida* a few points of biological interest have been raised such as the failure of the phagocyte to bind opsonin, the phagocytic uptake of *P. multocida* independent of opsonisation and the survival of *P. multocida* for at least 1 h in the hostile environment of the macrophage.

The similar rate of phagocytic uptake of both opsonised and non-opsonised bacteria indicated that opsonisation of *P. multocida* using heat-inactivated immune polyclonal antiserum was unsuccessful despite using serum raised to this specific strain. Formalin-induced alterations of the bacterial

surface components such as the trapping of carbohydrate and lipid molecules within a matrix of insoluble cross-linked proteins could conceivably interfere with antibody/phagocyte interaction, however, low concentrations of formalin used in the present work were unlikely to have affected the physiochemical and immunogenic properties of the bacteria (Nencioni *et al.*, 1991). It was not possible to control the incubation period for the opsonisation process as tightly as anticipated, and due to the nature of response curves whereby the margin of error was small, there was always a risk of low rates of opsonisation (section 2.9.5).

It is feasible that a change in the target antigen in a nutrient deficient environment *in vitro* may have arisen (Paustian *et al.*, 2001) or that the bacteria exhibited antigenic variation at the late log-to-stationary phase of growth, as has been demonstrated in *Neisseria* sp, (Serkin and Seifert, 1998). However, it seems unlikely that this could have occurred at the stage of growth that *P. multocida* were used at, which was mid-log phase.

Despite the apparent lack of opsonisation of *P. multocida*, there was evidence of bacterial internalisation. Macrophages are able to ingest bacteria and other pathogens by a variety of mechanisms. Opsonin-independent phagocytosis is elicited by the interaction of macrophage surface receptors with microbial surface components that include mannose receptors, type A scavenger receptors and integrins (Celli and Finlay, 2002). Another mechanism that can promote contact between bacteria and phagocytes is surface phagocytosis of immobilised bacteria a process that has been confirmed for *S. epidermidis* and *E. coli* by PMN and alveolar macrophages (Gordon *et al.*, 1989).

Fluorescent microscopy demonstrated that *P. multocida* had the capacity to survive within macrophages for a short period *in vitro*, illustrated by the negligible bactericidal activity of macrophages to *P. multocida* during a 1 h incubation period. Several mechanisms for intracellular survival of pathogens exist; escape into the cytoplasm, inhibition of phagolysosomal fusion and disabling of the respiratory burst.

Studies of phagocytosis with *Haemophilus somnus*, an organism that exhibits similar pathogenesis to that of *P. multocida*, revealed a survival tactic that involves the prevention of phagosome and lysosome fusion, thus inhibiting the respiratory burst (Gomis *et al.*, 1998). In this work a low to negligible LDCL response by lavaged macrophages in the presence of live bacteria indicated inhibition of the respiratory burst. A similar LDCL response from BAM has been reported for a live logarithmically growing or stationary phase of *H. somnus* (Gomis *et al.*, 1997).

The evidence presented in this work suggests that *P. multocida* may interfere with the oxidative dependent bactericidal ability of mononuclear cells. The low LDCL response to live non-opsonised *P. multocida* corresponds with results from adhesion slides, which show bacterial uptake but low

bactericidal activity. Measurement of the LDCL response following the addition of PMA to macrophages and live *P. multocida* would need to be performed to confirm the theory of pathogenic interference in reactive intermediate production; if there was no response curve the mechanism of respiratory burst has been disabled.

The environment, in which the macrophages and bacteria were suspended for both the cell-adhesion slide work (Goldner *et al.*, 1983) and the LDCL assay, must be taken into account when comparing the results between both these *in vitro* experimental setups. The cell-adhesion slide method requires RPMI medium during the incubation stages to provide macrophages with nutrient and help maintain viability throughout the procedure. The LDCL assay does not require medium, so it is plausible that the low LDCL response of macrophages to live *P. multocida* measured by the LDCL assay may have been due to the absence of medium, making the macrophages more susceptible to bacterial killing.

The rate of increase of the respiratory burst produced by phagocytes was greater for neutrophils than macrophages, suggesting that neutrophils produce precursors for reactive intermediates at a greater speed and quantity than do macrophages. In contrast, the rate of respiratory burst increase was gradual for macrophages regardless of the stimulus, indicating that hexose monophosphate shunt activity was depressed, delaying the production of oxygen intermediates.

Gross pathology results for calves (Group 2a) exposed to formalin-killed *P. multocida* A:3 showed that a greater area of lung was affected compared to that of control calves given PBS (Group 1a). It is unlikely that a neutrophilic response alone could be responsible for the tissue necrosis, as differential immune cell counts on lavaged cells indicated little difference in neutrophil population for Group 1 and 2 calves at day 1 and day 4 p.c. This observation may be attributed to the unexpected recovery of *P. multocida* from lung tissue, which was greater for Group 2a than Group 1a animals, reflecting a persistent underlying infection caused by a different strain of *P. multocida*. It would be of interest to perform PFGE on the bacteria recovered from the lungs of calves in Group 1a and Group 2a and characterise the adventitious pathogen and compare it with the homologous challenge used for Group 1b and Group 2b animals. Formalin-killed bacteria contain LPS which is responsible for the release of injurious agents such as reactive oxygen/nitrogen species and elastase from phagocytes. This toxic integral component of the *P. multocida* membrane may have been the likely cause for the greater area of lung consolidation recorded for Group 2a calves, than for Group 1a calves, in addition to the isolation of resident *P. multocida*.

Nasal swabbing was deemed an inaccurate and unreliable test for the detection of adventitious pathogens and only at PM was the bacterial status of the respiratory tract clarified, wherein 75 % of the calves that were given either PBS or formalin-killed bacteria tested positive for live *P. multocida*. It is conceivable that those animals that tested positive at PM had *P. multocida* residing

in the respiratory tract from the start of the experiment and undetected by swabbing of the nasal passages. Furthermore, the bronchoscope used for challenge and lavage may have introduced live bacteria from the URT to the LRT.

6.4.5. Conclusions

The investigation was unable to demonstrate a significant difference in blood levels of APP to between calves treated with formalin-killed *P. multocida*, compared to control animals and this finding showed with the low clinical response.

The opsonisation procedure appeared unsuccessful indicated by similar LDCL responses for antiserum alone and opsonised bacteria, however the phagocytic uptake of *P. multocida in vitro* still took place, as shown using cell-adhesion slides by the presence of *P. multocida* internalised within macrophages.

Comparable LDCL responses observed for both challenge groups showed formalin-killed *P. multocida* had no debilitating effect on the respiratory burst of the phagocytes.

Despite active phagocytosis *P. multocida* were able to survive intracellularly, as demonstrated by the AO/CV fluorescence technique. However, this process was observed only for a 60 m incubation period and it would be of interest to extend this period and monitor the ability for the bacteria to survive within the alveolar macrophage. Additionally, an accurate assessment of bactericidal activity was not achieved due to the Strugger effect, whereby dead bacteria appeared red in the first instance and rapidly turned green, despite the use of Cityfluor as antifade, making the accurate recording of dead (red) bacteria difficult.

The LDCL response of cells to *P. multocida* with opsonising antiserum or to opsonising antiserum alone was similar. Serum protein alone can cause the release of CL (Easmon *et al.*, 1980) and this phenomenon was observed and quantified in the present work.

The clinical, biochemical and cell-mediated responses noted from this study indicated that the host immune response to infection occurred mainly within the lung as there was no evidence of a systemic antibody response, although there was evidence of an APR. Future work should use the results of this study to improve the methodology and focus more on cytofluorometric techniques as these are likely to be simpler, rapid, more reproducible and objective (Chaka *et al.*, 1995; Raybourne *et al.*, 2001) in helping to elucidate the host-microbial interactions occurring at the site of infection or *in vitro*. Moreover it would be of interest to examine different dose formulations of formalin-killed *P. multocida*. In this experiment the same number of bacterial cells was used as for the live challenge (Chapter 4). The live dose, however, has the ability to multiply bacteria to levels

greater than that administered. In contrast formalin-killed bacteria are unable to multiply and for a fair comparison a larger dose of dead bacteria may have been needed. It would seem that Group 2 calves coped well with the exposure to bacterial antigens, since the clinical response was sizable but little difference was observed from control animals, raising the possibility that this approach may be useful in generating protection against a subsequent live infection.

Chapter 7. EFFECT OF LUNG EXPOSURE TO FORMALIN-KILLED *PASTEURELLA MULTOCIDA* BIOTYPE A:3 ON SUBSEQUENT RESPONSE TO HOMOLOGOUS LUNG CHALLENGE IN CALVES.

7.1. Introduction

The pathogenic mechanisms of fibrinous pneumonia in cattle due to *P. multocida* A:3 are poorly understood and the prevention and treatment of disease remains inadequate. To date no vaccine exists against *P. multocida* A:3, though many vaccines have been designed to reduce the incidence of pneumonic pasteurellosis caused by *M. haemolytica*, including whole cell, capsular, leukotoxin extract and iron-regulated vaccines (Panciera *et al.*, 1984b; Donachie *et al.*, 1983; Shewen and Wilkie, 1988; Gilmour *et al.*, 1991). These vaccines containing bacterial antigen offer variable levels of protection, due possibly to the mode of administration or type of adjuvant used, though it seems more plausible that the ability of the host to develop antibodies against IRP-OMP that are present in some vaccine formulations and not others would contribute to this variability (Confer *et al.*, 2001).

The use of formalin-killed *Pasteurella* spp., as a candidate vaccine antigen to control pneumonic pasteurellosis has been of limited value for *M. haemolytica* (Confer *et al.*, 1985) and to my knowledge no such studies have been associated with *P. multocida*. Several field trials were conducted to assess the effect of vaccination with killed *M. haemolytica* on resistance to subsequent experimental exposure with the live organism (Confer *et al.*, 1985, 1987). It was acknowledged that, despite the production of a serum antibody response, no protection was observed and lesion scores were comparable to control calves given PBS (Confer *et al.*, 1987; Mosier *et al.*, 1998). These results suggest that a systemic antibody response does not necessarily confer protection and may denote only exposure to somatic antigens, providing further support for the need to clarify pathogenic mechanisms of disease occurring within the lung. Furthermore, the inability of formalin-killed *P. multocida* to produce IRP would be a likely cause of a reduced antibody response in the work conducted by Confer *et al.*, (1985). In contrast, the administration of immune serum that is likely to contain antibodies to IRP, directly into the systemic system, has provided 94-100 % protection of recipients compared to control lambs against experimental pneumonic pasteurellosis (Jones *et al.*, 1989).

The potential for mucosal immunity to confer protection was considered in a study showing that initial priming of the nasal cavity by a natural infection enhanced the systemic immune response, leading to improved protection against experimental exposure of *Pasteurella* spp., (Conlon *et al.*, 1995). Mucosal and localised immunity within the upper respiratory tract and the lung, respectively, in the protection of ruminants against pneumonic pasteurellosis, may not be primed effectively by intramuscular or subcutaneous immunisation.

Repeated intranasal exposures to formalin-killed *M. haemolytica* A:2 have led to significant levels of secretory IgA, IgM and IgG in the lung fluid indicating the potential of killed cells as vaccine antigen (Zamri-Saad *et al.*, 1999). The efficacy of an intranasal instillation of formalin-killed *M. haemolytica* A:2 in goats to provide protection through mucosal immunity was demonstrated by the reduced severity and occurrence of lung lesions compared with unvaccinated animals (Effendy *et al.*, 1998). With this in mind, the treatment described in Chapter 6 may provide initial priming of the host lung immunity by lung exposure to formalin-killed *P. multocida*, enhancing the innate immune response and leading to better protection against a homologous challenge of *P. multocida*.

Aims of the study

Continuation of the work described in Chapter 6 involved an examination of whether prior exposure to formalin-killed *P. multocida* conferred protection against a live homologous challenge, as a possible vaccine strategy. The approach used to administer the inoculum would be different from that used by Effendy and colleagues (1998), with the expectation that administration of formalin-killed *P. multocida* directly into the lung would stimulate a localised immune response and provide a similar degree of protection to that observed with stimulation of the URT mucosa by intranasal instillation of formalin-killed *M. haemolytica*.

7.2. EXPERIMENTAL DESIGN AND METHODS

The animals used in this study were those assigned to Group 1b and Group 2b as explained in Chapter 6.

7.2.1. Preparation of live *P. multocida* inoculum

P. multocida A:3 was cultured to the log-phase stage of growth and diluted in PBS to give a total dose of 10^9 cfu in 300 ml (section 2.8.2.) The range of actual live bacterial doses, estimated retrospectively by plating out serial dilutions of each dose onto blood agar, was 1.5 to 2 x 10^9 cfu.

7.2.2. Experimental procedure

Calves belonging to Group 1b (435, 441, 443, 704, 706, 709) and Group 2b (172, 437, 440, 705, 707, 708) challenged previously on day 0 with either formalin-killed *P. multocida* (Group 2b) or PBS (Group 1b) were challenged intratracheally on day 21 with live *P. multocida* at 10^9 cfu in 300 ml PBS, followed by PM on day 28. Calves were monitored throughout the experiment for clinical signs of disease, using a scoring system established previously (section 2.8.5.) while access to veterinary care was available at all times.

7.2.3. Necropsy

Post-mortems were performed on day 28 p.c. after calves were killed by i.v., injection with 25 ml of Pentobarbitone Sodium B.P. (200 mg ml^{-1}; Animal Care). Details of necropsy and scoring of gross pathology are given in section 2.8.6.

Representative samples from the lymphoid system, respiratory system and other organs of significance were placed in two preservatives; 10 % formol-saline or zinc salts and prepared for histopathological examination using standard techniques.

7.2.4. Bacteriological examination

Small samples of tissue (approximately 1 cm cubes, about 1 g) were taken from four pre-selected lung sites (apical, cardiac, intermediate and diaphragmatic lobes), bronchial lymph nodes, caudal and mid mediastinal lymph nodes, tonsils, spleen, liver, kidney and heart. The samples were homogenised in 9 ml peptone water and diluted in 10-fold steps to 10^{-6}. Aliquots (10 µl) of each dilution from 10^{-6} to 10^{-2} were applied to SBA plates and incubated at 37°C for 16-20 h. Viable counts were determined and expressed as cfu g^{-1} of tissue (section 2.8.7).

7.2.5. Serology

Blood sampling from a jugular vein from 12 of the 20 calves resumed on day 21 prior to intratracheal challenge with live *P. multocida* A:3 4 h p.c. and daily thereafter until and including the day of necropsy (day 28). Plasma was separated and stored at -40°C until analysed for viral antibodies, IgG levels and APP (Hp, AGP and SAA) (section 2.6.2). Levels of viral antibody to BVDV, IBRV, RSV and PI-3 in sera prior to challenge and at PM were detected by an ELISA (section 2.6.4). Levels of antibody to *P. multocida* in the sera were measured by an ELISA (section 2.6.3) using microtitre plates coated with *P. multocida* envelope.

7.2.6. Statistical analysis

The AUC for the same time period as in Chapter 6 (-48 h to 120 h p.c; with 0 h being time of live challenge) was measured for the following parameters: Hp, SAA, AGP, respiratory rate and rectal temperature. Differences between groups in AUC were assessed for statistical significance by ANOVA. Treatment groups were fitted as a factor and the value of the variable on day 0 fitted as a covariate. An ANOVA, adjusted for covariate was also carried out for percentage of lung affected and mean bacterial burden per gram of lung tissue. Lung bacterial count was analysed on a log scale because of the skewed nature of the data.

7.3. RESULTS

Protection against a subsequent homologous live challenge of *P. multocida* A:3 was assessed by measuring pathophysiological responses between day 21 and day 28 p.c. Clinical measurements and blood sampling were recorded up to day 26 in order to make a direct comparison with the effects of formalin-killed challenge (Chapter 6).

7.3.1. Clinical observations

At day 21 p.c. all calves showed normal clinical signs and appeared healthy prior to and immediately after challenge with live *P. multocida* at 10^9 cfu in 300 ml PBS (Groups 1b and 2b). The challenge procedure was well tolerated by all animals, causing only mild coughing as observed on day 0. Within 5 h p.c. all animals became dull with signs of increased respiratory effort, mucopurulent nasal exudate and reduced appetite. Similar clinical observations after 5 h p.c. with live *P. multocida* A:3 were also reported (Chapter 4).

Mean rectal temperatures increased for all calves from a baseline value of 38.6°C (± 0.1) (Group 1b) or 39.0°C (± 0.2) (Group 2b) to a peak of 40°C at 10 h p.c. (± 0.4 Group 1b: ± 0.1 Group 2b) (Fig. 7.1a), returning to within the normal range (Chapter 4) by 24 h p.c. A secondary temperature response was shown by calves in both treatment groups, when an increase in mean rectal temperature of 0.7°C (Group 2b) and or 0.4°C (Group 1b) was observed at 24 h p.c. Group 2b calves had the higher mean rectal temperature between days 21 and 26 compared to Group 1b calves, although there was no statistically significant difference between the two groups. Rectal temperatures for Group 1b calves reached a peak value comparable to that observed for calves given 300 ml 10^9 cfu (Chapter 4), although the peak occurred at 10 h p.c in this study instead of 23 h p.c recorded (Chapter 4).

Prior to infection mean respiratory rates for Group 1b and 2b calves were pooled, 51 (± 3.0) and 48.5 (± 4.9) min^{-1} (Fig. 7.1b), representing a clinical score of 0. Rapid and marked rises in respiratory rates were observed in both treatment groups after infection, increasing to an average of 101 (± 5.7) min^{-1} at 5 h p.c., representing a clinical score of 3, in all infected animals. Calves in Group 1b and 2b exhibited a secondary response during which respiratory rates increased at 72 h and 24 h p.c., respectively and remained above the normal range until the scheduled PM date. There was no evidence of a difference in mean AUC of respiratory rate against time between Groups 1b and 2b. However, respiratory rates for both treatment groups were significantly higher ($p = 0.026$) than for animals challenged with PBS at day 0 (Group 1a, Chapter 6). Higher respiratory rates were observed at day 21 compared to day 0 for calves given live *P. multocida* challenge (Chapter 4).

