Immune responses of calves after vaccination with a live attenuated derivative of *Pasteurella multocida* B:2

Saeed Ataei Kachooei
D.V.M.

Division of Infection and Immunity,
Institute of Biomedical and Life Sciences,
University of Glasgow

July 2007

A thesis submitted for the degree of
Doctor of Philosophy

Declaration

I hereby declare that the work presented in this thesis is my own, except where otherwise cited or acknowledged. No part of this thesis has been presented for any other degree.

Saeed Ataei Kachooei

23rd July 2007

Dedication

To my wife, Shahin
who has always supported me and
to my daughters, Saba and Sahar who have always been source of happiness, life and love.
Also to my parents, who have always encouraged and supported me throughout my life.

Acknowledgements

I am very grateful to my supervisors Dr. John Coote and Dr. Roger Parton for their supervision and discussion during the course of this study. I would like to aknowledge them for critically reading and commenting on this thesis. I would like to express my most sincere thanks to Dr. J. Christopher Hodgson, who allowed me to conduct a major part of my research at Moredun Research Institute (MRI).

Special thanks are due to Dr. Robert Aitken my assessor for his encouragement, invaluable suggestions, comments, and friendship.

Many thanks to Dr. Mara Rocchi who allowed me to conduct FACS analysis experiments at Immunology Laboratory of MRI. I would like to thank Dr. Mark Dagliesh who provided technical help and material for immuno-histochemistry tests. I would also like to thank Mr. Gordon Moon and all staff of Bacteriology Laboratory staff at MRI.

I would like to thank Dr. Richard Burchmore from Proteomics Unit of Sir Henry Wellcome Functional Genomics Facility, University of Glasgow, for doing mass spectrometry assays.

I would also like to thank Prof. David Eckersall for performing SAA and Hp assays.

Many thanks also go to Mrs. Irene Houghton and all Infection and Immunity staff members for their technical help and collaboration during this project at University of Glasgow.

I wish to thank Dr. Jim Williams, Clinical Division of MRI, Dr. Harold Thompson and Mr. Richard Irvine, Veterinary Medicine school of University of Glasgow for providing calf blood.

I acknowledge the Razi Vaccine & Serum Research Institute, Ministriy of Health and Ministry of Agriculture of Islamic Republic of IRAN, who provided my scholarship and financially support this research work.

Finally, I would like to express my best gratitude and most sincere thanks to my father, mother, my wife and my children who encouraged and supported me throughout my career.

Abstract

The primary aim of this research was to investigate safety and potency of an *aroA* mutant of *Pasteurella multocida* serotype B:2 (JRMT12) as a live vaccine candidate in cattle against experimental haemorrhagic septicaemia (HS). To do this, three different experiments were set up to study the responses of calves to vaccination with the *aroA* strain and their response to challenge with the parent wild type 85020 strain one week after vaccination: 1) Different routes of vaccination, intranasal (IN) and intramuscular (IM) using two doses of 10^9 CFU of the *aroA* strain with a 4-week interval; 2) Different doses (10^7, 10^8 and 10^9 CFU) injected twice IM; 3) A single IM vaccination with 10^8 CFU.

Safety of JRMT12 was assessed by determination of clinical responses (rectal temperature and demeanour) and changes in serum amyloid A (SAA) and haptoblobin (Hp), taken as indicators of the progress and severity of infection. IN injection of calves with c.10^9 CFU of the mutant did not cause any clinical signs of HS. IM injection of c. 10^9 CFU of the mutant caused some typical clinical signs of the disease such as dullness and there was a clinical response manifested by a rise in rectal temperature and SAA levels. IM injection of calves with lower doses (c.10^8 and c.10^7 CFU) of JRMT12 did not cause disease symptoms but significantly increased the concentrations of SAA levels at 3 hours after both the primary and booster IM vaccinations. Our results showed that the mutant could be safely administered to calves.

Potency was assessed by measurement of serum antibodies (IgG and IgM) to *P. multocida* B:2 and by survival rate after subcutaneous challenge with the wild type strain. Vaccination with JRMT12 was able to induce a strong antibody response only after a second IM dose. Lower IgG titres were detected after the second vaccination with 10^8 or 10^7 CFU. A similar pattern of response was seen with IgM titres. IN-vaccinated calves showed no detectable antibody response to one or two doses of vaccine. All of the animals vaccinated twice IM with JRMT12 at doses of 10^9, 10^8 and 10^7 CFU were solidly immune to challenge with 10^7 CFU of *P. multocida* B:2 wild type. A single IM vaccination of calves with 10^8 CFU of JRMT12 did not confer protection against challenge. IM vaccination twice with 10^8 CFU was considered an optimum dose that fully protected calves but showed no obvious reactogenicity.

Immunogenic components of *P. multocida* to which antibody was raised during different stages of vaccination and challenge were detected by SDS-PAGE and immunoblotting. Antibody staining of the bands of 51, 37, 30, 26 and 16 kDa, only appeared with sera taken one week after the second vaccination. These 5 bands were thus identified as potentially

immunogenic components with roles in protection against challenge. For further identification of immunogenic components, outer-membrane proteins (OMPs) of the bacterium were prepared and two of them (37 and 30 kDa bands) were subjected to identification by mass spectrometry (MS). The 30 kDa was identified as OmpH and the 37 kDa band was identified as OmpA of *P. multocida* by MASCOT analysis of the data.

A possible contributory role of cellular immunity against HS was investigated in calves given a single IM dose of 10^8 CFU and in control calves after challenge. A lymphocyte stimulation assay was used to assess the effects of a CFE of *P. multocida* on peripheral blood mononuclear cells (PBMCs) isolated from calves at different times after challenge. The results were indicative of a possible immunosuppressive effect of challenge with *P. multocida* B:2 on calf PBMCs. Such an effect does not appear to have been reported previously, was in keeping with the lack of response to CFE observed with PBMCs from the in vivo experiment. The suppressive effect was further investigated by in vitro experiments. Calf PBMCs obtained from a normal calf were treated with CFE for 1 h before adding ConA. Addition of CFE at 50 μg/ml caused a 3-5 fold decrease in the proliferative response to ConA. The suppressive effect was only observed with CFE from two other *P. multocida* strains, a *P. multocida* serotype D strain (a causative agent of atrophic rhinitis in pigs) and, with lower suppressive activity, *P. multocida* serotype A3 (a causative agent of pneumonia in cattle). The active part of the suppressive agent(s) was likely to be protein. Heating at 80°C for 5 min completely destroyed the suppressive properties and an OMP preparation of *P. multocida* B:2 markedly suppressed the proliferative response of PBMCs to ConA. Dialysis of CFE with a cut off 10,000 Da did not alter its suppressive effects.

B cell and T cell subpopulations ($CD4^+$, $CD8^+$ and $\gamma\delta^+$) in normal calf PBMCs were identified and the changes in their relative proportions after different treatments was investigated using FACS analysis. The ratio of proliferating and non-proliferating fractions was also determined, to assess the proliferative status of each population. $CD4^+$ and $CD8^+$ T cells were the principal cells which proliferated in response to ConA and whose proliferation was suppressed by a CFE of *P. multocida*. The suppression of $CD4^+$ T cells was not due to an adverse toxic effect of CFE on the cells, as CFE alone did not decrease the number of these cells. ConA increased the percentage of proliferating $CD4^+$ T cells by 10-fold compared to the control cells, but CFE treatment before addition of ConA allowed only a 3-fold increase. Our in vitro experiments showed that *P. multocida* contains a component(s) with the potential to inhibit the proliferative reponse of $CD4^+$ and $CD8^+$ T cells.

List of contents

List of figures

List of tables

Abbreviations

aa	= amino acid
α_1-AGP	= α_1-acid glycoprotein
AGPT	= agar gel precipitation test
APC	= antigen presenting cell
APP	= acute-phase protein
APR	= acute phase response
APV	= alum-precipitated vaccine
AR	= atrophic rhinitis
aroA	= aromatic amino acid metabolism gene
BHI	= brain heart infusion
bp	= base pair
BSA	= bovine serum albumin
c.	= about
°C	= degrees Celsius
CFE	= cell-free extract
CFSE	= carboxyfluorescein diacetate succinimidyl ester
CFU	= colony forming unit
cm/mm	= centimetre/millimetre
CMI	= cell-mediated immunity
CO_2	= carbon dioxid
ConA	= concanavalin-A
CRP	= C-reactive protein
CSY	= casein sucrose yeast
CTL	= cytotoxic lymphocyte
DAB	= diaminobenzoic acid
Da/kDa	= dalton/kilodalton
DC	= dendritic cell
DNA	= deoxyribonucleic acid
DSA	= dextrose starch agar
DTH	= delayed-type hypersensitivity
EDTA	= ethylenediaminetetraacetic acid
ELISA	= enzyme-linked immunosorbent assay
EU	= ELISA units for Endotoxin units
FACS	= fluorescence activated cell sorter
FBS	= foetal bovine serum
FC	= fowl cholera
fh	= filamentous haemagglutinin
g/mg/µg	= grams/milligrams/micrograms
h	= hour(s)
HBSS	= Hank's balanced salt solution
HEPES	= N-2-hydroxyethylpiperazine-N-2-ethanosulphonic acid
Hp	= haptoglobin
HS	= haemorrhagic septicaemia
IFNγ	= interferon gamma
Ig	= immunoglobulin
IHA	= indirect haemagglutination
IL-(X)	= interleukin-(number)
IM	= intramuscular
IN	= intranasal
IROMP	= iron-dependent OMP
IU	= international units

l/ml/μl	= litres/millilitres/microlitres
LAL	= *Limulus* amoebocyte lysate
LB	= Luria-Bertani broth
LD_{50}	= lethal dose 50%
LPS	= lipopolysaccharide (endotoxin)
MHC	= major histocompatibility complex
M/mM/μM	= molar/millimolar/micromolar
min	= minute(s)
mRNA	= messenger RNA
Mw	= molecular weight
NaCl	= sodium chloride
NB	= nutrient broth
NK	= natural killer
NO	= nitric oxide
NOD	= nucleotide-binding oligomerisation domain
NSAID	= nonsteroidal ant-iinflamatory drug
OAV	= oil adjuvant vaccine
$OD_{X\ nm}$	= optical density at wavelength X nm
OMP	= outer-membrane protein
PABA	= para-aminobenzoic acid
PAGE	= polyacrilamide gel electrophoresis
PAR	= progressive atrophic rhinitis
PBMC	= peripheral blood mononuclear cell
PBS	= phosphate-buffered saline
PHA	= phytohaemagglutinin
PCR	= polymerase chain reaction
Pm	= *Pasteurella multocida*
PMN	= polymorphonuclear
PMPT	= passive mouse protection test
PMT	= *Pasteurella multocida* toxin
PRR	= pattern-recognition receptor
p.s.i.	= pound per square inch
PWM	= pokeweed mitogen
RNA	= ribonucleic acid
rpm	= revolutions per minute
RPMI	= Roswell Park Memorial Institute
rRNA	= ribosomal RNA
RT	= room temperature
SAA	= serum amyloid A
SBA	= sheep blood agar
sec	= second(s)
SDS	= sodium dodecyl sulphate
SIR	= subcutaneous inflammatory response
TCR	= T cell receptor
TEMED	= tetramethylethylenediamine
Th (1/2)	= helper T cell (1/2)
TLR	= toll-like receptor
TNF-α	= tumor necrosis factor alpha
tRNA	= transfer RNA
Tris	= Tris (hydroxymethyl) aminoethane
v/v	= volume by volume
w/v	= weight by volume

Chapter 1

INTRODUCTION

1.1 History and taxonomy of the family *Pasteurellaceae*

Members of bacterial family *Pasteurellaceae* are small (0.2-2 μm), Gram-negative, chemoorganotrophic, non-motile facultatively anaerobic coccobacilli or rods. They are, with few exceptions, fermentative and oxidase- and catalase-positive (Bisgaard, 1994). Organisms belonging to the family *Pasteurellaceae* can colonize mucosal surfaces of the respiratory, alimentary, genital tracts and cause diseases in different mammals, birds, and reptiles (Jacques, 2002). For a long time it was believed that the family consisted only of three genera: *Pasteurella* described by Trevisan 1887, *Actinobacillus* by Brumpt 1910, and *Haemophilus* by Winslow *et al.* 1917, but several other groups of organisms that exhibit complex phenotypic and genotypic relationships with these genera were also included (Boot & Bisgaard, 1995; De Alwis, 1999). After the redesignation of the organisms formely known as [*Pasteurella*] *haemolytica* into the new genus *Mannheimia* in 1999 (Angen *et al.*, 1999), substantial reclassification was made amongst the bovine *Pasteurellaceae*. Since then, researchers have added some new genera, such as *Histophilus* (Angen *et al.*, 2003), *Gallibacterium* (Christensen *et al.*, 2003; Christensen *et al.*, 2004a), *Actinobacillus* (Christensen & Bisgaard, 2004), *Volucribacter* (Christensen *et al.*, 2004b), *Nicoletella* (Kuhnert *et al.*, 2004), and *Avibacterium* (Blackall *et al.*, 2005) into the family. *Pasteurellaceae* now includes 11 genera (*Pasteurella, Actinobacillus, Haemophilus, Lonepinella, Mannheimia, Phocoenobacter, Gallibacterium, Histophilus, Volucribacter, Avibacterium, Nicoletella*) and 62 species. At least four genera and several more species are expected to be named soon (Olsen *et al.*, 2005).

1.1.1 Diseases caused by *Pasteurellaceae*

The *Pasteurellaceae* include a large number of important human pathogens, such as *Haemophilus influenzae, H.ducreyi* and *Actinobacillus actinomycetemcomitans*, and animal pathogens such as *Pasteurella multocida, Mannheimia haemolytica, Haemophilus paragallinarum, H. parasuis, H. somnus, Actinobacillus equuli, A. pleuropneumoniae*, and *A. suis*. Some of these pathogens are distributed widely, causing economically significant diseases in a variety of hosts (Jacques, 2002).

1.1.2 The genus *Pasteurella*

Classification of the genus *Pasteurella* is based on genetic relationships, determined by DNA:DNA hybridisation, rRNA:DNA hybridisation and 16S rRNA sequencing (De Alwis, 1999). On the basis of these methods, this genus consists of 11 species. *Pasteurella ,sensu stricto,* includes *Pasteurella multocida* with 3 subspecies (*multocida, septica,* and *gallicida), P. dagmatis, P. gallinarum, P. voluntium, P. stomatis, P. avium, P. langaa, P. anatis* and *Pasteurella* subspecies A and B (Boot & Bisgaard, 1995; Kawamoto *et al.*, 1990; Krause *et al.*, 1989).

1.1.3 The species *Pasteurella multocida*

Pasteurella multocida has been recognised as an important veterinary pathogen for over a century. Its importance as a human pathogen, causing respiratory diseases and infections of the central nervous system (Berge *et al.*, 2002; Capitini, 2002), has been increasingly recognised in the last 50 years (Johnson, 1994; Johnson *et al.*, 2000; Sheikh *et al.*, 1996). These result largely from animal bite wounds.

1.1.3.1 Cell morphology

P. multocida is a Gram-negative, non-motile, non-spore-forming, short rod or coccobacillus, 0.2-0.4 by 0.6-2.5 μm in size (De Alwis, 1999). Cells can occur singly, in pairs and occasionally as a chain or filaments, especially after repeated laboratory subcultures of old cultures or cultures grown under unfavourable conditions. In young cultures and animal tissues it shows the typical bipolar staining (safety pin shape), particularly with Leishman or methylene blue stain (Solano *et al.*, 1983). Virulent strains are usually capsulated and their capsules can be seen in organisms isolated from hosts and laboratory cultures supplemented with serum or blood (De Alwis, 1999).

1.1.3.2 Colonial characters

The organism grows on most of the common nutrient media. Special media such as brain-heart infusion (BHI) and dextrose-starch agar (DSA) provide relatively complete resources and result in better growth. Supplementing media with serum or blood enriches the medium and also supports good growth (OIE, 2000). Colonies from 18-24 hour cultures grown aerobically at 35-37°C on solid media are 1-3 mm in diameter. Freshly isolated colonies on tryptose agar or CSY (Casein, Sucrose, Yeast) agar enriched with blood are

approximately 2 mm in diameter after 24 hours at 37°C (Wijewardana, 1986). Colonies on blood agar are of approximately 1 mm in diameter and on plain unenriched media may be smaller (OIE, 2004). The morphology of colonies is mainly related to capsular type (Wijewardana, 1986). Colonies may vary in size, depending on the degree of capsulation. When freshly isolated or grown on media containing serum, they vary from larger greyish to smaller ones that give a yellowish-green or bluish-green iridescence when viewed in transmitted light. Rough colonies, that are generally the smallest, may be produced as result of loss of capsular material, due to long term storage or after several passages in synthetic media. This process is called dissociation and is associated with reduction of virulence and loss of antigenicity (Heddleston, 1966; Penn & Nagy, 1976). Capsule production, iridescence and virulence sometimes can be regained or enhanced by animal passage (Rimler & Rhoades, 1989).

1.1.3.3 The serotypes of *Pasteurella multocida*

A classification system (or typing) for host and disease specificity has been made based on antigenic differences in capsular polysaccharides and also differences in antigenic variety of lipopolysaccharides (LPS, also called O antigen). Based on capsular typing there are 5 different capsular types named A, B, D, E, and, F (Rimler *et al.*, 1989). The other method is "somatic typing" based on O antigen of the organism in which 16 different somatic types (numbered 1 to 16) have been distinguished (Heddleston *et al.*, 1972; Namioka & Murata, 1964). It is generally accepted that designation of serotypes should be based on a somatic:capsular combination. Among different systems, two systems commonly in use are the Namioka-Carter and the Carter-Heddleston systems. Asian and African haemorrhagic septicaemia-causing serotypes (Section 1.5.1) are designated 6:B and 6:E, respectively, in the former system, while in the latter system they are designated B:2 and E:2, respectively (OIE, 2000).

Generally, type A capsular *Pasteurella* are mainly associated with fowl cholera, rabbit snuffles (also called recurrent purulent rhinitis), and respiratory diseases of animals. Type D is the predominant type in atrophic rhinitis of pigs and occasionally has been isolated from the lungs of ruminants or other species. Type F is mainly isolated from turkeys. Types B and E are usually associated with haemorrhagic septicaemia of cattle and buffaloes.

1.2 Diseases in animals

Specific serotypes of *P. multocida* are associated with different diseases in cattle, buffalo, sheep, goats, camels, pigs, poultry and other animals.

1.2.1 Haemorrhagic septicaemia (HS)

HS is the most economically important disease of the group of diseases caused by different *Pasteurella.* The disease, unlike some other pasteurelloses where the agent plays a secondary role, is a primary pasteurellosis. It is an acute, fatal septicaemic disease of cattle, buffaloes, camels, feral ruminants and pigs (De Alwis, 1999). It is dealt with in more detail in section 1.5.

1.2.2 Fowl cholera (FC)

Fowl cholera is a primary pasteurellosis that in acute form results in septicaemia and death. Chronic forms of the disease are the predominant form in industrial poultry husbandry. FC is a noticeable cause of economic loss in the developed poultry industry and in traditional poultry husbandry (Rimler, 1997). Capsular serogroups A, B, D, E, and F have been reported; all but serogroup E have been isolated from avian hosts. A very wide range of birds including wild birds are susceptible to this disease. Serotypes A:1, A:3 and A:4 are the common cause of the FC in most countries, although all of the 16 somatic serotypes of the group A, and some types of group D have also been reported (Brogden *et al.*, 1978; De Alwis, 1993; OIE, 2004).

The records of FC date back to 1600. Many of the earliest studies on understanding the role of microorganisms in infectious diseases were with this disease. The use of attenuated agents as vaccine, for immunisation against diseases was invented by Louis Pasteur one hundred years ago, for this disease (De Alwis, 1999).

1.2.3 Atrophic rhinitis (AR)

Atrophic rhinitis or progressive atrophic rhinitis (PAR) is an infectious disease of swine characterised by purulent nasal discharge, shortening or twisting of the snout, atrophy of the turbinate (conchal) bones and reduced productivity. The most severe progressive form is caused by infection with toxigenic strains of *Pasteurella multocida* alone or in combination with *Bordetella bronchiseptica* (OIE, 2004). AR is an important disease in

intensive pig breeding. *Pasteurella multocida* type D and toxigenic strains of type A are associated with the disease (Davies *et al.*, 2003; Pijoan, 1988). *Bordetella bronchiseptica* as a normal inhabitant of the upper respiratory tract of the animal, is believed also to play a role in pathogenesis of the disease. Production of a dermonecrotic toxin is the main cause of the atrophy in turbinates. Concurrent infection with *B. bronchiseptica* or the action of certain irritants like aerial pollution creates favourable conditions for the proliferation of *P. multocida* (Chanter *et al.*, 1989; De Alwis, 1999; Hamilton-Miller & Shah, 1996).

1.2.4 Bovine pasteurellosis

Bovine pasteurellosis or pneumonic pasteurellosis is a pneumonia of mainly beef cattle that is caused by different viruses or stress as predisposing factors and *P. multocida* capsular serogroup A or *Mannheimia (Pasteurella) haemolytica* type A1 as secondary pathogen. There is no consistency in the somatic types involved. This is unlike haemorrhagic septicaemia, which is a primary pasteurellosis caused by specific serotypes (B:2 or E:2) of the *P. multocida.*

The disease has some other synonyms such as bovine enzootic pneumonia, shipping fever, transit fever, and bovine respiratory disease (BRD) complex that show the conditions that are relevant in specific circumstances. It is economically the most important disease of the beef and dairy industry of the United States, as it is believed that losses due to this disease are greater than those due to all other diseases put together. A challenge model has been developed, whereby challenged calves developed progressive pneumonic disease similar to that seen in natural cases of disease (Dowling *et al.*, 2002). The pasteurellae that cause pneumonic pasteurellosis are carried in the upper respiratory tract (URT) of calves. In the case of *Mannheimia (Pasteurella) haemolytica* type A1, the bacterium is not easily detected in the URT of healthy calves, but is shed and can be easily isolated in calves that are stressed in some way or affected with another concurrent infection. The URTs of stressed or otherwise diseased calves can be easily colonised by *Mannheimia (Pasteurella) haemolytica* A1. In the case of *P. multocida,* no such relationship between stress and ability to colonise has been observed (De Alwis, 1999).

1.2.5 Pasteurellosis in sheep and goats

Pasteurellosis of sheep and goats is a pneumonia in all ages of these animals, and a distinct septicaemic syndrome involving pleurisy and pericarditis in young lambs. The predominant organism that causes disease in sheep and goats is *Mannheimia (Pasteurella)*

haemolytica biotype A (Porter *et al.*, 1995). Experimental disease indicates an association between the incidence and severity of the disease and LPS chemotype and suggests an important role for LPS chemotype in determining host-species susceptibility to lung infection (Hodgson *et al.*, 2003). This is probably the most economically important bacterial disease of sheep and goats (De Alwis, 1999).

1.3 Molecular biology of *Pasteurella multocida*

Over a century has passed since the isolation of pasteurellae from birds by Pasteur in 1880 and from cattle by Kitt in 1885. During this time, considerable research has been done on members of the family but this has resulted in only very limited understanding of the organism. Substantial progress towards a better understanding has been made recently, with a number of groups studying different aspects of the molecular biology of *P. multocida* (Hunt *et al.*, 2000).

1.3.1 Non-protein cellular components

1.3.1.1 Capsule

Many strains express a polysaccharide capsule on their surface, and isolates can be differentiated serologically by capsular antigens into serogroups A, B, D, E, and F (Carter, 1967; Rimler & Rhoades, 1987). The capsule is composed of polysaccharides, lipopolysaccharides (LPS) and a variety of proteins. The capsule antigen of *P. multocida* responsible for serotype specificity is intimately associated with lipopolysaccharide (LPS) material. Both LPS and polysaccharides are absorbed onto erythrocytes and are believed to play a role in passive haemagglutination (Rimler & Rhoades, 1989). Both the capsule–specific antigen and LPS can be adsorbed onto erythrocytes from crude cell extracts. However, passive haemagglutination tests, with serum containing antibodies against the capsule-specific antigen and LPS, usually show a reaction only with the capsule-specific antigen (Rimler & Rhoades, 1989). Capsular antigens of *P. multocida* serotypes B and E have been shown to be immunogenic and protective in cattle against serotype E challenge (Nagy & Penn, 1976). However, non-capsulate and capsulate organisms of serotype A, strain X-73, were reported to be equally effective in immunising birds against fowl cholera (Heddleston, 1966). The capsule of serotype A strain is composed of hyaluronic acid and is believed to be an important virulence factor (Watt *et al.*, 2003). Acapsular mutants of serotype B:2 have also been prepared and shown to be avirulent and protective as a live vaccine (Boyce & Adler, 2000; Boyce & Adler, 2001).

The genes related to capsule synthesis have been identified in avian and bovine isolates (Boyce *et al.*, 2000a; Boyce *et al.*, 2000b; Chung *et al.*, 1998). The capsule biosynthetic locus of *P. multocida* B:2 consists of 15 genes, which can be grouped into three functional regions. Regions 1 and 3 contain genes proposed to encode proteins involved in capsule export, and region 2 contains genes proposed to encode proteins involved in polysaccharide biosynthesis (Boyce & Adler, 2000).

1.3.1.2 Lipopolysaccharide (LPS)

P. multocida LPS has chemical and biological properties such as endotoxic properties which are similar to those found in many species of Gram-negative bacteria. As antigens, LPSs have been associated with immune protection of animals and are believed to be the chemical basis for the specificity of the somatic typing system (Brogden & Rebers, 1978). LPSs have been purified from many different strains of *P. multocida*. In common with LPS from bacteria of the family *Enterobacteriaceae*, chemical analysis has shown that they contain lipid A, 2-keto-deoxyoctonate, L-glycero-D-mannoheptose, glucose and glucosamine. Other sugars encountered amongst LPSs from different strains were galactose, rhamnose, D-glycero-D-mannoheptose and galactosamine (Rimler *et al.*, 1984). Although in LPS isolated from two serotype A strains of *P. multocida*, a possible structure for the heptose region has been described, in which a triheptose unit linked to a 2-keto-3-deoxyoctulosonic acid residue the complete structure of *P. multocida* LPS has not been determined. A heptosyltransferase (an enzyme required for the addition of heptose to LPS) mutant of FC-associated *P. multocida* was prepared and shown to be attenuated in chicken (Harper *et al.*, 2003). Further studies showed that the predominant LPS glycoforms isolated from this mutant were severly truncated (Harper *et al.*, 2004).

LPS is believed to play an important role in the pathogenesis of diseases caused by different serotypes of *P. multocida*. In the HS-associated serotype B LPS is thought to be responsible for toxicity and to play an important role in the pathogenesis of the disease (Horadagoda *et al.*, 2002). Turkeys were relatively resistant to the lethal effects of LPS obtained from FC-related *P. multocida* serotype A (Rhoades & Rimler, 1987). Crude preparations of LPS have been associated with protection in experimental models but it has been shown that purified preparations are not immunogenic (Muniandy *et al.*, 1998).

1.3.2 Protein cellular components

It is generally believed that certain proteins may play an important role in protective mechanisms (Bosch *et al.*, 2004; Grossmann *et al.*, 1998; Lugtenberg *et al.*, 1986). With the exception of some strains in serotypes A and D, the protein components of *P. multocida* are non-toxic (De Alwis, 1999).

1.3.2.1 Outer-membrane proteins (OMPs)

The outer membrane of Gram-negative bacteria plays an important role in interaction of the organism with the environment. It is composed of phospholipids, LPS and OMPs. The LPS and phospholipids together compose a permeability barrier to hydrophobic compounds, and OMPs mainly function in transport of various molecules in and out of the organism and also participate in maintenance of outer membrane stability (Nikaido, 2003). It is generally believed that many virulence factors are surface located and many of them may play important roles in colonization and invasion. OMPs can also act as immunogenic components as they are in direct contact with host immune system during the process of infection (Greenwood *et al.*, 2002). Thus, OMPs have attracted major attention in the studies of host pathogen interactions. The sarcosine-insoluble membrane fraction (Sarkosyl extract) from different bacteria has been extensively used as an OMP preparation since it has been shown that it is highly enriched in OMPs (Davies *et al.*, 1990; Morton *et al.*, 1996; Ravaoarinoro *et al.*, 1994; Zhao *et al.*, 1995).