Overall clinical signs for Group 1b showed a similar trend to that of Group 2b animals, with marked, rapid responses within 5 h p.c. Thereafter, clinical symptoms for Group 1b began to resolve such that there was an obvious improvement in the health status of the calves until 72 h p.c., when there was a secondary clinical response to infection. The degree of clinical disease appeared greater for Group 2b animals between 48 h and 96 h p.c. as a result of a secondary clinical response. However, there was no statistically significant difference in the overall clinical response from the onset of exposure to *P. multocida* until 10 h p.c. between both treatment groups. From 24 h p.c. and until the end of the clinical monitoring phase, the clinical response appeared to be consistently higher for Group 2b animals compared to that of Group 1b, although none of these differences was statistically significant.

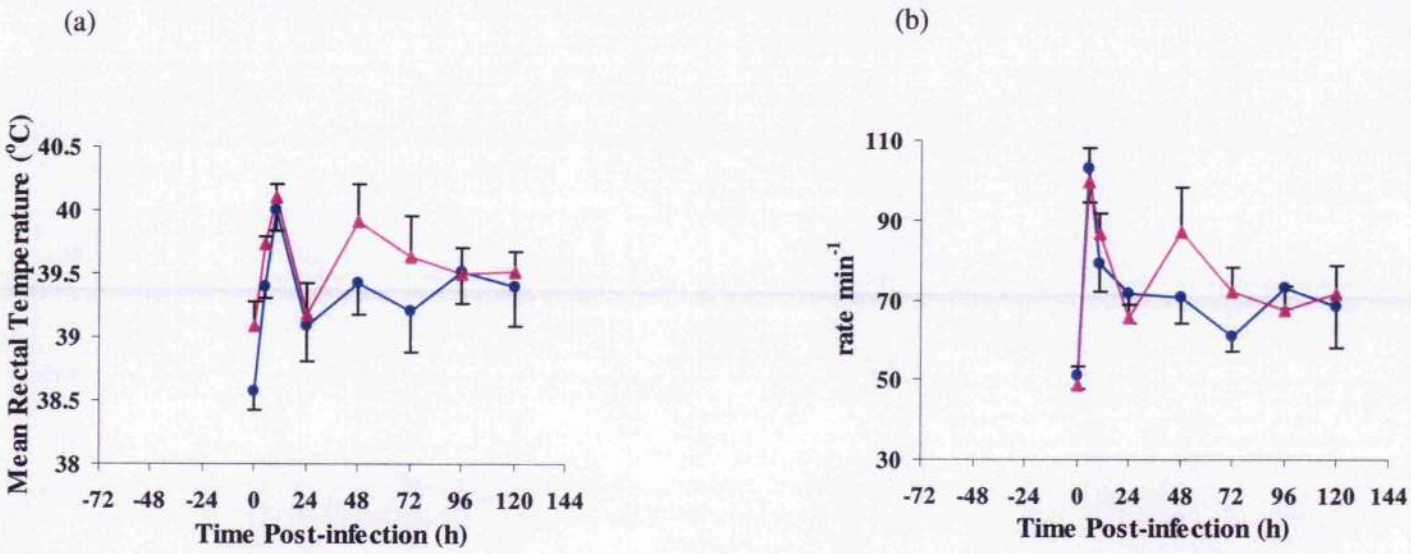


Fig. 7.1. Mean (±S.E) clinical measurements from calves inoculated with 10^9 cfu *P. multocida* A:3 in 300 ml PBS (a) Mean rectal temperature (°C); (b) Mean respiratory rate (rate min^{-1}). Data presented correspond to challenge groups 1b (●), 2b (▲).

7.3.2. Serology

7.3.2.1. Viral antibody analysis

Measurement of antibodies to viral antigens showed that 70 %, 95 %, 90 % and 70 % of pre-infection plasma samples contained antibodies to IBRV (Fig. 7.2), RSV (Fig. 7.3), PI-3 (Fig. 7.4), and BVDV (Fig. 7.5), respectively. For most calves the comparison of antibody level in samples taken before infection and immediately prior to PM showed a variable decrease in antibody, indicating that the calves were seronegative for these respiratory viruses. The decrease in antibody ranged from 39 to 100 % for BVDV, 21 to 62 % for PI-3, 12 to 100 % for RSV and 19 to 100 % for IBRV. However, though the RSV antibody titre for calf 530 (Group 1a) increased by 5 % during the experiment, the calf appeared clinically healthy.

7.3.2.2. IgG immune response

Mean plasma IgG titres at 1:100 dilution for all samples ranged from OD 1.41 (± 0.03) to 1.43 (± 0.03) on day 0 prior to challenge with either PBS or formalin-killed *P. multocida* A:3 (Fig. 7.6). Following challenge the IgG response was very similar for all treatment groups showing a mean increase in O.D of approximately 0.03, on day 21 which was sustained until the end of the sampling period on day 28.

7.3.2.3. Acute phase protein response

Mean plasma (± SE) Hp concentrations, prior to challenge with live *P. multocida* on day 21 were 136.7 (± 35.3) and 45 (± 10.6) μg ml^{-1} for Group 1b and 2b animals respectively and increased to 806 (± 253.3) μg ml^{-1} and 856 (± 300) μg ml^{-1} by 48 h p.c., in Group 1b and 2b animals respectively (Fig.7.7.a). Thereafter mean concentrations decreased gradually to 498 (± 228.5) μg ml^{-1} for Group 2b animals, whilst those in Group 1b animals showed a slight increase to 835 (± 314.5) μg ml^{-1} by the end of the sampling period (Fig.7.7.a). A t-test indicated no statistical significance in differences in mean Hp concentrations between groups 1b and 2b.

The rate of increase for mean plasma SAA concentrations was very rapid in both treatment groups and occurred between 5 and 24 h p.c. for Group 1b animals, increasing from 11.2 (± 1.9) to 101.3 (± 20) μg ml^{-1} (Fig.7.7.c) and between 0 and 10 h p.c. for Group 2b animals, increasing from 26.8 (± 4.3) to 67.4 (± 17.4) μg ml^{-1} (Fig.7.7.c).

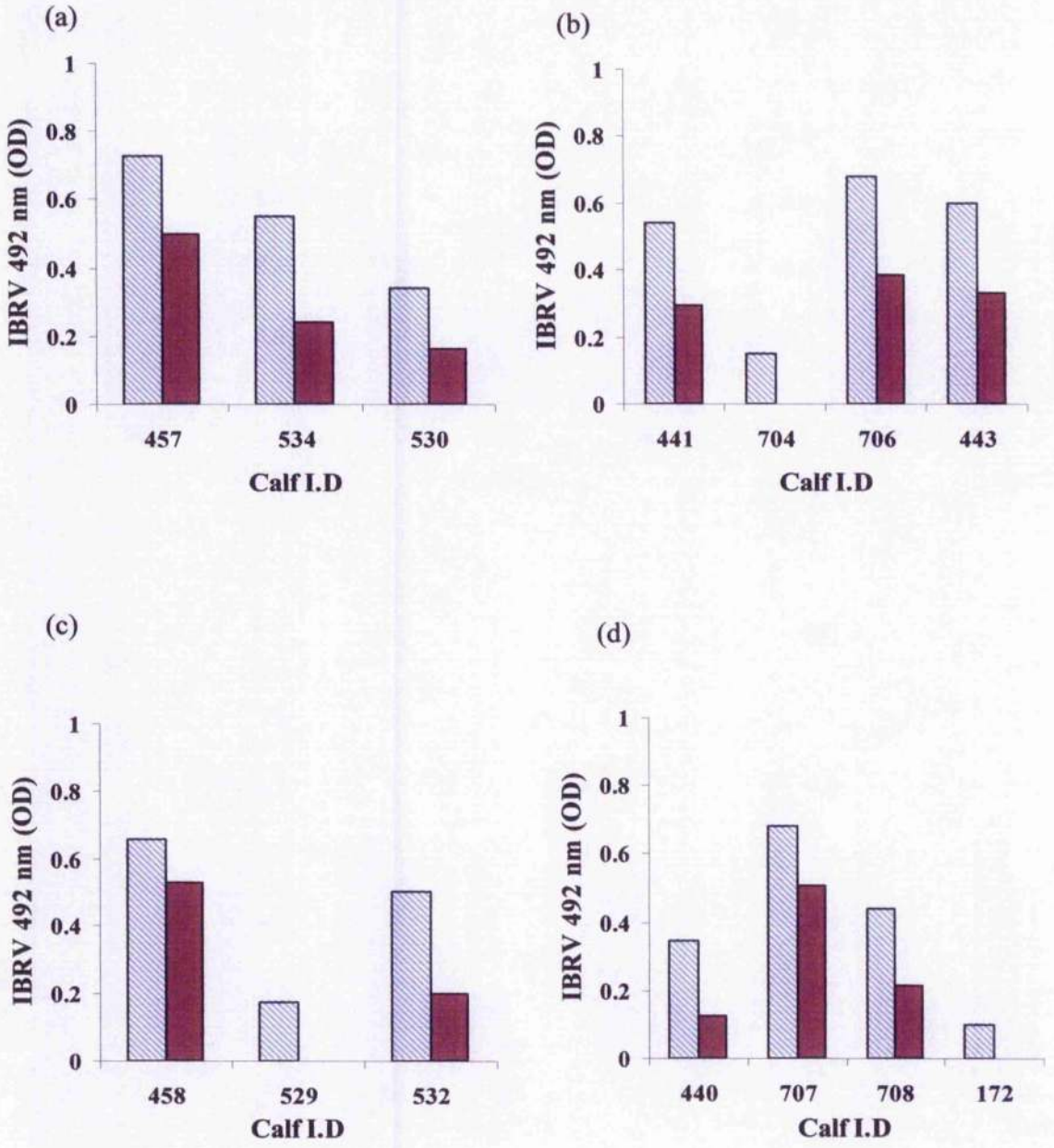


Fig. 7.2. IBRV antibody titres in pre-infection (▨) and post-mortem (■) (28 days) bleeds from calves inoculated with PBS, formalin-killed, *P. multocida* and *P. multocida* A:3 at 10^9 cfu in 300 ml PBS. Data presented corresponds to challenge groups; (a) Group 1a (300ml PBS) calves, no response for calf 459; (b) Group 1b (300ml PBS and 10^9 cfu live *P. multocida* in 300ml PBS) calves, no response for calf 709; (c) Group 2a (10^9 cfu formalin-killed *P. multocida* in 300ml PBS) calves; no response for calf 531; (d) Group 2b (10^9 cfu formalin-killed *P. multocida* and 10^9 cfu live *P. multocida* in 300ml PBS) calves, no response for calves 705 and 437.

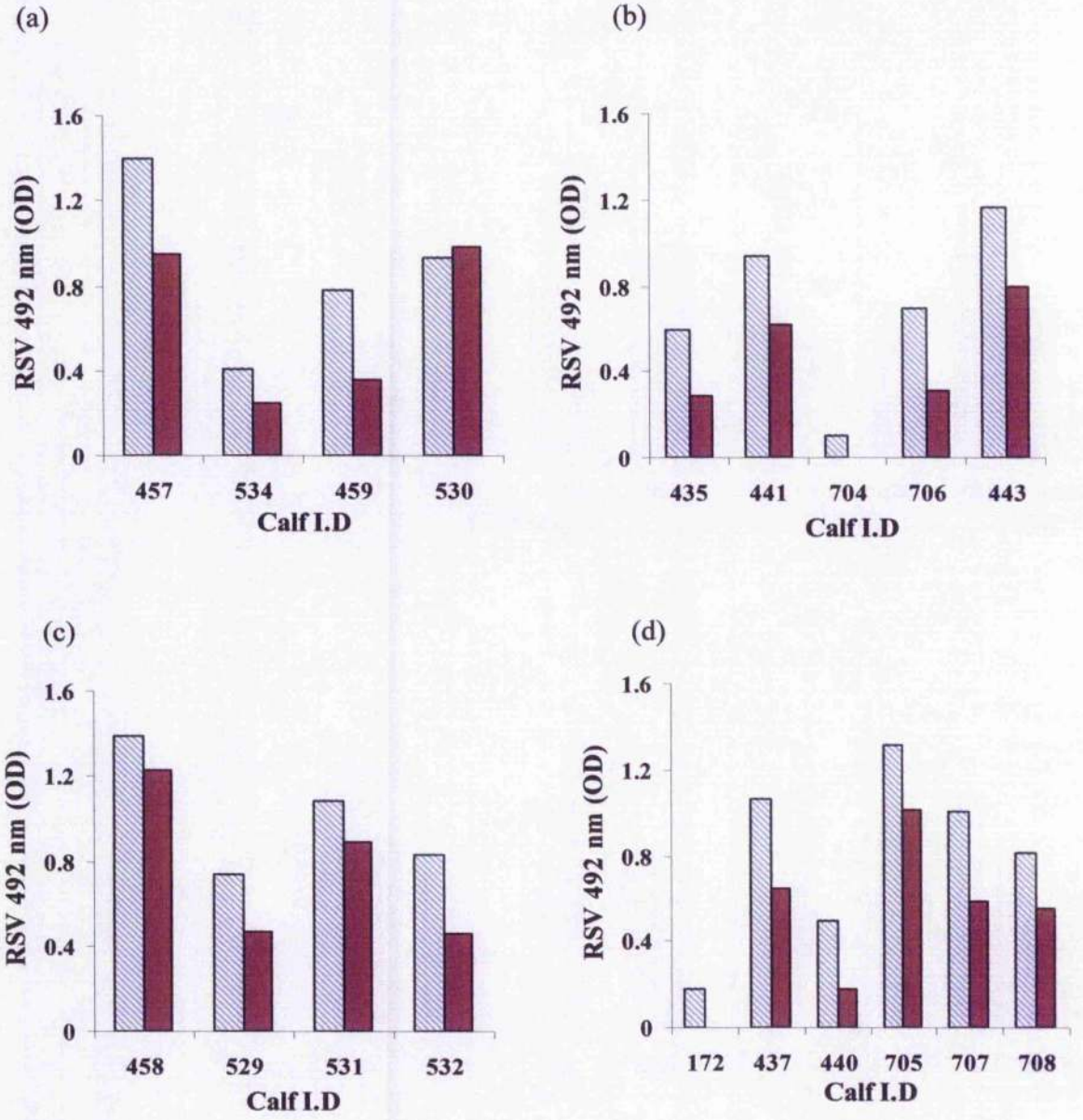


Fig. 7.3. RSV antibody titres in pre-infection (▨) and post-mortem (■) (28 days) bleeds from calves inoculated with PBS, formalin-killed *P. multocida* and *P. multocida* A:3 at 10^9cfu in 300 ml PBS. Data presented corresponds to challenge groups; (a) Group 1a (300ml PBS) calves; (b) Group 1b (300ml PBS and 10^9 cfu live *P. multocida* in 300ml PBS) calves, no response for calf 709; (c) Group 2a (10^9 cfu formalin-killed *P. multocida* in 300ml PBS) calves; (d) Group 2b (10^9 cfu formalin-killed *P. multocida* and 10^9 cfu live *P. multocida* in 300ml PBS) calves.

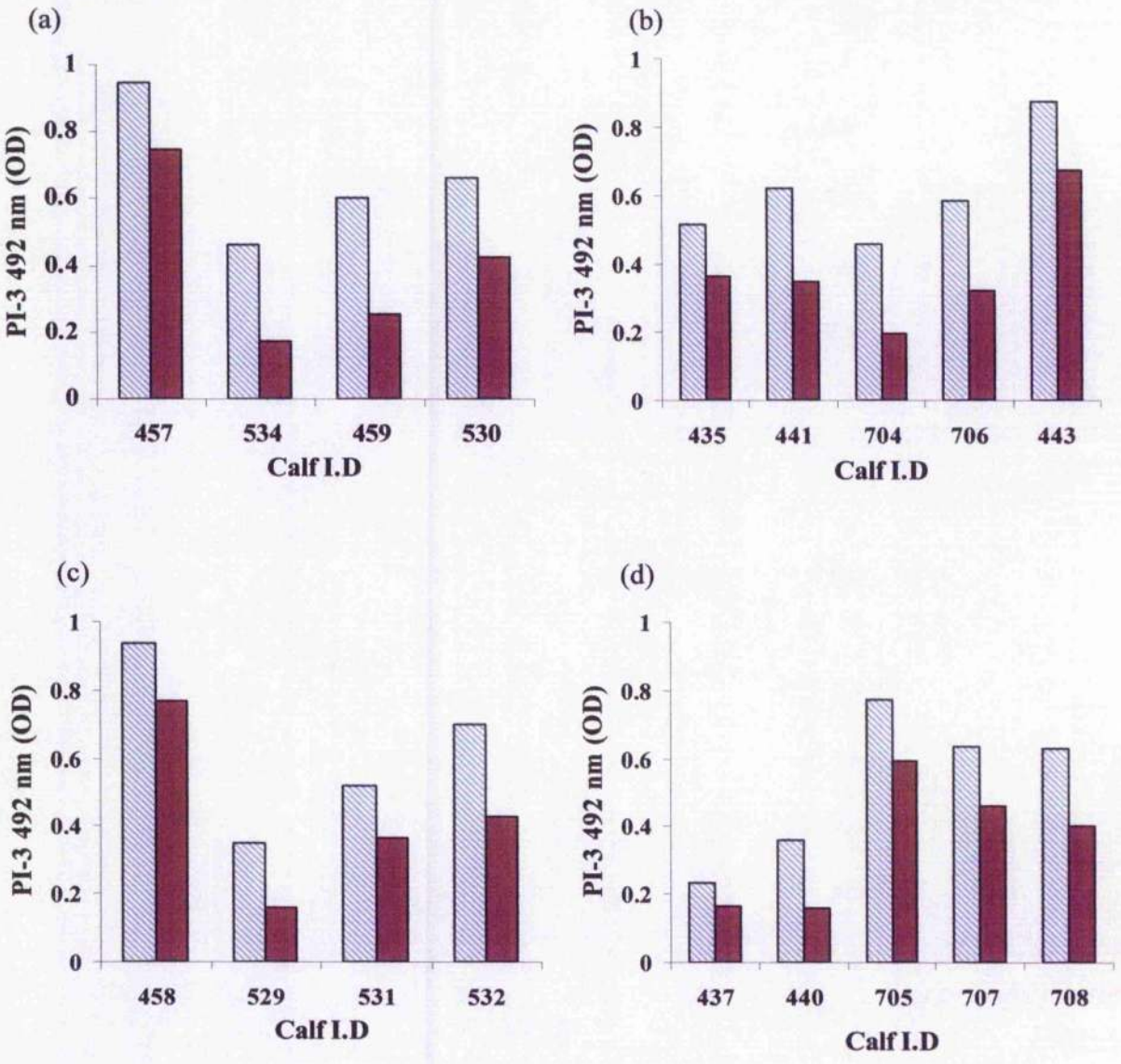


Fig. 7.4. PI-3 antibody titres in pre-infection (▨) and post-mortem (■) (28 days) bleeds from calves inoculated with PBS, formalin-killed *P. multocida* and live *P. multocida* A:3 at 10^9 cfu in 300 ml PBS. Data presented corresponds to challenge groups; (a) Group 1a (300ml PBS) calves; (b) Group 1b (300ml PBS and 10^9 cfu live *P. multocida* in 300ml PBS) calves, negative response for calf 709; (c) Group 2a (10^9 cfu formalin-killed *P. multocida* in 300ml PBS) calves; (d) Group 2b (10^9 cfu formalin-killed *P. multocida* and 10^9 cfu live *P. multocida* in 300ml PBS) calves, negative response for calf 172.

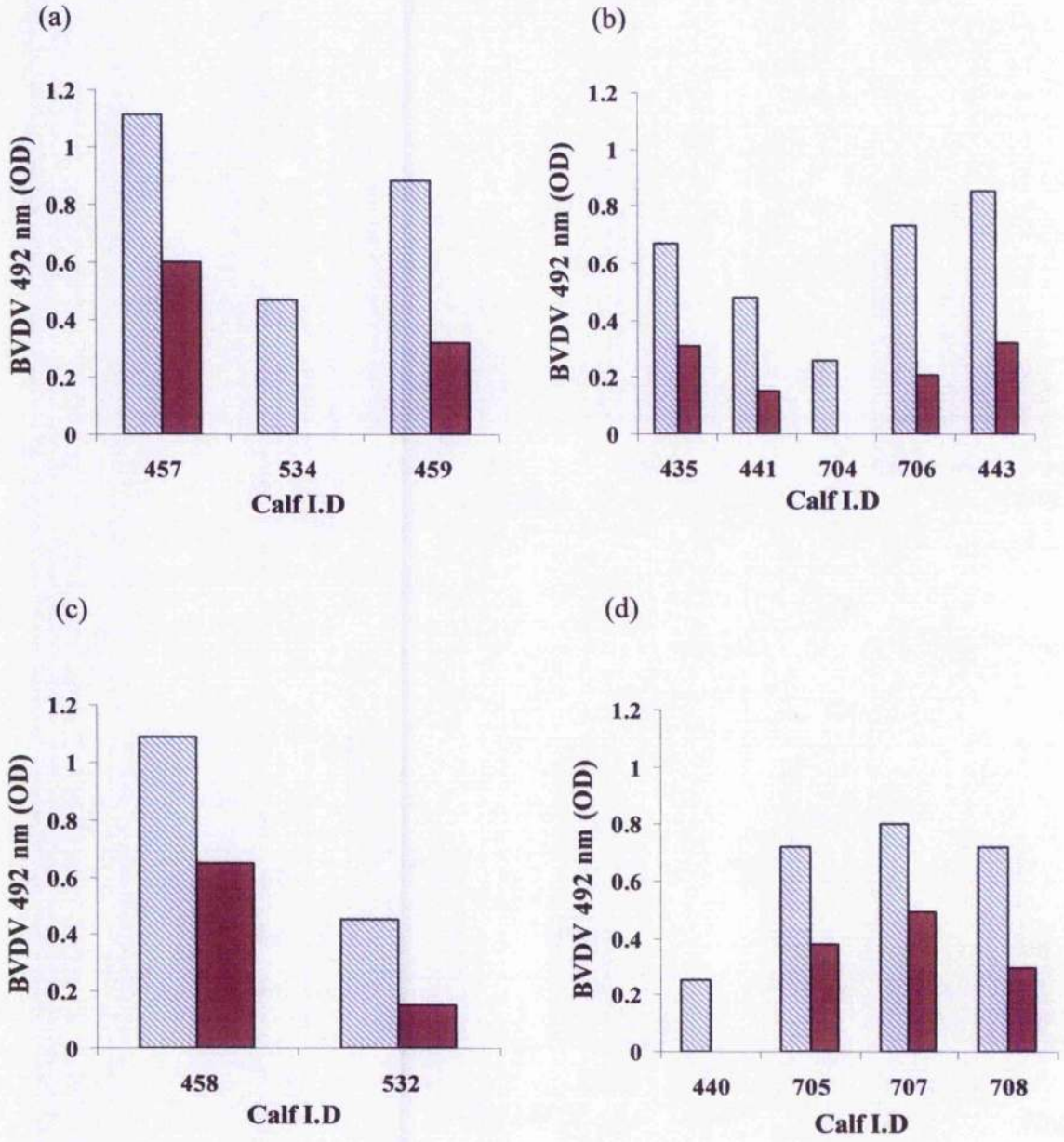


Fig. 7.5. BVDV antibody titres in pre-infection (▨) and post-mortem (■) (28 days) bleeds from calves inoculated with PBS, formalin-killed *P. multocida* and *P. multocida* A:3 at 10^9 cfu in 300 ml PBS. Data presented corresponds to challenge groups; (a) Group 1a (300ml PBS) calves, negative response for calf 530; (b) Group 1b (300ml PBS and 10^9 cfu live *P. multocida* in 300ml PBS) calves, negative response for calf 709; Group 2a (10^9 cfu formalin-killed *P. multocida* in 300ml PBS) calves, negative response for calves 529 and 531; (d) Group 2b (10^9 cfu formalin-killed *P. multocida* and 10^9 cfu live *P. multocida* in 300ml PBS) calves, negative response for calves 437 and 172.

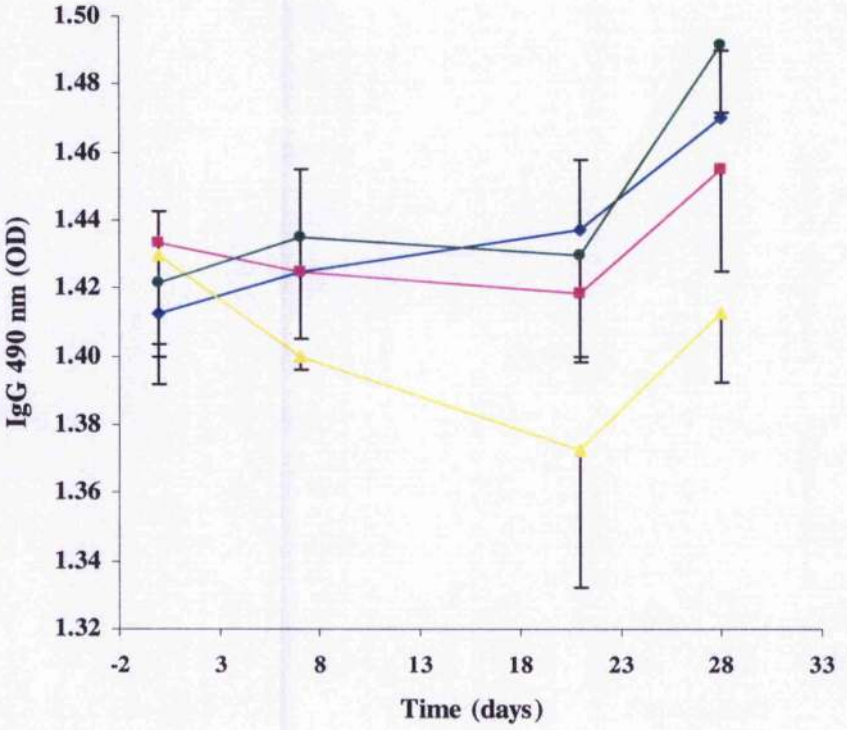


Fig. 7.6. Mean (± SE) plasma IgG titres in pre-infection (day 0), post-infection (day 7 and 21) and post-mortem (day 28) bleeds from calves inoculated with PBS, formalin-killed *P. multocida* and *P. multocida* A:3 at 10^9 cfu in 300 ml PBS . Data presented correspond to challenge groups. Group 1a: 300ml PBS (◆), Group 1b: 300ml PBS and 10^9 cfu live *P. multocida* in 300ml PBS (■), Group 2a: 10^9 cfu formalin-killed *P. multocida* in 300ml PBS (▲), Group 2b: 10^9 cfu formalin-killed *P. multocida* and 10^9 cfu live *P. multocida* in 300ml PBS (●).

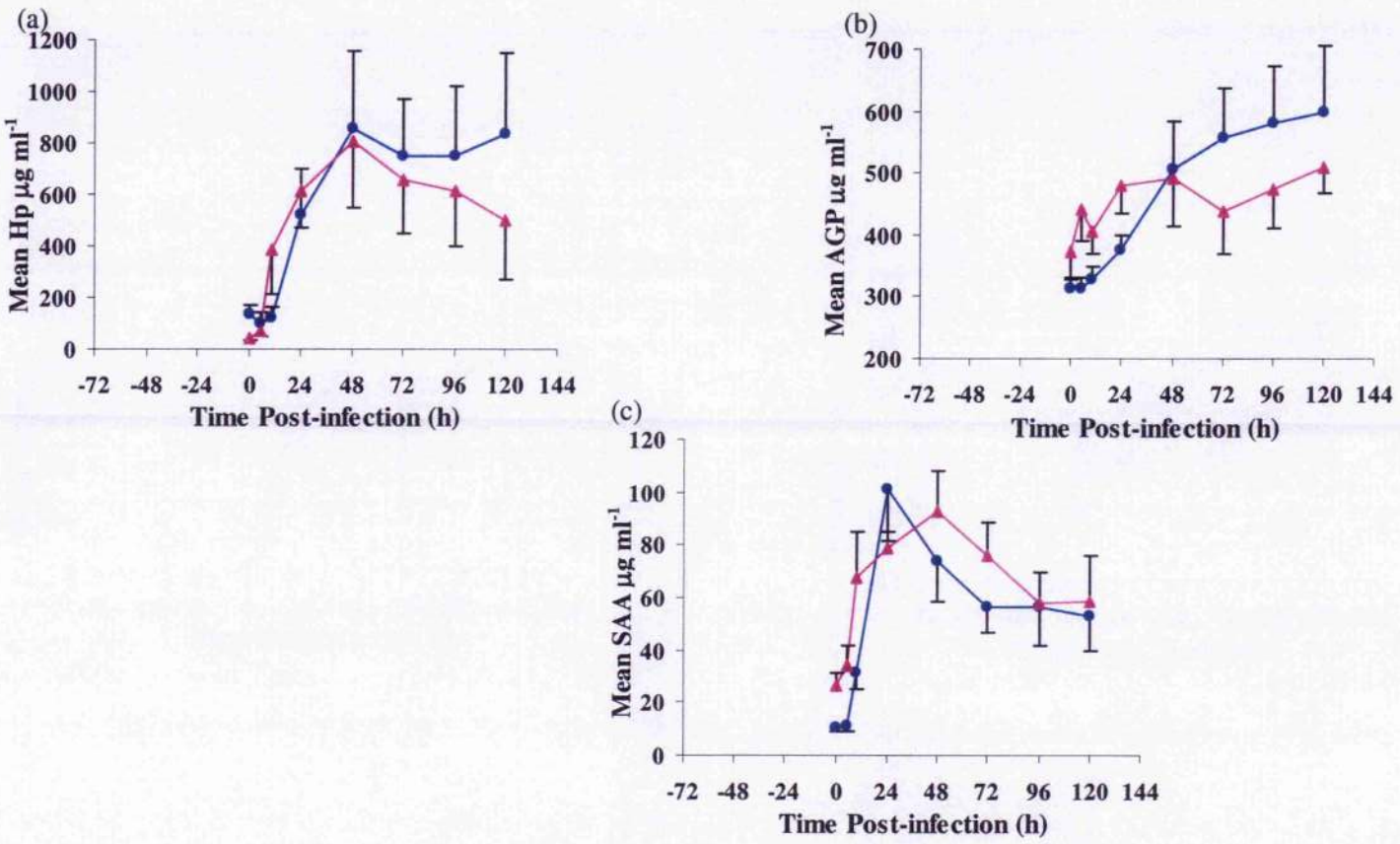


Fig.7.7. Mean (±S.E) plasma concentrations of haptoglobin (Hp), α_1 acid glycoprotein (AGP), and amyloid A (SAA) from calves inoculated with *P. multocida* A:3 at 10^9 cfu in 300 ml PBS. (a) Hp (µg ml^{-1}); (b) AGP (µg ml^{-1}); (c) SAA (µg ml^{-1}). Data presented corresponds to challenge groups 1b: 300ml PBS and 10^9 cfu live *P. multocida* in 300ml PBS (●) and 2b: 10^9 cfu formalin-killed *P. multocida* and 10^9 cfu live *P. multocida* in 300ml PBS (▲).

Thereafter the SAA increase in Group 2b was more gradual and sustained for longer to 48 h p.c, peaking at 92.8 (± 15.4) μg ml^{-1}. Thereafter SAA concentrations in both treatment groups decreased to about 55.7 (± 10.3) μg ml^{-1} at 96 h p.c., and remained at this level for the remainder of the experiment. There were differences in the SAA response between Groups 1b and 2b, although these differences were not statistically significant. However, the group of calves given only PBS (Chapter 6) had approximately half the mean SAA levels of Group 1b calves throughout the 5 day sampling periods between day 0 and day 5 and between day 21 and day 26.

Calves that had high levels of α_1AGP prior to exposure with live *P. multocida* tended to have high levels following infection. Increases in the mean plasma α_1AGP concentration for Group 1b animals were more gradual and sustained than those observed for Hp or SAA (Fig.7.7.b). All calves in Group 1b exhibited a steady increase in the α_1AGP response over the course of the experiment, similar to the α_1AGP response reported in Chapter 4, but for Group 2b calves (Fig.7.7.b) this rise was less sustained and reached an initial peak mean concentration of 492 (± 77.3) μg ml^{-1} at 48 h p.c., decreasing to 438 (± 70.1) μg ml^{-1} at 72 h p.c. Thereafter the mean α_1AGP concentration increased gradually to 510 (± 43.4) μg ml^{-1} by the end of the sampling period. There was no evidence that any of the differences in the α_1AGP response between Groups 1b and 2b were statistically significant.

There was no evidence of statistically significant differences in the responses of Hp, α_1AGP or SAA to treatment between Groups 1b and 2b animals. There was no evidence of a difference between the treatment groups in the change of these acute phase proteins from the period following challenge with formalin-killed *P. multocida* (Chapter 6) to challenge with live *P. multocida*.

7.3.3. Gross Pathology of the lung

Affected areas of lung tissue were collapsed, consolidated, varied from pink to purple in colour and often contained abscesses and thickened gelatinous pleura (Fig. 7.8). These observations were characteristic of all calves given the live challenge (Groups 1b and 2b) with the apical lobes being affected consistently (Fig. 7.8). On many occasions the apical lobes had fibrinous adhesions to the thoracic wall and the pericardium. Caudal lung lobes were variably affected with the lesions more scattered. The gross pathological features described here for Group 1b calves are similar to those described for calves given the same treatment (Chapter 4).

On sectioning of the affected areas the bronchial mucosa was oedematous, inflamed and often contained exudate within the airways. The mean percentage of surface lung affected was 23 % for calves in Group 2b and 16 % for calves in Group 1b and this difference was statistically significant

($p = 0.013$) (Table 7.1.). There was a statistically significant correlation between Hp and percentage of lung affected. Calves with high values of Hp tended to have greater lung damage ($p = 0.031$). Pleurisy was observed in all four groups, but there was no correlation between degree of pleurisy and treatment, though the extent of pleurisy was more pronounced in animals from Groups 1b and 2b (Table 7.1.) than those animals in Groups 1a and Group 2a (Chapter 6).

Bronchial and mediastinal lymph nodes from infected animals were often inflamed and in some cases (Calf 437 Group 2b) the cortex of the bronchial lymph nodes was barely discernible. In calf 706 (Group 1b) the pericardium appeared inflamed and contained excess yellow/green pericardial fluid. The tracheal mucosa of calves given the live challenge of *P. multocida* was yellow, inflamed and mucus was often present. The trachea appeared normal in control animals.

7.3.4. Bacteriology of the lung

P. multocida was recovered from the lungs of the 12 calves challenged intratracheally with live *P. multocida*, primarily in lobules exhibiting extensive consolidated lesions. The viable counts of tissue from individual calves ranged between 5.3×10^4 cfu g^{-1} (Group 2b) and 4.0×10^7 cfu g^{-1} (Group 1b) (Table 7.1.). On average, recovery of bacteria from lung tissue was lower in Group 2b than in Group 1b animals by approximately 1 log. The mean *P. multocida* lung count was similar for infected animals (Groups 1b, 2b) and there was no significant effect of treatment on bacterial load. However, calves with high temperatures also tended to have a higher bacterial count and this correlation was statistically significant ($p = 0.029$). There was no evidence of bacterial count being associated with respiratory rate, or any of the APP (Table 7.1.). *P. multocida* was also recovered from tracheal swabs of all calves, which was to be expected due to the inoculation of live bacteria. Bacteria were recovered from only one calf (Chapter 4) given 300 ml 10^9 cfu *P. multocida*, whereas in this study bacteria were recovered from all calves in Group 1b and Group 2b.