In *P. multocida* seven proteins have been shown to be present in outer membrane: OmpH-1 (Luo *et al.*, 1997), OmpA (Pm0786) (Gatto *et al.*, 2002), Omp87 (Pm1992) (Ruffolo & Adler, 1996), Omp16 (P6-like protein, Pm0966) (Kasten *et al.*, 1995), PlpB (Cooney & Lo, 1993), Lpp and GlpQ (Lo *et al.*, 2004). OmpW and Omp47 are possible orthologues of characterised OMPs in other species (Boyce *et al.*, 2006). Most of the recent studies on identification and characterization of OMPs of *P. multocida* has been focused on serotype A (causative agent of fowl cholera in chicken), since the whole genome of this serotype has been sequenced.

Whole-cell protein profiles of a wide range of HS-causing strains showed a high degree of homogeneity. B:2 strains showed a major protein band at 32 kDa while E:2 strains showed a major band at 37 kDa. Other bands at 27, 45 and 47 kDa were shared by all strains, irrespective of serotype (Johnson *et al.*, 1991). Proteins of 29 and 36 kDa in the

cytoplasmic and periplasmic fractions and a protein of 42 kDa in the membrane fraction were only partially protective in mice (Ramdani & Adler, 1994).

Certain outer membrane proteins have been reported to be immunogens for *P. multocida* serogroups B and E (Dawkins *et al.*, 1991a; Srivastava, 1998; Tomer *et al.*, 2002). In both A and B serotypes an 87 kDa OMP has been identified as an immunogenic component (Chaudhuri & Goswami, 2001; Ruffolo & Adler, 1996). An oil adjuvant vaccine, prepared from the OMP extract of *P. multocida* B:2, showed complete protection in buffalo calves. Immunoblotts indicated that the 44, 37 and 30 kDa fractions were major immunogens (Pati *et al.*, 1996). About 20 polypeptide bands ranging from 16 to 90 kDa were identified by SDS-PAGE profiles of OMP preparation from *P. multocida* serotype B:2. Three of them with molecular weight of 31, 33 and 37 kDa were considered to be the major OMPs, based on band thickness and intensity of staining. By immunoblotting studies, using whole-cell hyperimmune serum raised in rabbits as well as buffalo immune sera, it became evident that the polypeptide of 37 kDa was the most antigenic OMP in the profiles of all the isolates (Tomer *et al.*, 2002). Iron-dependent outer-membrane proteins (IROMPs) produced by both *P. multocida* and *M. haemolytica* are specific receptors for iron-binding host molecules, such as transferrin, lactoferrin, haemoglobin or haem, and are expressed in the absence of free iron. They play an important role in bacterial pathogenesis and are potential vaccine candidate antigens (Bosch *et al.*, 2004). The rapid spread and multiplication of invasive bacteria like *P. multocida*, requires efficient mechanisms for scavenging iron for growth (Veken *et al.*, 1996). Very low levels of free iron are available inside the host, due to the presence of iron-binding glycoproteins such as haptoglobin, transferrin, and lactoferrin. Generally two mechanisms of iron sequestering systems have been identified in bacteria. The first one is by secretion of siderophores to remove iron from the host iron-binding proteins. Then, the iron-siderophore complex can be captured and internalised by special receptors that are located in the outer membrane. *P. multocida* is able to secret a siderophore called multocidin (Subramaniam *et al.*, 1997). The second mechanism is through iron-binding proteins expressed on the outer membrane of the bacterium, which are able to bind to transferrin and lactoferrin. In the Asian HS-associated serotype of *P. multocida* an 82-kDa iron-regulated OMP has been found which specifically binds bovine transferrin. In contrast, *P. multocida* serotype B:3,4 that is associated with HS in feral ruminants, did not express transferrin-binding proteins (Veken *et al.*, 1996). It was found that convalescent-phase sera from buffaloes reacted against some iron-restricted and other proteins, indicating that these proteins were produced in vivo (Veken *et al.*, 1996). In addition to these mechanisms, some bacterial proteins have the ability to bind haemoglobin. This enables them to use heme as an iron source for their growth. HgbA is a

haemoglobin binding protein identified in *P. multocida* serogroup D from pig (Bosch *et al.*, 2002b) and HgbB was identified in a *P. multocida* A:1 fowl cholera-associated strain (Cox *et al.*, 2003). It has been shown, in a study on different HS-causing serotypes of *P. multocida,* that all three investigated serotypes, B:2, E:2 and B:3,4, bound haemoglobin while none of them bound lactoferrin. Transferrin binding was restricted to strain B:2 (Veken *et al.*, 1996). However, although almost all of these receptors from *P. multocida* were immunogenic, none of them protected mice against infection when used alone as a vaccine antigen (Bosch *et al.*, 2004).

The gene *psl* (P6-like protein product) was shown to be present in all 16 somatic serotypes (Kasten *et al.*, 1995). However, immunization of turkeys with recombinant P6 OMP failed to protect them against challenge despite the production of high titres of antibody to this protein (Kasten *et al.*, 1997b).

The *ompH* gene, encoding a porin that is a major structural protein of the outer membrane of *P. multocida*, is negatively regulated by iron and glucose (Bosch *et al.*, 2001; Luo *et al.*, 1997; Luo *et al.*, 1999). OmpH is a homologue of the P2 porin of *H. influenzae* and a monoclonal antibody against OmpH of *P. multocida* was passively able to protect mice against infection (Marandi & Mittal, 1996). OMP87 (oma87, Pm1992) has high similarity to the D15 OMP of *H. influenzae* (Ruffolo & Adler, 1996). This OMP from *H. influenza* was protective (Loosmore *et al.*, 1997). Recombinant OMP87 protein from *P. multocida* B:2 reacted with serum of vaccinated animals (Chaudhuri & Goswami, 2001).

OmpA is the major OMP of *P. multocida*. OmpA (35 kDa) is a homologue of Omp5 in *H. influenzae* and may be involved in adherence to host cells via heparin and/or fibronectin bridging (Dabo *et al.*, 2003). OmpA has significant identity to *E. coli* OmpA which functions as porin and plays important role in outer membrane stability (Nikaido, 2003). An *ompA*-like gene has been characterized in *P. multocida* serotype A3 that is associated with bovine pneumonic pasteurellosis (Dabo *et al.*, 2003).

A 39 kDa protein from *P. multocida* serotypes A:1 and A:3 induces active and passive protection in chicken or turkeys (Ibrahim *et al.*, 2000; Rimler, 2001). Correlation has been found between the amount of the 39 kDa protein in crude capsular extract (CCE) and pathogenicity and capsule thickness (Borrathybay *et al.*, 2003b). The 39 kDa molecule has recently been characterized as a lipoprotein and named Plp B (*Pasteurella* lipoprotein B) (Tabatabai & Zehr, 2004).

The *omp16* gene, encoding a 16 kDa OMP has been found among different serotypes of *P. multocida* (Goswami *et al.*, 2004). A 39 kDa protein is associated with capsular preparations and has been characterised as an adhesion factor (Ali *et al.*, 2004; Borrathybay *et al.*, 2003a).

1.3.2.2 Enzymes

P. multocida produces a number of enzymes, which are believed to play an important role in the pathogenesis of the diseases caused by this species.

Hyaluronidases are enzymes that are generally associated with invasive mechanisms in bacteria, helminths and snake venoms. Several Gram-positive bacteria produce the enzyme and it has been associated with their virulence (Rimler *et al.*, 1984; Rimler & Rhoades, 1994). Several studies documented that serogroup B strains isolated from cases of HS produced hyaluronidase (Carter & Chengappa, 1980; Rimler & Rhoades, 1994; Rimler, 2000). The role of the enzyme produced by type B strain in pathogenesis is uncertain, especially in view of the fact that it has not been demonstrated in serogroup E strains (De Alwis, 1995). Hyaluronidase production has been examined with different isolates from different hosts in different countries. These studies showed that all B:2 strains had activity against hyaluronic acid (Rimler & Rhoades, 1994).

Neuraminidase has been associated with virulence in other microorganisms but the association of *P. multocida* neuraminidase with a specific disease process or its role as a virulence factor is inconclusive as it was shown there was no correlation between virulence and activity of the enzyme in a mouse model (Drzeniek *et al.*, 1972). Serogroups A, B, D and E produce this enzyme (Drzeniek *et al.*, 1972; Muller & Krasemann, 1974; Scharmann & Blobel, 1972). Its activity is found to be highest in strains of serogroups of A and D (Drzeniek *et al.*, 1972).

1.3.2.4. Toxins

HS-causing B serotypes have not been shown to produce characterised exotoxins. However, cytotoxic effects of serotype B:2,5 on macrophages have been shown by *in vivo* and *in vitro* tests, and HS-associated strains were reported to produce a vacuolating cytotoxin (Shah *et al.*, 1996). No further reports on this toxin have been appeared.

Dermonecrotic toxin, also known as *P. multocida* toxin (PMT), is produced by *P. multocida* serotype D and is the principle virulence factor involved in the pathogenesis of swine progressive atrophic rhinitis (PAR) (Foged, 1991). PMT induces localised osteolysis in nasal turbinates through increased osteoclastic bone resorption (Hunt *et al.*, 2000). No correlation has been found between production of toxin and somatic serotype (Rimler & Brogden, 1986). Antiserum, made against the toxin from a swine serogroup D strain, showed neutralising activities against the lethal effects of the toxin from rabbit serogroup D and swine serogroup A and D strains from different geographic origins (Rimler *et al.*, 1989; Rimler & Rhoades, 1989).

The *toxA* gene has been cloned in *E. coli* and it has also been shown that the cloned gene is able to express functional PMT (Petersen *et al.*, 1991). Toxin related sequences, have not been characterised in other *P. multocida* serogroups (Hunt *et al.*, 2000). Attempts were made to express a protective epitope of PMT in *B. bronchiseptica* to create a single-component mucosal vaccine. This study showed that vaccination of mice with this live recombinant strain was not able to raise antibodies against PMT, although an antibody response to *B. bronchiseptica* was detected (Rajeev *et al.*, 2003). Intranasal challenge of mouse and pig with toxigenic strain of *P. multocida* serotype D causes suppression of antibody response to immunization with ovalbumin. Challenge with non-toxigenic strains did not cause this immunosuppressive effect. The study also showed that a CFE of the toxigenic strains, was also able to cause the suppression (Jordan *et al.*, 2003).

1.3.2.5 Bacteriocins

Bacteriocins are proteins produced by many species of bacteria and show bactericidal properties that are active against their own species or closely-related species (De Alwis, 1999). Some bovine and avian isolates of *P. multocida* are able to produce bacteriocins (Chengappa & Carter, 1977; Lee *et al.*, 1988; Lee *et al.*, 1991). In a study on serotypes A, B, and D, about half of the species were found to produce bacteriocins. The importance of bacteriocins in pathogenesis of the disease is not clear (De Alwis, 1999).

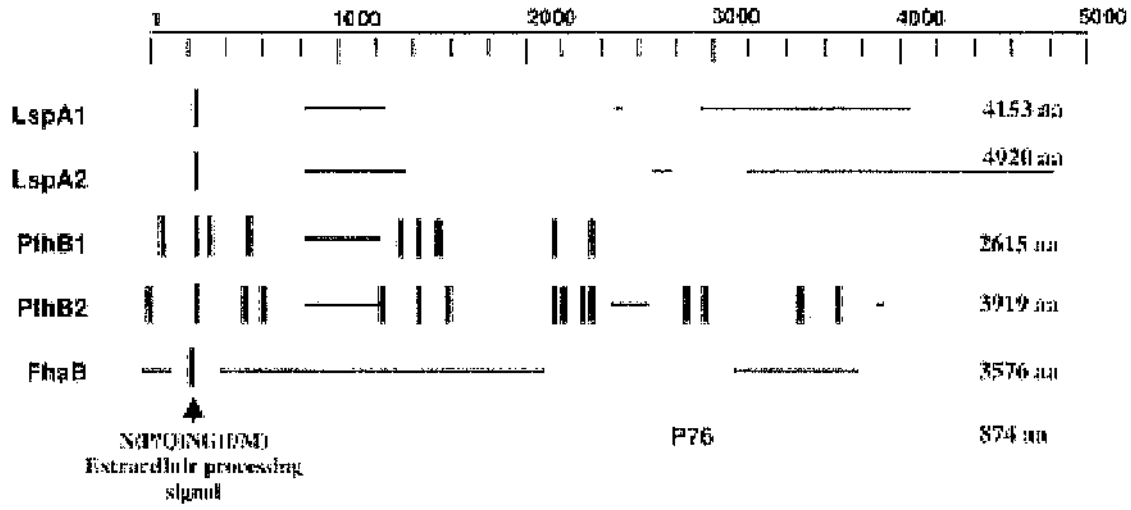
1.3.3 Common antigens

P. multocida contains some antigens which also can be found in other related Gram-negative bacteria. Antigenic relationships with *Haemophilus, Actinobacillus, Escherichia, Mannheimia* and *Neisseria* have been recorded (Bain & Knox, 1961; Prince & Smith, 1966; Schryvers *et al.*, 1986).

Both PfhB1 and PfhB2 (filamentous haemagglutinins) have been recognised as two new potential virulence factors in the genome sequence of the avian isolate *P. multocida* (Pm70). In particular, *P. multocida* has two genes *pfhB1* (7845 bp) and *pfhB2* (11757 bp) with significant homology (Figure 1) to the filamentous hemagglutinin gene in *Bordetella pertussis* (May *et al.*, 2001). They contain domains with strong homology to FhaB of *Bordetella pertussis*. FhaB governs the adherence of *B. pertussis* to host cells and is a major component of acellular vaccines for prevention of human whooping cough (see also 1.4.4). Similar proteins have also been described in several other pathogens, including *Haemophilus ducreyi* (LspA1 and LspA2), *Neisseria meningitidis*, *Serratia marcescens*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* (May *et al.*, 2001).

Figure 1 Homology of PfhB domains between *P. multocida, H. ducreyi,* and *B. pertussis*

Homology comparisons among PfhB1, PfhB2, from *P. multocida*, LspA1, LspA2, from *H. ducreyi*, FhaB from *B. pertussis*, and P76 from *Haemophilus somnus* are presented. Homologous domains are represented with the same coloured boxes and the direct repeats in p76 and PfhA2 are patterned in blue. The N(P/Q)NG(I/M) extracellular processing motif is indicated and the integrin-binding protein motifs are shown as dark purple lines (May *et al.*, 2001).



1.4 Genetics of *P. multocida*

The complete genome sequence of *P. multocida* serotype A:3 (Pm70), a common avian clone, has been published (May *et al.*, 2001). The genome of Pm70 is a single chromosome of 2,257,487 base pairs and contains 2,014 predicted coding regions, 6 ribosomal RNA operons, and 57 tRNAs (May *et al.*, 2001). The function of about half of the *Pasteurella mutocida* coding regions has been determined.

1.4.1 Virulence and virulence-associated genes

Identifying virulence genes has constituted a considerable proportion of studies on *P. multocida* in order to understand pathogenesis at the molecular level and also because of the potential for vaccine development. Despite considerable research, only a small number of virulence-associated genes have been definitively identified. These include those involved in the production of toxin, capsules, haemagglutinins and those involved in amino acid, nucleotide, and iron transport and metabolism (Boyce *et al.*, 2002).

Using signature-tagged mutagenesis, different genes have been identified that, when inactivated, cause a reduction in virulence. In serotypes of *P. multocida* associated with fowl cholera, 25 genes have been identified by this method in a mouse intraperitoneal model (Fuller *et al.*, 2000). In a similar study, 15 mutants have also been identified to be attenuated for chickens, 5 of which were also attenuated in the mouse. Some of the attenuated mutants were due to alterations in metabolic genes (Harper *et al.*, 2003).

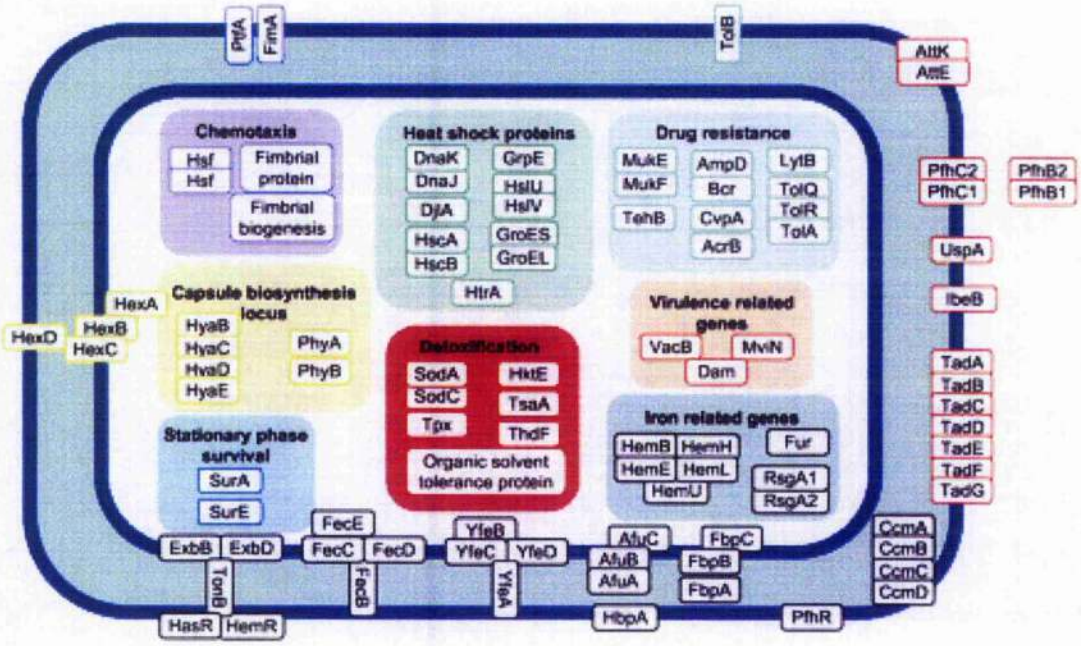
The *P. multocida dam* gene, encoding DNA adenine methylase, has been cloned and sequenced in fowl cholera associated serotypes. Similar to other Gram-negative species, this enzyme regulates the expression of virulence genes. In other bacterial species, Dam is important in regulating and coordinating several cell functions, including initiation of chromosome replication, DNA repair, and gene transcription. As a result of the role of Dam in DNA repair, bacteria with altered Dam activity have increased mutability. Its role in regulation of gene transcription, particularly of genes involved in pathogenicity, causes strains with altered Dam activity to be attenuated. It has been shown that alteration of Dam activity in *P. multocida* causes increased spontaneous mutation frequency and attenuation in mice (Chen *et al.*, 2003).

Several genes that may be involved in virulence and immunity in *P. multocida* and other bacterial pathogens were noted from the genome sequence, such as the type IV fimbrial

subunit gene *ptfA,* capsule biosynthesis genes, genes involved in iron acquisition, the *skp* gene and those encoding outer membrane porin *ompH* and the *omp*87 OMP. Thus, although the molecular basis for pathogenicity of *P. multocida* is not well understood, genomic analysis has identified 104 genes (about 7% of the coding density of the genome) that may be virulence-associated (Figure 2) (May *et al.*, 2001). However, the mechanisms that control expression of these potential virulence factors have not been precisely determined (Chen *et al.*, 2003).

Figure 2 **A comprehensive view of the biochemical processes involved in *P. multocida* pathogenicity**

Orthologues previously identified as virulence factors in other organisms are represented. Principal functional categories are shown in bold. Potential coding sequences related to these functions are arranged within each correspondingly coloured category (May *et al.*, 2001).



1.4.2 Iron-related genes

More than 2.5% of the *P. multocida* genome is devoted to genes encoding proteins homologous to known proteins involved in iron uptake or acquisition (May *et al.*, 2001). It has been shown that the expression of these genes increases under iron-limiting conditions (Paustian *et al.*, 2001). The *fur* gene of *P. multocida*, which encodes a predicted protein of 147 amino acids, has been cloned. The Fur protein, which exhibits Fe^{2+}-dependent DNA-binding activity, is the major regulator of genes encoding haem- or haemoglobin-binding proteins (Bosch *et al.*, 2001; Ekins & Niven, 2002). A *fur*-knockout mutant of *P. multocida* showed constitutive expression of high-molecular-mass proteins that have been associated with iron-uptake processes (Bosch *et al.*, 2001).

Expression of the *hgbA* gene produced two outer-membrane proteins, of 40 and 60 kDa in iron-depleted cultures using 2,2`-dipyridyl (DPD) as an iron chelator (Garrido *et al.*, 2003a). It is believed that *hgbA* is widespread in *P. multocida* strains regardless of their serotype or the animal from which they were isolated (Bosch *et al.*, 2002b). Although antibodies against the 40 and 60 kDa molecules were present in the serum of infected mice, they were not able to cause protection against challenge. It has been demonstrated that the *hbpA* gene is negatively regulated by iron, manganese and haemin through a *fur*-independent pathway (Garrido *et al.*, 2003a).

The *hgbB* gene from a fowl cholera-associated *P. multocida* strain, has been cloned and characterized. HgbB is an outer-membrane protein which shares 68 and 69% similarity to the haemoglobin/haemoglobin-haptoglobin binding protein HI0712 from *Haemophilus influenzae* Rd and HgpC from *H. influenzae* B, respectively. Inactivation of *hgbB* did not affect the ability of *P. multocida* to bind hemoglobin, nor its ability to produce disease in a mouse model. In addition, recombinant HgbB did not confer any protection against homologous or heterologous challenge (Cox *et al.*, 2003).

After iron has been captured, its transport into the cell requires the activity of the *exbB*, *exbD* and *tonB* gene products which supply the energy necessary for this process (Garrido *et al.*, 2003a). The expression of these genes in *P. multocida* is under iron control. It has been suggested that in fowl cholera-associated serotypes each of these three genes is necessary for virulence (Bosch *et al.*, 2002a).

The *P. multocida znuACB* genes encode a high-affinity zinc-uptake system. Contrary to that determined so far for all other *znuACB* bacterial systems known, *P. multocida znuACB*

genes are not under the control of the *zur* gene, which is absent in this bacterium, but rather are controlled by the iron-uptake regulator *(fur)* (Garrido *et al.*, 2003b).

1.4.3 Metabolic genes

There have been few metabolic genes cloned or characterized from *P. multocida*. A gene encoding the *P. multocida* adenylate cyclase has been described. It was very similar to its *E. coli* homologue with the deduced protein sequences indicating N-terminal catalytic and C-terminal regulatory domains (Mock *et al.*, 1991). The beta-subunit of the *P. multocida* tryptophan synthase has been cloned, with high level of similarity to homologues from other Gram-negative bacteria (Jablonski *et al.*, 1996). A *galE* mutant of *P. multocida* prepared by allelic exchange showed attenuation in the mouse. *galE* encodes an enzyme required for the epimerization of UDP-glucose to UDP-galactose prior to LPS assembly, and this mutant probably expresses an altered LPS (Harper *et al.*, 2004). The *galE* gene of *P. multocida* is able to complement a *galE* mutant of *Salmonella* (Fernandez de Henestrosa, 1997). The GalE protein is most closely related to a *H. influenzae* homologue with 85% identity. A transposon mutant deficient in heptosyl transferase III was shown to be attenuated in mice and chickens (Harper *et al.*, 2003). $waaQ_{PM}$, encoding a putative enzyme, is required for the addition of heptose to LPS. It was shown that the predominant LPS glycoforms of a $waaQ_{PM}$ mutant strain were severely truncated and the strain was attenuated. It was also noted that the attenuation was not due to increased serum sensitivity (Harper *et al.*, 2004).The gene *firA* encodes a glucosamine transferase which appears to be involved in the biosynthesis of Lipid A (Dicker & Seetharam, 1991).The *skp* gene has been cloned but its function is not very well understood. The *skp* and *firA* genes are believed to be a part of an operon governing the first steps of lipid A synthesis (Delamarche *et al.*, 1995; Manoha *et al.*, 1994).

Transposon insertion in the *purN* gene of a fowl cholera-associated *P. multocida* caused attenuation for both chickens and mice. This gene encodes the enzyme 5`-phosphoribosylglycinamide transformalase N, which catalyzes the fourth step of *de novo* purine biosynthesis (Harper *et al.*, 2003). The ability to synthesize purine nucleotides from simple precursors has been identified as being important for bacterial growth *in vivo*, and it has been reported recently that several of the genes involved in purine biosynthesis in *P. multocida* are up-regulated in bacteria harvested from the blood of infected chickens (Boyce *et al.*, 2002).

The gene encoding the *P. multocida* AroA (5-enolpyruvylshikimate-3-phosphate synthase) enzyme has been exploited to produce different attenuated mutants of avian and bovine serogroups. The enzyme catalyses the 6th step in the shikimate pathway for the biosynthesis of aromatic amino acids (L-phenylalanine, L-tyrosine and L-tryptophan). A defective *aroA* sequence creates a dependence for growth on aromatic compounds. Different deletion mutants of the *aroA* gene of *P. multocida* strains have been prepared (see also 1.12.4) using allelic exchange and shown to be attenuated for virulence in chicken or mouse (Homchampa *et al.*, 1992; Homchampa *et al.*, 1994; Homchampa *et al.*, 1997; Scott *et al.*, 1999; Tabatabaei, 2000; Tabatabaei *et al.*, 2002).

1.4.4. Genomic diversity and homology

The genetic map *of P. multocida* Pm70 represents the only *P. multocida* genome to have been sequenced. However genomic heterogeneity within *P. multocida* has been studied using different methods of molecular epidemiology such as repetitive sequence-based PCR (rep-PCR) and amplified fragment length polymorphism (AFLP) (Amonsin *et al.*, 2002), ribotyping (Blackall *et al.*, 1998), rRNA gene restriction site polymorphism analysis (ribotyping) and restriction endonuclease analysis (REA) (Amonsin *et al.*, 2002; Blackall *et al.*, 1998; Dabo *et al.*, 1999; Wang *et al.*, 2007). Avian strains showed considerable genomic diversity (Blackall *et al.*, 1998; Blackalu *et al.*, 2001). The generation of genetic maps of other serotypes of *P. multocida* to compare their organisation could help to understand whether the restriction polymorphism relates to a distinct association between the genome architecture and properties such as virulence, host specificity and other metabolic or pathological phenotypic aspects (Hunt *et al.*, 2000).

1.4.5 Gene regulation

To survive and multiply inside and outside the host, bacterial pathogens require the coordinated expression of a range of genes. By gene transcription experiments, it has been proved that genes are differentially regulated under different *in vitro* conditions and during different *in vivo* stages. It is likely that pathogens continually alter their gene expression profiles in response to the innate immune system and to the environment of their host, as they move from one host niche to another (Boyce *et al.*, 2002) or under different cultural conditions like low iron, different temperatures, different pHs, and different media (Xia *et al.*, 2002). Whole-genome microarrays of *P. multocida* serotype A (Pm70) have been utilized to compare levels of gene expression during growth in rich and minimal media. It has been shown that the expression of a total of 669 genes (one third of the genome) was

detectably altered over the course of the experiment. A large number (n = 439) of genes, including those involved in energy metabolism, transport, protein synthesis, and binding, were expressed at higher levels in rich medium. Genes with increased expression in minimal medium (n = 230) included those encoding amino acid biosynthesis and transport systems, outer membrane proteins, and heat shock proteins (Paustian *et al.*, 2002).

There are only a very limited amount of data available on how expression of bacterial virulence genes changes during stages within the host. A comparison has been made between gene expression profiles of *P. mutocida* harvested from the blood of septicaemic chickens in the last stages of fowl cholera and those from bacteria grown in rich medium. It was shown that 40 genes were differently expressed in all of three chickens. The majority of the up-regulated genes were involved in amino acid transport and metabolism and energy production pathways to cope with the host environment. Interestingly, bacteria from only two of the three infected animals had a gene expression profile highly similar to that observed during growth under iron-limiting conditions, suggesting that severe iron starvation may not always occur during *P. multocida* infection (Boyce *et al.*, 2002).

1.5 Distribution and importance of haemorrhagic septicaemia (HS)

1.5.1 Definition

HS, also called septicaemic pasteurellosis or barbone, is an acute septicaemic disease that occurs in cattle, yaks, camel, and water buffalo, and to a much smaller extent, pigs and horses. HS principally affects cattle and buffaloes. It is a primary pasteurellosis caused by serotypes B:2 or E:2 of *Pasteurella multocida* (Radostits *et al.*, 2000).

1.5.2 Global distribution

HS has been recorded in Asia, Africa, America and Europe. It has never occurred in Oceania (De Alwis, 1999). According to FAO (1991), some southern European states have recorded a low occurrence whilst low sporadic or endemic status has been reported in many south American states. According to Animal Health Yearbook 1994, there have been some reports of the disease from southern and eastern European countries but they were not confirmed by serotyping. In Africa, HS occurs in a number of countries in all regions of this continent (De Alwis, 1992).

HS is endemic in some areas of Asia and is the classical disease of southern Asia (Radostits *et al.*, 2000). It has also been reported to be endemic in the Near and Middle East. The disease was reported from Iran in 1930s, and a serious study of the disease was made at the Razi Vaccine and Serum Research Institute in 1935. This resulted in the introduction of a saponin-adjuvanted vaccine in 1938 using a native isolated strain (Delpy & Rastegar, 1938; Kaweh *et al.*, 1960). HS occurs in South and Southeast Asia, including Indonesia, Myanmar, Laos, Cambodia, the Philippines, Thailand and Malaysia. The disease was first reported in 1880 in Malaysia (Benkirane, 2002). In Sri Lanka, it was reported in 1911 for the first time. Routine vaccination was introduced in 1957 after the epidemics in the mid-1950s (Wijewardana, 1986). HS is believed to have been introduced into the Philippines in buffaloes imported from Hong Kong in 1902 and in a shipment of cattle imported from Shanghai in 1903. In Japan the disease was reported in 1923 for the first time but there is no report of HS after 1954 (De Alwis, 1999).

In North America, HS is not endemic although there have been some reports of the disease in bison. Some cases of sporadic outbreaks have been recorded in dairy and beef cattle in different areas of the USA (Chengappa *et al.*, 1982; Heddleston *et al.*, 1967). In Canada, an outbreak was reported in beef calves in 1994 (Rimler & Wilson, 1994). In some South American countries HS is likely to occur due to the significant population of water buffalo and conditions similar to those in tropical areas of Asia.

1.5.3 Economic importance

The actual economic losses due to HS are difficult to determine. The problem is that many factors need to be considered in computing economic losses (De Alwis, 1999). HS is a disease that is most prevalent in situations where husbandry practices are poor and, concurrently, disease surveillance systems are often not well developed. Reported losses, therefore, merely reflect the trend whereas actual losses may be considerably higher. Very few countries have made actual estimates of economic losses (De Alwis, 1992). Sudden epidemics do occur occasionally in non-endemic areas, causing heavy losses in all age groups of animals. In endemic areas, most adult animals have acquired natural immunity, and the disease is prevalent among young animals in the 6 months to 2 years age group. Such losses, though insidious in nature and difficult to estimate, are economically significant (De Alwis, 1992).

1.5.3.1 Losses in Asia

HS is economically the most important disease of cattle and buffaloes, particularly in Asia and to a lesser extend in Africa (Benkirane, 2002). According to FAO reports, Asia harbours 447 million cattle and 161 million buffaloes, 33% and 95% of the world's populations of these species, respectively (FAO, 2003). The high population of buffaloes in Asia and the higher susceptibility of this species to HS, have made more significant the economic losses due to HS in Asia. 37% of the milk in Asia is produced by buffaloes but, in India, where the production of milk is the highest, this amount is 50%. It was estimated that, in India, during the past four decades 46-55% of all bovine deaths were due to HS (Dutta *et al.*, 1990). In Myanmar about 50% of the governmental measures in animal disease control is spent on HS (Johnson *et al.*, 1989). According to an FAO report in 1979 in Pakistan 34.1% of all deaths in susceptible animals were caused by HS and economic losses in 1978 were estimated at US$189 million (De Alwis, 1999). In most of the Asian countries, cattle and buffaloes are used for draft power mainly in the rice fields. Concurrent seasonal outbreaks of the disease in farms, during the harvest season, highlight the significance of the economic losses caused by HS in these countries. In most estimates of losses, only direct losses, i.e. value of animals that die of HS, has been taken into account. A true estimate of losses should cover all of the factors, which also constitute the above mentioned indirect losses (Benkirane, 2002).

1.5.3.2 Losses in Africa

Africa has less of the world's cattle and buffalo populations, with only 15% and 2% for cattle and buffaloes, respectively. As a result, the disease is of less economic importance in Africa than in Asia. There are some other diseases that are economically more important than HS. These include the African endemic diseases, such as trypanosomiasis, theilleriosis and contagious bovine pleuropneumonia; and also rinderpest and FMD (Foot and Mouth Disease), which are common in both Africa and Asia (De Alwis, 1999).

1.6 Haemorrhagic septicaemia: clinical aspects

1.6.1 The disease

Haemorrhagic septicaemia is an acute, highly fatal disease characterised by terminal septicaemia, principally affecting cattle and buffaloes. It is caused by two serotypes of the bacterium *Pasteurella multocida* known as the Asian and African serotypes, designated

6:B and 6:E by the Namioka-Carter system and more recently B:2 and E:2 by the Carter-Heddleston system, respectively (OIE, 2004).

Using modern techniques of serotyping, it has been indicated that type B strains of *P. multocida*, other than serotype B:2, have also been involved in some cases of HS (Rimler & Wilson, 1994). In view of the wider spectrum of serotypes of the B and E groups, and the involvement of species of animals other than cattle and buffaloes it may be more appropriate to give the disease a broader definition as: an acute, fatal, septicaemic disease caused by strains of *P. multocida* belonging to the serogroups B or E, commonly in cattle and buffaloes and also in pigs and feral ruminants (De Alwis, 1999).

Unlike other pasteurelloses, where the pasteurella plays a secondary role, HS is a primary pasteurellosis. The disease is experimentally reproducible using pure cultures of the causative organism alone and is preventable by vaccines incorporating the specific serotypes. Thus, HS is a specific form of pasteurellosis, occurring mainly in cattle and buffaloes. This is similar to the situation of typhoid in humans, and pollorum in poultry, which are both caused by a specific strain of *Salmonella* in a specific host species (De Alwis, 1992).

1.6.2 Source of infection

Pasteurella multocida dose not survive for a long time in the environmental conditions outside the host. Thus, the environment could not be the major source of infection although the organism survives for relatively longer in moist conditions (De Alwis, 1999). It has been shown that the organism survives in sterilised soil for 2-3 weeks but in the natural mud where animals live this survival period is not more than 24 hours (De Alwis, 1999). It is believed that pasteurellae can survive in animal tissues and perhaps in carcases for a few days. Thus, carcases dumped into rivers are likely to be a method of spreading the infection. However, no permanent source of infection has been established outside the host (De Alwis, 1999).

Outbreaks of HS are more likely to occur when clinically affected or carrier animals are introduced into the herd. In experimental development of the disease, 10^7 to 10^{12} colony forming units (CFU) are required to produce the infection by the natural routes (De Alwis *et al.*, 1990). In the natural transmission of the infection it is not clearly understood if or how such a large dose of the bacterium is transmitted to an animal. It has been suggested that unknown circumstances may alter the susceptibility of animals so that a smaller

number of bacteria can cause the disease. Alternatively, it can be speculated that bacteria grown *in vitro* are not as virulent as those multiplied in the host (De Alwis, 1999). Isolates from clinical cases and from latent carrier animals showed no difference in virulence for mice (Wijewardana, 1986) but, as the presence of host-specific virulence factors has been shown (Harper *et al.*, 2003), this remains to be investigated in the natural hosts.

1.6.3 Route of infection

Natural routes of infection are by inhalation and/or ingestion of contaminated materials. The disease is reproducible using oral drenching or aerosol sprays, although experimental infections have shown that large numbers of organisms are required to set up an infection by the intra-nasal or the oral route. However, the dose required to produce clinical disease is not always reproducibly the same and also results with a given dose are not always the same (De Alwis, 1994).

Artificial routes of infection are used in most experiments because they are more reproducible. Subcutaneous injection of 10^4 to 10^7 CFU is able to cause the disease. Different routes of experimental infection are loosely related to the course of the disease, clinical syndrome and the extent of pathological lesions. Generally, intranasal and oral infections result in a longer course of the disease and more profound lesions. On the other hand subcutaneous inoculation of the agent results in a rapid onset of disease, a shorter course and less marked pathological lesions (De Alwis, 1999).

1.6.4 Clinical signs

Haemorrhagic septicaemia is usually manifest by a rapid course, sudden onset of fever, profuse salivation, severe depression and death in about 24 hours. These are the usual symptoms observed in the disease. Oedema in the submandibular region is common and in later stages the affected animal may show severe dyspnoea and alimentary tract disorders. In outbreaks, sudden death without any previous signs is usually reported (Horadagoda *et al.*, 2002).

The disease is more acute and has a shorter course in buffaloes than cattle (De Alwis, 1994). Generally as a syndrome, during the progression of the disease, three phases can be distinguished. In the first phase which is the phase of temperature elevation (often unnoticed) loss of appetite, depression, general apathy and increased temperature are dominant signs. The fever (41-42°C) lasts throughout the course, but the temperature drops

to subnormal levels during the terminal phase, a few hours before death. In the second phase respiratory signs such as increased respiratory rate, laboured breathing, clear nasal discharge and salivation are dominant. Submandibular oedema may appear in this stage. Nasal discharges become mucopurulent with increased severity of the disease. In the third phase recumbency is the most obvious sign and terminal septicaemia occurs and death follows in almost all of the cases (OIE, 2004). The incubation period is usually 1-3 days, and the course of the disease may range from sudden death, with no observable clinical signs, to a protracted course extending up to 5 days (Radostits *et al.*, 2000).

At post-mortem examination, the first obvious lesion is subcutaneous oedema, particularly in the submandibular and brisket region. Other lesions include petechial to ecchymotic haemorrhages, congestion and/or consolidation of lungs, fibrinous pneumonia, pleurisy and pericarditis (OIE, 2004).

1.6.5 Pathogenesis of HS

It appears that after entry into an unexposed susceptible animal, the initial site of multiplication is the tonsillar region. The host defence mechanisms, both specific and non-specific, will interact with the pathogen which will lead either to clinical disease or arrested infection in which the animal becomes an immune carrier. The dose of infecting organisms, amongst other factors, is an important determinant of outcome, at least in experimental infection (De Alwis, 1999; Horadagoda *et al.*, 2001; Radostits *et al.*, 2000).

There is little evidence that HS-causing strains of *P. multocida* produce specific exotoxins that are required for pathogenesis. However, it has been shown that sera from buffaloes recovering from HS are able to inhibit the vacuolating cytotoxic activity of the organism and of culture supernatants known to contain free toxin (Shah *et al.*, 1996). The production of hyaluronidase by HS-causing strains of serotype B:2 has been implicated in pathogenesis (Carter & Chengappa, 1980), but other B type strains such as B:3,4, which are able to produce a syndrome similar to HS, are not able to produce hyaluronidase. Therefore, the importance of this enzyme is not clear (De Alwis, 1999; Rimler & Rhoades, 1994). An 82-kDa iron-regulated OMP, has been detected in serotype B:2 which specifically binds bovine transferrin, but the protein has not been detected in serotype B:3,4. (Veken *et al.*, 1996).

The role of endotoxin of *P. multocida* in the pathogenesis of the disease is important (Heddleston & Rebers, 1975; Horadagoda *et al.*, 2002; Musa *et al.*, 1972). It has been

shown that administration of endotoxin to buffalo calves produces a clinical syndrome similar to HS. Endotoxin can also promote an acute phase response (APR) including production of tumour necrosis factor-α (TNFα). The APR is a non-specific, systemic host response that is cytokine-mediated. It results from tissue injury or infection and is an integral part of the host defence mechanism (Horadagoda *et al.*, 2002). Therefore, LPS can facilitate the multiplication and spread of the bacterium in the host, and further reinforces the role of the capsule in virulence mechanism (Boyce & Adler, 2000; De Alwis, 1999). Many of the putative virulence-associated components were identified in *P. multocida* serotype A chicken isolates such as fimbriae and filamentous hemagglutinin (Fuller *et al.*, 2000; Harper *et al.*, 2003) and it is not clear in many cases whether they are also present in the HS-associated strains (Section 1.4.1).

1.6.6 Bacteriology

Tonsillar tissues are believed to be the initial site of multiplication after the entry of the pasteurella organisms into the host. *Pasteurella* can be isolated from blood only during the terminal stages of the disease. At the time of death, around 10^5-10^6 CFU/ml appear in the blood. Multiplication of the bacterium occurs in the carcass. Under tropical conditions, bacterial counts in the carcass's blood can reach to 10^{11}-10^{12} CFU/ml (De Alwis, 1999).

1.7 Epidemiology

HS, in most countries of Asia and sub-Saharan Africa is an endemic disease. According to the incidence and distribution of the disease, Asian countries have been classified into three categories; countries where the disease is endemic or sporadic, countries with clinically suspected but not confirmed cases of the disease, or countries free of the disease (Benkirane, 2002).

1.7.1 Seasonal distribution

The distribution of HS is related to climatic conditions, husbandry practices, and the type of animals reared. HS is generally associated with wet, humid weather conditions and most outbreaks occur during the wet seasons. Outbreaks can occur at all times of the year but those occurring during wet seasons tend to spread, presumably due to the longer survival of the organism under moist conditions (Radostits *et al.*, 2000). In the endemic areas, outbreaks usually coincide with onset of rains which is normally preceded by a long dry period (Francis *et al.*, 1980). The correlation with wet weather conditions has been proved

by studies in India and Pakistan. Seasonal occurrence has been well documented in India, with the majority of the outbreaks occurring during wet seasons from July to September, with the peak in August. Seasonal occurrence has also been confirmed in Pakistan (Sheikh *et al.*, 1996). In India, the disease occurred more frequently in states that received a higher annual mean rainfall (Dutta *et al.*, 1990).

1.7.2 Serotype and strain distribution

The Asian serotypes belong to capsular type B only whereas the African serotypes are types B and E (OIE, 2004). Egypt and Sudan have reported the presence of both E and B serotypes (Hassanin *et al.*, 1995; Shigidi & Mustafa, 1979) and from Cameroon there is one record of an outbreak caused by the B strain (Martrenchar & Njanpop, 1994). There are indications that serotype B may be present in some East African countries (De Alwis, 1999).

North American isolates are recorded as B:2 serotypes. Re-examination of these isolates using serotyping and fingerprinting methods revealed that a bison strain isolated from Montana in 1965 was serotype B:3,4, not B:2 as earlier reported (Rimler & Wilson, 1994). Other serotypes associated with sporadic septicaemic pasteurellosis resembling HS in feral ruminants include serotype B:1 isolated from antelope in the United States, and B:4 from bison in Canada. Genetic fingerprinting has established that the North American B:2 strains possesses a DNA profile different from that of the Asian B:2 strains (De Alwis, 1999).

HS has not been reported from United Kingdom. However, from deer with a septicaemic disease a strain has been isolated which has been identified as serotype B:3,4. This strain has been used as a live vaccine in a large-scale trial (Section 1.12.2) (Jones & Hussaini, 1982; Myint *et al.*, 1987).

From an epidemiological standpoint, DNA fingerprinting serves as an additional tool to serotyping methods in strain identification (Wilson *et al.*, 1992). Recently, a global collection of over 200 isolates associated with classical HS disease has been examined using DNA fingerprinting along with other serotyping methods. According to this, a large number of DNA fingerprint profiles have been identified amongst HS-associated group B isolates (Rimler, 2000).

1.7.3 Host susceptibility

Buffaloes are generally believed to be more susceptible to HS than cattle, and in this species, the disease course is shorter. The overall mean case-fatality rate for buffaloes is nearly three times as high as in cattle (Radostits *et al.*, 2000). In Sri Lanka it has been shown that whilst the herd infection rate was not significantly different in cattle and buffalo herds, the morbidity rates within affected buffalo herds was considerably higher (De Alwis *et al.*, 1990; Wijewardana, 1986). The buffalo is highly sensitive to circulatory LPS and this may be the reason for higher mortality rate in buffalo than in cattle (Horadagoda *et al.*, 2002). Reports are available on the association of HS serotypes with disease in other species. Sporadic outbreaks of HS have been reported among pigs caused by the Asian B:2 serotype (Verma, 1988). It has been shown in Sri Lanka that goats kept in close contact with buffaloes clinically affected with HS developed neither disease nor immune carrier status. In fact, experimentally, large doses of organism (10^4 to 10^{12} CFU) were able to cause disease only in 10% of goats (Wijewardana, 1986). Infection with B:2 serotype has been recorded among elephants associated with outbreaks in cattle and buffaloes in Sri Lanka (de Alwis & Sumanadasa, 1982). HS has been reported among camels in Sudan (De Alwis, 1999; Hassanin *et al.*, 1995). In the United States, septicaemic disease caused by serotype B:1 has been recorded in antelopes and that caused by B:3,4 in elks. In Canada, the disease caused by B:4 has been recorded in bison (Rimler & Wilson, 1994).

1.7.4 Morbidity and mortality rate

Both morbidity and mortality rates vary between 50 and 100% and animals that recover require a long convalescence. Morbidity and mortality are influenced by a variety of factors and their interactions. These factors are 1) host species and age; 2) immunological factors; 3) endemic and non-endemic areas; 4) husbandry methods (Radostits *et al.*, 2000). Relatively higher morbidity rates have been recognised among young animals aged 6 months to two years. In Sri Lanka for example, 65% and 77% of all HS death among cattle and buffaloes, respectively, were in animals under 2 years of age (Wijewardana, 1986).

When clinical disease is established, case fatality approaches 100% if treatment is not carried out sufficiently early (in the pyrexic stage). Morbidity is high in non-endemic areas when HS occurs as sporadic outbreaks and also when it is first introduced in clean areas. Morbidity in endemic areas is related to herd immunity status, the greater the percentage of immune to non-immune animals, the lower will be the morbidity (Radostits *et al.*, 2000).

1.7.5 Carrier animals

The presence of the HS organisms in the nasopharynx of healthy cattle and buffaloes is well documented (De Alwis *et al.*, 1990; Wijewardana, 1986). Further investigations showed that the presence of nasopharyngeal carrier animals was related to recent outbreaks of the disease, indicating that the percentage of nasopharyngeal carriers is related to recent exposure to disease. Approximately 45% of healthy cattle in herds associated with the disease harbour the organism in comparison to 3-5% in cattle from herds unassociated with the disease (Radostits *et al.*, 2000).

Pasteurellae are not confined to the nasopharynx and associated lymph nodes of animals could harbour the organism (Wijewardana, 1986). It has been observed that some animals display a positive status, then negative, and subsequently positive again, indicating an intermittent appearance of the organism in the nasopharynx. It has been concluded that two types of carrier status exist - latent and active. In the active carriers the bacterium is present in the nasopharynx and from this site is shed in nasal secretions. Latent carriers harbour the organism in tonsils in which the bacterium is believed to multiply and intermittently spill over into the nasopharynx (De Alwis *et al.*, 1990). It has been shown by immunochemical techniques that the exact place in which Pasteurella is localized is the crypt of the tonsil and not the tonsilar tissue (Horadagoda & Belak, 1990). The mechanisms involved in converting a latent carrier to active carrier have not been revealed yet (De Alwis, 1999).

1.7.6 Epidemiological cycle

A presumptive epidemiological cycle (Figure 3) for haemorrhagic septicaemia has been suggested (De Alwis, 1994). The old theory that, under stress, carrier animals break down into clinical cases does not appear to hold, since all carrier animals also have high antibody levels believed to be protective (De Alwis, 1999). In an endemic area, after one outbreak of HS, a large number of surviving animals become latent carriers. They intermittently shed the organisms, the frequency diminishing with time. Since the herd immunity is high, there would be no new clinical cases. The first clinical case occurs when a shedder comes into contact with a susceptible animal, which is a new animal to a herd, such as a newborn after the previous outbreak, or as an introduced animal from elsewhere (De Alwis, 1994).

Movement of animals has frequently been associated with epidemics. Movements can precipitate disease in two ways. Firstly, the animals being moved may be carriers and able to infect susceptible stock. Secondly, the animals being moved may be susceptible and

then may be infected from native immune carriers. In either case explosive outbreaks could result (De Alwis, 1999).

1.8 Diagnosis of HS

1.8.1 Clinical diagnosis

A clinical diagnosis can be made on the basis of characteristic signs, gross pathological lesions, herd history, morbidity and mortality patterns, species susceptibility, and age group affected. A tentative clinical diagnosis is generally important because the immediate investigation of control measures to prevent spread of disease is based on a field diagnosis (OIE, 2004). As HS occurs mostly under poor husbandry conditions, no clinical signs whatever may be observed in the first case of an outbreak (Radostits *et al.*, 2000). Since morbidity and mortality rates are highly variable and are dependent on a number of factors, such as the presence of susceptible animals, the previous occurrence of HS in the herd and climatic conditions, whether it is an enzootic area, the age group and species affected, whether the animals had been vaccinated, and finally their interactions, they must be viewed against the background of these surrounding circumstances (De Alwis, 1994).

1.8.2 Laboratory diagnosis

Laboratory confirmation of diagnosis is by isolation in culture or in mice and identification of the agent by biochemical and serological methods as type B or E. An outline of a scheme for routine laboratory tests for diagnosis of HS from test materials (De Alwis, 1994), is shown in Figure 4. Laboratories in countries where HS is endemic receive a variety of specimen types and quality for diagnosis. A wide range of laboratory diagnostic tests have been developed which mainly include: 1) culture on specific media for isolation; 2) biochemical and serological tests for identification and serotyping; 3) nonserological tests to complement presumptive identification of serotype; 4) molecular methods for strain identification of serotypes (OIE, 2000).

Figure 3 **Presumptive epidemiological cycle for haemorrhagic septicaemia (De Alwis, 1994)**

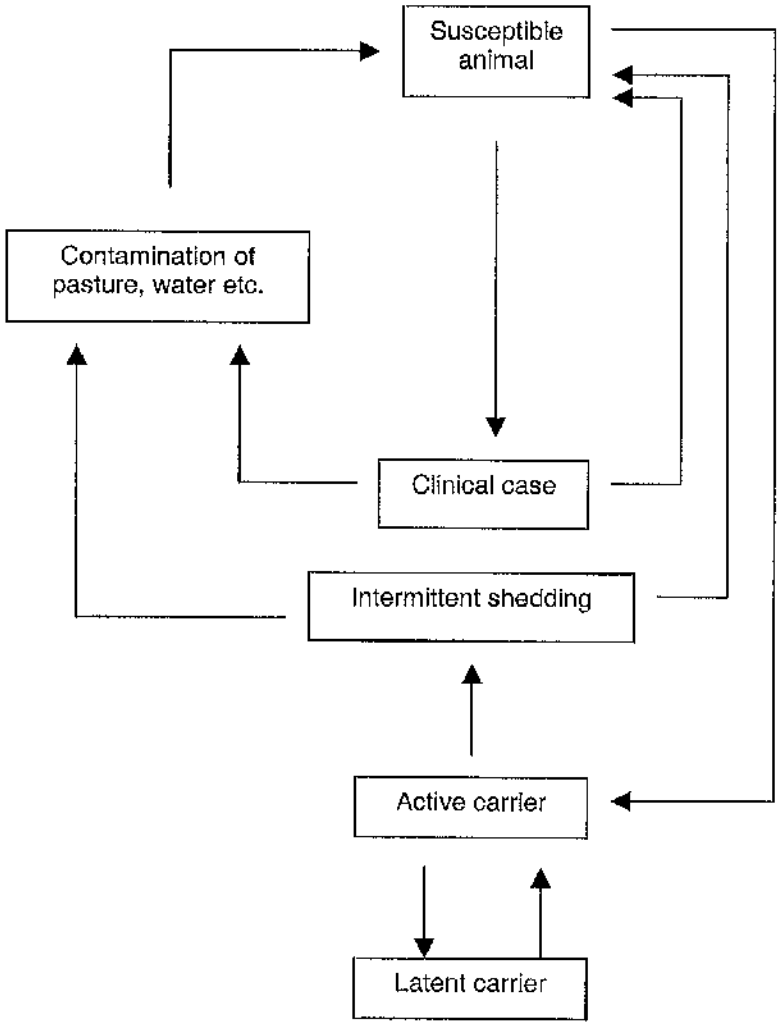
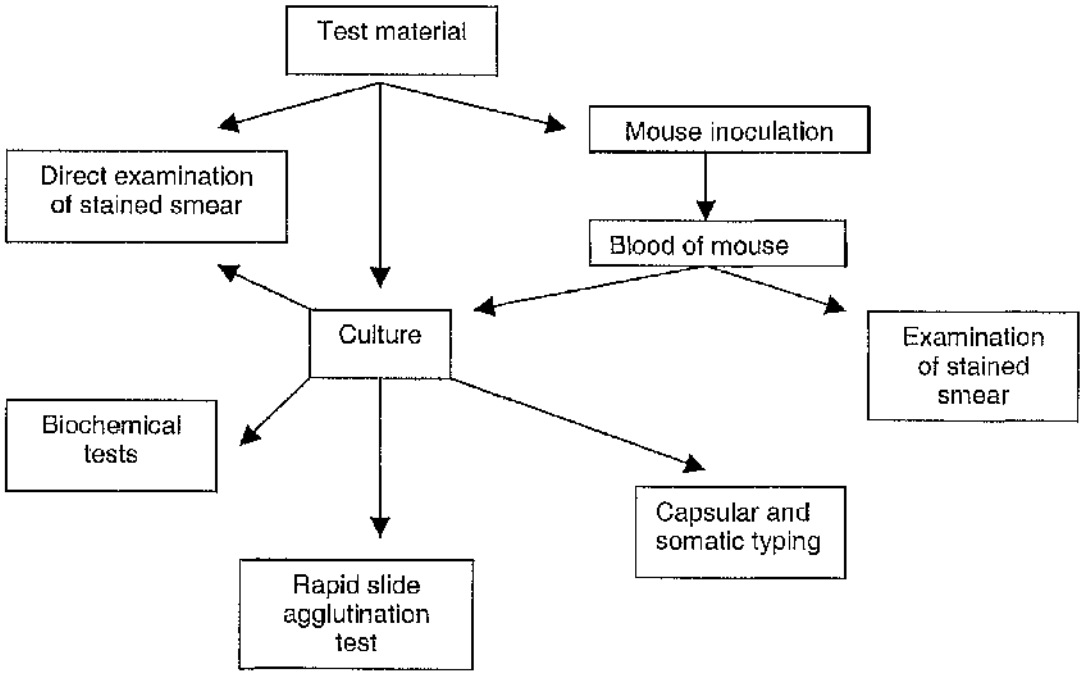


Figure 4 **Scheme for routine laboratory diagnisis of haemorrhagic septicaemia (De Alwis, 1994)**



1.8.2.1 Microbiological tests

The conventional method of laboratory diagnosis is based on isolation of the organism from animal tissues and identification by biochemical and serological methods. The organism can be cultured from blood or nasal swab from an animal within a few hours of death. From older carcasses, a long bone is used for culture from the bone marrow (Radostits *et al.*, 2000). Pasteurellae grow on ordinary media such as nutrient agar, or enriched media such as tryptose agar and CSY agar (Casein Sucrose Yeast), with or without 5% sterile blood. Direct culture yields results only with fresh material. Contaminants and post-mortem invaders, when present, overgrow the pasteurellae (OIE, 2004). Biological screening (mouse inoculation) is used to isolate *P. multocida* from contaminated samples. In this method a saline suspension of the sample (0.2 ml of eluted blood swabs or a portion of bone marrow in saline) is used for subcutaneous inoculation of a mouse. If *P. multocida* types that cause HS are present, the mouse will die within 24-36 hours. Smears of the heart blood of the infected mouse will show an abundance of bipolar staining coccobacilli when stained with Gram, Leishman or methylene blue stains. The blood of the mouse can also be cultured, and will yield pure cultures (De Alwis, 1994).

Cultures are oxidase and catalase positive, produce indole and reduce nitrates, but fail to produce urease, utilise citrate, grow on McConkey agar medium or liquify gelatine. Growth on triple sugar iron (TSI) agar helps to differentiate pasteurellae from the common Gram-negative enteric bacteria. *Pasteurella* gives a slow acid reaction with no gas, and no detectable production of hydrogen sulphide in this medium (OIE, 2004).