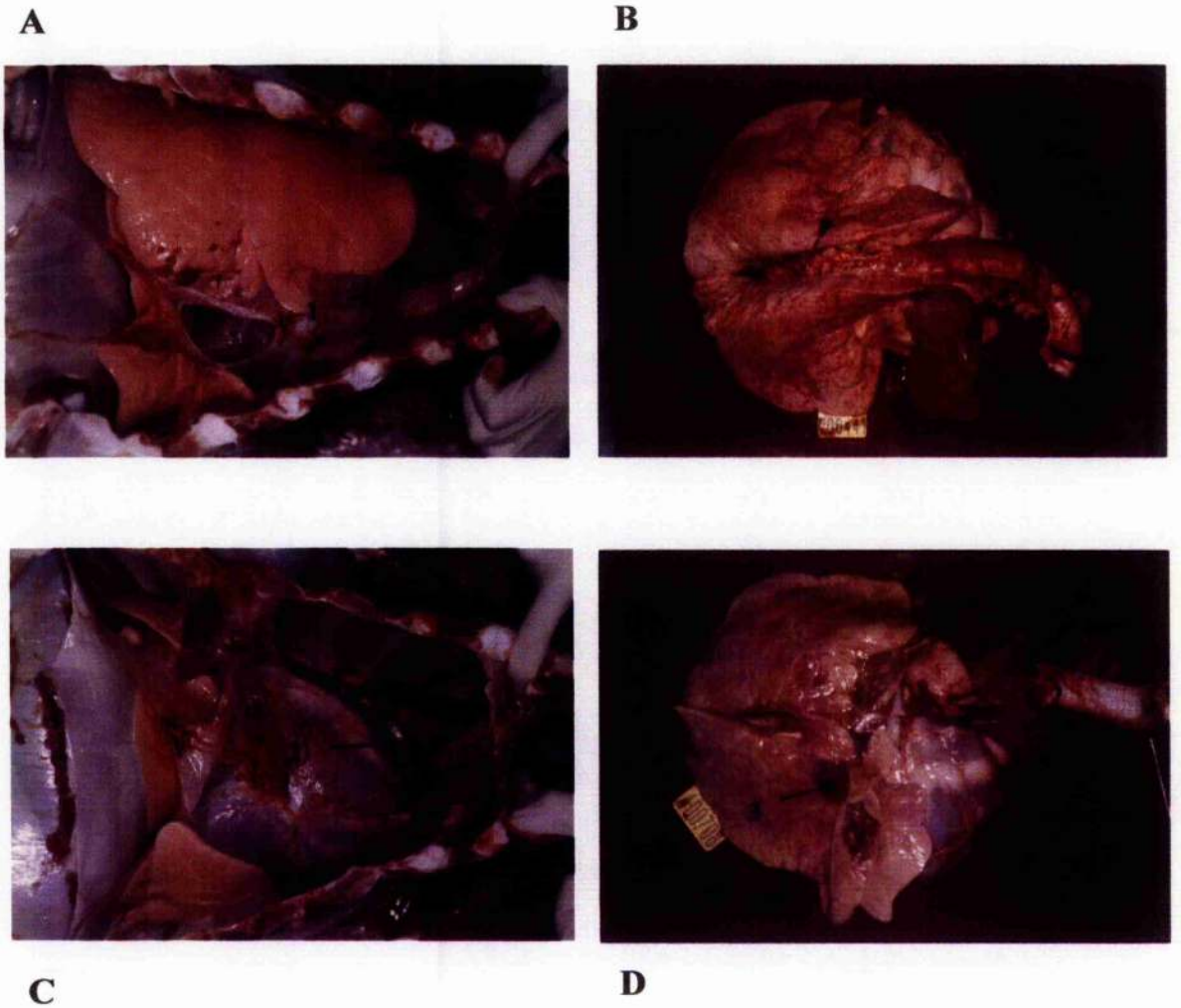


Fig. 7.8. Gross pathological changes observed for calves in Group 1b and Group 2b given homologous challenge on day 21 following challenge with either PBS or formalin-killed *P. multocida*. **A:** Dorsal surface of lungs *in situ* from Calf 441 (Group 1b) and **B:** Dorsal view of the same lungs removed from the thoracic cavity; consolidation of the right apical lobe is appreciable in both photographs (1). **C:** Dorsal surface of lungs *in situ* from Calf 708 (Group 2b) and **D:** Ventral view of the same lungs removed from the thoracic cavity. Consolidation of the right apical lobe is evident in both photographs (2) and some flecks of necrotic tissue in the upper area of the caudal lobe (3).

Table 7.1. Percentage of lung surface affected by lesions, pleurisy scores and bacterial recoveries from lung tissue (cfu g^{-1}) of Group 1b and Group 2b calves.

Group	Calf	% lung surface affected	Pleurisy	Viable lung count (cfu g^{-1})
1b	435	18	1	1.2×10^5
	441	12	0	1.3×10^6
	704	3	2	1.2×10^5
	706	28	1	4.0×10^7
	443	10	0	4.2×10^5
	709	28	0	4.0×10^6
2b	707	15	0	1.1×10^6
	172	21	1	1.1×10^6
	708	21	0	4.8×10^5
	440	24	1	1.6×10^6
	705	31	1	5.3×10^4
	437	27	1	1.1×10^5

7.3.5. Histopathology

Lesions observed in the lung samples varied from mild-moderate to severe multifocal fibrinopurulent bronchopneumonia. Lesions were well compartmentalised by the prominent inter-lobular septa present in bovine lung. The predominant inflammatory cell was the neutrophil indicating the lesions were acute in nature.

All six calves assigned to Group 2b showed evidence of acute, severe broncho-interstitial pneumonia in the right apical lobe (Fig. 7.9). Only half of the calves had lesions in the left apical lung lobe and these ranged in severity from mild to severe with half having mildly affected caudal lung lobes (Fig. 7.9). BALT appeared to be very active in some sections from calves 172 and 437. Mild to severe alveolar oedema was present in 50 % of calves and a high level of neutrophils present in airways, alveolar air spaces, migrating through the respiratory epithelium of all 6 calves. All six calves assigned to Group 1b showed evidence of acute, severe to very severe broncho-

interstitial pneumonia with the right and left apical lung lobes affected consistently (Fig.7.10). BALT appeared stimulated in 5 of the 6 calves in various lung lobes with no evidence of severe depletion of lymphoid cells. Alveolar oedema was present occasionally, mostly mild but severe in the left apical lobe of calf 435. Occasional foci of neutrophils in airways, alveolar air spaces and respiratory epithelium were present (Fig.7.10). Bacteria were not recovered from pericardial or pleural fluid, or from heart blood. Bronchial (right and left) and mediastinal (mid and caudal) lymph nodes appeared active in all calves as denoted by the presence of a large number of secondary follicles. PMN leukocytes were present along the cortical trabeculae and in the medullary cords of the lymph nodes. No significant histopathological changes were observed in any of the samples taken from the spleen, heart, liver or kidney.

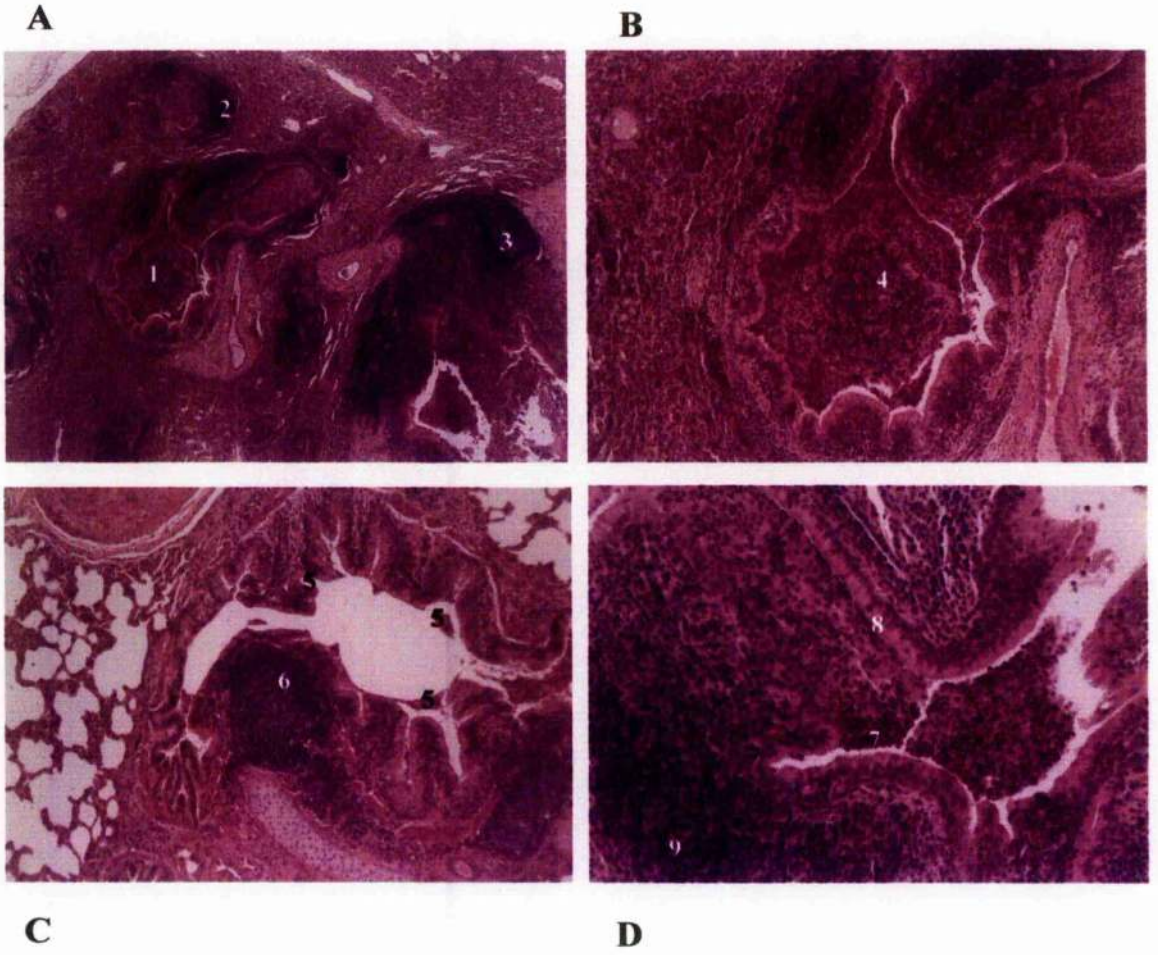


Fig. 7.9. Histopathological changes observed in Group 2b calves challenged with 10^9 cfu formalin-killed *P. multocida* in 300ml PBS and also with 10^9 cfu live *P. multocida* in 300ml PBS, 3 weeks later. **A:** Calf 172 28 d p.c., right apical lobe (H&E, original magnification x 40); severe bronchopneumonia as denoted by collapse and consolidation of lung parenchyma and airway lumen completely full of inflammatory exudate (1). Note the presence of BALT (2), some containing germinal centres (3). **B:** Calf 172 28 d p.c., right apical lobe (H&E, original magnification x 100) illustrating complete obliteration of the luminal airspace by exudates (4). **C:** Calf 437 28 d p.c., left caudal lung lobe (H&E, original magnification x 100); note mild inflammatory cell infiltration into the bronchial airway (5). Note the presence of BALT (6). **D:** Calf 437 28 d p.c., right apical lobe (H&E, original magnification x 200); airway inflammatory exudate consists of mixed, predominately PMN, inflammatory cells with a significant population of what appears to be activated macrophages (7). Note the presence of PMN streaming through the intact respiratory epithelium (8) and the sub-epithelial mass of lymphoid cells that comprise the BALT (9).

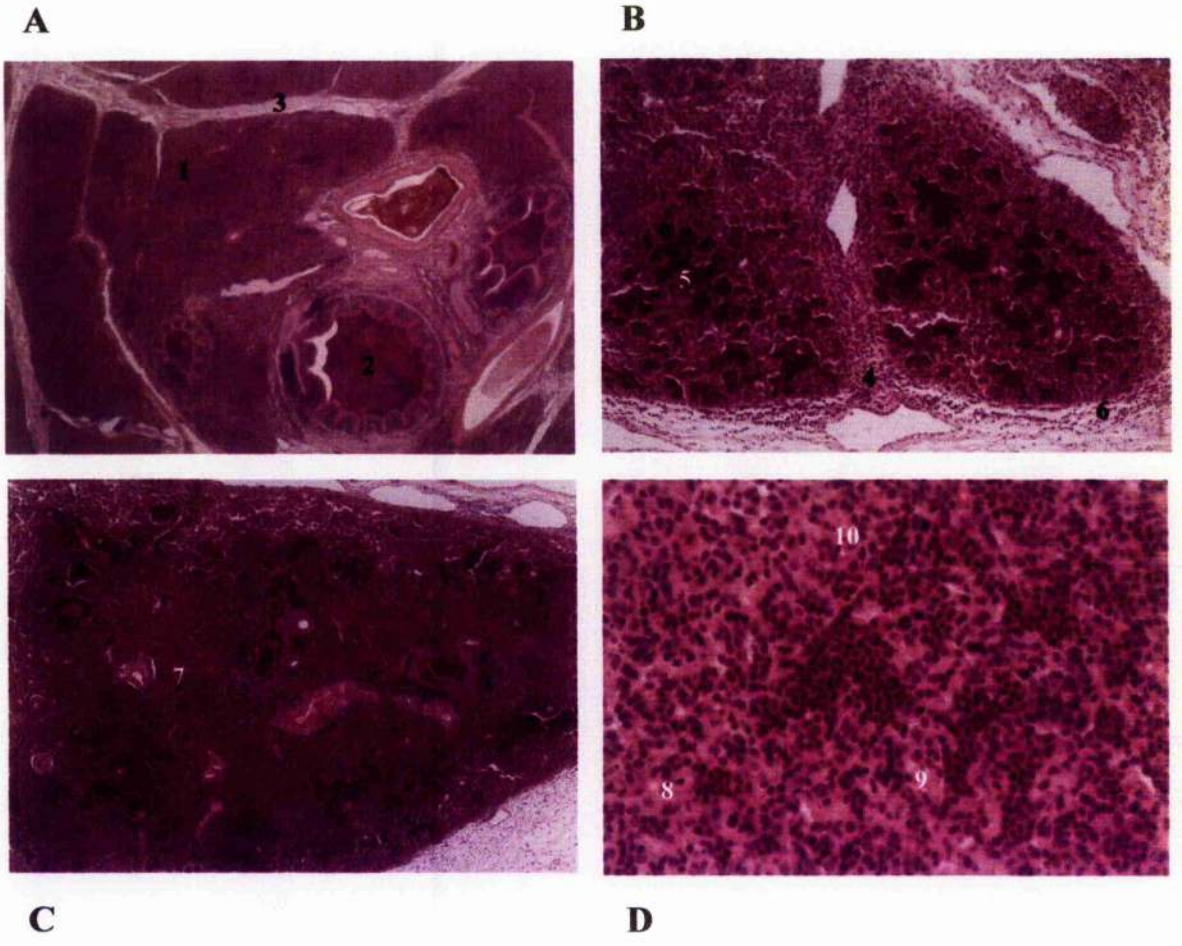


Fig.7.10. Histopathological changes observed in Group 1b calves infected with 10^9 cfu live *P. multocida* in 300ml PBS. **A:** Calf 443 28 d p.c., left apical lobe illustrating severe broncho-pneumonia (H&E, original magnification x 40); large areas of collapsed and consolidated alveoli (1) are present along with a severe inflammatory cell infiltration while bronchi and bronchiolar airspaces are obliterated due to the presence of inflammatory cells (2) and interlobular septa are swollen and oedematous (3). **B:** Calf 441 28 d p.c., right apical lobe (H&E, original magnification x 100); inflammatory cells are PMN (4), while alveolar air spaces contain many PMN (5) and interlobular septa appear swollen and oedematous (6). **C:** Calf 441 28 d p.c. right apical lobe illustrating severe broncho-pneumonia (H&E, original magnification x 40); alveoli are collapsed, consolidated and contain inflammatory cells (7) and all airways contain inflammatory exudates. **D:** Calf 443 28 d p.c., left apical lobe (H&E, original magnification x 400); inflammatory cells can be identified as predominately PMN (8), while monocytes (9) and activated macrophages (10) are also present.

7.3.6. Nasal swab analysis

Nasal swab cultures from 10 of the 12 calves prior to live challenge were positive on day 21 of the experiment, before exposure to a homologous challenge of *P. multocida* (Table 7.2).

Table 7.2. *P. multocida* detection from nasal swabs taken periodically prior to challenge with live *P. multocida* A:3 and at PM (day 28) for calves in Group 1b and Group 2b.

Group	Calf I.D	Prior to challenge	Week 1	Week 2	Week 3: Prior to challenge with live *P. multocida*	Week 4: PM
1b	435	-	+	-	+	+
	441	-	+	-	-	+
	704	-	-	-	+	+
	706	-	+	-	+	+
	443	-	+	-	+	+
	709	-	+	+	+	+
2b	707	-	+	-	-	+
	172	-	+	+	+	+
	708	-	-	-	+	+
	440	-	+	-	+	+
	705	-	-	+	+	+
	437	-	-	-	+	+

7.4. Discussion

The study was designed to evaluate the protective effects of formalin-killed *P. multocida* against a homologous (live) challenge as assessed by measuring pathophysiological changes. In addition, comparisons were made between the pathophysiological responses following homologous challenge in Group 1b calves and the pathophysiological responses following live challenge (300ml at 10^9 cfu) in Chapter 4.

Clinical responses to live *P. multocida* such as pyrexia and increases in respiratory rate were comparable to those responses observed previously (Chapter 4), except for rectal temperature which was slightly lower (40.2°C) in this study. There was no difference in the clinical response for the initial phase of the experiment (0-24 h p.c.) in calves belonging to groups 1b and 2b indicating that exposure to formalin-killed *P. multocida* failed to stimulate the appropriate immune response necessary to provide protection for calves on exposure to live *P. multocida*. Furthermore, clinical signs for Group 2b calves seemed to be more severe than for Group 1b from 24 h p.c. until the end of the experiment. The sizable clinical response and percentage of surface lung affected for Group 2b calves compared to Group 1b calves indicated that the formalin-killed *P. multocida* challenge exacerbated the clinical response to exposure to homologous challenge. At this point one would consider that formalin-killed *P. multocida* was detrimental to the host, yet a log lower bacterial burden was recorded for the calves in Group 2b compared with calves in Group 1b. One may postulate that calves in Group 2b had a lower proportion of apoptotic neutrophils than calves in Group 1b and that the presence of a higher proportion of neutrophils is likely to result in the removal of more bacteria while causing extensive lung damage. The fraction of apoptotic neutrophils in the recovered BALF was not assessed in this study though this would be of value for future work.