1.8.2.2 Serological tests

Different serological methods have been used for identification of pasteurellae which are principally based on the detection of the capsular and somatic antigens. Conventional serological tests which are currently in use in diagnostic laboratories include: rapid slide agglutination test, indirect haemagglutination test (IHAT) and agar gel precipitation test (AGPT). Several other serological tests have been developed which are mainly used in research programmes. These include AGPT for capsular typing, counter-immunoelectrophoresis for somatic typing, coagglutination test for differentiation between type B and E, agglutination test for somatic typing and enzyme-linked immunosorbent assay for identification of *P. multocida* that cause HS (OIE, 2004).

The rapid slide agglutination test is a simple method for capsular typing of pasteurellae using fresh cultures. A single colony is mixed with a drop of saline on a slide, a drop of antiserum is added, and the slide is warmed gently. A coarse, floccular agglutination within 30 seconds, is indicative of a positive reaction (Namioka & Murata, 1961).

The indirect haemagglutination test (IHA) is also a method for capsular typing of pasteurellae. The capsular extract, which is also called polysaccharide capsule, (see section 2.3.4), of the unknown strain is used to coat (sensitise) red blood cells (RBCs). Agglutination of the coated erythrocytes (haemagglutination) by hyper-immune antisera specific for capsular types A, B, D, E and F is considered positive for the corresponding type (OIE, 2000). The original method was described by Carter and modified by Sawada (Carter, 1955; Sawada *et al.*, 1982).

The agar gel precipitation test (AGPT) is a method for somatic typing of pasteurellae (De Alwis, 1999). The somatic antigen is a heat-treated extraction of pasteurella grown on a solid medium. In the double diffusion technique, wells are punched in the solid agar in a circular pattern with one centre well surrounded by six peripheral wells. The test antigen is placed in the centre well and antisera which are prepared in chickens against 16 different types are placed in the peripheral wells. All HS-associated serotypes will react with type 2 antiserum. Cross reactions may occur with type 5 (OIE, 2000). The test was invented by Heddleston and modified by Wijewardena (Heddleston *et al.*, 1972; Wijewardana, 1982).

1.8.2.3 Non-serological tests

Several non-immunological tests have been developed for the rapid identification of the *P. mutocida* strains. These include the acriflavine flocculation test described by Carter and Subronto (Carter & Subronto, 1973) for identification of type D strains, the hyaluronidase decapsulation test for type A strains (Carter & Rundell, 1975) and the hyaluronidase production test for the rapid identification of HS-related type B strains (Carter & Chengappa, 1980). Two methods have been used to assess the production of hyaluronidase by *Pasteurella*. In one method, a hyaluronic acid producing culture of *Streptococcus equi* or a capsulated mucoid *P. multocida* type A is streaked across an appropriate solid medium. *Pasteurella* cultures to be tested are streaked across at right angles. The plates are then incubated for 18 hours at 37° C. Hyaluronidase production is indicated by reduction in size of hyaluronic acid producer colonies which are located adjacent to the *Pasteurella* colonies (OIE, 2004).

1.8.2.4 Molecular methods

In recent years, identification and characterisation has favoured analyses that reflect one of the most fundamental properties of an organism, its genomic information. Molecular approaches such as DNA hybridisation and nucleic acid amplification have allowed bacterial detection directly from clinical samples, dramatically reducing the time required for identification. Molecular technology has significantly influenced the identification and characterisation of *P. multocida* (Hunt *et al.*, 2000). These nucleic acid recognition methods not only differentiate the serotypes that cause HS but also help in differentiation of strains within serotypes (Wilson *et al.*, 1992).

Polymerase chain reaction (PCR) tests have been developed for the diagnosis of *P. multocida* and HS-causing pasteurellae (Brickell *et al.*, 1998; Kasten *et al.*, 1997a; Townsend *et al.*, 1998a). In a PCR test, primer sequences are designed to enable identification of the pathogen at any level of specificity such as strain, serotype, species etc.. The *P. multocida*-specific PCR described by Townsend (1998) identifies all subspecies of *P. multocida*; subsp. Multocida, subsp. Gallicida, and subsp. Septica through specific amplification of a 460 bp DNA fragment. Type B cultures with the predominant somatic antigen being either type 2 or 5 are identified by the amplification of a 620 bp fragment (OIE, 2004). It has also been indicated that the putative transcriptional regulator genes Pm0762 and Pm1231 are species-specific, and that PCR methods targeting these genes provide a useful means of rapidly and precisely differentiating *P. multocida* from other bacteria (Liu *et al.*, 2004). HS-causing type-B-specific PCR primers can also be used in a multiplex PCR with the *P. multocida*-specific primers and confirm the identity and provide a presumptive serotype within 3-4 hours, in comparison with biochemical analysis and conventional serotyping, which can take up to 2 weeks (OIE, 2004). HS-causing isolates of *P. multocida* have also been analysed by a PCR-based fingerprinting method known as repetitive extragenic palindromic (REP) PCR. The analysis of HS-causing *P. multocida* strains provided evidence of a disease-associated REP profile with a high degree of homogeneity observed among the strains regardless of whether the capsular serotype was B or E (Townsend *et al.*, 1997). These profiles were clearly distinct from serologically similar strains that do not cause HS, but exhibited a degree of relatedness to strains that cause clinically similar septicaemic disease such as B:1, B:2, B:3, B:4. These findings have shown the possibility of a disease-specific test rather than a serotype-specific test (De Alwis, 1999).

Once identification has been made, further differentiation of isolates is possible, by genotypic fingerprinting methods. Restriction endonuclease analysis (REA), has been carried out on a wide range of HS causing strains. *Hha*I and *Hpa*II have been used to digest the DNA. The resulting images of band patterns (DNA fingerprint profiles) may be analysed by computer. Using *Hha*I, 13 unique fingerprint profiles were characterised among 54 serotype 2 isolates. In contrast, while a single *Hha*I profile was observed among 13 serogroup E isolates, differentiation of these strains was possible following *Hpa*II digestion (Rimler, 2000; Wilson *et al.*, 1992). Ribotyping and field alteration gel electrophoresis (FAGE) have been used to analyse the DNA of strains of *P. multocida* that cause HS, after digestion with restriction enzymes (Blackall *et al.*, 1995; Townsend *et al.*, 1998b). FAGE has displayed a greater degree of discrimination between strains, compared with ribotyping. Both of these methods are useful tools for differentiation between HS-causing and avirulent strains (OIE, 2000). Random amplified polymorphic DNA (RAPD) analysis and arbitrarily primed PCR (AP-PCR), have been shown to be useful for epidemiological studies of *P. multocida* isolates from rabbits and for differentiating post-vaccination isolates of *P. multocida* obtained from turkeys, respectively. However, RAPD and AP-PCR analysis of HS-causing *P. multocida* isolates have not been described (OIE, 2000). Using repetitive sequence PCR, all HS-causing strains that were analysed demonstrated similar profiles, although this method provided a useful discrimination of avian and swine isolates (Townsend *et al.*, 1997; Townsend *et al.*, 1998b).

1.8.2.5 Antibody detection in host

Antibody detection is not used for routine diagnosis of HS. The epidemiological finding that most exposed but clinically unaffected animals develop high antibody titre unattainable by vaccination, is of diagnostic value in situations where deaths have occurred in a very short time and no carcasses are available for collection of material for laboratory tests. In other circumstances, disease-reporting systems may be poorly developed and consequently reporting is delayed and no material is available for culture and isolation at the time of investigation. In these situations, antibody levels in surviving animals can be assayed as an indicator of HS infection (De Alwis, 1994).

Numerous serological tests have been described for the detection of antibodies in naturally immune, infected and vaccinated animals. These include the indirect haemagglutination (IHA) test (Carter, 1964; Nagy & Penn, 1976; Verma & Jaiswal, 1997), immunoblotting techniques (Johnson *et al.*, 1989; Pati *et al.*, 1996; Tomer *et al.*, 2002), passive mouse protection (PMP) test (Carter, 1964; Chandrasekaran *et al.*, 1994a), and enzyme-linked

immunosorbent assay (ELISA) test (Carter, 1964; Nagy & Penn, 1976; Verma & Jaiswal, 1997).

The original IHA test (Carter, 1955) was later modified by him, using formalinised human 'O' cells (Carter, 1962), then by Wijewardana *et al.* (1976) using sheep erythrocytes (Wijewardana, 1986), and later by Sawada using glutaraldehyde-treated sheep erythrocytes (Sawada *et al.*, 1985). This test can be used for diagnostic purposes because it is generally believed that high titres (1/160 up to 1/1280 or higher) detected by the IHA test among in-contact animals surviving in affected herds are indicative of recent exposure to HS. In different experiments on buffaloes and cattle, the IHA test has been used to assess potency of different kind of vaccines.

The application of an ELISA technique has been described for the detection of HS antibodies (Chandrasekaran *et al.*, 1994a; Dawkins *et al.*, 1991b; Johnson *et al.*, 1989; Muneer *et al.*, 1994). Chandrasekaran *et al.* used three different kinds of antigens in an ELISA test to measure antibody titres after vaccination of buffaloes (Chandrasekaran *et al.*, 1994a) and Pati *et al.* used a sonicated preparation of *P. multocida*. Antibody titres detectable in vaccinated buffalo calves using an IHA test were lower than the ELISA titres but the pattern of the antibody response was similar (Pati *et al.*, 1996). In another study on vaccinated calves, humoral immune responses were measured by the IHA test and the ELISA. Statistically, ELISA values were found to be superior to IHA values because of the small coefficient of variance (Verma & Jaiswal, 1997). In another study, it was shown that there was no relationship between PMP and IHA, and protection of buffaloes against challenge (Chandrasekaran *et al.*, 1994a).

Immunoblotting has been used to detect the immunogenic proteins, especially outer membrane proteins (OMP) of *P. multocida*, along with other serological procedures. In a study by Johnson *et al.* (1989), antibodies from vaccinated cattle strongly labelled five to six of the 40 protein bands in *P. multocida*, in addition to the response to lipopolysaccharide. In another study, Pati *et al.* (1996) observed ten major polypeptide bands in OMP of 88 to 25 kDa. Immunoblotting suggested that the polypeptides of MW 44, 37 and 30 kDa were the major immunogens, of which two (37 and 30 kDa) were type-specific to serotype B:2 (Pati *et al.*, 1996). Recently, it has been shown by Tomer *et al.* (2002) that a polypeptide of 37 kDa was the most antigenic OMP in the profiles of all the investigated *P. multocida* B:2 isolates.

1.9 Treatment and prevention

The rapid onset of HS and its short course leaves little opportunity for treatment, which is effective only if carried out in the early stages of the disease. For this reason, vaccination is generally accepted as a means of disease control.

1.9.1. Treatment

Once visible signs appear, treatment is of little value. Antibiotic therapy can only be used as a practical approach in an outbreak by checking the rectal temperatures of all in-contact animals in a diseased herd and using the drug immediately. The oldest therapy recommended was intravenous treatment with sulphonamides. Intravenous infusion of sulfadimidin sodium at a dosage of 1 ml per 2.3 kg bodyweight has been recommended. The large volume required in this treatment procedure has made the use of sulphonamides difficult (De Alwis, 1992). Other antibiotics have been tested and found to be effective against HS and penicillin, ampicillin and oxytetracycline appear to be the most useful. However the sensitivity patterns are likely to vary from one country to another depending on the prevailing drug usage practices (Radostits *et al.*, 2000).

Treatment with hyperimmune serum (serum therapy) has been attempted experimentally but appears to have no practical value (De Alwis, 1999).

1.9.2 Prevention

In all countries where HS occurs, vaccination is adopted as the method of control. There are three categories of measures for prevention and control of HS. 1. Measures to be adopted in endemic countries on a prophylactic basis. 2. Measures to be taken in the event of an outbreak. 3. Measures necessary for prevention of spread across regional or national borders. In all of these measures vaccination is the most important component (De Alwis, 1999).

1.10 HS vaccines

1.10.1 History

The work of Louis Pasteur on chicken cholera opened the way to vaccine development in the laboratory. In April 1880, Pasteur reported to the French Academy of Science that

"...chicken cholera is produced by a microscopic parasite [now known as *Pasteurella multocida*], that there exists an attenuated virus [Pasteur was using "virus" in the ancient sense of the word] of that disease, and that one or more inoculations of this attenuated virus can preserve the animals from the mortal effects of a later inoculation...let me be permitted to use the word 'vaccinate' to express the act of inoculating a chicken with the attenuated virus" (Plotkin, 2003).

Historically, Baldrey in 1907 prepared a killed vaccine (0.25% lysol-inactivated broth culture) for HS (De Alwis, 1999). This first attempt of vaccination offered immunity against HS for 6 weeks. Delpy and Rastegar in 1938 prepared a vaccine that consisted of a suspension of lysed bacteria in 5% saponin. Dhana in 1959 prepared a vaccine from capsular extract of the organism. Chengappa and Carter in 1979 introduced a streptomycin-dependent (Str^D) mutant of the agent as a live vaccine. Myint in 1987 used a strain isolated from deer for vaccination as a live vaccine. Muniandy in 1993 used an OMP preparation of the organism as subunit vaccine. Tabatabaei *et al.* in 2000 prepared an *aroA* mutant of the agent and showed its attenuation and protection in mouse.

1.10.2 Available HS vaccines

Currently there are four different types of HS vaccines. These vaccines are commercially available and are being used in different countries and under different regimens.

1.10.2.1 Plain bacterins

Bacterins which consist of a suspension of killed whole cells are the simplest form of vaccine. To provide sufficient immunity with bacterins, repeated vaccination is required. Inactivated broth culture is the commonest type of bacterin, also called broth bacterins, but may cause shock reactions in a small percentage of animals. These reactions are presumably due to free endotoxin present in the preparation and are more probable with dense formalinised bacterial suspensions. Another disadvantage with plain bacterins is that the antibody response to them is poor and only provides immunity for about six weeks (OIE, 2004). Due to the shock encountered in some animals vaccinated with broth bacterins, an agar-wash formalin-killed vaccine (harvested from surface of solid medium). Some of these vaccines with higher concentration of cells are able to provide immunity for up to 4 months (Verma & Jaiswal, 1998).

1.10.2.2 Alum-precipitated vaccine (APV)

APV appears to be the most popular vaccine used in Asia and Africa (Verma & Jaiswal, 1998). It consists of a bacterin to which potash alum has been added to give a final concentration of 1% (w/v) alum. Again, the disadvantages of such vaccines are short duration of immunity, which is only 3-4 months, and shock reactions can occur. They tend to precipitate during storage so it is important to mix them thoroughly before use. If this precaution is not taken, animals receiving vaccine from the bottom of the bottle will receive a large dose of bacteria and the chances of shock will be increased (OIE, 2004).

1.10.2.3 Aluminium hydroxide gel vaccine

In Thailand and Laos, an aluminium hydroxide gel vaccine is used extensively (Verma & Jaiswal, 1998). This vaccine has common properties with the alum-precipitated vaccine. Immunity up to 6 months is claimed and vaccination is practised twice a year (De Alwis, 1999). Further vaccination did not confer a substantial immunity of more than 90 days even using Vitamin E and levamisole as immunomodulators (Verma & Jaiswal, 1998).

1.10.2.4 Oil adjuvant vaccine (OAV)

An oil adjuvant vaccine for HS was first developed in 1955. It consists of a water in oil emulsion, where the aqueous phase consists of a dense broth culture and the oil phase a light mineral oil (OIE, 2004). The choice of emulsifying agent is very important in influencing the type of emulsion that results (Verma & Jaiswal, 1998). The longest duration of immunity, which is 6-9 months, is conferred by OAV. Two initial doses followed by annual revaccination is reckoned to provide adequate immunity. This vaccine is the one recommended for general use. The optimum age for primary vaccination is 4-6 months as it has been found that the response of younger animals is poor (OIE, 2004). The OAV possesses a thick viscosity, and it is therefore difficult to administer, sometimes causing swelling at the site of inoculation (Verma & Jaiswal, 1998).

1.10.3 Deficiencies of available vaccines

It has been over 100 years since the discovery of prophylactic vaccination of cattle against HS with killed vaccines and around 60 years since the first uses of different kinds of adjuvanted vaccines. During this long period it has been proved that using these vaccines provide some control of HS. On the other hand deficiencies of these vaccines have been

recorded including, short duration of immunity, high viscosity, reactogenicity, short shelf time. Broth bacterins confer only an immunity of very short duration, and dense bacterin may cause an endotoxic shock. The alum precipitated and aluminium hydroxide gel vaccines are believed to confer immunity for only 3-4 months and need to be given twice yearly. Oil adjuvant vaccines suffer from limited duration of stability and high viscosity (Verma & Jaiswal, 1998).

1.11 Experimental vaccines

Although several new vaccines have been prepared, none of them has become established as a practical vaccine in the field. Some of these vaccine trials have focused on use of other adjuvants especially with low viscosity, like the production of double emulsions. Most of the vaccine trials relating to improvement of the vaccine can be categorised under two main headings: 1) Development of media to provide growth conditions that would cause the fullest expression of antigenic components that play an important role in protection; 2) Development of live vaccine by production of suitable avirulent, protective mutants using genetic manipulation. Such organisms, that can be administered by any route, should serve as ideal vaccine candidates as they will mimic the normal routes of infection and promote similar immune responses, at least initially, as the native strain.

1.11.1 The ideal vaccine

It is generally believed that an ideal HS vaccine should have the following characteristics (De Alwis, 1999):

-High level of immunity with minimum delay after vaccination and lasting for at least a year
-No adverse reaction
-Easy to handle in the field with a consistency that makes it easy to administer
-Stable for use in the tropics
-Easy and economical to produce

None of the vaccines developed so far attain all these requirements. Although different types of new vaccines have been developed by different groups of researchers, none of them has been able to replace any of the routine vaccines in the field.

1.11.2 OMP-enriched vaccine

It has been shown that vaccines made from *P. multocida* serotype B:2 grown on iron-restricted media (containing the iron chelator 2.2-dipyridyl) give better protection than those grown in iron-replete media (containing 0.1 M $FeCl_3$). This superior protection has been attributed to a high molecular mass protein of over 84 kDa, which is expressed in abundance under iron-restricted conditions. Another protein (116 kDa) was found to be produced in vivo and when grown under iron-restricted conditions in vitro but was absent from cultures grown in normal media. Protein profiles by SDS-PAGE and immunoblotting, using serum from buffaloes naturally immune as a result of experimental exposure to a virulent B:2 strain, recognised the 116 kDa, whereas sera from buffaloes immunised with the inactivated oil adjuvant vaccine failed to do so (De Alwis, 1999). Convalescent-phase sera from buffaloes reacted against some iron-restricted and other proteins, indicating that these proteins were produced in vivo (Veken *et al.*, 1996).

Although much work has been done on the characterisation of high molecular mass OMPs produced in vivo, and in iron-restricted media in vitro, by type A strains of *P. multocida* associated with avian pasteurellosis and bovine pneumonia, the experiments have not resulted in the production of a commercial vaccine for these strains (Bosch *et al.*, 2004). A commercial ovine pasteurellosis vaccine against *Mannheimia (Pasteurella) haemolytica* (Ovipast Plus^R, Intervet), is prepared by growing the organism in iron-restricted media and causes strong protection in sheep (Gilmour *et al.*, 1991; Porter *et al.*, 1995).

1.11.3 Subunit vaccines

In order to generate a vaccine of superior quality, several studies have been done on different cellular components of *Pasteurella multocida*, including outer-membrane proteins (OMPs), lipopolysaccharides (LPS), polysaccharide capsules and proteins to evaluate their potential as protective immunogens (Chaudhuri & Goswami, 2001; Dawkins *et al.*, 1991b; Nagy & Penn, 1976; Ramdani & Adler, 1991). It has been concluded that no single component is totally responsible for immunity. All fractions, LPS, proteins and polysaccharides evidently contribute towards immunity. The complete set of immunogens would be present in whole bacterial cells grown in vivo or cultivated in vitro in a medium that provides all essential in vivo conditions for the full expression of relevant immunogenic components. It appears that there are many common components in the different types of *P. multocida* that play a role in protection (Frost & Adler, 2000; Hunt *et al.*, 2000).

1.11.3.1 OMP vaccines

Since OMPs of *P. multocida* have been identified as major immunogens (Chevalier *et al.*, 1992; Lu *et al.*, 1988; Lu *et al.*, 1991a; Lu *et al.*, 1991b), a number of reports have focused on the role of OMPs in eliciting protective immune responses (Vasfi Marandi *et al.*, 1996; Vasfi Marandi & Mittal, 1997). OMP extracts from *P. multocida* strain P52 (the Indian vaccine strain of serotype B:2) have been investigated for their use as subunit vaccines. Using SDS-PAGE, 10 major polypeptide bands of molecular weight ranging from 88 to 25 kDa have been detected. Using immunoblots, it has been indicated that the 44, 37 and 30 kDa fractions were major immunogens. Buffalo calves in two groups were vaccinated using a whole-cell, oil-adjuvanted vaccine and an oil adjuvant vaccine prepared from the OMP extract. Antibody responses were tested, by IHA, ELISA and passive mouse protection (PMP) tests. Following challenge, all the five OMP vaccinated animals survived whereas only 2 out of the three HS oil adjuvant vaccinated animals withstood the challenge. It was suggested that this OMP preparation could be useful as a vaccine against HS (Pati *et al.*, 1996). Although administration of an OMP-enriched crude preparation from *P.multocida* B:2 was protective in buffalo calves (Pati *et al.*, 1996), none of the purified OMPs was identified as a protective antigen. The 37 kDa OMP appeared to be one of the most antigenic components and was common between vaccine and field isolates (Tomer *et al.*, 2002).

1.11.3.2 Lipopolysaccharides (LPS)

Lipopolysaccharide (LPS) preparations of *Pasteurella multocida* type B:2 were shown to be protective against experimentally-induced pasteurellosis in mice (Ramdani & Adler, 1991). However, the observed protection was abrogated if such LPS was digested with proteinase K prior to use in immunization, indicating that the protectivity of LPS extracts prepared by the Westphal method was actually due to contaminating OMPs in the preparation (Muniandy *et al.*, 1998). The role of LPS in immunity has been studied using monoclonal antibodies and active immunisation experiments. Both procedures conferred only partial passive protection in mice, indicating that LPS plays a partial role in immunity to infection (Adler *et al.*, 1996). The O-antigen polysaccharide side-chain (OS) of LPS did not appear to contribute to the observed protection as judged by the fact that immunisation of mice with purified OS or OS-protein conjugates, failed to confer full protection against challenge with homologous virulent organisms. This was despite generation of significant levels of OS-specific antibodies, predominantly either of the IgM or IgG isotypes, in immunised mice (Muniandy *et al.*, 1998). Although LPS possesses antigenic properties, it

has not been accepted as a vaccine candidate for cattle due to strong toxicity and anaphylactic effects.

1.11.3.3 Capsular polysaccharides

Capsular preparations have been used for experimental vaccination of cattle (Dhanda, 1959) but have not resulted in a practical vaccine. In a later study, aluminium hydroxide adjuvanted capsular preparations of types B and E were used to immunise cattle. All animals which received the highest vaccine dose survived and all unimmunised control animals died after intravenous challenge by a virulent type E strain and a vaccine dose-response relationship was obtained. Dose-dependent serological responses were observed which were similar for both antigens. The results of PMP and IHA tests (type E) on the sera of immunised cattle corresponded with the degree of protection against challenge (Nagy & Penn, 1976). Oil-adjuvanted capsular extracts prepared by solvent precipitation from the supernatant fluid of fermenter-grown type B has been used for vaccination of cattle but did not yield any encouraging results (Nagy, 1979; Verma & Jaiswal, 1998). The differences in protection conferred by different capsular preparations might be due to the contamination of capsular extracts with other cellular components.

1.11.3.4 Protein vaccines

Proteins are generally likely to play an important role in protective mechanism against HS. Soluble cytoplasmic or periplasmic proteins of serogroups B and E are non-toxic. These fractions that were able to cause 25-60% protection in a mouse model (Ramdani & Adler, 1994).

1.12 Live vaccines

It is generally accepted that natural exposure to live organisms (such as in actual infection) produces greater immunity than immunisation through inactivated vaccines. This has been specially shown in HS, where the naturally-acquired immunity in exposed animals that results from arrested infection has been found to be superior to vaccinal immunity (De Alwis, 1982). If we can find an organism that has all the properties of the field strains, including all the antigen components, but has lost its virulence, it will constitute the basis for an ideal live vaccine. An ideal live vaccine should have the following characteristics: 1) it must have all, or at least most of the protective antigens present in the field strains; 2) it must grow readily in ordinary culture media; 3) it should be avirulent for cattle and

buffaloes, or it may have low virulence (the lethal dose must be considerably higher than immunising dose); 4) it should be able to multiply sufficiently in vivo following vaccination, producing a full complement of the important immunogens, thereby stimulating an adequate immune response (De Alwis, 1999).

Strains satisfying these criteria may occur in nature, or may be produced in the laboratory by exposure to mutagenic agents or by gene manipulation techniques.

1.12.1 Streptomycin-dependent (Str^D) mutant

Using N-methyl-N nitro-N-nitrosoguanidine (NMG), an HS-causing type B strain of *P. multocida* (strain R-473) was mutagenised to produce a streptomycin-dependent (Str^D) mutant. This mutant was used for vaccination of the mouse and rabbit as animal models. It was shown that single vaccination was only fully protective in rabbit against a homologous challenge 21 days after vaccination (Wei & Carter, 1978).

A number of Str^D mutants have been obtained from Sri Lankan strains of HS-causing *P. multocida* serotype B:2. These mutants were avirulent to mice, when inoculated alone, but some mutants killed mice when inoculated with streptomycin. Although most of the mutants were stable, some of them produced streptomycin-independent revertants. It was shown that the rate of reversion varied between different mutants and most of them were highly virulent for mice (De Alwis *et al.*, 1980). One of these mutants has been used to immunise calves subcutaneously or intramuscularly with one or two doses in Sri Lanka. No adverse reactions were observed in any of the vaccinated animals. It was shown that a single dose of vaccine conferred immunity in 66.6% to 83.3% of cattle and 100% of buffalo calves, and a booster dose given three weeks later enhanced the immunity in cattle. One of the disadvantages of the vaccine was the large number of cells of this mutant strain that was needed to produce a protective response. The absence of in vivo multiplication of the organism in the absence of streptomycin was suggested as one possible reason (De Alwis & Carter, 1980). The Str^D mutants have not led to the development of a practical vaccine but they have revealed that live strains of *P. multocida* can be used for vaccination.

1.12.2 Deer strain live vaccine

A *P. multocida* serotype B:3,4 was isolated from a fallow deer (*Dama dama*) in England after an outbreak of septicaemic disease (Jones & Hussaini, 1982). It was reported to have a low virulence for cattle possibly due to the lack of transferrin-binding proteins (Veken *et*

al., 1996). It has also been used as an experimental live vaccine, containing 2×10^7 viable organisms per dose, in cattle and buffaloes. The strain was able to raise antibodies in cattle that protected mice against serotype B:2 infection. Six months after vaccination, five of six calves were protected against serotype B:2 challenge. It protected calves for up to 9 months after vaccination against a challenge with serotype B:2. The live vaccine was reported to be more efficacious than an alum-precipitated vaccine in protecting calves against B:2 challenge (Myint *et al.*, 1987). A lyophilised form of the vaccine induced protection in three of five subcutaneously-vaccinated and three of four intradermally-vaccinated young cattle after 12 months, when challenged with the B:2 serotype. Eleven buffaloes vaccinated subcutaneously and two vaccinated intradermally survived the same challenge 13 months after vaccination (Myint & Carter, 1989). In a field experiment, 1415 cattle and 303 buffaloes were vaccinated subcutaneously. No disease was recorded in the cattle after vaccination but three buffaloes died soon after vaccination (Myint, 1990). It was shown that the strain was able to cause death in a small proportion of young animals when administered subcutaneously. The effect of the route of vaccination was evaluated in a field study by subcutaneous vaccination of 8231 cattle and buffaloes and intranasal vaccination of 674 animals (Carter *et al.*, 1991). The intranasal route of vaccination was not protective against the standard subcutaneous challenge. Therefore, after field experiments, the safety of this vaccine has been questioned for primary vaccination of young buffalo calves (Carter *et al.*, 1991; Myint, 1990). But, encouraging results have been obtained when the deer strain was administered intranasally as an aerosol spray (possibly due to access to lower parts of respiratory tract) in a population of cattle and buffaloes over six months of age in Myanmar (Myint *et al.*, 2005). However, there is some doubt about efficacy of vaccine, as the study did not include non-vaccinated animals as a control group. The vaccine should only be administered intranasally and for animals over six months of age. In Denmark, *P. multocida* serotype B:3,4 was isolated from Danish fallow deer (*Dama dama*) with haemorrhagic septicaemia. Restriction endonuclease analysis using *HhaI* as restriction endonuclease, showed that all isolates had a profile identical to that of the fallow deer isolate from the United Kingdom. Cultures from the palatine tonsils of apparently healthy fallow deer from the same area showed a carrier rate of 27% (isolated from 6 animals) among apparently normal animals from the same population (Aalbaek *et al.*, 1999). This report showed that the deer strain vaccine is a potential pathogen, thus vaccination sould be done only under controlled conditions to prevent the spread of the organism.

1.12.3 Acapsular mutants

A *cexA* (gene involved in the export of capsule) mutant (PBA875) of *P. multocida* serotype B:2 has been constructed by allelic replacement (Boyce & Adler, 2000). It was shown that immunisation with high, but not low, doses of the mutant confered significant protection against wild-type challenge in a mouse model (Boyce & Adler, 2001).

Another acapsular mutant (AL18) of *P. multocida* serotype B:2 has been constructed by inactivation of the *bcbH* gene (a gene predicted to be involved in polysaccharide biosynthesis). Immunisation of mice with the mutant was able to confer significant protection against wild-type challenge, while immunisation with similar doses of either killed wild-type or killed mutant failed to confer protection (Boyce & Adler, 2001).

The protection afforded both of these acapsular vaccines was less than 100% and it has been suggested that this is due to the rapid removal of acapsular bacteria from the blood, a view which has been supported by the strong dependence of the level of protection on vaccine dose (Boyce & Adler, 2001).

1.12.4 *aroA* mutant vaccine

In 1950, for the first time it was demonstrated that attenuated auxotrophs of *Salmonella typhi* that required aromatic compounds for growth were avirulent in a mouse model (Brown *et al.*, 1987; Li *et al.*, 1999). Mutants in *aroD* or *aroA* genes are attenuated by a blockage in biosynthesis of aromatic metabolites such as chorismic acid, a precursor of p-aminobenzoic acid (PABA). This metabolite is required for the biosynthesis of tetrahydrofolate, and is a precursor of L-tyrosine, L-phenylalanine and L-tryptophan. Since mammals acquire the folic acid precursors and aromatic amino acids through dietary nutrients, intermediary metabolism does not include a PABA biosynthetic pathway. *Salmonella* cannot assimilate the folic acid that is available in the mammalian cytoplasm, and aromatic amino acids are in short supply. Multiplication of intracellular *aro* mutants is therefore limited (Hale, 1995). After the first attempt to introduce *aroA Salmonella typhimurium* as a vaccine candidate (Brown *et al.*, 1987), the capacity of live salmonella vaccines to elicit immune responses to guest antigens from viruses, bacteria and parasites was demonstrated. Also, they may be used as delivery systems for other substances such as interleukins, by oral administration (Hormaeche, 1995).

AroA mutants of a wide range of pathogenic bacteria have now been created to be exploited as live vaccines. Some of these mutants are: *Aeromonas hydrophila* (Hernanz Moral *et al.*, 1998; Vivas *et al.*, 2004; Vivas *et al.*, 2005), *Aeromonas salmonicida* (Marsden *et al.*, 1996), *Shigella flexneri* (Kotloff *et al.*, 1996; Noriega *et al.*, 1994; Verma & Lindberg, 1991), *Shigella dysenteriae* (Walker & Verma, 1997), *Yersinia pestis* (Oyston *et al.*, 1996), *Yersinia enterocolitica* (Bowe *et al.*, 1989; O'Gaora *et al.*, 1990), *Neisseria gonorrhoeae* (Chamberlain *et al.*, 1993), *Pseudomonas aeruginosa* (Priebe & Schwarz, 2003). Following exposure to aerosols of *aroA* mutants of *Bordetella bronchiseptica*, mice developed a limited lung infection and were subsequently protected against respiratory challenge with the virulent parental strain (Kabay *et al.*, 1992).

An *aroA* mutant of *Mannheimia (Pasteurella) haemolytica* biotype A, serotype 1 has been constructed. This mutant was highly attenuated in a mouse septicaemic model. Mice immunized intraperitoneally with two doses of the mutant were protected against a lethal parental strain challenge (Homchampa *et al.*, 1994).

aroA mutants from a fowl cholera-associated *Pasteurella multocida*, serotype A:1 (X-73) and serotype A:3 (P-1059) have also been constructed and designated PMP1 and PMP3, respectively. In a mouse model, PMP1 and PMP3 showed high attenuation and were able to protect against lethal challenge. One of the mutants, PMP3, was able to confer immunity against heterologous challenge with serotype A:1 or A:4 (Homchampa *et al.*, 1992; Homchampa *et al.*, 1997). These mutants also protected chickens against intratracheal challenge with wild type. At least 10^6 CFU of PMP1 and 10^8 CFU of PMP3 were required to provide complete protection against challenge. Doses of 10^9 CFU of the mutants caused endotoxin reactions. Both vaccine strains provided cross-protection with a heterologous challenge strain serotype 4 (Scott *et al.*, 1999).

aroA mutants of HS-associated *P. multocida* serotype B:2 strains have also been constructed by allelic exchange in our laboratory (Tabatabaei *et al.*, 2002). These derivates have been confirmed as highly attenuated for virulence in a mouse model of HS. Intraperitoneal injection of mice with 1 CFU/mouse with *P. multocida* 85020 parent strain (Section 2.1.1) killed 2 of 3 injected mice, but intraperitoneal injection with 2.6×10^7 CFU/mouse of the mutant did not kill any of 5 injected mice. It was shown by mouse challenge experiments that intraperitoneal or intranasal vaccination of an *aroA* strain was able to create complete protection against homologous challenge. All of the 5 mice injected intraperitoneally with 2.6×10^7 CFU/mouse survived the intraperitoneal challenge with 1000 LD_{50}s with parent strain two weeks after vaccination. The experiments also showed

that one intranasal inoculation of mice with 3.8×10^8 CFU/mouse was protective against intraperitoneal challenge in 2 of 4 mice (Tabatabaei, 2000). The virulence and protection experiments and investigation of immune responses in natural host, form part of this thesis (Section 1.14).

1.13 Immunity to HS

Immunity against pathogens is a consequence of activation of the immune system, which is an organization of cells and molecules with specialized functions (Roitt *et al.*, 1998). The physiological function of the immune system is defence against infectious microbes (Abbas & Lichtman, 2003). The immune system is composed of two interrelated parts, the innate and adaptive systems, whose response depends on the type of microbial invader (Kaufmann *et al.*, 2002). Innate immunity (also called natural or native immunity) consists of cellular and biochemical defence mechanisms that are in place even before infections and respond rapidly. These mechanisms, which react only to microbes and not to non-infectious agents, essentially respond in the same way to repeated infections (Abbas & Lichtman, 2003). Adaptive immunity (also called specific or acquired immunity) develops as a response to infection and increases in magnitude and defensive capabilities with each successive exposure to a particular microbe. The adaptive immune response has the ability to recognize and react to a large number of microbial and non-microbial substances. The components of adaptive immunity are lymphocytes and their products (Abbas & Lichtman, 2003).

Recognition of the microbial pathogen is the first step of immune response. To achieve this, innate immunity has evolved a set of germ line-encoded proteins, named pattern-recognition receptors (PRRs), which are either free in the plasma (including collectins, pentraxins, and complement) or membrane bound (such as Toll-like receptors [TLRs] and nucleotide-binding oligomerisation domain [NOD] receptors), and are capable of recognizing conserved microbial molecules, termed pathogen-associated microbial patterns (PAMPs). In bacterial pathogens, these molecules include lipopolysaccharide (LPS) of the Gram-negative bacteria and lipoteichoic acid of Gram-positive bacteria (Kaufmann *et al.*, 2002). Although innate and adaptive immunity are two distinguishable components of the immune system, there are two important links between them. First, the innate immune response to microbes stimulates the adaptive immune response and influences the nature of the adaptive response. Second, the adaptive immune responses uses many of the effector mechanisms of innate immunity to eliminate microbes, and they often function by

enhancing the antimicrobial activities of the defence mechanisms of innate immunity (Abbas & Lichtman, 2003). The successful elimination of most pathogens requires interaction between these two arms of the immune system. The initial inflammatory reaction to infection, which is a part of the non-specific response, sets a suitable environment for mounting an adaptive response, by increasing blood flow and recruitment of polymorphonuclear leukocytes ([PMNs], also called neutrophils), monocytes/macrophages and lymphocytes to the site of infection. IL-2 secreted by activated $CD4^+$ T cells recruit the antigen-presenting cells (APCs) (Section 1.13.2) in phagocytosing and processing the pathogen (Hodgson, 2001). These are examples of active interaction between innate and adaptive immunity.

Significant gaps exist in the current understanding of the nature of immunity against HS. Further information, especially related to vaccine development, would be helpful in the control of the disease (De Alwis, 1999). Most of the current knowledge has been obtained through studies on immune responses against different vaccines, mostly on the basis of data on morbidity/survival (Verma & Jaiswal, 1998).

1.13.1 Innate immunity

Innate immunity uses different cellular and molecular components in defences against pathogens (Abbas & Lichtman, 2003). In order to control the replication and spread of microbes, the innate immunity must be able to recognize and respond to infection rapidly, since most microbial pathogens can proliferate far more quickly than the host can mount an adaptive response (Kaufmann *et al.*, 2002). This is more prominent in acute diseases. The innate immune response to intracellular bacteria is mainly cellular, while both molecular and cellular mechanisms are involved in innate immunity against extracellular bacteria (Abbas & Lichtman, 2003). Cellular defences consist of phagocytic cells (PMNs, monocytes, and macrophages), and natural killer cells (NK). Some of the molecular components of innate immunity are blood proteins, including members of the complement system, acute phase proteins (APPs), and other mediators of inflammation. Another group of molecules is the cytokines, which regulate and coordinate many of the activities of the cells of innate immunity. Innate immunity also uses other mechanisms such as physical and chemical barriers, such as epithelia and antimicrobial substances produced at epithelial surfaces to prevent the entrance or spread of pathogens through body (Abbas & Lichtman, 2003).

Mice infected intraperitoneally with as few as 20 CFUs of HS-associated *P. multocida* B:2 showed an overwhelming septicaemia in less than 30 hours. The kinetics of infection demonstrated a very rapid in vivo multiplication rate. There was no evidence of inhibition of bacterial cell growth by natural host defence mechanisms, even with the very small inocula used (Ramdani *et al.*, 1990). Similar experiments on mice showed that the LD_{50} varied according to the different routes of challenge. The LD_{50} dose (dose which kills 50% of inoculated animals) of *P. multocida* 85020 (an HS-causing strain isolated in Sri Lanka) inoculated by the IP and SC routes was <20 CFU/mouse, whereas when delivered by the IN route the LD_{50} was c. 10^3 CFU/mouse (Tabatabaei *et al.*, 2002). Generally, oral and IN inoculation of the agent causes a longer course of the disease (Section 1.6.3). In natural hosts SC injection of 10^4 to 10^7 CFU causes the disease but, using the oral or IN route, high doses of inoculum are required (De Alwis, 1999). After intranasal infection of eight buffaloes with $2.5\text{-}3.4 \times 10^9$ CFU only three animals succumbed to the disease 26-30 hours later and five survived (Horadagoda *et al.*, 2001). This is also suggestive that the dose required to cause the disease varied individually and results with a given dose may not be consistent. These demonstrations indicate that innate immunity may play an important role in natural infection (by oral or IN routes) in natural hosts.

1.13.1.1 Complement

The complement system, when it was identified more than hundred years ago, was known as part of the antibody response that destroys bacteria by lysis. The system is composed of about twenty serum proteins which interact with each other or with other cellular components of immune system such as phagocytes and T cells (Kemper & Atkinson, 2007). Complement, along with other plasma derived PRRs, coats the microbes (opsonisation), allowing recognition and binding by opsonic receptors on host phagocytes (Kaufmann *et al.*, 2002). The complement system can be activated through three different pathways: the classical pathway, which is triggered directly by pathogen or indirectly by antibody binding to the pathogen surface; the lectin pathway; and the alternative pathway. All three pathways can be initiated independently of antibody as part of innate immunity. Finally, all three pathways culminate in the formation of the C3 and C5 convertases that, in turn, generate the anaphylatoxins C3a and C5a, the membrane-attack complex (MAC; C5b-C9) and the opsonin C3b (Marion *et al.*, 1984). Activation of the complement system in response to extracellular bacteria may be through the alternative pathway when the opsonin C3b binds to the surface of the microbe (Abbas & Lichtman, 2003). The complement system can also affect T-cell responses through the direct modulation of T-

cells or indirectly through the alteration of immunomodulatory cells, particularly APCs (Kemper & Atkinson, 2007).

The destructive effects of fresh serum on Gram-negative bacteria is assumed to be one of the consequences of the activity of complement (Taylor, 1983). In *P. multocida* serotype A, the causative agent of fowl cholera, it has been shown that a virulent strain was resistant to turkey serum while an avirulent strain was serum-susceptible (Hansen & Hirsh, 1989; Lee *et al.*, 1988). Similar results have been reported by other studies and serum resistance has been considered as a tool to assess the virulence in FC-associated strains (Morishita *et al.*, 1990). However, lesions have also been reported in chickens infected by serum-sensitive strains (Muhairwa *et al.*, 2002) and the relationship between virulence and serum resistance is complicated in the chicken (Diallo & Frost, 2000). Different monoclonal antibodies reacting with the LPS of the bacterium could opsonise *P. multocida* for phagocytosis by mouse macrophages, but were not bactericidal in the presence of complement (Adler *et al.*, 1996; Ramdani & Adler, 1991). Experiments on sensitivity of *P. multocida* B:2 to naïve calf serum show that the organism is quite resistant to complement-mediated killing. This study showed that both the wild type and an acapsular mutant grew rapidly in either fresh or heat-treated calf serum. It was concluded that capsule does not play any role in resistance to complement. Similar results were obtained for growth in mouse serum (Boyce & Adler, 2000).

1.13.1.2 Cytokines

Cytokines are proteins secreted by the cells of innate and adaptive immunity and form a complex network that play crucial roles in both innate and adaptive immunity. Generally they can be listed under pro- and anti-inflammatory categories (Kaufmann *et al.*, 2002). Chemokines (name is a contraction of chemotactic cytokine) are a large family of structurally homologous cytokines that stimulate leukocyte movement and regulate the migration of them from the blood to tissues (Abbas & Lichtman, 2003). Since in different texts there is some overlap in classification of the cytokines and chemokines, all of them are mentioned here under one category. The main cytokines that contribute to innate immune reactions against bacterial infections are TNF, IL-1, IL-12, IFN-γ, and IL-18. In infections by bacteria, macrophages respond to bacterial endotoxins and perhaps to other bacterial products by producing TNF (also called tumor necrosis factor-alpha [TNF-α], for historical reasons and to distinguish it from the closely-related TNF-β, or lymphotoxin), interleukin-1 (IL-1), and chemokines. TNF-α and IL-1 are pro-inflammatory cytokines that mediate and regulate innate immunity. They are principal mediators of the acute

inflammatory response to Gram-negative bacteria and responsible for many of the systemic complications of severe infections (Abbas & Lichtman, 2003). Macrophages also respond to LPS-producing bacteria and intracellular bacteria by secreting IL-12, which induces the local production of IFN-γ from T-cells and NK cells. The IFN-γ then activates the macrophages to destroy phagocytosed bacteria. IL-12 also stimulates the subsequent adaptive immune response. These actions of IL-12 are completed by IL-18. These cytokines secreted by macrophages, especially TNF-α, IL-1, and IL-12, are also responsible for the systemic manifestations of infection. IL-12 is produced by macrophages in response to many microbes. Large amounts of IL-12 are produced in severe Gram-negative sepsis, resulting in the production of TNF, the principal mediator of septic shock. IL-12 antagonists prevent lethality in experimental models of LPS-induced septic shock. TNF-α has different biological actions at different concentrations. At low concentrations, TNF-α acts on leukocytes and endothelium to induce acute inflammation. At moderate concentrations, it mediates the systemic effects of inflammation (fever, secretion of APPs by liver and production of leukocytes by bone marrow), and at high concentrations, it causes the pathologic abnormalities of septic shock (Abbas & Lichtman, 2003).

The toxic effects of LPS-containing antigen prepared from virulent serotype B:2 strains of *P. multocida* have been shown in laboratory animals and calves (Heddleston *et al.*, 1967). Administration of LPS has been demonstrated to induce the secretion of several cytokines including IL-1, TNF-α, and IL-6 (Chen *et al.*, 1992). It has been shown that intravenous injection of *P. multocida* LPS is capable of inducing an immediate (with a peak 1-2 hours post-injection) TNF response in the buffalo (Horadagoda *et al.*, 2002). A primary role for LPS in the induction of HS in the calf was indicated by the consequent development of a rapid and severe endotoxemia in the terminal stages of the disease. However, TNF-α was only detected in serum in one of the three diseased animals. The early release of TNF-α, 2-3 hours after exposure to LPS, and restriction of the production to local levels were suggested as possible reasons for the lack of detection of TNF-α (Horadagoda *et al.*, 2001). Individual animal differences, similar to those noted in calves by others following LPS injection, has been suggested as the possible reason (Horadagoda *et al.*, 2002). In this experiment, leukocytosis was observed only in animals that developed a severe endotoxaemia and eventually died of HS and not in those that survived. According to this observation, it was suggested that endotoxin is initially restricted within the lung lesions during the course of infection, only to be released into the circulation in a fatal overwhelming dose, perhaps after bacterial growth exeeds a certain threshold (Horadagoda *et al.*, 2001). Considerable species variation in the sensitivity to LPS has been reported, with ruminants and pigs being more sensitive compared to dogs and rodents (Olson *et al.*,

1995). It has been concluded that buffaloes are more sensitive to circulatory LPS and this has been suggested as a possible explanation for the higher mortality rate in buffaloes than in cattle due to HS (Horadagoda *et al.*, 2002).

1.13.1.3 Acute phase proteins (APPs)

Acute phase proteins such as C-reative protein (CRP), fibrinogen, serum amyloid A (SAA), haptoglobin (Hp), and α_1-acid glycoprotein (α_1-AGP) are proteins secreted by the liver into the blood plasma in the acute-phase response (Abbas & Lichtman, 2003). The acute-phase response, which is characterized by pyrexia, leukocytosis, endocrine changes and the redistribution of trace elements, plays a crucial role in containing the body damage and assisting the processes of healing. One of the changes in the acute-phase response includes changes in the concentration of APPs, some of which decrease in concentration (negative APPs), such as albumin or transferrin, and others of which increase in concentration (positive APPs), such as CRP, SAA, Hp, α_1-AGP, and ceruloplasmin (Ceron *et al.*, 2005). Several APPs which were originally identified in humans have been identified in different animals, although there are some degrees of variation in the response of individual proteins between different species (Eckersall & Conner, 1988; Murata *et al.*, 2004). Clinically, the measurement of plasma concentrations of APPs are used to assess the severity of inflammation. They are preferable to haematological assays for this purpose as they are more sensitive and also the APP assay can be done on frozen samples. Haptoglobin (Hp), the most widely studied APP in cattle, is a protein that binds free haemoglobin in the circulation. Hp has been identified as a reliable index to show the level of inflammation in both experimental and clinical investigations (Horadagoda *et al.*, 1999). Serum amyloid A (SAA) is also a major APP in humans, cattle, and laboratory animals. α_1-AGP, which like most of the APPs is a glycoprotein, has been identified as an inflammatory marker in cattle (Horadagoda *et al.*, 1993; Horadagoda *et al.*, 1994). α_1-AGP is a moderate APP in both humans and cattle and its serum levels only increase two- to four-fold in response to tissue damage (Motoi *et al.*, 1992).

In a study on calves, the involvement of APPs in pathogenesis and resolution of pneumonia was investigated after intratracheal challenge with *P. multocida* A:3. Plasma concentrations of SAA increased rapidly between 5 and 23 hours in response to challenge and decreased dramatically between 72 and 96 hours after challenge. Hp concentrations also increased significantly compare to mean values of samples before challenge and fell gradually thereafter. Increases in the α_1-AGP concentrations were more gradual than those observed for SAA or Hp. The α_1-AGP response reached its highest levels at 48 hours after

challenge and decreased thereafter with a slight recovery between 72-96 hours (Dowling *et al.*, 2002). The study indicated a role for the investigated APPs in inflammatory responses of cattle to infection, and was used in a further study as markers of the onset and progress of experimental HS in calves (Hodgson *et al.*, 2005).

1.13.1.4 Phagocytes

PMNs are the main phagocytic cells in blood. They are the major phagocytic cells controlling the growth of extracellular bacteria as well as some facultative intracellular pathogens. PMNs and activated macrophages utilize both oxygen-dependent (respiratory burst) and oxygen-independent (using proteolytic enzymes) mechanisms to kill bacteria. Oxygen-dependent mechanisms of respiratory burst are initiated by activators such as bacteria and LPS. Most bacteria are sensitive to toxic intermediates released by phagocytic cells but some of them show different levels of sensitivities to these mediators. The level of sensitivity of different species may affect the prognosis of the disease in the host.

In mammals the main phagocytic cells, PMNs, monocytes and macrophages, are also called professional phagocytes because of their ability to ingest a variety of particles and they are responsible for recognition of microbes by presenting them to T cells. Paraprofessional phagocytes include dendritic cells (DC) with selective phagocytic ability and along with macrophages, are important in inducing the adaptive response by presenting antigen. DCs originate from both myeloid and lymphoid precursors, the former are involved in the induction of the adaptive immune response (Kaufmann *et al.*, 2002). Cattle macrophages and PMNs possess a unique Fc receptor that is structurally different from other Fc receptors. This receptor may represent a special adaptation to the structure of IgG2 since bovine IgG2 has a very small hinge region (Tizard, 2000).

In atrophic rhinitis-related strains of *P. multocida,* the OmpH of 37.5 kDa was able to induce a dose-dependent increase in oxidative burst of bovine PMNs isolated from peripheral blood (Galdiero *et al.*, 1998). The major OMPs isolated from *M. haemolytica* were also able to induce alterations of the biological activity of bovine PMNs (Iovane *et al.*, 1998a). Phagocytosed *P. multocida* serotype A:3 (bovine pulmonary strain) was able to survive in bovine monocytes (Dowling *et al.*, 2004). An avian isolate of *P. multocida* (fowl cholera causing strain) has also been shown to persist in avian monocytes because of an outer membrane protein (Truscott & Hirsh, 1988). An attempt was made to investigate the effect of HS-related *P. multocida* on certain microbicidal reactive oxygen and nitrogen intermediates released by PMNs from vaccinated animals. The PMNs from the peripheral

blood of both control and experimental buffaloes vaccinated against HS were isolated. PMNs from control animals upon activation with *P. multocida* LPS and live *P. multocida* cells generated higher levels of hydrogen peroxide (H_2O_2) and nitric oxide (NO^-) than the non-activated cells ($P < 0.01$). In the presence of *P. multocida* LPS, PMNs from animals vaccinated against haemorrhagic septicaemia generated significantly higher H_2O_2 ($P < 0.05$) and NO- ($P < 0.01$) than the PMNs from control animals. The study suggested that buffalo PMNs possessed a potent oxidant defence system even in the presence of *P. multocida* (Roy *et al.*, 1996). However, HS-causing strains of *P. multocida* B:2 are cytotoxic for murine macrophages. Intraperitoneal injection of mice causes cytoplasmic vacuolation, lysis and death in peritoneal macrophages. In vitro incubation of a mouse macrophages cell line with HS-causing strains or with their culture supernatants was also able to induce vacuolation. Pre-incubation of cells with antiserum obtained from an infected buffalo, inhibited the vacuolation (Shah *et al.*, 1996). OMPs of *P. multocida* B:2 are antiphagocytic. They are able to interfere with phagocytosis of opsonised *Candida albicans* by murine peritoneal cells in vivo. It has been suggested that antiphagocytic mechanisms induced by the OMPs enhance the virulence of the organism (Srivastava, 1998). The polysaccharide capsule of *P. multocida* B:2 has also been considered as an antiphagocytic agent, since acapsular mutants are readily taken up by mouse peritoneal macrophages, while there is a significant resistance to phagocytosis in wild-type strains (Boyce & Adler, 2000).

There is limited evidence of intracellular survival of *P. multocida* and mechanisms involved in interference with the respiratory burst (Hodgson *et al.*, 2006). Several studies have investigated the effects of pasteurellae on mononuclear phagocytes. Some pathogens have aquired the ability to alter killing mechanisms of macrophages. Many of the studies have studied the whole organisms while some others have used different components of the organisms for study. Opsonising antibodies against *P. multocida* B:2 facilitate phagocytosis of the bacterium. These investigations, which could also be categorised under adaptive immunity (Section 1.13.2), have revealed that, despite the resistance to complement activity and other anti-phagocytic properties, opsonisation of the organism with antibodies enhances the up-take of the organism (Adler *et al.*, 1996; Ramdani & Adler, 1991). *P. multocida* B:2 has been shown to be phagocytosed inefficiently in a mouse model but to survive inside macrophages without multiplication (Adler *et al.*, 1996). In another study using acapsular mutants of *P. multocida* B:2 it was shown that the mutants were phagocytosed more rapidly than the wild type by murine peritoneal macrophages. It was also reported that the internalized mutant cells survived for at least 30 minutes in mouse macrophages (Boyce & Adler, 2000). It was reported in another study

that *P. multocida* B:2 is able to survive at least for 2 hours in a murine macrophage cell line (J774) (Tabatabaei, 2000).

1.13.2 Adaptive immunity

There are two types of adaptive immune responses; humoral (also called antibody-mediated) immunity and cell-mediated (also called cellular) immunity. Humoral immunity is mediated by antibodies in the blood and at mucosal surfaces that are produced by B lymphocytes (B cells). Cell-mediated immunity is mediated by T lymphocytes (also called T cells). Adaptive immune responses are generated to specific antigens of pathogens that are captured and displayed by antigen-presenting cells (APCs) (Abbas & Lichtman, 2003). Different types of cells function as APCs at different stages of cellular and humoral responses. The most highly specialized APCs are DCs, which capture microbial pathogens that enter from the external environment, process them to small fragments, transport them to adjacent lymphoid organs, and present them to T cell receptors (TCRs) on naïve T cells to start an immune response. TCR, which is structurally very similar to Fab fragment of an immunoglobulin molecule, consists of two different polypeptide chains, termed α and β, linked by disulphide bond. A minority of T cells bear an alternative, but structurally similar receptor made up of a different pair of polypeptide chains designed γ and δ (Marion *et al.*, 1984). The function of $\gamma\delta^+$ T cells in immune responses is not entirely clear. $\gamma\delta^+$ T cells share many functions with $\alpha\beta^+$ T cells particularly in their role as inducers of inflammation by IFN-γ production. On the other hand, they are different from $\alpha\beta^+$ T cells in the way by which their receptor interacts with antigen and the types of antigens that stimulate them. γδ TCR is not able to recognize antigen in the form of peptide-MHC complex but they express receptors recognise and react with some microbial phospholipids antigen molecules, such as isopentenylpyrophosphate (IPP), found on *Mycobacteria* and other bacteria and parasites. The specifity of these receptors for different non-peptide molecules on different pathogens shows variation between different species, suggesting that the γδ response may be directed against pathogens commonly encountered by a given species (Goldsby *et al.*, 2003; Herzig *et al.*, 2006). In young ruminants $\gamma\delta^+$ T cells predominate (45-50% of peripheral blood T cells) over $\alpha\beta^+$ T cells (5-30% of peripheral T cells) (Tizard, 2000), but this proportion decreases by adulthood (to 5% of total PBMCs) (Herzig *et al.*, 2006). TCRs (here the term TCR is used as αβ-TCR as this is the only TCR capable of recognizing antigen) only recognize the antigen as peptide fragments bound to a MHC (major histocompatibility complex) molecule displayed on the surface of APC that is called peptide-MHC complex. There are two classes of MHC molecule (named MHC class I and MHC class II), which differ in their structure and expression pattern on body tissues.