The APR exhibited by animals (Chapter 4) given the same treatment as calves in Group 1b in this study, demonstrated a far more gradual increase in SAA concentration that peaked at 23 h p.c., though the time to peak was the same in both cases. However, the SAA concentration was lower by 400 mg l^{-1} for calves in chapter 4 than that recorded in this study. Calves (Chapter 4) given 300 ml 10^9 cfu *P. multocida* showed a very slow rise in α_1AGP which was quite different to that described in this study for Group 1b calves. Calves (Chapter 4) given 300 ml 10^9 cfu *P. multocida* demonstrated a more gradual increase in Hp concentration than that observed in Group 1b in this study, and the peak Hp concentration was higher and occurred at the same point (48 h p.c.) as that observed in this study. Overall, the levels of Hp and SAA detected in the plasma reported in Chapter 4 were greater; in contrast mean AGP levels in Chapter 4 were lower, perhaps due to between-animal variation in the innate APR. The lack of a significant difference in APR between

the vaccine group 2b and the control Group 1b was evidence that prior exposure to formalin-killed *P. multocida* alone had little effect on the systemic system.

The fact that these clinical and APR occurred in Group 2b animals following exposure to a homologous challenge indicated that formalin-killed *P. multocida* did not prime the immune system as anticipated initially, leading to uncertainty with regard to the method of bacterin production, the quality of the bacterin formulation and the mode of administration. It is possible that formalin treatment may have destroyed the conformational structure of the epitopes responsible for induction of immunity. This has been the possible cause for failure of formalin-inactivated BHV-1 vaccines, wherein certain BHV-1 glycoprotein epitopes are highly susceptible to destruction by formalin treatment (Duque *et al.*, 1989). However, it has been shown that low concentrations of formaldehyde, as used in this study, maintain the antigenic integrity of many important epitopes involved in inducing protection although the concentration of induced pathogen-neutralising antibodies is low (Nencioni *et al.*, 1991). It is feasible, therefore, that the formalin-killed challenge contained an insufficient concentration of protective antigens and that one or more repeat instillations of bacterin may be required to achieve and maintain the optimum level of protective antigens and stimulate the immune response.

Evidence, in this study, for the bacterin's inability to produce a protective immune response stems from the serum IgG response. Prior to challenge in 8 week old conventionally reared calves, antibody to respiratory viruses and *P. multocida* was detected and the age of the animals indicated that this was due to immunity passively acquired from the mother via colostrum and or their own immune response, which is often well developed at 8 weeks of age. In all samples the antibody levels to the viruses (BVDV, PI-3, RSV and IBRV) decreased with time, indicating that the viral antibody at the start of the experiment was attributable to passive transfer and not to an active response to infection.

Following challenge with formalin-killed *P. multocida* there was a low and biologically insignificant change (0.03 OD) over the four weeks of the experiment in the high level of IgG detected prior to challenge with live *P. multocida*. Work by Black *et al.*, (1985) showed an optimum level of systemic antibody when vaccination is expected to work, above which the presence of maternally derived antibodies (MDA) may suppress the response to vaccination. The lack of an IgG response in this study may have been due to high levels of MDA prior to challenge with formalin-killed *P. multocida* that could have suppressed the development of the young calf's active immune responses to these antigens (Black *et al.*, 1985). For future studies it would be of benefit to define the level at which antibody provides protection and to challenge the calves below this point, reducing the risk of MDA interfering with the active response to vaccination.

The route of challenge adopted in this study may be a contributory factor to the lack of an IgG response. Effendy *et al.*, (1998) demonstrated that repeated intranasal exposures to formalin-killed *M. haemolytica* A:2, followed by an intratracheal challenge, led to the local production of IgG and IgA and stimulated also a systemic antibody response. Intratracheal instillation of formalin-killed bacteria as used in this study may have bypassed the priming of the mucosal epithelial layer of the URT which, when analysing the work conducted by Effendy and others (1998), seems to be a requirement for stimulation of systemic immunity. When evaluating the nature of formalin-killed bacteria, which are fixed and have no independent movement or opportunity to disseminate via, for example, penetrating the mucosal epithelial barrier, one would expect the immune response to be highly localised and the release of antibodies, primarily IgA into the lung and not into the systemic system (Wilkie, 1982). In this experiment the route of infection was the same as that used in Chapter 4 so that a direct comparison could be made between the pathophysiological responses attributed to live and killed *P. multocida*.

Lung pathology of animals given formalin-killed *P. multocida* was attributable to the host response to foreign material. However, *P. multocida* in the range of 1.3×10^3 and 1.2×10^7 cfu ml^{-1} was recovered from these animals, suggesting that the role of natural infection must be addressed since these calves were conventionally derived, with access to colostrum and the risk of natural exposure to *Pasteurella* spp. It has been reported that prior natural exposure to *Pasteurella* spp. enhanced resistance to an experimental infection of pneumonic pasteurella (Confer *et al.*, 1984). In the current work, the challenge dose of live *P. multocida* increased considerably the surface area of lung affected in animals primed with formalin-killed *P. multocida* (Group 2b), probably due to the ability of *P. multocida* to proliferate and disseminate leading to extensive lung pathology.

Group 1b calves showed a log higher lung bacterial burden, than did Group 2b calves. This result seems to indicate that the lung clearance mechanism for Group 2b calves is more efficient at removing bacteria than for Group 1b animals. A possible advantage of administering formalin-killed *P. multocida* but this result was not statistically significant. However, the restrictive sampling technique on lung tissue for recovery of bacteria may have omitted areas containing a high bacterial load. This was discussed in some detail earlier (Chapter 4) and may provide an explanation for this 1 log difference in bacterial recovery between Groups 1b and 2b, thereby ruling out a possible protective factor of formalin-killed *P. multocida* challenge.

P. multocida was recovered from the lungs of all calves in Group1b but this was not the case for calves given similar treatment previously (Chapter 4), where bacteria were recovered from only one calf. It may be possible that the presence of a persistent underlying infection in Group 1b calves exacerbated the pathology resulting from the live challenge with *P. multocida* by

overloading the alveolar macrophages with bacteria and contributing to the reduced lung clearance in this study.

A significant, positive correlation ($P<0.05$) was found between lung damage and concentration of plasma Hp for calves in Group 2b. One may postulate from this result that lung damage could be responsible for the local production of Hp. The synthesis of Hp in the alveolar fluid within the lung has been reported and moreover provides a source of antioxidant and antimicrobial activity (Yang *et al.*, 1995). The mechanism of Hp induced antimicrobial activity has been demonstrated in transgenic mice where Hp was over-expressed in alveolar macrophages (Yang *et al.*, 2003). These mice demonstrated an increase in haemoglobin clearance leading to attenuated blood-induced lung injury and inflammation attributable to a lung bacterial infection.

If killed products of *P. multocida* are to become effective vaccine antigens, it would be of benefit to focus research on identifying and characterising the protective antigens (Confer *et al.*, 1985). Furthermore, the choice of adjuvant is vital to ensure a good immune response (Confer *et al.*, 1985). Studies using different adjuvants have demonstrated a significant difference in the serologic responses in calves. For example, a greater serum antibody response was observed in calves vaccinated with bacterin, initially with Freund's complete adjuvant followed 7 d later with Freund's incomplete adjuvant resulting in enhanced resistance against exposure to live *M. haemolytica*, when compared to aluminium hydroxide in gel adjuvant (Confer *et al.*, 1987). A similar result has been demonstrated in lambs vaccinated with capsular *M. haemolytica* combined with different adjuvants (Wells *et al.*, 1979). An adjuvant was omitted from the bacterin formulation in this study as the main aim was to make a direct comparison between responses to challenge with live (Chapter 4) or killed (Chapter 6) *P. multocida*.

In summary, the observed pathophysiological responses indicated that the formalin-killed *P. multocida* treatment was ineffective in protecting the animal from a live formulation of *P. multocida*. Instead it appeared to make the animal more susceptible to live challenge as shown by the percentage of surface lung area affected. However, the discovery that calves in Group 2b had a log lower bacterial load than did calves in Group 1b indicated that formalin-killed *P. multocida* may have induced the production of local antibody, neutralising the presence of immunogens and, furthermore, the proportion of apoptotic and killer neutrophils may favour greater bacterial clearance for Group 2b animals. However, it would seem reasonable given the lack of conferred protection in this study that the lung would not be the preferred route of immunization. From the results documented by Effendy *et al.*, (1998) and Zamri-Saad *et al.*, (1999), repeated intranasal instillations of formalin-killed bacteria would seen to be the preferred route of challenge in order to enhance the systemic immune response and to lead to better protection against exposure to a homologous challenge.

Chapter 8. General Discussion

The mechanisms by which *P. multocida* A:3 causes disease are not fully characterised. The aims of this thesis were to develop a progressive and reproducible model of pneumonic pasteurellosis in conventional calves, comparable with the clinical and pathological responses observed in field cases that would allow also a more detailed characterisation of disease pathogenesis. Using the model, analyses of the pathophysiological responses to live and formalin-killed formulations of *P. multocida* were conducted insight into key mechanisms of host-pathogen interactions were gained by the investigation of cellular and secretory responses to infection within the lung.

This general discussion will summarise the key findings of the work in the context of current knowledge, emphasising their specific and general relevance to the topic of pneumonic disease. For the questions which remain unanswered, suggestions are made which may provide the basis for future work.

The increasing incidence of *P. multocida* isolation in cases of bovine pneumonic pasteurellosis and from calves that appear healthy indicates the need for detailed research into what is an emerging disease. Characterisation of the bacterium and, specifically, how it interacts with the host will help us understand the pathogenesis of the disease and provide the information necessary to design novel methods of detection and control.

LPS is considered an important virulence factor (Adler *et al.*, 1999). Therefore, a preliminary epidemiological study was conducted to characterise the LPS from *P. multocida* A:3 and from the MRI challenge strain (671/90). This LPS, and that of all field isolates, was found to be of the rough form, indicating that none of the collected strains were able to synthesise the variable region, the O-antigen. This discovery that may facilitate the development of cross-reactive vaccines targeted against the more conserved core region of LPS. However, LPS chemotype profiling of the core region of these isolates exhibited 6 LPS core banding patterns, while immunoblot studies revealed the potential for low molecular weight core bands to remain concealed from the host surveillance system. This finding demonstrates the potential for diversity and a strategy for the pathogen to evade host immune defence components. There was no evidence that a particular LPS structure was associated with either commensal or pathogenic *P. multocida* strains.

Modification of LPS in Gram-negative bacteria plays a vital role during infection in the evasion of immune system recognition, resistance to host antimicrobial proteins and maintenance of virulent genes (Gunn and Miller, 1996). *Neisseria gonorrhoeae* are known to avoid immune recognition by the transfer of sialyl groups to the surface of its LPS and this inhibits any possible reaction with

bactericidal antibody (Parsons *et al.*, 1989). Structural modifications of *Salmonella typhimurium* LPS, such as fatty acid additions to the core and lipid A regions and acetylation of the O-antigen are induced by a two component regulator PhoPQ, which regulates genes for the intracellular survival of *S. typhimurium* in macrophages and resistance to cationic peptides (Guo *et al.*, 1997). A PhoPQ system has also been identified in *Yersinia pestis* which expresses lipo-oligosaccharide (LOS) and alterations to the core region of LOS may be required for the survival of *Yersinia pestis* (Hitchen *et al.*, 2002). No such PhoPQ regulatory system has been identified for *P. multocida* LPS, which may provide key clues for the control of *P. multocida* pathogenesis and lead to novel approaches for the prevention of pasteurellosis.

Pulsed-field gel electrophoresis characterised the genetic aspect of the bacterium and revealed the potential for much genetic diversity. Thus it is unlikely that any one simple control strategy would prove effective. It is anticipated that the findings presented in Chapter 3 will initiate a larger epidemiological study that will stimulate further investigation in this area.

An experimental model of BPP was a first and essential step to simulate cases of BPP occurring in the field in order for studies of pathogenesis to proceed. Despite several attempts reported in the literature (Ames *et al.*, 1985; Gourlay *et al.*, 1989; Chengappa *et al.*, 1989) to produce a suitable animal model of *P. multocida* infection there was no reliable, reproducible model that closely mimicked the field situation. The approach adopted was to use groups of animals each subjected to challenge with 4 different combinations of the MRI rough *P. multocida* type A:3 strain and volume of bacterial suspension. The results presented in Chapter 4 show that 300 ml at 10^9 cfu of *P. multocida* suspension produced progressive disease, eliciting a moderate to severe clinical, biochemical and pathological response lasting 24 to 48 hours and representative of the situation observed in field cases. Clinical signs of disease showed a rapid onset, appearing by 5 h p.c., and a short course, with fever, cough, respiratory distress and dyspnoea being common symptoms amongst infected animals. Calves that recovered from the bacterial infection often had persisting ill health that, in a commercial context, would be expected to reduce the milk output and meat quality of the animal. Levels of APP were determined as they have been shown to be highly sensitive indicators of bacterial infection (Horadogoda *et al.*, 1994; Godson *et al.*, 1996). This work showed for the first time that significant increases in the concentrations of APP, in particular Hp, were associated with *P. multocida* A:3 challenge. A similar Hp response has been observed following the intravenous inoculation of *P. multocida* serotype B:2 endotoxin in buffalo (Horadogoda *et al.*, 2002). The tip of the bronchoscope was placed at the bifurcation of the trachea when dosing the calves, making it likely that the greater volume of bacterial suspension disseminated affecting a greater area of lung than did the lesser volume (60 ml) used (Donachie, unpublished observations). The experimental model (Dowling *et al.*, 2002) has made a significant contribution to the work described in the thesis and the consistency of the pathophysiological responses between the animal

experiments reflect the reliability and reproducibility of the model which is of importance for future experimental studies.

Subsequent work (Chapter 5) confirmed the reliability and reproducibility of the animal model in terms of clinical and pathological responses to infection. Control animals were incorporated into the study to determine whether a large volume of PBS alone and the invasive challenge procedure using the bronchoscope would have any adverse effects on the clinical response of the animals or whether the responses observed (Chapter 4 and Chapter 5) could be attributed primarily to *P. multocida*. The latter was shown to be the case as calves that were challenged with PBS alone exhibited only a slight rise in mean rectal temperature and respiratory rate and these remained within the normal range. In addition, lung and nasopharyngeal samples were free of *P. multocida*.

Reproducing the model (Chapter 5) and the establishment of a proteomics unit at MRI provided an ideal opportunity to analyse the constituents of the pulmonary lung fluid prior to infection and at day 1 and day 4 following experimental infection with *P. multocida*. The choice of these sampling days was made for the following reasons. Firstly, due to the timing of the clinical response described in Chapter 4, it was appropriate for the lavage to take place on the day following challenge. Secondly, the HO project licence required a minimum of 3 days between lavages, this limited the opportunities for examining the pulmonary defence system during progression of the disease but was considered necessary with respect to animal welfare. Nevertheless, this use of live lavage was entirely novel and provided information for the first time on events within the lung during the development of pneumonic pasteurellosis.

It would be of great value for vaccine development to be able to identify host and bacterial proteins within lung fluid. Bacterial proteins may have the potential to play a significant role as protective antigens in the pathogenesis of pneumonic disease. Immune sera raised against *P. multocida* A:3 could be used to probe for the presence of bacterial proteins by western blotting of 2-DE gels. Host proteins identified during the inflammatory response included Annexin I and V, apolipoprotein A-I precursor, antioxidant protein 2, serotransferrin precursor and antimicrobial peptides. However, these proteins were not associated specifically with *P. multocida* because they were also found in the pulmonary fluid of calves administered PBS. No bacterial proteins were identified using the *P. multocida* database (http://lenti.med.umn.edu/pub/PM70/).

Albumin comprised almost half of the host protein found in the lung and often masked the recovery of high molecular weight lung fluid proteins, thus obscuring an area of gel that contains proteins with potentially vital roles in the pathogenesis of disease. Future studies on BALF analysis will concentrate on the removal of albumin from the lavage sample by running the samples through either an affinity column with bound antibodies to bovine albumin (Lopez, *et al.*, 2002) or a column (affinity-blue) with immobilin dye which is known to bind albumin, thus allowing other

proteins of a similar mass and pI to appear on the 2D-gel. The use of 11 cm gels restricted the amount of protein extract to be loaded onto gels and decreased the probability of detecting low abundance proteins and the resolution of the protein spots. In retrospect, these problems could have been overcome by using larger 18 cm gels with a narrow pH gradient. Moreover, larger gels would have allowed more efficient protein separation (Sabounchi-Schutt *et al.*, 2003).

The animal model was used to study and compare host responses to both live and killed *P. multocida* A:3 challenge. It was important to see if 300 ml at 10^9 cfu of formalin-killed *P. multocida* was able to cause lung and systemic pathophysiological responses comparable to that of live *P. multocida* suspended in the same volume of PBS and at the same dose of bacteria. So as to differentiate between diseases caused by bacterial components expressed *in vivo*. This has already shown to be important with IRP of *M. haemolytica* and supplements other approaches such as signature tagged mutagenesis (Fuller *et al.*, 2000) and *in vivo* expression technology (Hunt *et al.*, 2001).

This work (Chapter 6) was designed to provide new information on the innate immune response of the host on which future control strategies could be based. A marked clinical response occurred after the formalin-killed challenge, though a similar response also was observed in control calves. This response was not recorded in control calves administered 300 ml of PBS alone (Chapter 5) suggesting that these discernible responses for both challenge groups were due either to environmental conditions or to the presence of *P. multocida* throughout the respiratory tract that was not detected by nasal swabbing prior to day 0 of challenge. Fluctuations in environmental temperature occurred prior to and during the course of the experiment (Chapter 6), and may have compromised the animals respiratory defence system prior to challenge. Previous studies have demonstrated that clinically healthy calves up to 6 weeks of age were unable to tolerate acute changes in ambient temperature, from 5 to 35°C (Elmer and Reinhold, 2002). The main clinical findings of that study were significant increases in body temperature and respiratory rate. Microbiological examination of lung tissue from calves exposed to 5°C confirmed that mainly *Mycoplasma* sp, were found while *P. multocida* and *M. haemolytica* were the only bacteria found in the lung tissue of calves exposed to 35°C (Reinhold and Elmer, 2002). It has been suggested by Jones, (1987) that rapid proliferation of *Pasteurella* spp. in the nasopharynx is often accompanied by a change in climate (Jones, 1987). It would be of benefit for future animal experiments to prevent fluctuations of the environmental temperatures by placing the calves in an enclosed room with good ventilation and stable temperature.

Chapter 6 demonstrated clearly that SAA is a highly responsive APP and, along with Hp, exhibits marked responses to *P. multocida* infection similar to those observed during a *M. haemolytica* pneumonic infection (Horadogoda *et al.*, 1994). Statistically significant differences in APP

responses were not found between control and infected animals because variation between the small groups of animals was large. Future experimental studies would require larger groups of animals for a significant effect to be documented. In man, SAA is a marker of inflammation, tissue trauma and acute disorders such as myocardial infarctions and acute pancreatitis (Mayer *et al.*, 2002). In domestic animals, SAA plays a role in the transport of bacterial endotoxins and lipid metabolism (Urieli-Shoval *et al.*, 2000). Both SAA and Hp are detected early in the onset of disease, suggesting a possible release of these proteins from local cells such as alveolar macrophages. An increase in Hp mRNA within the lung following a bacterial infection has been reported (Yang *et al.*, 1995). It is conceivable that *P. multocida* LPS could stimulate alveolar macrophages to release proinflammatory mediators such as IL-6, IL-1 and TNF-α which may stimulate pulmonary mucosal epithelial cells or alveolar macrophages to release APP *in situ*, instead of the cytokines stimulating the hepatocytes to release the APP into the systemic system. It would be of value to measure the levels of APP within BALF following experimental introduction of *P. multocida* into the lower respiratory tract and to identify the origin of their release by the development of a PCR assay, whereby mRNA could be extracted from an array of pulmonary cells and amplified against an APP primer. In addition, cytokine mRNA profiles could be examined to help try and understand the role of cytokine-mediated release of APP.

Measurement of lung phagocyte function in the presence of formalin-killed *P. multocida in vivo* and live *P. multocida in vitro* characterised possible mechanisms of host pathogen interactions (Chapter 6). The recovery of fresh cellular material from the lower respiratory tract by bronchoalveolar lavage and examination of this material through cytospin preparations made it possible to identify the stage of inflammation and differentiate between infected and normal animals. The recovery of lung fluid a few days before experimental challenge did provide a good indication of the clinical status of experimental animals. In this study, the percentage of mixed cells isolated were 25 % neutrophils, which was 15 % higher than normal bovine neutrophil levels (Allen *et al.*, 1992b), and the remaining 75 % were macrophages. This may indicate that these particular animals had an underlying subclinical infection prior to the challenge.

The nasopharynx, where the bacterium often colonises, was swabbed on a weekly basis prior to challenge but during this period *P. multocida* was not detected (Barbour *et al.*, 1997; Brogden *et al.*, 1998). Additionally, bacteriological examination of the lung fluid prior to challenge may have indicated the presence of *P. multocida*, which if in the LRT, are often pathogenic. Evidence for the survival of *M. haemolytica* in tracheobronchial washings has been shown, especially in bovine species where long-term survival was observed (Rowe *et al.*, 2001), but no such studies have been conducted for *P. multocida*. Environmental differences between the URT and LRT may be responsible for the ability of *P. multocida* to cause disease in the LRT. It would be important for future *in vitro* studies to incubate bacteria in fluid extracted from nasopharynx and from lung, in the

presence of both iron replete and iron deplete conditions for the pathogen, to assess the survival and proliferation potential of *P. multocida* in the URT and LRT.

The presence of gross and microscopic pathological changes following formalin-killed *P. multocida* challenge was thought, initially, to be due to the high degree of neutrophil recruitment, as uncontrolled neutrophil recruitment to the site of tissue injury has been implicated in causing further damage (Soethout *et al.*, 2002). However, similar high neutrophil recruitment was also observed following PBS challenge and it has been demonstrated in domestic animals that pulmonary lavage alone can stimulate neutrophil migration through endothelial cells (Cohen and Batra, 1980). The procedure of challenge and lung lavage, alongside bacterial infection, as indicated by the recovery of *P. multocida* from swabbing of the nasopharynx following challenge was therefore likely to be the cause of the increase in neutrophils.

At necropsy it was possible to recover bacteria from both infected (Group 2a, 10^9 cfu formalin-killed *P. multocida* 300ml PBS) and control (Group 1a, 300ml PBS) groups which were surprising considering neither of these groups were challenged with live *P. multocida*. A greater amount of bacteria recovered were from lungs in the formalin-killed *P. multocida* group which correlated with the extent of lung damage, although the correlation was not statistically significant. Perhaps *P. multocida* resided before the start of the experiment in the tonsillar crypts of those calves from which it was recovered at PM and the use of standard nasal swabs did not detect *P. multocida*, whereas tracheal swabs may have been more efficient. Alternatively, the bronchoscope could have introduced *P. multocida* from the URT into the LRT. It may have been better to use an alternative BAL technique such as transtracheal sampling that, although it requires surgical preparation of the ventral surface of the neck, ensures that samples are collected without contamination from the URT (Caldow, 2001). However, the nature of this technique is invasive and may not be suitable for routine examination of the lung for investigating bacterial diseases.

Interactions between cells comprising the first line of pulmonary defence and *P. multocida* have not been well characterised compared with other respiratory pathogens such as *M. haemolytica* and *Haemophilus somnus* (Richards and Renshaw, 1989 and Gomis *et al.*, 1997). The work presented in this thesis has demonstrated the ability of alveolar macrophages to phagocytose *P. multocida in vitro* independent of an opsonin, as well as to demonstrate the survival of this pathogen in the hostile environment of the macrophage for up to 1 h. The latter finding suggests that as one of the pathogen's survival strategies to evade the bactericidal properties of the host, *P. multocida* may have prevented the fusion of the phagosome and lysosome, thereby disabling the initiation of the respiratory burst. The pathogen, *H. somnus* is able to survive and multiply within bovine phagocytes *in vitro* despite the activation of macrophage function through *E. coli* LPS and recombinant bovine cytokines. This demonstrates the ability of *H. somnus* to escape from the

bactericidal effects of the macrophage leading to dissemination of the pathogen in the body (Gomis *et al.*, 1997 and Gomis *et al.*, 1998). Furthermore, recent findings have confirmed the ability for *E. coli* K1 OmpA (+) to enter, survive and replicate intracellularly in monocytes and macrophages of newborn rats. Immunohistochemistry studies demonstrated the ability of *E. coli* to multiply rapidly within a single phagosome and burst the cell, leading to bacteremia (Sukumaran *et al.*, 2003).

The LDCL assay confirmed the inactivity of the respiratory burst when macrophages were incubated with *P. multocida* when only a small CL response was observed, comparable to the control (McCoys medium). To investigate this further it would be of benefit to incubate cells simultaneously with bacteria and PMA, (Jian *et al.*, 1995) and thus determine whether *P. multocida in vitro* had already disabled the respiratory burst. *In vivo* formalin-killed *P. multocida* neither enhanced nor disabled the respiratory burst of the lung phagocytes, as demonstrated by the similar large LDCL responses observed on the addition of PMA *in vitro* to bronchoalveolar cells recovered from calves challenged with PBS.

It would be of value for future work to examine different dose formulations of formalin-killed *P. multocida* and to investigate the optimal dose that would cause a significant clinical response from control animals. In Chapters 4 and 5 the bacteria were live and were thus likely to proliferate to a concentration higher than that in the administered bacterial suspension, therefore, a higher dose of bacterin than that used for live bacteria would be required to cause comparable disease.

Calves given formalin-killed *P. multocida* appeared to cope well with bacterin challenge, indicating a possible method for generating protection against exposure to a homologous challenge. Bacterin vaccines for *M. haemolytica* have demonstrated partial protection (Shewen and Wilkie, 1988; Gilmour *et al.*, 1991) but to date no vaccine exists for *P. multocida* and so the generation of an adaptive response to a homologous challenge was investigated. It was expected that the pathophysiological responses in calves given only homologous challenge would be more severe than those calves given homologous challenge after exposure to the bacterin. However, surprisingly, a greater area of lung damage was observed in those calves treated with bacterin prior to homologous challenge, suggesting that the bacterin compromised the animals' defences to the homologous challenge rather than enhancing protection. If the bacterin was responsible for predisposing the animal to infection then one would expect the bronchoalveolar cells to have been disabled by formalin-killed *P. multocida*. However, this was clearly not the case, as mentioned previously. A more feasible explanation lay in the fact that calves given the bacterin had an underlying *P. multocida* infection, as demonstrated by the recovery of bacteria at PM, and this may have exacerbated the effects of the bacterin. Furthermore, the formalin-killed *P. multocida* challenge may have contained LPS that interacts with toll-like receptor-4 to stimulate the expression of cytokines or directly stimulate neutrophils to secrete reactive oxygen intermediates

and granule contents such as elastase and proteolyic enzymes (Fittschen *et al.*, 1988). These toxic products are known to cause injury to adjacent cells and connective tissue structures, resulting in a more exacerbated pathological response in Group 2b calves than that observed for Group 1b calves. It would be interesting to measure the level of LPS potency in the formalin-killed *P. multocida* formulation. Localised IgG would have been produced by Group 2b calves following formalin-killed *P. multocida* to opsonise the homologous challenge, and promote phagocytosis. This thesis has provided evidence for the intracellular survival of *P. multocida* and future findings may support the lysis of the macrophage by the bacteria and in so doing release high levels of reactive oxygen intermediates causing host tissue damage. This mode of pathogenesis may provide an explanation as to why a more exacerbated pathological response was observed for Group 2b calves than that observed for Group 1b calves, as their immune system was not primed by formalin-killed *P. multocida* prior to the homologous challenge. The measurement of localised antibody production following formalin-killed *P. multocida* challenge was beyond the scope of this thesis and would be considered for future studies.

A significant positive correlation was observed between lung damage and concentrations of Hp. This novel finding suggests that local cytokine production within the area of lung damage could have mediated the synthesis of Hp *in situ*.

The systemic IgG level of all calves remained elevated, on a par with the high levels of serum neutralising antibody generated in calves infected with *P. multocida* A:3 prior to and during the experiment, probably residual from colostrum transfer. However there was no evidence of an increase or decrease in systemic IgG following exposure to formalin-killed *P. multocida*. It would be of interest for future work to assess the levels of IgG and IgA in lung fluid, as local antibody production following formalin-killed *M. haemolytica* has been observed in BALF recovered from goats (Zamri-Saad *et al.*, 1999). One would expect the immune response to be highly localised and that the release of antibodies, primarily IgA, would be into the lung and not into the systemic system (Wilkie, 1982). It is important to stress that the primary aim of the work in Chapters 6 and 7 was not to develop a bacterin vaccine but to provide a direct comparison between responses to dead and live challenge with *P. multocida*. The experiment would have been designed differently if the primary objective was vaccine development. For example, an adjuvant would have been used to complement the immune response, the vaccine would have been administered either intranasally or subcutaneously to stimulate the mucosal and systemic immune response, and repeat bacterin challenge would have been used to maximally stimulate an immune response. An obvious alternative for future work, associated with the production of a vaccine against *P. multocida*, would be to base the research on iron modulated bacterial protein expression technology, which has assisted successfully the development of a vaccine against *M. haemolytica* A:2 infection in sheep

and *M. haemolytica* A:1 infection in cattle at MRI (Gilmour *et al.*, 1991; Donachie, unpublished observations).

It became apparent during the latter stages of the study that farms with calves free of *P. multocida* were becoming more difficult to find. When selecting calves for experimental work in Chapters 4 and 5 at the early stage of the study, we were privileged to have a unique farm near MRI that reared calves consistently free of *Pasteurella* spp., a unique situation that was not appreciated fully until the farm closed down after the Foot and Mouth outbreak in 2001, when we were forced to search for a farm with *Pasteurella* spp. free calves within southern Scotland. This proved to be extremely difficult because it was common to recover *P. multocida* from approximately 50 % of calves within a few days of birth, indicating that transmission of *P. multocida* was rapid and delayed the start of the final animal experiment (Chapter 6 and Chapter 7). Detection was carried out using nasal swabs smeared on vancomycin SBA plates and in many cases *P. multocida* was recovered, along with *Proteus* and *E. coli*. In light of this, it is of great importance for future studies that a large scale epidemiological study is conducted to assess accurately the extent and variety of serotypes present in Scottish herds. Ideally this study would be performed by swabbing the tonsillar crypts in addition to the nasopharynx, which should increase the probability of recovering *P. multocida*. Cultures testing positive for *P. multocida* would then be subjected to an array of serological, biochemical and genotypic tests to ascertain strain diversity or relatedness. This would improve our understanding of the epidemiology of pneumonic pasteurellosis caused by this bacterium and provide information on the most appropriate serotypes to be used for successful vaccination studies against *P. multocida*. Furthermore, the serotypes isolated would need to be correlated with health status and disease patterns of the animal, to provide more concise information needed when selecting potential vaccine candidates if a particular serotype could be associated consistently with either diseased or healthy animals. The potential for key bacterial surface structures, such as LPS that interact with the host system, to undergo antigenic variation may hinder vaccine development. An understanding therefore of the diversity of such antigens will aid control strategies.

Official figures from VIDA, UK showed an increased incidence in pneumonic pasteurellosis caused by *P. multocida* as compared with *M. haemolytica*, from 1999 to 2001 (figures for 2002 are unavailable). This could be attributed to changes in farming practices brought about by changes in climatic conditions, as data retrieved from VIDA in 2001 showed a distinct seasonal pattern with the majority of pneumonic cases occurring in January, February and December. The low incidence in the autumn was likely due to relatively warm and dry conditions that allowed livestock to remain at pasture until much later in the year, with typical winter weather only occurring from December onwards (VIDA, UK).

Changes in climatic conditions can alter plans for lambing or calving and often force the farmer to remove livestock from pasture and house them in small areas. Studies in ruminants at pasture have shown that pneumonic pasteurellosis tends to spread slowly and the mortality rate is lower than in feeder calves kept in confined quarters. This stems from the aetiology of the disease whereby animals become stressed due to overcrowding and poor ventilation, leading to suppression of the immune system, rendering the animals susceptible to *P. multocida* infection. Risk factors to be considered include the mixing of calves of different ages, vaccination status, and movement of calves within and between farms, housing from pasture, weaning, dietary changes, decline in MDA status and other stresses such as castration (Gibbs, 2001). However, while any of the above factors may be associated with an outbreak of pneumonia in calves, it seems unlikely that alterations in farming practices alone are responsible for the increased prevalence of *P. multocida*. Rather it is more than likely due to the evolution of virulent attributes of *P. multocida* that favour the survival of the pathogen in a hostile host environment. *P. multocida* seems to differ from *M. haemolytica* in the sense that it possesses an array of virulence factors that all contribute to the survival and proliferation of the organism, as opposed to *M. haemolytica* that perhaps has a smaller number of virulence factors. Leukotoxin of *M. haemolytica* is the main recognised virulence factor (Jeyaseelan *et al.*, 2002) but, more recently, LPS chemotype, specifically the rough form of *M. haemolytica* A:1 has been associated with the severity of ovine pneumonic pasteurellosis (Hodgson *et al.*, 2003). *P. multocida* does not possess leukotoxin and this present study indicates that smooth LPS forms are rare. These differences between *M. haemolytica* and *P. multocida* emphasise the nature of the latter heterogeneous species that infects a broad spectrum of wild and domestic animals (Adler *et al.*, 1999), which makes the development of a vaccine against *P. multocida* more of a challenge.

The work reported in this thesis led to a successful, progressive and reproducible animal model that has provided a detailed picture of the clinical and pathological responses typical of BPP. This model of pneumonic pasteurellosis is currently being used by Intervet to assist with vaccine trials. In addition, the measurement of Hp and SAA has shown them to be useful markers for the disease. Phagocytic studies have demonstrated the ability of *P. multocida* A:3 to survive within the hostile environment of the alveolar macrophage for up to one hour *in vitro*, this novel finding may highlight a hitherto strategy unknown of *P. multocida* for evading the immune surveillance system *in vivo*. The challenge of conventional calves with formalin-killed *P. multocida* seemed to predispose the animals to a homologous challenge instead of conferring protection which indicates that a bacterin challenge via the lung would not be the route to adopt for vaccination programmes. The LPS from *P. multocida* A:3, a potentially important virulence factor, was characterised and found to be of the rough form, indicating loss of the O-antigen which was thought previously to be responsible for variation amongst bacterial isolates. However this thesis has shown the potential for variation within the core region of LPS, denoted by 6 LPS core banding patterns amongst 30

isolates. This novel discovery may hinder the development of vaccines targeted against LPS. Pulsed-field gel electrophoresis of DNA cut by enzymes with infrequently predicted restriction sites revealed the potential for much genetic diversity. This study has provided a better understanding of the disease process and the host interaction with *P. multocida* A:3. Despite an apparent increase in *P. multocida* virulence, the findings presented in this thesis, in conjunction with modern genetic tools and the recent sequencing of the entire genome of a turkey capsular serotype A strain (Pm 70) of *P. multocida* (May *et al.*, 2002) ensure that more informed approaches to studies of the host-pathogen interaction of value to vaccine development will be possible.

Appendix 1. SOLUTIONS AND REAGENTS

1.1. Carbohydrate media

1.1.1. Peptone water sugar base

Bacto peptone	10 g
NaCl	5g
Bromocresol purple solution (0.5% (w/v) in 95% ethanol	10 ml
Distilled water	1000 ml

Adjust pH to 6.8, autoclave at 121°C for 15 minutes.

1.1.2. Preparation of ONPG Broth

ONPG solution:

O-nitro-phenyl-D-galactopyranoside (ONPG)	0.6 g
0.01M Na_2HPO_4	100 ml

Adjust pH to 7.5 and filter sterilise.

Peptone water:

Bacto peptone	10 g
NaCl	5 g
Distilled water	1000 ml

Autoclave at 121°C for 15 minutes.
Complete medium: ONPG solution, 25ml and Peptone water, 75 ml
Mix aseptically and dispense in 2.5 ml volumes (stable for one month at 4°C)

1.2. Blood Agar

1.2.1. 5 % Sheep blood agar plates

Blood agar base No.2 (Oxoid)	500 ml
Sterile sheep blood	25 ml

Melt the blood agar base completely in the microwave (reducing the power to 50 % after the first 5 m allows the entire agar to melt without boiling over). Allow to cool at 56°C for 30 m, and then add aseptically sheep blood to 500 ml agar base. Swirl gently to mix and pour carefully avoiding air bubbles, into sterile Petri dishes in approximately 20 ml amounts. If bubbles appear in the Petri dish, pass a Bunsen flame over the agar to remove the bubbles prior to the agar setting. When

plates are dry (within 15 min) incubate a test plate overnight to check sterility and store the rest in sealed bags at 4°C up to 4 weeks.