MHC class I molecules are expressed by most of the somatic cells but MHC class II molecules are normally expressed by APCs (Goldsby *et al.*, 2003).

Mature $\alpha\beta^+$ T cells express either CD4 or CD8 molecules and, together with CD3, they form an essential part of the TCR complex. $CD4^+$ T cells usually act as helper T cells and recognize antigen presented by MHC class II molecules on APCs (as macrophages, DCs, and B cells). By contrast, $CD8^+$ T cells are usually cytotoxic and recognize antigen-derived peptides presented by MHC class I molecules on somatic cells (Marion *et al.*, 1984). For an efficient stimulation of T cells, activated DCs also express co-stimulatory molecules called B7-1, B7-2 (also called CD80 and CD86, respectively), and CD40. The co-stimulatory molecules B7 and CD40 boost the stimulation when bound to corresponding T cells receptors CD28 and CD154, respectively (Roitt *et al.*, 1998). After activation, $CD8^+$ T cells, acquire cytotoxic activity and are called cytolytic T lymphocytes (CTLs), which are able to destroy host cells that are infected. Activated $CD4^+$ T cells can be divided into two major sub-populations, called type 1 helper (Th1) and type 2 helper (Th2) T cells. Th1 T cells, which mainly function against intracellular pathogens, are activated by antigen and, in the presence of IFN-γ and/or IL-12, express IL-2 and IFN-γ. Th2 T cells, which function against some helminth parasites and stimulate allergic reactions, are activated by antigen, IL-4 or IL-10 and express IL-4 and IL-6 (Hodgson, 2001).

These two different $CD4^+$ T cell-mediated responses, have been identified in mouse and humans and it is believed that the type of response influences the outcome of infection-resolution or exacerbation. However, this model of $CD4^+$ T cell activation is different in the bovine as it was shown that the T cell response can not be categorized under this model (Brown *et al.*, 1998). It was also suggested that, the Th1/Th2 paradigm might be different from mouse and human. For instance, in sheep and cattle, at the site of intracellular microbes (with the possible exception of the skin) and helminth parasite infections, T cells produce cytokines related to both Th1 and Th2 profiles (Hodgson, 2001). A high proportion of peripheral blood lymphocytes (15-30%), in young ruminants do not show any of the CD4 or CD8 markers (called $CD4^-CD8^-$ T cells) and in newborn calves this proportion is very high (up to 80%). Thus the circulating T cells in ruminants ($\gamma\delta^+$, $CD4^-$, $CD8^-$, $WC1^+$ [cattle species-specific cell-surface antigen found on a major subset of $\gamma\delta^+$ T cells with no homologue in human or mouse]) are very different from T cells found in blood of human and mouse ($\alpha\beta^+$, $WC1^-$, $CD4^+$, $CD8^-$) (Tizard, 2000).

In chicken and turkeys infected with *P. multocida,* both humoral and cellular immune mechanisms are involved in protection (Baba *et al.*, 1978; Baba, 1984; Maheswaran *et al.*,

1976). Although in HS, humoral immunity is believed to be the main component of the immunity, the contributory role of cell-mediated immunity cannot be discounted (De Alwis 1999).

1.13.2.1 Humoral immunity

Humoral immunity is the principal protective immunity against extracellular bacteria. Antibody produced in response to extracellular bacteria is mainly directed against antigenic components of the bacterial cell wall and secreted or cell-associated toxins, which maybe proteins or polysaccharides. Protection against polysaccharide-rich encapsulated bacteria is a major function of humoral immunity. Neutralisation, opsonisation and phagocytosis, and complement activation through the classical pathway, are effector mechanisms of antibodies to fight these infections. Antigenic proteins of extracellular bacteria are also able to activate $CD4^+$ helper T cells. Activated $CD4^+$ cells produce cytokines that stimulate antibody production by B cells, induce local inflammation (by secretion of TNF), and enhance the phagocytic activity of macrophages (by secretion of IFN-γ). Activated macrophages produce cytokines which causes inflammation and in the some severe cases result in septic shock. Septic shock is the most severe cytokine-induced pathological consequence of infection by Gram-negative and some Gram-positive bacteria in which TNF is the principal mediator (Abbas & Lichtman, 2003).

It is generally believed that immunity to HS is predominantly humoral and it can be transferred with serum of immune animals to naïve animals. Although very little is known about what constitutes a protective response, measuring the antibody levels has been used as a tool to measure immunity in vaccinated animals (Verma & Jaiswal, 1998). The tests that have been mainly used for this purpose include the passive mouse protection test (PMPT), indirect haemagglutination (IHA) test, immunoblotting technique, and antibody enzyme-linked immunosorbent assay (ELISA), but studies on buffaloes have revealed no strong relationship between PMPT, IHA (Section 1.8.2.5) and protection (Chandrasekaran *et al.*, 1994a; Chandrasekaran *et al.*, 1994b).

ELISA and immunoblotting tests have been used to examine the antibody responses of cattle vaccinated against HS-causing strains of *P. multocida*. The study revealed that, in addition to the response to lipopolysaccharide, antibodies from the vaccinated animals reacted strongly with five to six of the 40 protein bands in this organism (Johnson *et al.*, 1989). Monoclonal antibodies against LPS could opsonise *P. multocida* serotype B:2 for

phagocytosis by mouse macrophages, but were not bactericidal in the presence of complement (Adler *et al.*, 1996).

The humoral responses of buffaloes was characterized after immunization with four different killed HS vaccines including; non-adjuvanted broth bacterin, alum-precipitated, oil adjuvant and a double emulsion. There appeared to be a relationship between ELISA antibody titres and active protection of animals. Three different preparations of *P. multocida* B:2 including formalin killed and boiled whole cells, and LPS were used as antigens in the ELISA system. Regardless of the different kinds of antigen used, the mean antibody levels rose significantly over a 3-month period in buffaloes immunised with the adjuvanted vaccines (Chandrasekaran *et al.*, 1994b). Generally, bovine IgG consists of three subclasses: IgG1, IgG2a, and IgG2b. IgG1 constitutes about 50% of the serum IgG and is remarkable for being the predominant immunoglobulin in cow's milk rather than IgA (Tizard, 2000). Preliminary analysis of the antibody isotype in the pre-challenge sera of the buffaloes in the above study at 6 months post-immunisation revealed the predominant isotypes to be IgG1 and IgG2 with the percentage of 48% and 42%, with IgM and IgA at 7% and 3% respectively (Chandrasekaran *et al.*, 1994b). It was not possible to assign any relative protective role to these immunoglobulins isolated from immune animals because of the lack of a reliable and reproducible data from the passive mouse protection test. Because of the lack of complete protection in some of the vaccinated buffaloes, it was suggested that there may be a minimum threshold of antibody level necessary for protection against challenge (Chandrasekaran *et al.*, 1994b).

No minimum level of antibody necessary for protection has been distinguished by other studies. One reason for this is the differences in the methods of antigen preparation used in the ELISA. Another reason could be the individual differences between animals, especially between cross-bred animals due to genetic differences, in mounting an immune response (Verma & Jaiswal, 1998), as it has been shown in cattle that serum IgG2 concentrations vary greatly among different individuals and IgG2 levels are highly heritable (Tizard, 2000). A strong relationship between IgG but not IgM antibody levels and resistance to challenge was revealed in another study on vaccinated buffaloes using different HS vaccines. It was shown that animals immunised with an oil-adjuvanted vaccine developed a high level of IgG and IgM as compared to the other groups of animals vaccinated with bacterin or non-vaccinated animals. The IgG class was detected as the principal antibody response with only mild IgM responses. It was shown that the response increased gradually until day 75, then dropped slightly and maintained its level for about one year. At the time of challenge (250 days post-vaccination) the level of IgM class antibodies had decreased

considerably while the IgG class response was still at a high level (Shah & de Graaf, 1997). In both of the above-mentioned studies, the increase in antibody titre after vaccination was only gradual and did not reach maximum until 2-3 months after vaccination.

In another study, passive immune cross-protection in mice produced by rabbit antisera was used to determine the immunological relationship between strains associated with HS and fowl cholera (FC). Various degrees of cross-protection were seen among the strains. Antiserum against a serotype B:3,4 strain protected against strains capable of causing HS (serotypes B:1, B:2, B:3,4, B:4 and E:2) and FC (serotypes A:1, A:3 and A:5). Antiserum against an FC strain (serotype A:5) similarly protected against strains capable of causing HS and FC. Interestingly, antigenic analyses indicated that cross-protection was not necessarily induced by serotype-specific capsular or somatic antigens or major porin proteins. SDS-PAGE and immunoblots of whole cell lysates of the different HS and FC strains using the various antisera showed many protein-staining bands with similar mobilities and antigenic activity. These cross-reactive antigenic bands occurred in the 20- to 120-kDa range. Furthermore, it was shown that adsorption of antiserum with a heterologous serotype removed its reactivity with most of these bands, as well as its ability to cross-protect (Rimler, 1996).

1.13.2.2 Cell-mediated immunity (CMI)

Cell-mediated immunity is the major adaptive immune response against intracellular bacteria. CMI mainly consists of two types of reactions: lysis of infected cells and macrophage activation. The lysis of infected cells is carried out by CTLs. Macrophage activation, which results in killing of phagocytosed bacteria, is through T cell-derived signals; production of IFN-γ and CD40 ligand (Abbas & Lichtman, 2003). In some cases the macrophage activation also causes tissue injury in the host. This tissue injury can be manifested as delayed-type hypersensitivity (DTH) reactions against bacterial protein antigens (Kaufmann *et al.*, 2002).

The role of cell-mediated immune responses has not received much attention in research on HS. This might be because most of the knowledge on immune responses is from vaccination studies using killed vaccines, where a strong cellular response would not be expected. The other reason for this is insufficiencies in reagents commercially available for analyzing bovine blood cells. Thus, the contribution of cell-mediated immunity in the protection against HS merits further investigation. However, the contribution of CMI was

studied in an extensive experiment on buffaloes where cutaneous delayed-type hypersensitivity (DTH) was used to assess the cellular response against four different kinds of inactivated vaccines. Vaccinated animals developed a substantial to significant cutaneous DTH response. Histopathological examination of selected biopsy specimens taken from the site of inoculation showed a predominance of infiltrating lymphocytes, thus indicating a probable DTH response. An apparent relationship was demonstrated between the DTH response and protection of buffaloes in different vaccination groups. However, it was suggested that, because the antigen used for intradermal inoculation was an ultrasonicate of cells (intradermal inoculation in the caudal fold of 0.1 ml at a concentration of 220 µg protein per ml) which contained a wide range of possible protective and non-protective antigens, it was not possible to reach a definitive conclusion about the extent of the contribution of the cellular response in protection (Chandrasekaran *et al.*, 1994b).

In a study on water buffaloes, the capacity to mount humoral and cell-mediated immune responses against a HS aluminium-precipitated vaccine was suppressed due to infection with *Trypanosoma evansi*. The subcutaneous inflammatory response (SIR) to primary and secondary vaccination at the vaccination site, which was measured as a cellular response, showed significant reduction in animals experimentally infected with *T. evansi*. On the other hand, no significant differences were observed in antibody responses against *P. multocida*. It was concluded that reduction of local inflammatory reactions due to HS vaccination was due to reduced levels or reduced reactivity of certain T cell phenotypes, and that the reduced skin induration would suggest diminished levels of protection against *P. multocida* (Holland *et al.*, 2001). However, in this study the protection against challenge, which could have been very informative, was not determined.

The role of CMI in protection of cattle against *P. multocida* after vaccination was investigated in another study. The leukocyte migration inhibition test (LMIT) was used as an indicator of cellular immunity in calves vaccinated with a killed oil-adjuvanted vaccine. LMIT showed more than 20% migration inhibition during all pre- and post-challenge periods in vaccinated animals suggesting an involvement of cell-mediated immune mechanisms in protection (Verma & Jaiswal, 1997). It was suggested that both humoral and cell-mediated immune responses contribute to protection in vaccinated calves (Verma & Jaiswal, 1997). Overall, however, the contribution and extent of CMI in protection against *P. mutocida* serotype B:2 is not completely understood. A fatal human case of *Pasteurella* sepsis has been reported in a patient with hairy-cell leukemia (CLL, a lymphoproliferative disorder), which can be indicative of the importance of cellular

immunity against the bacterium, since the disease has usually been associated with increased rates of infection caused by microorganisms normally controlled by CMI (Athar *et al.*, 2003).

1.13.3 In vitro lymphocytic responses

To study the modulatory effects of *Pasteurella* and other bacterial pathogens on immune response, several in vitro experiments have investigated the stimulation, proliferation, and cytokine responses of mononuclear cells after treatment with different components of the bacteria.

1.13.3.1 Lymphocyte stimulation assay

The lymphocyte stimulation assay (also called antigen-stimulated lymphocyte proliferation or lymphoblastogenesis) is one of the most useful assays for assessment of cellular immune response. In the body, stimulation of primed (activated) lymphocytes, occurs in lymphoid tissues in response to antigen presented to them. However the ability of isolated lymphocytes (such as PBMCs) to be stimulated (expressed as proliferation and/or cytokine secretion) when cultured in the presence of the antigen to which then are activated has been exploited as a tool to assess cellular response in vitro.

The proliferative response of activated peripheral blood mononuclear cells (PBMCs) to antigen has been extensively used to study cellular immunity, as PBMCs from infected or immune hosts will proliferate in response to antigen obtained from the organism they have been exposed to. In a study on HS- and shipping fever-related *Pasteurella* it was reported that PBMCs from HS-immunised cattle exhibited higher stimulation indices when incubated with antigen preparations from homologous strains than with the heterologous shipping fever strain. Conversely, PBMCs from cattle immunised with the shipping fever strain of *P. multocida* exhibited a higher stimulation index when incubated with an antigen preparation from the homologous strain than with antigen preparation from heterologous HS strains (Maheswaran & Thies, 1979).

To investigate the cellular response of calves in a pneumonia model caused by *M. haemolytica*, proliferative responses and IFN-γ production of PBMCs and lymph node cells were assessed after vaccination with live and inactivated vaccines or intratracheal infection. Different antigen preparations, including capsular polysaccharide (CP), lipopolysaccharide (LPS), lipopolysaccharide-associated protein (LAP), bacterial cell

sonicate (SON) and outer membrane protein (OMP) were used for the lymphocyte stimulation assay. Of them, OMP was found to be the best component for this purpose and no stimulation was observed using capsular polysaccharide. Lymph node cells from calves in the live vaccine group and infected group showed strong proliferation and IFN-γ production after stimulation. In the infection group, a stronger cellular respose was observed in cells obtained from lymph nodes nearer the site of bacterial administration. In the live vaccine group, a stronger response was observed in distant lymph nodes compared to lymph nodes near to infection site. In PBMCs obtained from animals intratracheally inoculated before challenge, no proliferation or IFN-γ production was detected in any of the groups. In an attempt to explain this unresponsiveness of PBMCs, it was suggested that reactive lymphocytes were absent in the blood and they possibly were partitioned in the lymph nodes. Another suggested possible reason was that the antigen presenting cells in lymph nodes acted more effectively than those in peripheral blood in the in vitro assay (DeBey *et al.*, 1996). However, in this experiment the PBMC stimulation assay was not done in parallel with cells obtained from lymph nodes on the day of necropsy and therefore it is quite difficult to compare the responsiveness of these two sets of cells. In another study, PBMCs from *M. haemolytica*-infected calves gave a poor lymphoproliferative response to leukotoxin despite their high serum antibody levels against the leukotoxin and *M. haemolytica* (Hughes *et al.*, 1992).

1.13.3.2 In vitro proliferative responses

The in vitro proliferative response of PBMCs to mitogens has also been extensively used to study cellular immunity and investigation of T and B cell functions. Mitogens are lymphoid proliferative agents, which can non-specifically induce cell division. They are polyclonoal activators as they activate most T and/or B cells. Lectins, which are carbohydrate-binding proteins, are commonly used mitogens. Three of the most commonly used lectins are: 1) concanavalin A (ConA), a T cell mitogen derived from jack bean seeds; 2) phytohaemagglutinin (PHA), a T cell mitogen derived from kidney beans; 3) pokeweed mitogen (PWM), a mitogen for both T and B cells derived from pokeweed. LPS although not a lectin, has mitogenic activity on B cells.

Mitogen proliferation of lymphocytes in vitro, which is done by cross-linking the BCRs (B cell receptors) or TCRs (T cell receptors), is believed to mimic stimulation by specific antigens fairly closely. The use of these agents in vitro has shown that activation of T and B cells leads to the production of cytokines and their receptors, which together drive the cells through their cell cycle (proliferation) and ultimately to maturation and to the

production of effector cells (Roitt *et al.*, 1998). Concanavalin A (Con A) is a lectin with potent T cell mitogenic activity and has been extensively used to study in vitro lymphocyte responses. In cattle, blood lymphocytes acquired the ability to respond to mitogens between 75 and 80 days age of the foetus, but this ability is lost temporarily around the time of birth as a result of high serum steroid levels (Tizard, 2000).

In different studies, the inhibition of proliferative response of lymphocytes to mitogens has been investigated as suppression of cellular immunity due to infection caused by pathogens. In a study on sheep, it was shown that infection with *Trypanosoma evansi* suppressed the proliferative responses of PBMCs to ConA and LPS. This study also showed that the proliferative response of PBMCs obtained from *M. haemolytica*-vaccinated animals to corresponding antigen (assessed by lymphocyte stimulation assay) was suppressed by infection with *T. evansi*. Interestingly, it was shown that treatment of animals with a trypanocide restored the proliferative ability of the cells (Onah *et al.*, 1998). In another study on cattle, the proliferative response to lectins and *S. aureus* antigens in lymphocytes obtained from mammary glands infected with *S. aureus* was less than the response of cells from healthy animals (Park *et al.*, 1993).

The suppressive effects of some bacteria or their components have also been investigated using in vitro systems. A study on the suppressive properties of *Helicobacter pylori* showed that the lysate of the bacterium was able to inhibit mitogen-induced proliferation of human PBMCs (Knipp *et al.*, 1993). Further studies on murine spleen and mesenteric lymph node cells showed that the pre-exposure (incubation for 2 h) of cells to CFE of the bacterium had the same inhibitory effects on T cell proliferative agents (Con A, phorbol-myristate-acetate and ionomycin, and the anti-CD3epsilon monoclonal antibody). Heating of the CFE at 80°C for 30 minutes, completely abolished its inhibitory action (Chen *et al.*, 2000). Vacuolating cytotoxin (VacA) was identified by further studies as the suppressive agent (Gebert *et al.*, 2003) and the mechanism involved is believed to be independent of IL-2 (Sundrud *et al.*, 2004). A study on *Mycoplasma bovis* (a pathogen of cattle that causes pneumonia, arthritis, and mastitis), showed that the organism was able to induce apoptosis in bovine lymphocytes (Vanden Bush & Rosenbusch, 2002). Further study revealed immunosuppressive properties of a protein component of the organism with anti-lymphoproliferative effects on cattle PBMCs. This study also introduced a 26-amino acid recombinant peptide from *Mycoplasma bovis*, which showed suppressive effects and was able to suppress the ConA-induced proliferation of bovine lymphocytes (Vanden Bush & Rosenbusch, 2004). No similar studies, on the immunomodulatory effects of *P. multocida*,

especially serotype B:2 or its structural components, on proliferation of lymphocytes have been reported.

1.13.3.3 In vitro cytokine responses

Immunoregulatory cytokines play an important role in the immune response against different pathogens. Several studies have used in vitro systems to investigate changes in cytokines after exposure of immune cells to different microbial pathogens or their components. In vitro systems have also been used to study the profiles of cytokines and their function as a model of protective response.

The effect of *P. multocida* porin and LPS on mouse splenocyte cultures has been studied by analysing the expression and release of IL-1α, IL-6, TNF-α, IL-4, IFN-γ, IL-10 and IL-12 cytokines (Iovane *et al.*, 1998b). Treatment of splenocytes with either purified porin or LPS for 3 hours caused an increase in intensity of the signal from IL-1α, IL-6, TNF-α, IFN-γ and IL-12 mRNA while no effect was detected on IL-4 and IL-10 mRNA under the same conditions. These purified components also showed a dose-dependent regulatory effect on the induction and release of pro-inflammatory cytokines (IL-1α, IL-6, and TNF-α) after 24 hours and immunoregulatory cytokines (IFN-γ and IL-12) after 48 hours. Both of the LPS and porin induced release of IL-1α, IL-6, TNF-α, IFN-γ and IL-12 in cultures. Stimulation with LPS induced the release of IL-1α, IL-6 and TNF-α, which began at 3 hours and was completed after 12 hours. LPS induction of IFN-γ and IL-12 began after 12 hours and was completed at 36 hours. Stimulation with porin caused IL-1α, IL-6 and TNF-α release after 6 hours and was completed after 24 hours. Porin induction of IFN-γ and IL-12 reached a significant level after 24 hours and was completed after 48 hours. It was concluded that the stimulation with LPS is a faster process than porin and it happens at a greater rate (Iovane *et al.*, 1998b).

1.13.4 Naturally-acquired immunity

Naturally-acquired immunity is the immunity against HS that occurs on exposure to the organism but without any vaccination. This status was recorded for the first time among buffaloes in Thailand and it was concluded that about 10% of the buffalo herds were naturally immune (Bain, 1954). Further studies in Sri Lanka showed that naturally-acquired immunity of animals resulted from natural exposure to infection and the proportion of immunity varied from herd to herd and also from time to time (de Alwis & Sumanadasa, 1982). After outbreaks of HS, antibody levels rise in two to four weeks and

persist in some animals for over a year. The percentage of naturally-immune animals is highest in endemic areas and least in non-endemic areas (De Alwis, 1982). Natural immunity has also been reported in some areas where HS does not exist, including Australia and United States (De Alwis, 1994). In a study in USA, antibodies against HS-related serotypes B and E were demonstrated among a high percentage of sera from domestic feeder calves. The antibodies were demonstrated by passive mouse protection (PMP), agglutination, and indirect haemagglutination (IHA) tests. In the PMP test, injection of mice with sera obtained from calves caused protection against challenge with serotypes B and E in 81% and 91 % of cases respectively. IHA and agglutination tests demonstrated antibody in nearly all sera. It was concluded that the source of these antibodies was not HS-related serotypes, as serotype E has been isolated only in Africa and there is only one report of serotype B isolation from cattle in the United States (Sawada *et al.*, 1985). Since, these organisms were not considered likely sources of the antigenic stimulation that provoked production of these antibodies, it is believed that in these situations immunity may have been due to exposure to other antigenically-related *P. multocida* (De Alwis, 1999). Thus, it can be inferred from the studies on naturally-acquired immunity against HS that this immunity could be attributed to two different sources: to protective antibodies that develop as a result of non-fatal exposure to the organism, and to the exposure to antigenically-related organisms, sometimes called non-specific immunity, particularly in areas where HS does not exist.

1.14 Objectives and experimental approach of this study

The primary objective of this study was to investigate safety and potency of an *aroA* mutant of *P. multocida* serotype B:2 (JRMT12, section 2.1.1) as a live vaccine candidate in cattle against experimental HS. To do this, three different experiments were set up to study the responses of 2-4 week old calves to vaccination with the *aroA* strain and their response to challenge with the parent wild type 85020 strain one week after vaccination: 1) Different routes of vaccination, IN and IM, using two doses of 10^9 CFU of the *aroA* strain with a 4-week interval; 2) Different doses (10^7, 10^8 and 10^9 CFU) injected twice IM; 3) A single IM vaccination with 10^8 CFU.

Safety of JRMT12 was assessed by determination of clinical responses (rectal temperature, dullness, respiration, appetite, incidence of nasal/oral discharges) and changes in SAA and Hp, taken as indicators of the progress and severity of infection. Potency was assessed by

measurement of serum antibodies (IgG and IgM) to *P. multocida* B:2 and by survival rate after subcutaneous challenge with the wild type strain.

Immunogenic components of *P. multocida* to which antibody were raised during different stages of vaccination and challenge were detected by SDS-PAGE and immunoblotting. For further identification of immunogenic components, OMPs of the bacterium were prepared and subjected to identification by mass spectrometry (MS). Changes in OMPs prepared from *P. multocida* B:2 grown under iron-depleted conditions and the possibility of presence of immunogenic components were also investigated.

A possible contributory role of cellular immunity against HS was investigated in calves given a single IM dose of 10^8 CFU and in control calves after challenge. A lymphocyte stimulation assay was used to assess the effects of a cell-free extract (CFE) of *P. multocida* on PBMCs isolated from calves at different times after challenge. Following the observation of inhibitory effect of challenge with *P. multocida* B;2 on calf PBMCs, further in vitro experiments were carried out to investigate the possibility of suppressive properties of CFE on PBMCs obtained from normal calves. Further investigations were focused on preliminary characterisation of the suppressive agent(s) and identification of the suppressed cell population(s) of PBMCs. FACS analysis, was used to determine the populations of PBMCs, which were affected by treatment with CFE. B cell and T cell subpopulations ($CD4^+$, $CD8^+$ and $\gamma\delta^+$) in normal calf PBMCs were identified and the changes in their relative proportions after different treatments were also investigated.

Chapter 2

MATERIALS and METHODS

2.1 General bacteriological procedures

2.1.1 Source of bacteria

Bacterial strains used in the experiments were obtained from culture collections of the Division of Infection and Immunity, University of Glasgow or the Moredun Research Institute, Edinburgh (Table 1). *Pasteurella multocida* serotype B:2 strain 85020 was originally imported from Sri Lanka under licence PO/95/22. JRMT12 was an *aroA* mutant constructed from *P. multocida* B:2 strain 85020 (Tabatabaei *et al.*, 2002).

2.1.2 Growth media and bacterial storage

Media used for growth of bacteria were Brain Heart Infusion (BHI, Oxoid) broth (Appendix A.1), Nutrient Broth (NB, Oxoid) (Appendix A.2), or Sheep Blood Agar (SBA, Oxoid) [supplemented with 5% (v/v) defibrinated sheep blood (B&E Laboratories, Scotland)] (Appendix A.3). All strains were stored at -70°C in 1 ml aliquots in 50% (v/v) autoclaved glycerol in BHI broth. All media were sterilised by autoclaving at 15 p.s.i., 121°C for 15 min except where stated. Heat-labile ingredients such as antibiotics were sterilised by filtration through a sterile 0.2-μm pore size membrane (Sartorius). Glassware was sterilised by dry heating to 160 °C for 2 h.

Table 1 Different bacteria used in experiments

Species or Strain	Origin	Supplied by
Pasteurella multocida serotype B:2 (85020)	Bovine haemorrhagic septicaemia	Division of Infection and Immunity, University of Glasgow (Dr. John Coote)
JRMT12 (an *aroA* mutant of *Pasteurella multocida* B:2 85020)	Bovine haemorrhagic septicaemia	Division of Infection and Immunity, University of Glasgow, (Dr. John Coote)
Pasteurella multocida serotype A3 (MRI, 671/90)	Bovine pneumonia	Moredun Research Institute, (Dr. J. Christopher Hodgson)
Pasteurella multocida serotype D	Swine atrophic rhinitis	Division of Infection and Immunity, University of Glasgow, (Dr. Robert L. Davies)
Pasteurella multocida serotype A	Ovine pneumonia	Division of Infection and Immunity, University of Glasgow, (Dr. Robert L. Davies)
Pasteurella multocida serotype A	Avian fowl cholera	Division of Infection and Immunity, University of Glasgow, (Dr. Robert L. Davies)
Mannheimia haemolytica serotype A1	Bovine pneumonia	Division of Infection and Immunity, University of Glasgow, (Dr. Robert L. Davies)
Mannheimia haemolytica serotype A2	Ovine pneumonia	Division of Infection and immunity, University of Glasgow, (Dr. Robert L. Davies)
Escherichia coli Strain K12		Division of infection and Immunity, University of Glasgow, (Dr. John Coote)
Staphylococcus aureus		Division of Infection and Immunity, University of Glasgow, (Dr. John Coote)

2.1.3 Growth of *P. multocida*

P. multocida strains, from -70°C glycerol stock, were inoculated in 1 ml aliquots into 9 ml of BHI broth, NB or onto SBA. Incubation was for 16-18 h at 37°C statically. For large-scale culture for preparation of different components of the bacteria, 16 h broths were inoculated into 250 ml amounts of BHI broth in 1-L flasks (5-10 percent inoculum) and incubated in the same conditions statically.

For growth of *P. multocida* strain 85020 under iron-limited conditions, 2.2'-dipyridyl was added to BHI broth at 150μM concentration (Appendix A7). In preliminary experiments, the growth rate was assessed at five different concentrations (120, 140, 150, 170, and 220 μM) of dipyridyl. The 150μM concentration of dipyridyl was highest concentration at which *P. multocida* grew.

2.1.4 Preparation and administration of vaccination and challenge doses

To keep the vaccination and challenge doses uniform, 1 ml aliquots of a single batch of a stock bacterial suspension containing either the *P. multocida* 85020 wild type or JRMT12 mutant strain (Table 1), from the –70°C freezer, were inoculated into 9 ml of BHI broth, and incubated overnight (16 h statically) at 37°C, and 3 ml of the resulting culture was transferred to 27 ml of BHI broth in 50-ml flasks. The flasks were incubated at 37°C with shaking at 200 rpm for 2.5 h (strain 85020) or 3 h (strain JRMT12) to obtain cultures containing around 10^9 CFU/ml as measured by previous experiments where bacterial counts were confirmed by spreading dilutions on SBA. Cells were collected by centrifugation, resuspended in PBS, and diluted in PBS to the desired vaccination or challenge doses, confirmed by viable counts on SBA. For administration of vaccination doses in IM-vaccinated groups, desired doses of *P. multocida* JRMT12 mutant strain were prepared in 5 ml of PBS as two 2.5 ml doses and injected into semi-tendinosus muscle of each hind limb. In IN-vaccinated group the 5 ml dose was inoculated into nostrils of calves while the head was kept up. For administration of the challenge dose, the desired dose of *P. multocida* strain 85020 was prepared in 5 ml of PBS as two 2.5 doses injected SC, one over each prescapular region.

Intratracheal challenge of calves with *Pasteurella multocida* serotype A3 was carried out according to the procedure of Dowling (Dowling *et al.*, 2002). The challenge dose was prepared in 300 ml of PBS containing 10^9 CFU of *P. multocida* strain MRI-671/90 (Table 1). A one ml aliquot of a stock culture of the strain was inoculated into 10 ml of nutrient

broth (NB) (Appendix A2), and incubated at 37°C statically overnight. Aliquots of 0.5 ml of this overnight culture were inoculated into 10 ml of NB and incubated at 37°C with shaking for 3.5 h. Cultures were diluted with pre-warmed (37°C) PBS to produce challenge inocula containing an estimated 10^9 CFU in 300 ml volumes. Actual challenge doses were estimated retrospectively by plating out serial dilutions of each dose onto SBA.

2.2 Animal procedures at Moredun Research Institute

All animal experimental protocols were approved by the Moredun Research Institute Animals Experiments Committee and authorized under the United Kingdom Animals (Scientific Procedures) Act of 1986. The work with calves was performed by Dr. J.C. Hodgson (Bacteriology Laboratory, Moredun Research Institute) and my involvement in the conduct of the experiments was done under his supervision.

During the course of animal experiments, any calf showing pyrexia was treated with intravenous Flunixin meglumin (Finadyne; Schering-Plough Animal Health, Welwyn Garden City, United Kingdom), a nonsteroidal anti-inflammatory drug (NSAID), at 2.2 mg/kg. At the end of the experiments, the calves were killed humanely within the containment facility by intravenous injection of 25 ml of Pentobarbitone sodium B.P. (200 mg/ml; Animal care, York, United Kingdom), surface disinfected, and bagged before transport to the post-mortem suite for gross pathological examination and removal of tissues for bacteriology and histopathology. Any of the animals showing clinical signs of pain or very high stages of sickness, after challenge or at any stages of the experiments, were killed for humane reasons.

2.2.1 Selection of *P. multocida*-free calves

Infection of the upper respiratory tract of calves with *Pasteurella multocida* serogroup A is common in the United Kingdom (Dowling *et al.*, 2002). To reduce the possibility of an active humoral immune response compromising the vaccination studies, only *P. multocida*-free calves were selected for experiments. To check the calves, nasal swabs (CultureSwab Plus; BD Biosciences, Oxford, United Kingdom) were obtained from calves within 24 h of birth by Dr. Hodgson and assessed for growth of *P. multocida* after being plated on SBA containing vancomycin (1 mg/ml) (Appendix A.5) to prevent the growth of Gram-positive bacteria. Chosen calves that had been shown to be free of *P. multocida* infection were treated at 2 days of age with enrofloxacin at 2.5 mg/kg of body weight (Baytril Max; Bayer Animal Health, Newbury, United Kingdom) by subcutaneous injection, housed in air-

filtered isolation pens, offered milk and, at 7 weeks old, weaned onto a diet of calf creep feed and hay.

2.2.2 Conditions for containment of calves

Throughout the experiments the animals were maintained at a high standard of care and sanitation with their health and well-being assessed daily; veterinary assistance from the Clinical Division of the Moredun Research Institute was available at all times, and feed and water were provided *ad libitum*. The conditions for containment and handling of animals inoculated with either the mutant or parent strain of *P. multocida* B:2, which has not been reported from the United kingdom, were set at containment category 2 (Health and Safety Executive Advisory Committee on Dangerous Pathogens). The animal accommodation used for the experiments provided this level of containment, but as added safety precautions to prevent the escape of *P. multocida* B:2 into the environment, ventilation was adjusted so that the rooms were under negative pressure during the experiments, drains were blocked, matting and absorbent bedding were provided and renewed regularly, and all waste materials were bagged, surface disinfected, and incinerated.

2.2.3. Experimental design

Three sets of experiments were designed to assess safety and potency of different doses of JRMT12 by different routes of vaccination. In each experiment, the calves were allocated randomly to different groups of vaccinated and control animals. The number of animals in each group was restricted to the minimum likely to generate a statistically significant result.

2.2.3.1 First experiment

The object of this experiment was to investigate whether vaccination of calves twice either intramuscularly or intranasally with 10^9 CFU of *P. multocida* B:2 mutant strain JRMT12 would provide protection against subcutaneous challenge with 10^7 CFU of the *P. multocida* B:2 parent strain 85020.

Number of calves	Treatment			Purpose
	1st vaccination (2 weeks old)	2nd vaccination (at 6 weeks old)	Challenge (at 8 weeks old)	
3	-	-	10^7 cfu	Challenge controls
4	Intranasal, (10^9 cfu) Actual dose*: (6×10^8 cfu)	Intranasal (10^9 cfu) Actual dose: (10^9 cfu)	10^7 cfu	To determine if two intranasal vaccinations with 10^9 cfu dose protects against challenge
4	Intramuscular, (10^9 cfu) Actual dose:(6×10^8 cfu)	Intramuscular,(10^9 cfu) Actual dose:(10^9 cfu)	10^7 cfu	To determine if two intramuscular vaccinations with 10^9 cfu dose protects against challenge

* Actual dose: determined by spreading dilutions on SBA.

2.2.3.2 Second experiment

The object of this experiment was to investigate whether vaccination of calves twice intramuscularly with 10^9, 10^8, or 10^7 CFU of *P. multocida* B:2 mutant strain JRMT12 would provide protection against subcutaneous challenge with 10^7 CFU *P. multocida* B:2 parent strain 85020, in the absence of an inflammatory response.

Number of calves	Treatment			Purpose
	1st vaccination (at 4 weeks old)	2nd vaccination (at 8 weeks old)	Challenge (at 10 weeks old)	
3	-	-	10^7cfu	Challenge controls
5	Intramuscular: (10^9 cfu) Actual dose: (1.1×10^9)	Intramuscular, (10^9 cfu) Actual dose: (8×10^8)	10^7 cfu	Confirm protection obtained in first Expt. with 10^9 cfu
5	Intramuscular: (10^8 cfu) Actual dose: (6.3×10^7)	Intramuscular(10^8 cfu) Actual dose: (1×10^8)	10^7cfu	Determine if 10^8 cfu dose protects
5	Intramuscular: (10^7 cfu) Actual dose: (8.5×10^6)	Intramuscular, (10^7 cfu) Actual dose: (1.1×10^7 cfu)	10^7cfu	Determine if 10^7 cfu dose protects

2.2.3.3 Third experiment

The object of this experiment was to investigate whether a single vaccination of calves intramuscularly with 10^8 cfu of *P. multocida* B:2 mutant strain JRMT12 at 4 weeks of age would provide protection against subcutaneous challenge at 8 weeks of age with 10^7 cfu *P. multocida* B:2 parent strain 85020.

Number of calves	Treatment		Purpose
	Vaccination (at 4 weeks old)	Challenge (at 8 weeks old)	
5	-	10^7 cfu	Challenge controls
4	Intramuscular: (10^8 cfu) Actual dose: (5×10^7 cfu)	10^7 cfu	Determine if single vaccination protects against challenge

2.2.4 Clinical responses

Following vaccination or challenge, clinical responses including: rectal temperature, dullness, respiration (rate and nature), appetite, incidence of nasal/oral discharges, of animals were monitored and recorded at 2-3 h intervals or as necessary, by experienced observers of the Clinical Division of Moredun Research Institute. Clinical reaction to vaccination and challenge was expressed as a clinical score that was assessed by general demeanour (scored zero to 3; calf normal scored as 0; calf dull, slow to move, appears inattentive scored as 1; calf depressed and reluctant to stand unassisted scored as 2; calf severely depressed, recumbent, unable to rise scored as 3).

2.2.5 Blood analysis

Blood samples (10 ml) were collected by **Dr.** Hodgson from the jugular vein of animals into plain and heparinised Vacutainers (Becton Dickinson, Meylan, France). Blood samples were collected from all calves before vaccination, after each vaccination and at weekly intervals in between. Blood samples were also collected before challenge, and at different time points after challenge. Sera or plasmas were separated and stored at -40°C for further investigation.

2.2.6 Bacteriological examination

Tissue samples of size of around 1 cm^3 were taken post-mortem by Dr. M. Dagleish (Department of Pathology, Moredun Research Institute) from lung, kidney, heart, spleen, liver, brain, and different lymph nodes and were homogenised in 9 ml of peptone-water (Appendix A.4). Aliquots of 10 µl were spread on SBA containing 1mg/ml vancomycin (Appendix A.5) and incubated for 16-20 hours at 37°C prior to phenotypic and numerical analysis. Blood samples (10 µl) were cultured in the same way. *P. multocida* serotypes A colonies were distinguished from *P. multocida* B:2 by a mucoid consistency and creamy colour.

2.2.7 Necropsy

All animals were subjected to post-mortem examination by Dr. Dagleish at the Clinical and Pathology Departments of Moredun Research Institute. Gross pathology was assessed, and tissue samples from different organs including lung, liver, kidney, spleen, brain, tonsil, thymus, and lymph nodes (prescapular, bronchial, mid- and caudal mediastinal, mesenteric, submandibular, axillary, retropharyngeal, and prefemoral) were preserved in saline containing 4% (w/v) formaldehyde: formol-saline and prepared for histopathological examination using standard techniques (embedded in paraffin wax, sectioned, mounted on microscope slides, and stained with haematoxylin and eosin [H&E]).

2.3 Laboratory procedures

2.3.1 Preparation of bacterial cell-free extract (CFE)

CFEs of different bacterial strains were prepared from 18 h broth cultures. A purity test was done by Gram staining a smear preparation of the culture and direct checking by light microscopy. Cells were collected by centrifugation at 3,000 × g for 30 min at 4°C (RC-5B, Sorvall) and washed three times with PBS (Appendix A.6). A thick suspension of bacteria in PBS was lysed by sonication using a Vibra Cell ultrasonic processor (Jencons-PLS, Leighton Buzzard, United Kingdom) for three 60-s bursts with intermittent cooling on ice. The broken-cell suspension was centrifuged at 3,000 × *g* for 30 min at 4°C, and the supernate was filtered through a 0.2-μm-pore-size membrane (Sartorius). The protein concentration was determined by Bradford assay (Section 2.3.6.1) and adjusted to a desired concentration by adding sterile PBS.

2.3.2 Dialysis of CFE

CFE obtained from *P. multocida* 85020 was dialysed using dialysis tubing with a molecular weight cut off of 10,000 Da (Medicell International Ltd.). The tubing was boiled in 0.5 mM EDTA (pH 8.0) for 30 min and stored in 70% (v/v) ethanol at 4°C until ready to be used. The tubing was rinsed thoroughly with distilled water before use. 5 ml of the CFE was dialysed in 1000 ml PBS (Appendix A.6) twice overnight at 4°C with constant stirring. The dialysed preparation was kept at -70°C until use.

2.3.3 Outer-membrane protein (OMP) preparation

OMPs were prepared by Sarkosyl extraction according to Davies with modifications (Davies *et al.*, 1992). Bacteria were harvested from 18 h cultures of BHI broth (Appendix A.1) then washed three times with PBS (pH 7.2) by centrifugation at 3,000 × g for 30 min at 4°C. The cells were suspended in cold 20 mM-Tris/HCl (pH 7.2) buffer and sonicated on ice for three 60-s bursts with intermittent cooling on ice with a Vibra Cell ultrasonic processor (Jencons-PLS, Leighton Buzzard, United Kingdom). The suspension was centrifuged at 10,000×g for 30 min at 4°C to remove unbroken cells. The supernate was then centrifuged at 100,000×g for 1 h at 4°C (Combi, Sorvall) to pellet the cell envelopes. The pellet was resuspended in 0.5% (w/v) *N*-lauroylsarcosine (Sarkosyl, Sigma) (Appendix B.8) and left for 20 min at room temperature to solublise the cytoplasmic membranes. Outer-membrane proteins (sarcosine-insoluble membrane fraction) were then pelleted by centrifugation at 100,000×g for 30 min at 4°C. The pellet was washed once in distilled water and resuspended in PBS (pH 7.2) at a protein concentration of 2 mg/ml, assessed by Bradford assay (Section 2.3.6.1), and stored at -70°C.

Outer-membrane proteins were also prepared from the *P. multocida* strain 85020 grown under iron-limited conditions (Section 2.1.3). The same procedure detailed above was used for the OMP preparation.

2.3.4 Polysaccharide capsule and culture supernate preparation

Polysaccharide capsule of *P. multocida* 85020 was isolated based on the procedure of Carter (OIE, 2004). An overnight culture in BHI was centrifuged at 3,000 × g for 30 min at 4°C. The culture supernate was filtered through a 0.2 µm-pore-size membrane (Sartorius), a sample was removed for estimation of protein concentration by Bradford assay (Section 2.3.6.1) and the remainder was kept at –70°C until used. The pellet was washed three times with PBS (pH 7.2) by centrifugation at 3,000 × g for 30 min at 4°C. The cells were suspended in PBS and heated in a water bath at 56°C for 30 min, centrifuged at 3,000 × g for 15 min at 4°C and the clear supernatant fluid separated and filtered through 0.2 µm-pore-size membrane. This preparation was used as polysaccharide capsule and kept at –70°C until used.

2.3.5 LPS from *E. coli*

Purified LPS was extracted from *E. coli*, and was kindly supplied by Dr. G. Cheung (Division of Infection and Immunity, University of Glasgow). Briefly, a 3 h culture of *E coli* grown in LB at 37°C with shaking at 200 rpm was harvested by centrifugation at 13,700 × g for 30 min (Sorvall, RC-5B). A thick suspension of the resulting bacteria was freeze-dried (Christ technologies, alpha 1-4) and then, to each gram of dry weight, 10 ml of PBS containing 5mM EDTA was added and incubated for 2 h at 37°C. Lysozyme (Sigma) was added at 0.02 g for each gram of dry weight and incubated at 4°C for 16 h, with constant stirring. The solution was further incubated at 37°C for 20 min and then an equal volume of 20 mM magnesium chloride was added. RNAase (Sigma) and DNAase (Sigma) were added to the mixture, both at final concentrations of 1 μg/ml, and incubated for 10 min at 37°C then at 60°C for a further 10 min. An equal volume of pre-heated (70°C) phenol solution, equilibrated with 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 7.5-7.8) (Sigma), was added to the mixture and incubated for 20 min at 70°C with constant stirring. The aqueous/phenol mixture was placed on ice for 20 min with stirring, then overnight at 4°C with no stirring. The upper aqueous phase (containing LPS) was removed and dialysed (MW cut off 12-14 kDa) against water for 3 days (Section 2.3.2). The dialysed LPS material was centrifuged at 4,220 × g (Sorvall, RC-5B) for 15 min at 4°C. The supernate was collected and freeze-dried and the fine LPS powder was stored at 4°C. Once reconstituted with endotoxin-free water (Sigma), the LPS was stored at –20°C. The LPS content was assessed by the LAL-Chromogenic method (LAL reagent, BioWhittaker, UK) and expressed in Endotoxin Units (EU)/ml.

2.3.6 Quantification of proteins

All of the CFEs, OMPs, and other bacterial preparations were subjected to protein quantification to measure the protein content and set to the desired concentrations. Two methods were used for this purpose. Bradford assay was used to measure the protein content of all preparations and, if the protein concentration was less than 200 μg/ml, the sample was then subjected to modified Lowry assay.

2.3.6.1. Bradford assay

The Bradford reagent (BioRad) was used to provide a quantitative measurement of protein concentration of samples containing soluble proteins (excluding OMP preparations). When Coomassie dye binds protein in an acidic environment, an immediate absorbance shift

occurs from 465 nm to 595 nm with a simultaneous colour change of the reagent from green/blue to blue. The Bradford's reagent was diluted 1 in 5 with distilled water and filtered through a 0.45 μm Sartorius filter before use. The method needs a standard curve which is established with different concentrations of bovine serum albumin (BSA) (Sigma) (Appendix B.7) in PBS. A solution of 2 mg/ml of BSA was prepared in PBS and 2-fold dilutions of this in PBS were prepared in a microtitre plate. PBS alone served as a negative control and blank. To a new flat-bottomed 96-well microtitre plate, 25 μl of each dilutions of BSA and sample were mixed with 200 μl of diluted Bradford's reagent. After incubation for 10 min at room temperature, the absorbance values were read at $OD_{620\ nm}$ in a plate reader (Dynex technologies). Once the blank OD values had been subtracted from all OD values, the protein concentrations of the samples were determined from the BSA standard curve.

2.3.6.2. Modified Lowry assay

This assay, which is appropriate for quantification of soluble and non-soluble proteins, relies on the formation of a protein-copper complex (Biuret reaction) and the reduction of the phosphomolybdate-phosphotungstate reagent (Folin-Ciocalteu phenol reagent, Sigma) (Appendix B 6.3) by the tyrosine and tryptophan residues of the protein. The method also needs a standard curve, which is generally established with BSA (Appendix B.7). Protein standards containing 25 to 200 μg/ml of BSA in PBS were used. Dilutions of the sample of unknown protein concentrations were prepared in duplicate. 100 parts of reagent A was mixed with 1 part of reagent B (Appendix B 6.2) to form reagent C (the alkaline copper reagent) (Appendix B 6.3). A sample volume of 1 ml containing 20 to 200 μg/ml of protein was added to 3 ml of reagent C and then incubated at room temperature for 10 to 60 min. The samples were then mixed vigorously with 0.3 ml of diluted Folin-Ciocalteu (Sigma) phenol reagent (Appendix B 6.3) and incubated for 45 min at room temperature. The OD was read at 660 nm by spectrophotometer (Gilbert 250), and the results expressed in μg/ml of protein.

2.3.7 Acute phase protein (APP) assay

Serum amyloid A (SAA) and haptoglobin (Hp) were measured by Prof. David Eckersall in calf serum samples obtained at different stages of vaccination and challenge. Serum samples were supplied to the Centre for Integrated Diagnostinc Systems (CIDS) of University of Glasgow. The assay method was based on an ELISA for SAA and a

biochemical haemoglobin binding assay for Hp according to Horadagoda method (Horadagoda *et al.*, 1999).

2.3.8 Serum sensitivity assay

The serum sensitivity assay was done according to Boyce and Adler with modifications (Boyce & Adler, 2000). Guinea pig complement serum (Sigma) and sera from young calves (fresh normal calf serum [Bov.Ser]) were used as the complement source. Jugular blood from normal young calves collected (kindly permitted by Dr. Harold Thompson and helped by Mr. Richard Irvin) in sterile glass containers when the calves were put down at the Department of Pathology, School of Veterinary Medicine, University of Glasgow. Collected blood was then left at room temperature for 4 h and the serum was separated, aliquoted and kept at – 20°C. The assay was performed in 96-well, tissue culture-grade, flat-bottomed, micro-plates (Costar, USA). To prepare a bacterial suspension, a few separate colonies from an overnight culture of *P. multocida* 85020 on SBA (Appendix A.3) were inoculated into 9 ml of BHI broth and incubated statically overnight at 37°C. An inoculum of 0.5 ml was transferred into 9 ml of pre-warmed BHI broth (Appendix A.1) and incubated at 37°C with shaking at 200 rpm for 4 h. The cells were collected by centrifugation at 3,000 x *g* for 30 min at 4°C and then washed once with PBS (pH 7.2) and re-suspended in PBS. A bacterial suspension of 2×10^6 CFU/ml in PBS was prepared from *P. multocida* 85020 or *E. coli* and used as the bacterial suspension. Different test sera (25 µl) were added in duplicate in the wells of sterile micro-plates, except when evaluating the effect of complement alone, where the serum was replaced with PBS. Bacterial inoculum (50 µl) was then added to each well. The complement source (25 µl) was added, except to the control wells for evaluation of the effect of test sera on *P. multocida* in the absence of complement and the complement control wells in which heat-inactivated (56°C for 30 min) complement was added. Plates were then covered with a lid and incubated at 37°C for the required time. Diluted (10^{-1} and 10^{-2}) and undiluted samples (10µl) from each well were plated on SBA in 10 ×10 cm square plates (Bibby Sterilin, United Kingdom) at the beginning (time zero) and at one h after incubation. The plates were allowed to dry and then incubated overnight at 37°C. The count of each countable test was calculated as the mean number of colonies derived from in each of the three different dilutions. The killing effect of each serum was calculated as a bactericidal index: CFU/ml after 1 h incubation divided by CFU/ml at time zero. Data presented as the average of two assays ± standard deviation.

2.3.9 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE for analysing *P. multocida* CFEs and outer membrane proteins was done according to the method of Laemmli (Laemmli, 1970). Samples were diluted 1:6 with 5× loading buffer (Appendix B.1) and boiled for 5 min. A vertical gel electrophoresis tank using the Mini Protean 3™ slab gel electrophoresis system (Mini Protean, Bio-Rad, Richmond) was used with 4% polyacrylamide gel as stacking gel (Appendix B.2) and 10% polyacrylamide gel as resolving gel. Resolving gels (Appendix) were poured between the assembled glass plates with a 1.5-cm space left on top for the later addition of the stacking gel. Immediately, absolute ethanol was poured on top of the gel to eliminate any bubbles and form a horizontal interface for addition of the stacking gel. Once set, the ethanol was removed and then a stacking gel (Appendix B.2) was poured onto the polymerised resolving gel and a comb with 10 wells was placed into the gel solution which was allowed to set. The wells were rinsed out with distilled water and any residual polymerised gel on the face of the assembled glass plates was removed using tissues. The electrophoresis tank and gels were assembled according to the manufacturer's instructions. Electrophoresis was performed in 1× electrode running buffer (Appendix B.4) with a constant voltage of 100 V for 2 h or until the dye front ran off the gel. Protein bands were then visualised by staining with Coomassie blue (Appendix B.5) for a minimum of 30 min on a rotating platform. After incubation, the Coomassie blue stain was decanted and the gel was destained (Appendix B.5) several times, for a minimum of 30 min in between changes, on a rotating platform until protein bands could be visualised. At this stage, the gel was immersed in water and photographed using a transilluminator with camera attached.

2.3.10 Immunoblotting

Following SDS-PAGE, polypeptide bands were transferred to Hybond-C Extra membrane, (Amersham Biosciences) using the Mini Protean 3™ electrophoresis tank containing the Mini Transblot™ electroblot apparatus (BioRad) according to the method of the manufacturer. The stacking gel was removed and the gel was placed in an electroblotting apparatus. This was placed into the electrophoresis tank containing 1× transfer buffer (Appendix C.1) and an ice pack. After transfer for 1 h at 100 volts, the nitrocellulose membrane was soaked in Ponceau S solution (Sigma) for several minutes and then rinsed with distilled water to confirm the transfer of proteins onto the membrane. The membranes were incubated for 1 h in blocking buffer (PBS-SMT) (Appendix C.3) with shaking at room temperature. Membranes were then incubated overnight with a 1/250 dilution of

immune calf serum in PBST diluent (Appendix C.4) as primary antibody at 4°C on a shaker. After washing 3 times with PBST (Appendix C.2) for 10 min each, the membranes were incubated on a shaker for 1 h in 1/1000 dilution of sheep anti-bovine IgG-horseradish peroxidase (HRP) conjugate, as secondary antibody (Serotec Ltd., Kidlington, United Kingdom). They were then washed 3 times with PBS for 10 min and the substrate (3-diaminobenzidine tetrahydrochloride (0.05% w/v) (Sigma) in Tris buffer pH 7.4, containing hydrogen peroxide) (Sigma) was added for colour development (substrate solution) of reactive bands (Appendix C.5). After the appearance of bands the reaction was stopped by washing with excess water.

2.3.11 Protein identification by mass spectrometry

Designated bands were manually excised from a SDS-PAGE gel and placed in 1.5 ml eppendorf tubes which were sent to the Sir Henry Wellcome Functional Genomics Laboratory at University of Glasgow. Mass spectrometry analysis was performed by Dr. Richard Burchmore following standard protocols for LC-MS/Ms (Mutapi *et al.*, 2005). Gel pieces were cut into several pieces and then washed in 500 µl of 100 mM ammonium bicarbonate for 30 min to 1 h. Gel pieces were washed in 50% acetonitrile, 100 mM ammonium bicarbonate for 30 min to 1 h. To reduce samples, 150 µl of 100 mM ammonium bicarbonate and 10 µl of 45 mM DTT were added and incubated at 60°C for 30 min. To alkylate samples, 10 µl of 100 mM iodoacetamide was added and incubated at room temperature in the dark for 30 min. Solvent was discarded and gel pieces were washed then in 500 µl of 50% acetonitrile, 100 mM ammonium bicarbonate with shaking for 1 h. The supernate was discarded and 50 µl of acetonitrile was added for 10 min to dehydrate the gel pieces. Solvent was removed and gel pieces were dried in a vacuum centrifuge. A sufficient amount of 0.2 µg/µl sequencing grade modified Porcine Trypsin (Promega) in 25 mM ammonium bicarbonate was added to each gel piece and left for 15 min to rehydrate. Finally, rehydrated gel pieces were covered by 25 mM ammonium bicarbonate and incubated at 37 °C overnight for digestion of proteins. Peptides were extracted by addition of acetonitrile and dried before analysis by LC-MS/MS. All peptide samples were separated on an LC system (Famos / Switchos / Ultimate, LC Packings) before being analysed by electrospray ionisation (ESI) mass spectrometry on a QSTAR® XL Hybrid LC/MS/MS System. Peptide mixtures, extracted from trypsinized gel bands, were separated on a single dimension using a Pepmap C18 reversed phase column (LC Packings), using a 5 - 85% v/v acetonitrile gradient (in 0.5% v/v formic acid) run over 45 min. The flow rate was maintained at 0.2 µl / min. Mass spectrometric analysis was performed using a 3 sec survey MS scan followed by up to four MS/MS analyses of the

most abundant peptides (3 sec per peak) in Information Dependent Acquisition (IDA) mode, choosing 2^+ to 4^+ ions above threshold of 30 counts, with dynamic exclusion for 180 sec. MS/.MS ions data was extracted using Analyst QS software and submitted to the Mascot search engine (Matrix Science) for protein identification (Appendix G).