1.2.2. Vancomycin blood agar

Blood agar base No.2 (Oxoid)	500 ml
Vancomycin solution (1mg ml^{-1} in DW, filter sterilised)	5ml

Prepare the blood agar as above. Add vancomycin solution (final concentration 10 μg ml^{-1}). Mix well and pour into sterile Petri dishes.

1.3. Pulsed-field gel electrophoresis reagents

1.3.1. Pett IV Buffer

Tris (pH 7.4)	10 ml of a 1 M stock
Sodium chloride (MWt 58.44g)	58.44 g
dH_2O	990 ml

Dissolve the above and store at RT

1.3.2. EC Lysis solution

Tris-HCl (pH 7.6)	6 ml of 1 M stock
1 M Sodium chloride (MWt 58.44)	58.4 g
EDTA (pH 7.5)	100 ml of 0.5 M stock
Deoxycholic Acid (Na) (MWt 392.6)	2 g
N-Lauroyl Sarcosine (MWt 293.4)	5 g
Polyoxyethylele 20 Cetyl Ether Brij 58	5 g
dH_2O	894 ml

Dissolve the above. Autoclave at 121°C for 15 m. Store at RT.
Before use add fresh lysozyme at 1 mg ml^{-1} and RNAse at 20 μl ml^{-1} (add 2 μl of 10 mg ml^{-1} stock solution ml^{-1}).

1.3.3. ESP buffer

EDTA (pH 9-9.5)	100 ml of 0.5 M stock
N-Lauroyl Sarcosine (MWt 293.4)	1 g
Proteinase K	0.1 g

Incubate solution at 37°C for 2 h. Freeze in 1 ml aliquots at -20°C

1.3.4. TE Buffer

5 ml 1 M Tris (pH 8) stock solution:-giving a final concentration 10mM Tris

1 ml 0.5 M EDTA (pH 8) stock solution:-giving a final concentration 1mM EDTA

Add to 494 ml DW. Autoclave at 121°C for 15 m and store at RT.

1.3.5. 0.1 M PMSF

Resuspend 17.5 mg of PMSF (MWt 174.2) in 1 ml of isopropanol. Make fresh each time.

1.3.6. 10 x TBE Buffer

Tris (MWt 121.1)	108 g
Boric acid (MWt 61.83)	55 g
EDTA (pH 8)	40 ml 0.5 M stock solution

Add to 800 ml DW. Mix and make up to 1 litre with DW. Autoclave at 121°C for 15 m and store at RT.

1.3.7. 0.5 x TBE buffer

Mix 50 ml of 10 x TBE with 950 ml DW. Store at RT.

1.3.8. 0.5 M EDTA

Add 93.06 g EDTA (MWt 372.24) to 400 ml DW and mix on a magnetic stirrer. Adjust the pH to 8.0 with NaOH and make up to 500 ml with DW. Autoclave at 121°C for 15 m and store at RT. EDTA will not dissolve until pH is adjusted to 8.0

1.3.9. 1 M Tris

Add 121.1 g Tris to 800 ml DW. Adjust to desired pH with concentrated HCl. Make up to 1 litre with DW. Autoclave at 121°C for 15 m and store at RT. For 10 mM Tris (MWt 121.1) 5 ml of 1M Tris pH 8.0 stock solution.

1.3.10. 2 % Low gelling agarose for plugs

Add 2 g of low gelling temperature agarose to 98 ml of DW. Dissolve in a microwave. Cool to 30-40°C in a waterbath for use or store at RT.

1.3.11. 1 % Agarose for PFGE gel

Add 3 g of agarose to 300 ml of 0.5 x TBE. Dissolve in a microwave. Cool to 50°C in a waterbath for use or store at RT.

1.4. Immunoblotting solutions

1.4.1. Transfer buffer

25 mM Tris (MWt 121.1)	15 g
192 mM Glycine (MWt 75.07)	72.06 g
25 % (v/v) Methanol	1 250 ml

Mix and make up to 5 litres with DW. Store at RT.

1.4.2. High salt blocking buffer

0.5 M NaCl	29.22 g
0.5 % Tween-80 (v/v)	5 ml
PBS	1000 ml

Mix contents thoroughly and store at RT.

1.4.3. 0.1 M Tris (pH 7.6)

Add 12.1 g Tris (MWt 121.1) to 800 ml DW. Adjust to pII 7.6 with concentrated HCl. Make up to 1 litre with DW. Store at RT.

1.4.4. 5 mg ml^{-1} diaminobenzidine

3, 3'-diaminobenzadine	100 mg
0.1 M Tris (pH 7.6)	20 ml

Dissolve and immediately dispense into 1 ml and store at -20°C

1.4.5. DAB HRP substrate

5 mg ml^{-1} diaminobenzidine	1 ml
0.1 M Tris (pH 7.6)	19 ml
0.15 % hydrogen peroxide (30 %, MWt 34.01)	100 µl

Mix and use immediately.

1.5. ELISA reagents

1.5.1. Carbonate bi-carbonate buffer

Na_2CO_3 (MWt 105.99) (15 mM)	0.795 g
$NaHCO_3$ (MWt 84.01)	1.465 g

Dissolve in 400 ml of DW. Adjust pH to 9.6 and make up to 500 ml with DW. Store at 4°C for up to one month.

1.5.2. ELISA wash buffer

10 x PBS	1 litre
Tween 20 (0.05 %)	5 ml
DW	9 litres

Mix and store at RT.

1.5.3. 0.2 M Phosphate solution

Add 14.2 g Na_2HPO_4 (MWt 141.96) to 500 ml DW. Store at RT.

1.5.4. Citric acid solution

Add 9.6 g Citric acid (MWt 210.14) to 500 ml DW. Store at RT.

1.5.5. OPD substrate solution

0.09 M Citric acid solution	12.1 ml
0.2 M Phosphate solution	12.9 ml
DW	25 ml
OPD (MWt 181.1)	40 mg
30 % H_2O_2	40 µl

Mix citric acid and phosphate solutions together, then add DW and OPD. Finally add hydrogen peroxide and mix well. Use immediately.

1.5.6. 2.5 M Sulphuric acid

Slowly add 136.25 ml concentrated H_2SO4 to 863.75 ml DW. Important to add acid to water, not water to acid. Fume hood, eye protection and gloves. Mix and store at RT.

1.6. Viral ELISA Reagents

1.6.1. ELISA diluent (PBS/Tween-20/EDTA/0.5 % (v/v) ovalbumin)

1 x PBS / 0.05 % Tween-20	180 ml
0.1M EDTA (MWT 372.2)	2 ml
Ovalbumin (5 % w/v)	1 g

Add ovalbumin to 20 ml of PBST and mix to dissolve, filter through a Whatman No.1 filter and Buchner funnel, make up fresh and discard remainder.

1.6.2. ELISA substrate (Orthophenylenediamine)

0.1M Citric acid (MWT 210.4)	121.5 ml
0.2M Di-sodium hydrogen orthophosphate (MWT 141.96)	128.5 ml
DW	250 ml

pH 5.0 using either of the above reagents and store at 4°C. Before use add the following to 100 ml.

Orthophenylenediamine (OPD) tablets	80 mg
30 % Hydrogen peroxide (MWT 34.01)	80 µl

1.7. Proteomic reagents

1.7.1. Rehydration buffer

8 M Urea (MWt 60.06)	12 g
2% (w/v) CHAPS	5 g
0.5% (v/v) IPG buffer (same pH range as the IPG strip)	125 µl
bromophenol blue	a few grains
DW	25 ml

1.7.2. Equilibration buffer

6 M Urea (MWt 60.06)	72.07 g
30 % (v/v) glycerol	69 ml
2 % (w/v) SDS (MWt 288.38)	4 g
50 mM Tris-HCl (pH 8.8)	6.7 ml
Bromophenol blue	a few grains
DW	200 ml

1.8. Phagocytic reagents

1.8.1. Nigrosin

Stock solution: 1 % (w/v) nigrosin in DW
Diluting solution: 2 % (v/v) 0.4 ml FCS in 19.6 ml PBS
Working solution: 0.1 % nigrosin (1 part stock solution to 9 parts diluting solution), filter, sterilise and store at -20°C in 90 µl aliquots.

1.8.2. Percoll reagent for the separation of bronchoalveolar cells

Percoll dilution to 1.075 g ml^{-1}: 53.2 ml Percoll stock solution, 10 ml of 10 x PBS and make up to 100 ml final volume with DW.

Percoll dilution to 1.055 g ml^{-1}: 37.8 ml Percoll stock solution, 10 ml of 10 x PBS and make up to 100 ml final volume with DW.

1.8.3. Fluorescent dyes

Crystal violet: Stock solution is 1 % methyl violet stored at RT. The working solution is a 1/20 dilution of the stock solution in PBS.

Acridine orange: Stock solution is 10 mg ml^{-1} stored at 4°C and the working solution is 14.4 mg/100ml of Gey's balanced salt solution (GBSS).

1.8.4. RPMI (without antibiotics)

RPMI 1640 (without L-Glutamine)	40.65 ml
FBS (heat inactivated for 1 h at 56°C)	5 ml
1 % (v/v) Glutamine	1 ml
1 % (v/v) HEPES (2.5M)	600 µl
Mercaptoethanol	250 µl

Mix the contents together using aseptic techniques in a sterile class II cabinet and adjust the pH to 7.2 using 7.5 % Sodium bicarbonate. Store at 4°C.

1.8.5. Freeze mixture for liquid nitrogen storage of lung cells

RPMI (10 % stock solution see appendix 1.8.4)	8 ml
DMSO	2 ml
FBS (heat inactivated)	10 ml

Mix the contents together using aseptic techniques in a sterile class II cabinet. Store at - 20°C.

1.9. Serology reagents

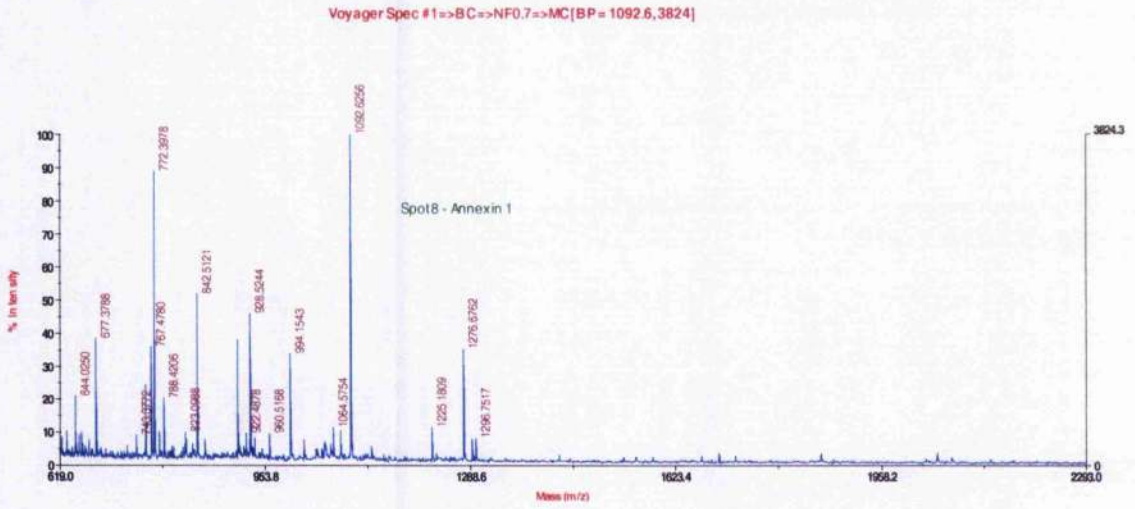
1.9.1. EDTA

Disodium EDTA	2 g
Sodium Chloride	0.8 g

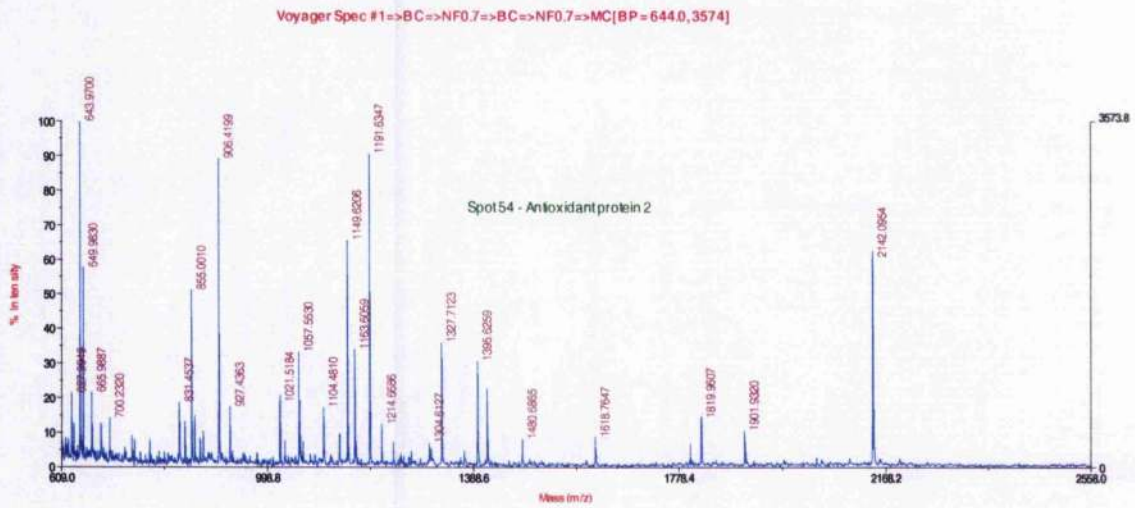
Adjust to pH 7.4 with sodium hydroxide and make up the solution to a final volume of 100 ml with DW.

Appendix 2. MALDI FINGERPRINTS OF SAMPLES PRODUCING SIGNIFICANT HITS IN THE SWISSPROT DATABASE.

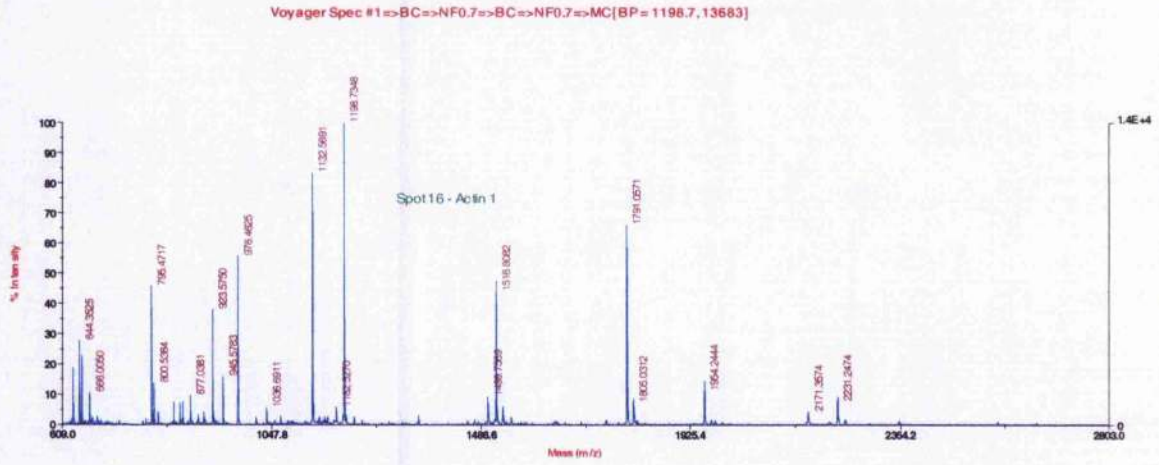
2.1. Spot 8 Annexin I



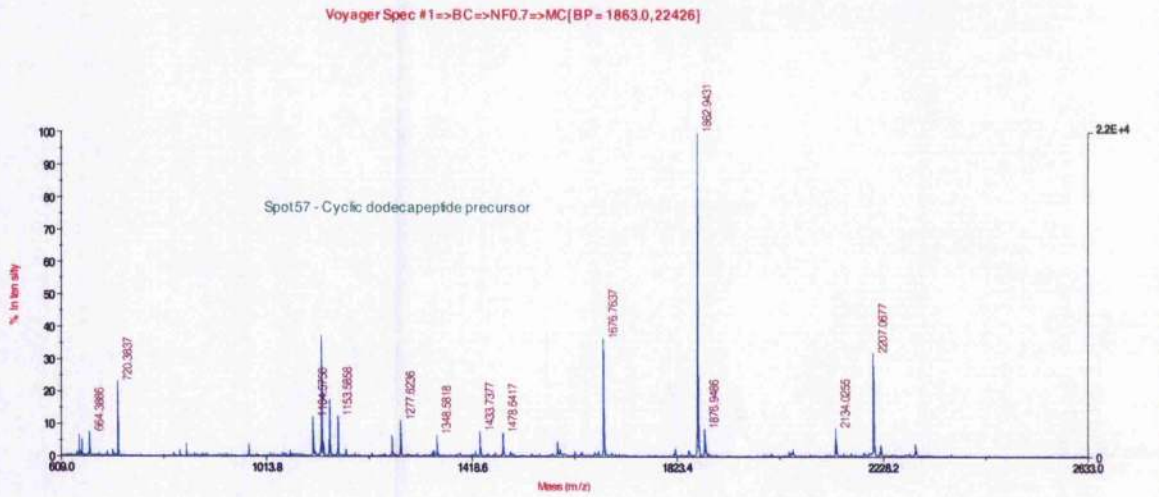
2.2. Spot 54 Antioxidant protein2



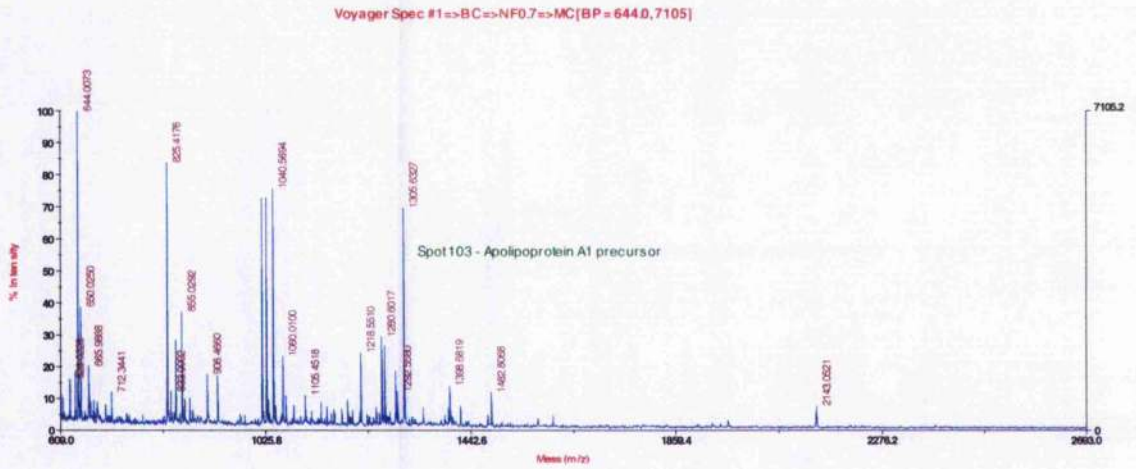
2.3. Spot 116 Actin 1



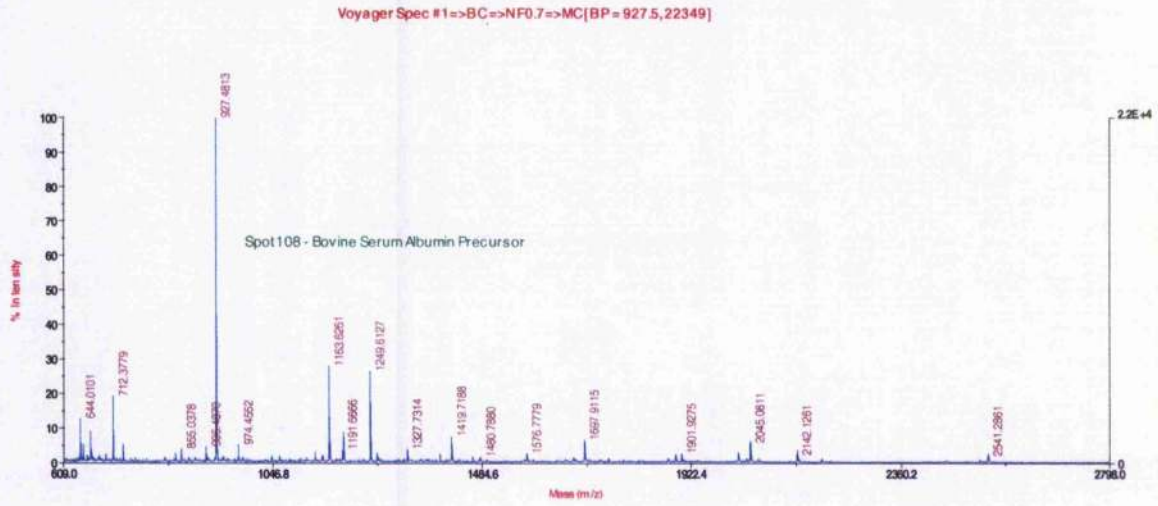
2.4. Spot 57 Cyclic dodecapeptide precursor



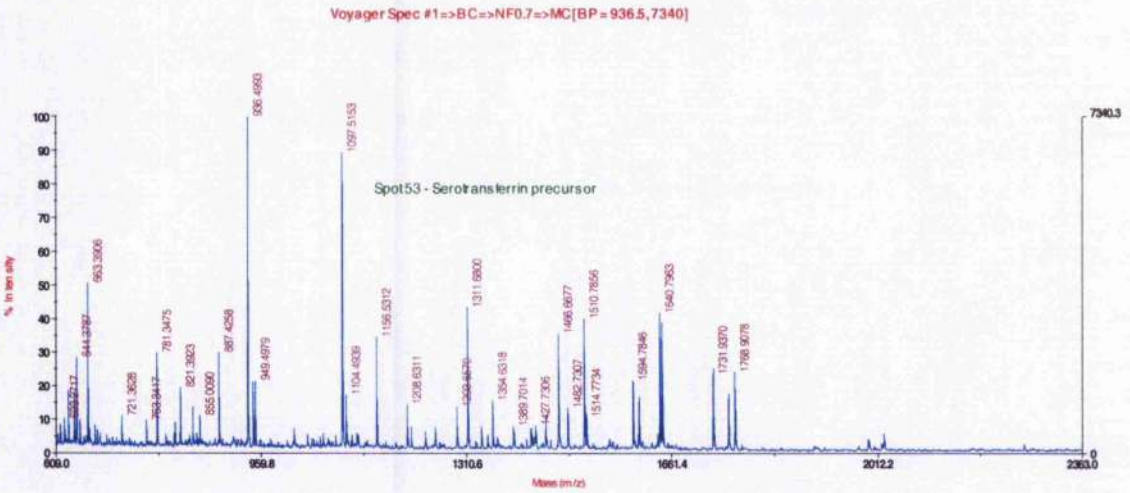
2.5. Spot 103 Apolipoprotein A1 precursor



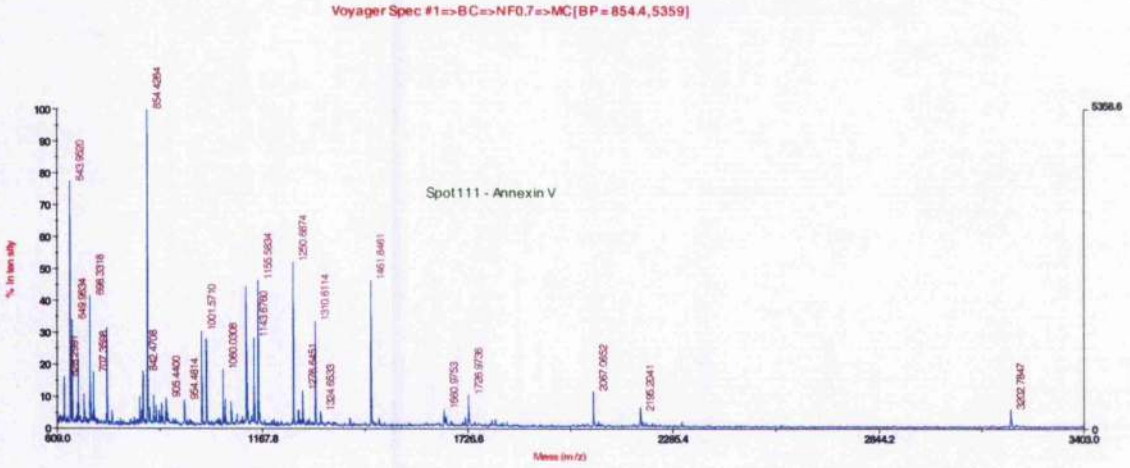
2.6. Spot 108 Bovine serum albumin precursor



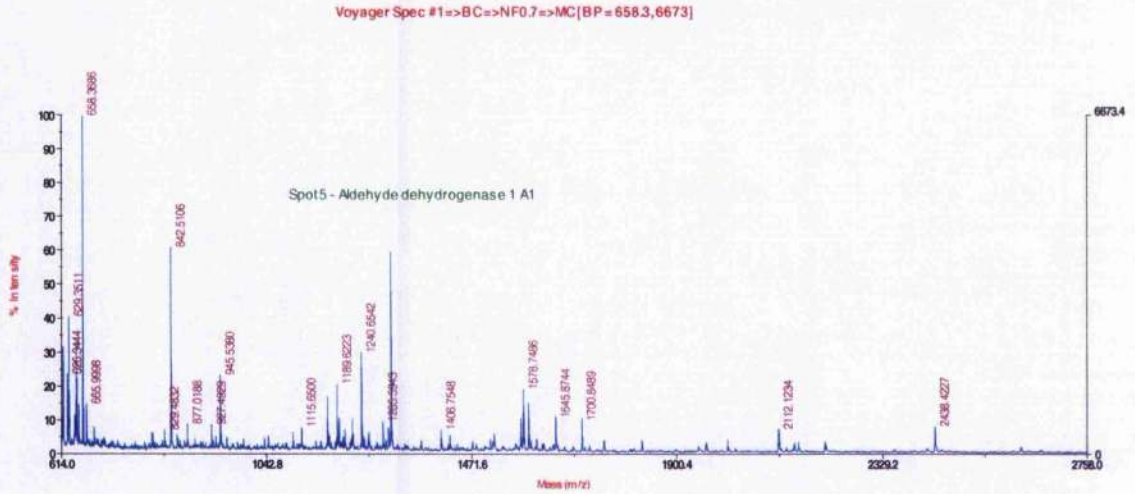
2.7. Spot 53 Serotransferrin precursor



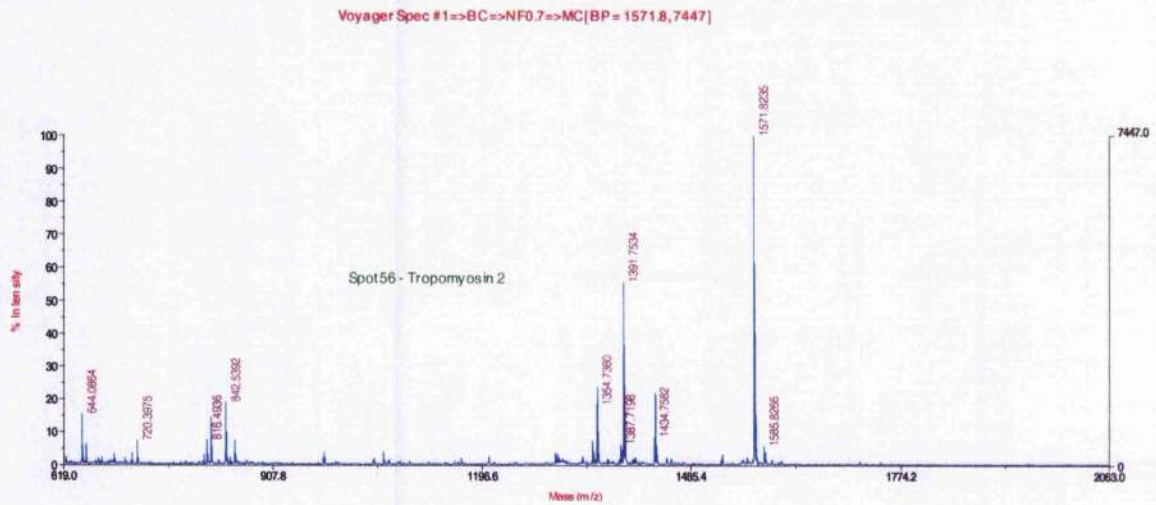
2.8. Spot 111 Annexin V



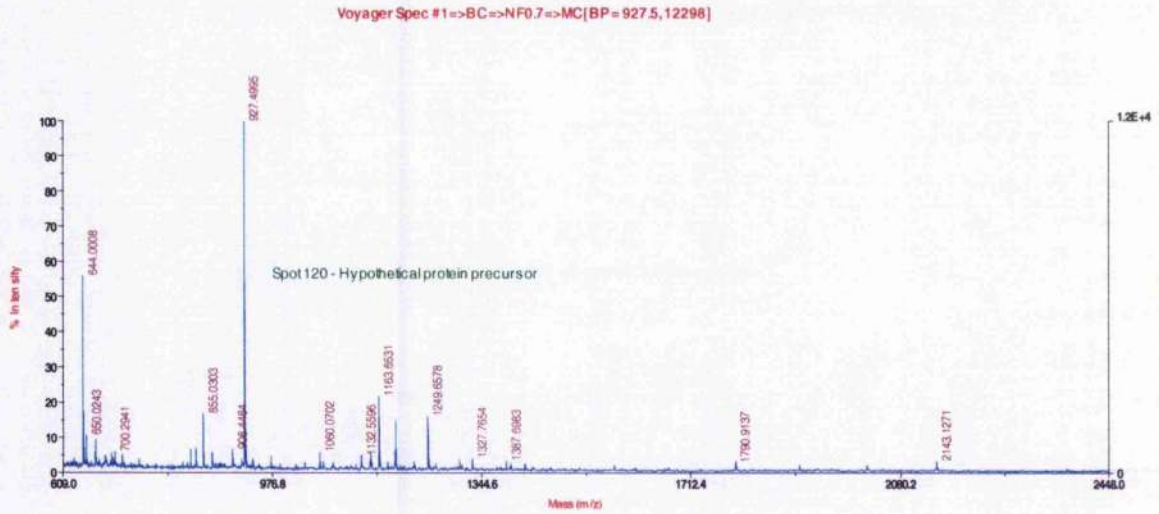
2.9. Spot 5 Aldehyde dehydrogenase 1 A1



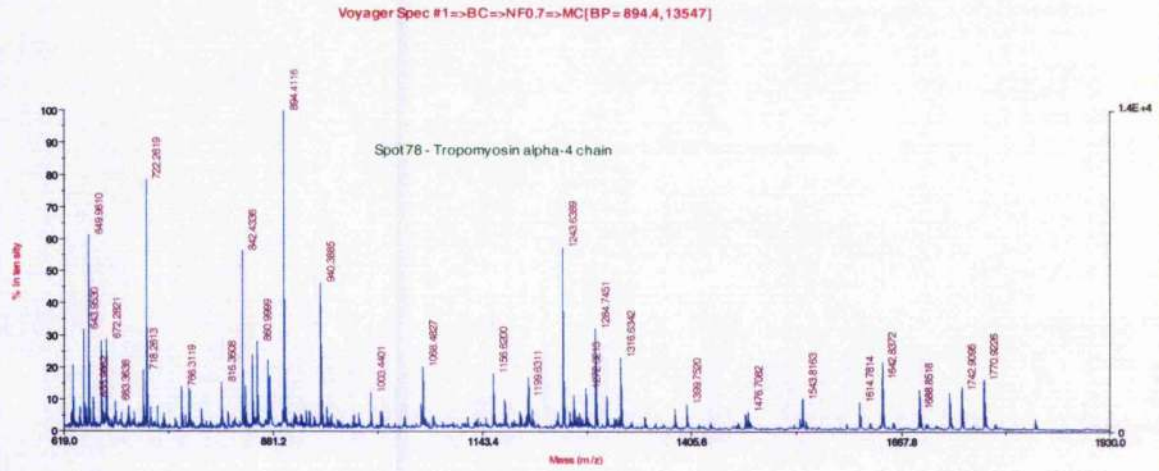
2.10. Spot 56 Tropomyosin 2



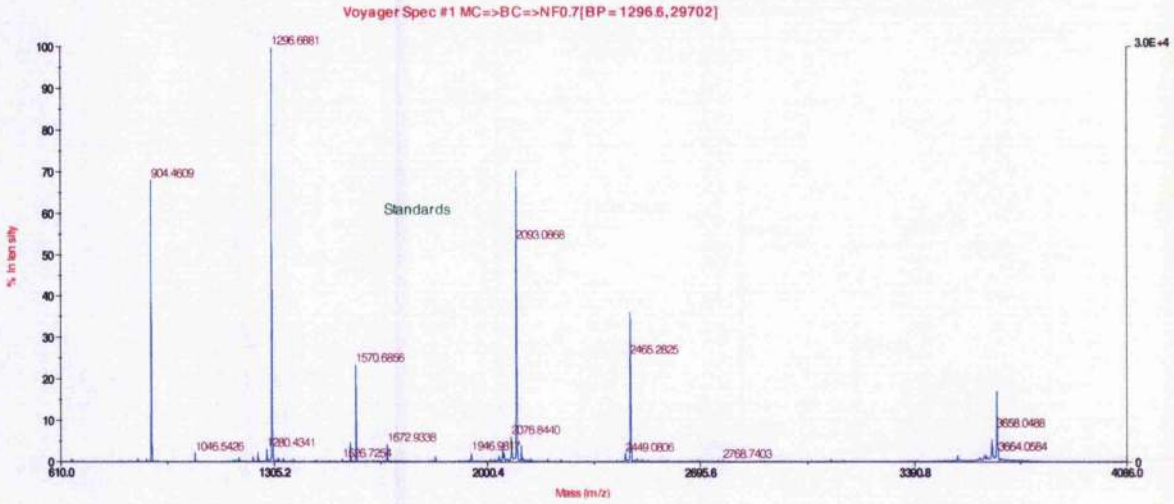
2.11. Spot 120 Hypothetical protein precursor



2.12. Spot 78 Tropomyosin alpha-4 chain



2.13. The fingerprint generated by mass spectrometry of standards



Appendix 3. PUBLICATIONS ARISING FROM THIS THESIS

3.1. Scientific Abstracts

Dowling, A., Hodgson, J. C., Schock, A., Quirie, M., Eckersall, P. D. and McKendrick, I. J. (2000). Development of a model of pneumonia in calves infected with *Pasteurella multocida. Research in Veterinary Science*, **68**, Suppl A, 3.

Dowling, A., Quirie, M. & Hodgson, J.C (2002) LPS chemotypes and molecular characterisation of *Pasteurella multocida* isolates from calves during a pasteurellosis outbreak. *Journal of Endotoxin Research* **8,** 162.

Dowling, A., Hodgson, J.C., Dalgleish, M., Eckersall, P.D & Sales, J (2003) Pathophysiological and immune cell responses to lung challenge with formalin-killed *Pasteurella multocida* A:3 in calves. *Research in Veterinary Science* **74,** Suppl A, 27.

3.2. Scientific Papers

Dowling, A., Hodgson, J. C., Schock, A., Donachie, W., Eckersall, P. D. & McKendrick, I. J. (2002). Experimental induction of pneumonic pasteurellosis in calves by intratracheal infection with *Pasteurella multocida* biotype A:3. *Research in Veterinary Science* **73,** 37-44.

Dowling, A., Hodgson, J. C., Schock, A., Quirie, M., Eckersall, P. D. and McKendrick, I. J. (2001) Development of a model of pneumonia in calves infected with *Pasteurella multocida.* Report of First European Colloquium for Standardisation of Acute Phase Proteins, *Veterinary Clinical Pathology*, **30**, 2-7.

3.3. Posters

Dowling, A., Quirie, M & Hodgson, J.C (2002) Phenotypic and genotypic characterisation of *Pasteurella multocida* isolated from conventional calves during a natural outbreak. Proceedings of International Pasteurellaceae Society Conference, Banff, Canada. Ed R Lo.

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