2.3.12 ELISA for IgG and IgM antibodies to *P. multocida* B:2

The ELISA for detection of antibody to *P. multocida* B:2 CFE (Section 2.3.1) was based on that of Pati with modifications (Pati *et al.*, 1996). High-binding, 96-well ELISA plates (Greiner Bio-One Ltd., Stonehouse, United Kingdom) were coated with a CFE from the wild-type 85020 strain. For this, 1 ml of frozen stock culture was thawed, inoculated into 9 ml of BHI broth, and incubated statically at 37°C for 18 h. Cells were collected by centrifugation at 3,000 × *g* for 30 min at 4°C and washed three times with PBS, and a suspension of 7×10^9 CFU/ml was prepared. A CFE was prepared from the suspension. The extract was diluted 1 in 8 with carbonate-bicarbonate buffer, pH 9.6 (Sigma) (Appendix D1), to give a final concentration of 60 μg of protein/ml assessed by Modified Lowry (Section 2.3.6.2). Plates were filled with 50 μl per well of CFE and left overnight at 4°C, after which the plates were washed three times with PBST (Appendix D.2) and then incubated for 2 h at room temperature with 200 μl of 0.1% (w/v) gelatin (Sigma) (Appendix D.3) in PBS per well as a blocking buffer. Following further washing with PBS, 50 μl of 1-in-100 dilutions of serum samples were added to the wells, and doubling dilutions were prepared in duplicate in PBS. The plates were incubated at 37°C for 1 h and then washed three times with PBST, followed by incubation with 50 μl of a 1-in-10,000 dilution of sheep anti-bovine immunoglobulin G (IgG) or IgM-horseradish peroxidase (HRP) conjugate (Serotec Ltd., Kidlington, United Kingdom) at 37°C for 1 h. After a final three-wash step with PBST, bound conjugate was detected using 50 μl of the peroxidase substrate Fast OPD (Sigma) per well in the dark for 30 min. The reaction was stopped by the addition of 50 μl of stop solution (Appendix D.4) per well. Optical density values were measured at 492 nm in an Anthos ELISA reader (Labtech, United Kingdom). Antibody titers were determined as the number of ELISA units of undiluted calf serum per ml by comparison with values obtained for sera with intermediate IgG or IgM titers (from the survivor of the intranasal-vaccinated group at 7 days postchallenge for IgG and from an intramuscular-vaccinated calf on the day of challenge for IgM), each arbitrarily assigned the reference value of 1,000 ELISA units/ml. The antibody titers (in ELISA units/ml) of samples were calculated as the ratio of test serum titre to reference serum titre × 1,000.

2.3.13 Isolation and preparation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated by the procedure of Vanden Bush with some modifications (Vanden Bush & Rosenbusch, 2002). Blood samples were provided by Dr. Hodgson. Samples were collected from the jugular vein of calves in heparinised Vacutainers (Becton Dickinson, Meylan, France) containing 170 I.U. heparin and kept on ice until being centrifuged at 1730 × g for 20 min at 20°C (Heraeus multifuge 3 S-R). The buffy coat was transferred into a universal tube containing Hank's balanced salt solution (HBSS, Invitrogen, United Kingdom) without Ca^{2+} and Mg^{2+}, at room temperature. Then the mixture was layered carefully on top of an equal volume of NycoPrep^{TM}1.077 A (Fresenius Kabi Norge, Norway) and centrifuged at 600 × g, for 20 min, at 20°C. The interface opaque band was transferred to HBSS without Ca^{2+} and Mg^{2+} and washed once with HBSS by centrifugation at 200 × g at 4°C for 10 min. To lyse the contaminating erythrocytes, the pellet was re-suspended in red blood cell lysis buffer (Appendix E.3) and kept for 10 min on ice and then washed once with HBSS by centrifugation at 200 × g at 4°C for 10 min. The pellet was suspended in complete RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (Bio West), 2 mM L-glutamine (Gibco), 100 U of penicillin per ml, 100 µg of streptomycin per ml, 0.5% (w/v) nystatin, 1% (w/v) gentamycin, and 30mM HEPES (Sigma).to reach a final concentration of 1.0×10^6 live cells/ml, as estimated by trypan blue staining and haemocytometer cell count, (Life Technologies) (Appendix E.1).

2.3.14 Lymphocyte stimulation assay

The assay was based on that of Vanden Bush with some modifications (Vanden Bush & Rosenbusch, 2004). PBMCs (2×10^5) were mixed in flat-bottom 96-well plates (Costar, USA) in triplicate with 50µg/ml antigen, 5µg/ml concanavalin A (Con A) (Sigma) (Appendix E.2) or left as control cultures in a total volume of 220 µl. Wells containing Con A were prepared by adding 10 µl of RPMI containing a concentration of 100 µg/ml of Con A. To the wells to be stimulated with antigen, 10 µl of RPMI containing the required concentration of CFE was added before adding PBMCs (section 2.3.1). As control, 10 µl of RPMI without ConA was added to 200 µl of cell suspension. Plates were incubated at 37°C in a humidified atmosphere of 5% (v/v) CO_2 and 95% (v/v) air (Wolf laboratories, Galaxy R). On the third day of incubation, each well was pulsed with 37 KBq [methyl-^{3}H] thymidine (Amersham) in 20 µl RPMI and incubated for another 18 hour. The cultures were then harvested onto printed filtermat (glass fiber filter size 90 × 120 mm) (Wallac,

Finland) by a semi-automated 96-well plate harvester (Tomtec M111) and allowed to dry at 37°C. Dried filtermats then were bagged (Sample bag, PerkinElmer) with 4 ml of liquid scintillation cocktail (Betaplate Scint, PerkinElmer) and sealed. The amount of incorporated [methyl-^{3}H] thymidine was determined by a scintillation counter (Microbeta TriLux, PerkinElmer) and expressed as counts per minute (cpm). The stimulation index (si) was expressed as: cpm of treated cultures/cpm of control cultures without treatment.

2.3.15 Lymphocyte proliferation inhibition assay

To investigate inhibitory effects of *P. multocida* B:2 and other bacteria on proliferative response of PBMCs to ConA, the required concentration of a CFE was added to the wells and after adding the PBMCs, the cells were incubated for 1 h in the CO_2 incubator. After incubation, 10 µl of RPMI containing a concentration of 100 µg/ml of ConA was added to the wells. The proliferative response of PBMCs, was then assessed in the same way as the lymphocyte proliferation assay (Section 2.3.14).

2.3.16 Fluorescence-activated cell sorter (FACS) analysis

A FACS Calibur cytometer (Becton Dickinson) was used for the assay and analysis was performed using CellQuest or ModFit LT™ software. Principally, cells are passed through a laser beam and the scatters of fluorescent cells are used for detection of size (forward scatter) and density (side scatter) of cells. Status of cells can also be detected based on these data for example, it could be concluded that a cell was proliferative with a smaller size identified based on their size. This analysis was carried out under the supervision of Dr. Mara Rocchi at the Moredun Research Institute. Acquisition of 10,000 events was carried out on control PBMCs and cells cultured after different treatments with different bacterial preparations. The results are expressed as percentage of each cell population in PBMCs under different treatments.

2.3.17 CFSE loading of cells

PBMCs were loaded with CFSE (carboxyfluorescein diacetate succinimidyl ester) (Appendix F.1) for analysis of different generations of each cell population, according to Sathiyaseelan and Baldwin with modifications (Sathiyaseelan & Baldwin, 2000). In CFSE-loaded cells, CFSE binds to cytoplasmic proteins and the loaded cells are detectable based on the level of fluorescence activity. After each division in CFSE-loaded cells, the quantity of CFSE and consequently the fluorescence activity is halved. Thus, different generations

show different CFSE levels and all the cells in each generation contain the same level of CFSE. Briefly, a suspensions of 2×10^7 PBMCs/ml were washed 3 times in wash buffer (Appendix F.2) then re-suspended very gently at a 2-fold concentration in cold PBS and left to reach room temperature gradually. An equal volume of CFSE-PBS (Appendix F.1) was added to the cell suspension and mixed gently. The cells were covered by a lid and incubated at 37°C for 5 min in the CO_2 incubator. The reaction was stopped by adding an equal volume of ice-cold FBS (BioWest). Cells were centrifuged at 600 × g for 10 min at 4°C and the supernatant was discarded. The pellet was then re-suspended gently and washed three times in 10 ml of complete RPMI medium (Appendix E.1). Viable counting was done by a haemocytometer after trypan blue (Sigma) staining of the sample, and the cells re-suspended at an appropriate concentration in complete RPMI medium.

2.3.18 Immunofluorescence and flow cytometry

CFSE-loaded and un-loaded PBMCs were counter-stained by indirect immunofluorescence for surface antigens based on the procedure of Baldwin (Baldwin *et al.*, 2002). For surface antigen staining, the following mouse IgG monoclonal antibodies were used: IL-A12 to detect CD4, IL-A105 to detect CD8, IL-A29 to detect WC1 molecule of γδ T cells, IL-A30 to detect the IgM molecule of B cells. Goat FITC-conjugated (fluorescein isothiocyanate) anti-mouse IgG was used as the secondary antibody for detecting cell-surface antigens bound to primary antibodies, and also as negative control (Section 3.4.5.1). PBMCs were washed in FACS buffer (Appendix F.3) and re-suspended at 2×10^6 cell/ml in FACS buffer. Aliquots of 50 µl were dispensed into wells of U-bottomed 96-well plates (Costar, USA). Then, 50 µl of primary antibody at the appropriate dilution in FACS buffer was added to each well and incubated at 4°C for 30 min. 150 µl of FACS buffer was added and the cells centrifuged in pre-chilled tubes at 4°C for 30 s at 1000 × g. Cells were resuspended in 50 µl goat anti-mouse secondary antibody, at appropriate dilution, and incubated for 30 min at 4°C in dark. 150 µl FACS buffer were added and cells were washed once as before. Cells were washed again in 200 µl PBS by centrifugation at 4°C for 30 s at 1000 × g, and resuspended in 200 µl FACS Fix buffer (Appendix F.4). Plates were sealed in a FACS tube and stored at 4°C in the dark until FACS analysis (Section 2.3.18).

2.4 Statistical methods

The results for rectal temperatures were obtained at intervals after each vaccination and post-challenge and analysed separately for each period by fitting linear mixed models with

calf fitted as a random effect. The effect of treatment on rectal temperatures were analysed as differences from the baseline temperature at time zero. For the analysis of clinical scores in the first experiment, both the mean and maximum score attained by each calf during each treatment were calculated, and the difference in the proportion of calves from different groups that had maximum scores of either 0 or >0 was investigated using Fisher's exact test. The differences in mean scores over each 12 h surveillance period were analysed using either the Mann-Whitney U test (for comparision between two groups) or the Kruskal-Wallis test (for three groups), in these tests allowance was made for the small sample size. Readings for the IgM and IgG titres and SAA levels showed increased variability with higher values and so a log transformation was used on the data before fitting the repeated measure models. Serum Hp concentrations in all groups were often zero and because of this, small sample size and a high correlation between successive time-points, it was not sensible to fit a repeated measures model either to the raw data or to the data treated as a binary variable. Instead, the effect of treatment on Hp concentrations was compared using a Kruska-Wallis test base on a median for each group of the maximum Hp value observed for each calf. These statistical methods were applied with the help of Dr. Jill Sales from Biomathematics and Statistics Scotland.

Chapter 3

RESULTS

3.1 Responses of calves to administration of JRMT12 by different routes of vaccination (First experiment)

In this experiment (Section 2.2.3.1), two groups of four 2-week old calves were vaccinated twice intramuscularly (IM) or intranasally (IN) with a 4-week interval using c. 10^9 CFU of the JRMT12 *aroA* strain. Two weeks after the second vaccination, all of the vaccinated and three non-vaccinated control calves were challenged subcutaneously with c. 10^7 CFU *P. multocida* B:2 wild strain 85020.

3.1.1 Clinical response to vaccination and challenge

3.1.1.1 Mean rectal temperature

At 3 and 7 h after the first vaccination with the JRMT12 strain (Figure 5A), the mean rectal temperatures (±pooled standard errors of the mean [SEM]) of IM-vaccinated animals showed an average rise of 0.7°C (± 0.17). By comparison, IN-vaccinated animals showed no significant change from a mean (± SEM) rectal temperature of 39.2°C (± 0.14) at time zero, and this difference between groups was statistically significant ($p = 0.011$).

At 4 and 6 h after the second vaccination (Figure 5B), the mean rectal temperatures (± pooled SEM) of IM- and IN-vaccinated animals showed an average rise of 1.6 and 0.3 °C (± 0.13°C), respectively, and this difference between groups was statistically significant ($p < 0.001$).

Between 3 and 12 h after challenge with the wild-type 85020 strain (Figure 5C), the rectal temperatures for IN-vaccinated and control calves showed similar patterns of response, during which the mean values rose by 1.2 and 0.9°C (± 0.12°C), respectively. By comparison, IM-vaccinated animals showed no significant change from a mean rectal temperature of 39.1°C (± 0.16) at time zero, and the difference between this group and the IN-vaccinated and control groups was statistically significant ($p<0.001$).

Figure 5 Changes in rectal temperatures with different routes of vaccination and after challenge (First experiment)

Rectal temperatures of calves in intramuscularly-vaccinated (IM), intranasally-vaccinated (IN), and non-vaccinated (control) groups were measured at different intervals after first vaccination (5A), second vaccination (5B), and after challenge with the wild type strain (5C). Results are the mean rectal temperatures of animals in each group, with SEM as error bars.

Figure 5A Rectal temperature changes after first vaccination

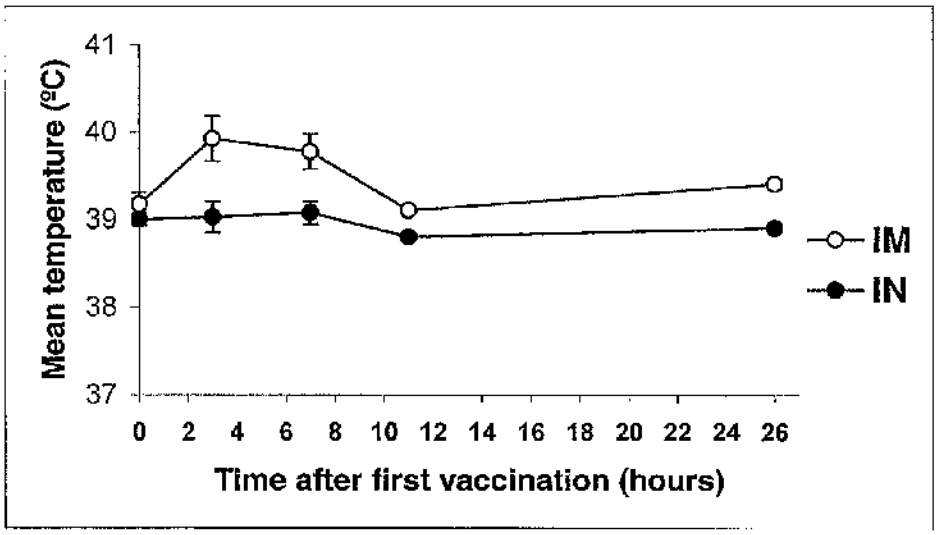


Figure 5B Rectal temperature changes after second vaccination

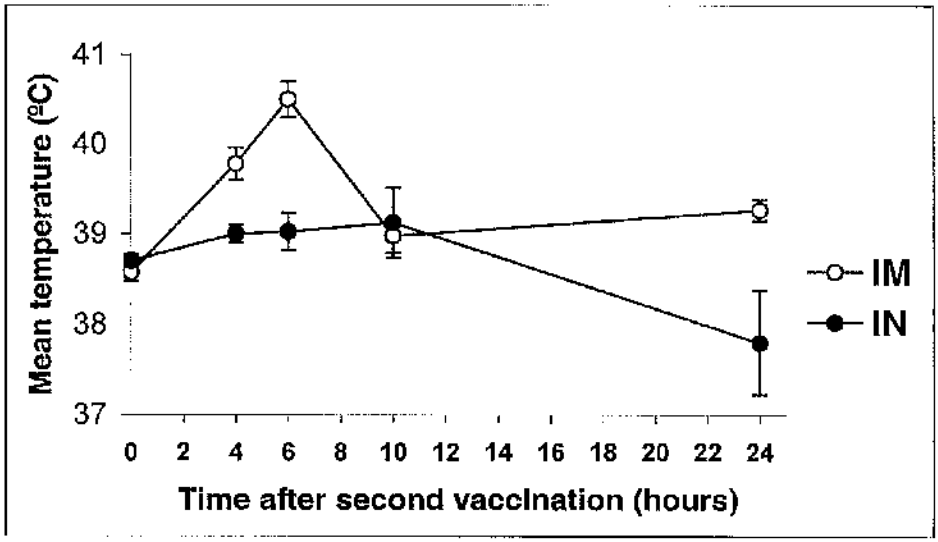
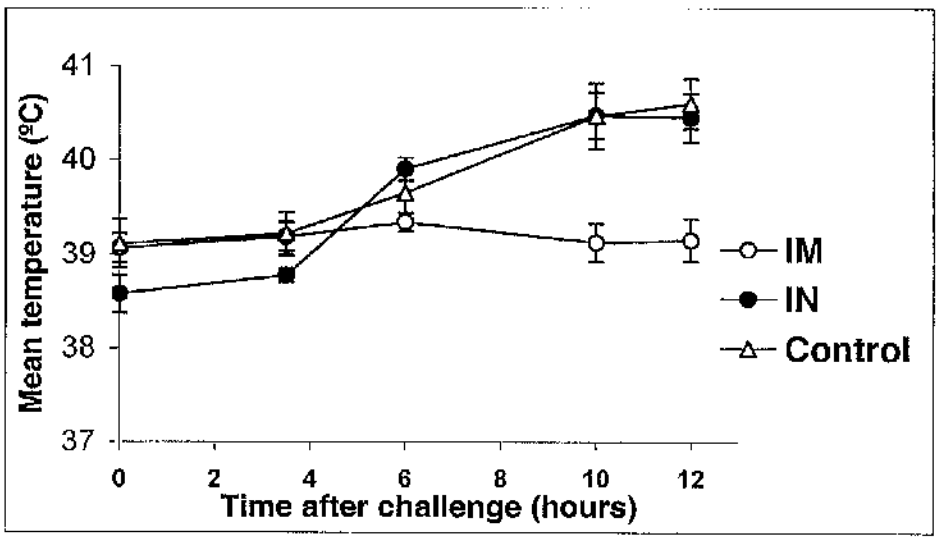


Figure 5C Rectal temperature changes after challenge



3.1.3.2 Changes in clinical score

During the period 0 to 11 h after the first vaccination, the mean clinical score for IM vaccinated animals was 0.8 (range, 0 to 2), whereas that for IN-vaccinated animals was 0, and this difference was significant ($p < 0.05$). The corresponding value for IM-vaccinated animals after the second vaccination was 0.6 (range, 0 to 1), whereas clinical scores of all IN vaccinated animals were 0, apart from one calf that had a score of 2 at 11 h post-vaccination. Because of this individual, the difference between the mean clinical scores for the two groups was not statistically significant. Because the IM-vaccinated group showed elevated temperature and change in demeanor, they were treated with NSAID (Section 2.2). Within 4 to 5 h of treating IM-vaccinated calves with NSAID (7 h after the first vaccination and 6 h after the second vaccination), rectal temperatures returned to pre-treatment (time zero) values and clinical scores returned to 0 (Section 2.2.4).

Mean clinical scores 3 to 12 h post-challenge were 0.8 (range, 0 to 2) and 1.0 (range, 0 to 2) for IN-vaccinated and control animals, respectively, whereas that for IM-vaccinated animals was 0.1 due to a score of 1 on one occasion, and this difference was significant ($p < 0.01$). Animals that were dull and febrile were treated with NSAID between 9 and 12 h post-challenge, but in three IN-vaccinated and two control calves, temperatures and clinical scores continued to increase and, for humane reasons, they were killed within 14 h post-challenge. The temperatures of each remaining calf from these groups returned to normal within 2 h of NSAID administration, and the clinical scores returned to 0 within 24 to 48 h post-challenge. None of the calves in the IM-vaccinated group required treatment with NSAID, and all survived challenge.

Post-mortem examination carried out by clinical department of Moredun Research Institute confirmed the gross pathological signs of HS in two control and three IN-vaccinated calves that were humanely put down within 14 h of challenge. Post-mortem examination of IM-vaccinated calves showed that there was no pathological signs of HS.

3.3.1.2 Changes in Serum Amyloid A (SAA)

Sera were supplied to CIDS (Centre for Integrated Diagnostic Systems) of University of Glasgow for determination of SAA levels. The concentrations of SAA in sera of individual calves varied considerably. The patterns of response in SAA concentrations after first vaccination (Figure 6A) were different in IM and IN-vaccinated calves; the concentrations increased significantly ($p < 0.05$) in IM vaccinated calves but showed no significant

changes in IN-vaccinated calves. SAA levels were also measured in IN and IM groups on the day of the second vaccination (day 28) and 24 h later. In IM-vaccinated group SAA levels increased significantly 24 h after second vaccination (from 39.3 ± 12.6 to 660 ± 32.7) but in IN-vaccinated group slightly decreased (from 21.3 ± 8 to 14.4 ± 1.7) (data not shown). The SAA concentrations declined in IM-vaccinated calves after 24 h (Figure 6A) but increased again significantly ($p < 0.001$) in response to the second vaccination. No significant changes due to the second vaccination were noted for IN-vaccinated calves.

The mean of SAA levels before challenge were highest in the control calves, followed by IM vaccinated and then by IN-vaccinated calves (Figure 6B). The mean SAA concentration in IN-vaccinated animals increased 13-fold by 10 h after challenge but by only 66 and 11 % in control and IM-vaccinated calves, respectively. This difference between IN-vaccinated calves and the others was statistically significant ($p < 0.01$). At 23 h post-challenge, SAA values exceeded 158 µg/ml in the two calves, one each from the IN-vaccinated and control groups, that had survived, whereas the maximum increase observed for calves in the IM-vaccinated group was only three fold (to 37 ±15.6 µg/ml). High SAA concentrations took 72 to 140 h to return to pre-challenge levels in the surviving calves (Figure 6B).

Figure 6 Changes in (SAA) levels after different routes of vaccinations and challenge (First experiment)

Calves were vaccinated twice with a 4-week interval. SAA concentrations were measured in sera collected on the day of first vaccination (day 0), and at intervals thereafter, and on the day of the second vaccination (day 28) and 24 h later (A). Two weeks after the second vaccination, the animals were challenged with the wild-type strain. SAA levels were measured in sera collected on the day of challenge (day 42) and at intervals thereafter. For control and IN-vaccinated groups at 14 h after challenge 3 calves were put down in IN-vaccinated and 2 in control group and the data onward represents results for one calf in each of these groups (B). Results represent the mean SAA concentrations of animals in each group with SEM as error bars.

Figure 6A Changes in SAA levels after vaccination

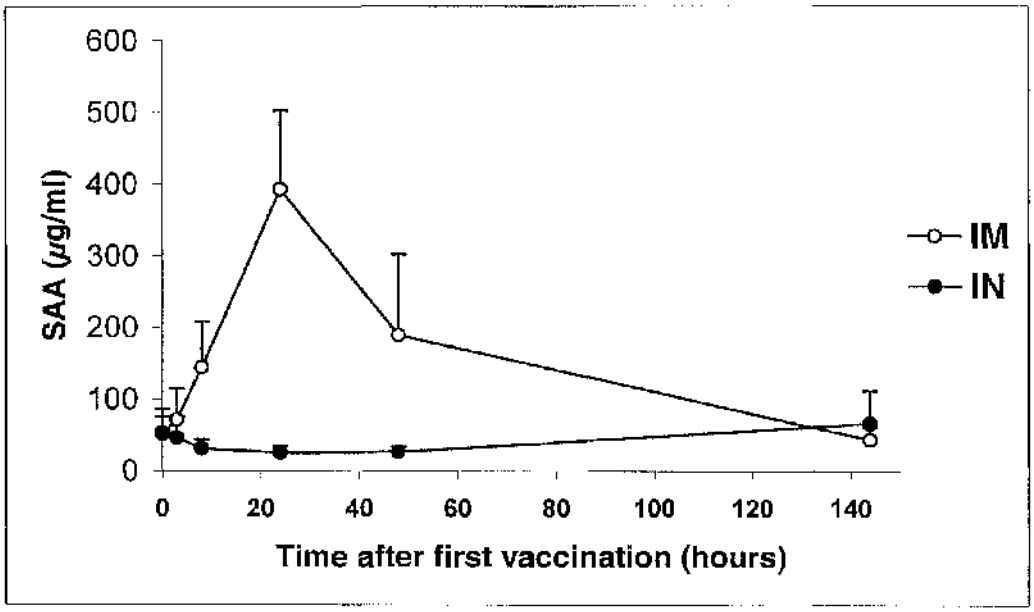
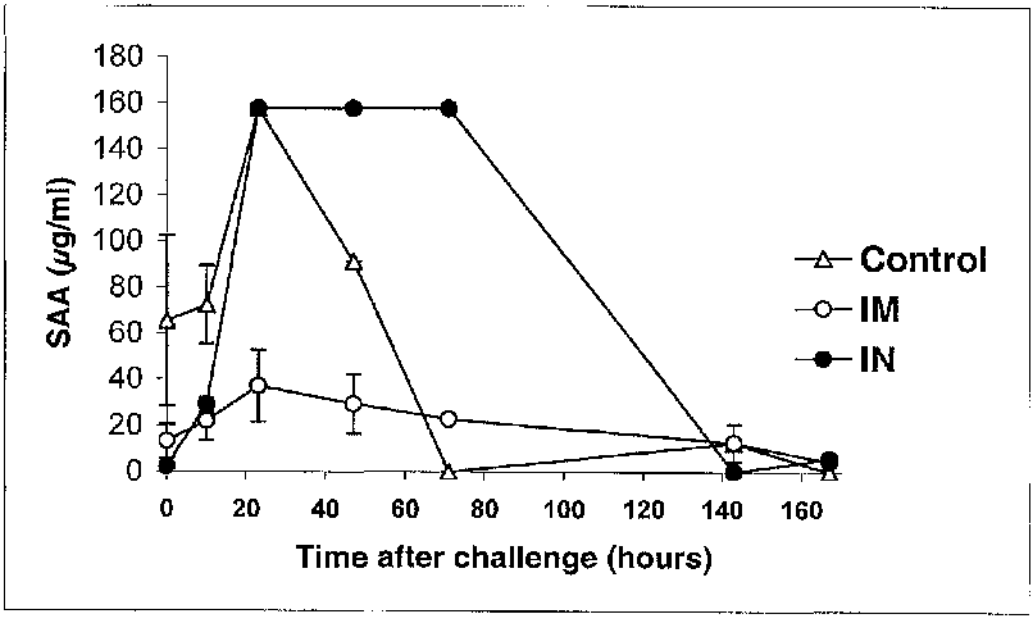


Figure 6B Changes in SAA after challenge



3.1.3 Antibody responses to vaccination and challenge

An ELISA system was used with a CFE of *P. multocida* serotype B:2 as coating antigen to measure the serum IgG and IgM levels at different stages before and after vaccination and after challenge.

3.1.3.1 Serum IgG levels

IgG titres to the *P. multocida* antigen preparation obtained from individual calves varied considerably. There was a significant difference in the pattern of change in the mean IgG titres between IM-vaccinated group and IN-vaccinated and control groups ($p < 0.001$).The mean (± SEM) ELISA data for IgG showed little response 28 days after first vaccination (Figure 7). At day 35 (7 days after second vaccination) the sera from IM-vaccinated calves had markedly increased IgG titres, which had increased further by day 42 (the day of challenge). There was no evidence of an increase in IgG titre in IN-vaccinated and control animals.

The data showed that IgG titres in IM-vaccinated calves at 7 days post-challenge (5285 ± 878.6 ELISA units/ml) had increased compared to the values obtained on the day of challenge (4522 ± 900.5 ELISA units/ml). Only one calf survived the challenge in each of the IN-vaccinated and control groups at 7 days post-challenge, so the ELISA data 7 days post-challenge are not included in (Figure 7). The IgG titres in the surviving calves in the IN and control group were 1000 and 263 ELISA units/ml, respectively.

3.1.3.2 Serum IgM levels

IgM antibody titres to the *P. multocida* antigen preparation of individual calves also varied considerably. Data for IgM in pre-vaccinated calves showed a wide range of titres, from 67 to 1548 ELISA units/ml in individual animals but by 14 and 28 days post-vaccination, these values had fallen (Figure 8). As with IgG, the IgM titres had increased markedly by 7 days after the second vaccination in the IM-vaccinated animals but remained low in the IN-vaccinated and control calves. The pattern of responses was significantly different ($p < 0.001$) between the IM-vaccinated group with the mean level in the IN-vaccinated and control groups. The mean IgM titre in the IN-vaccinated calves remained fairly constant over the course of the experiment.

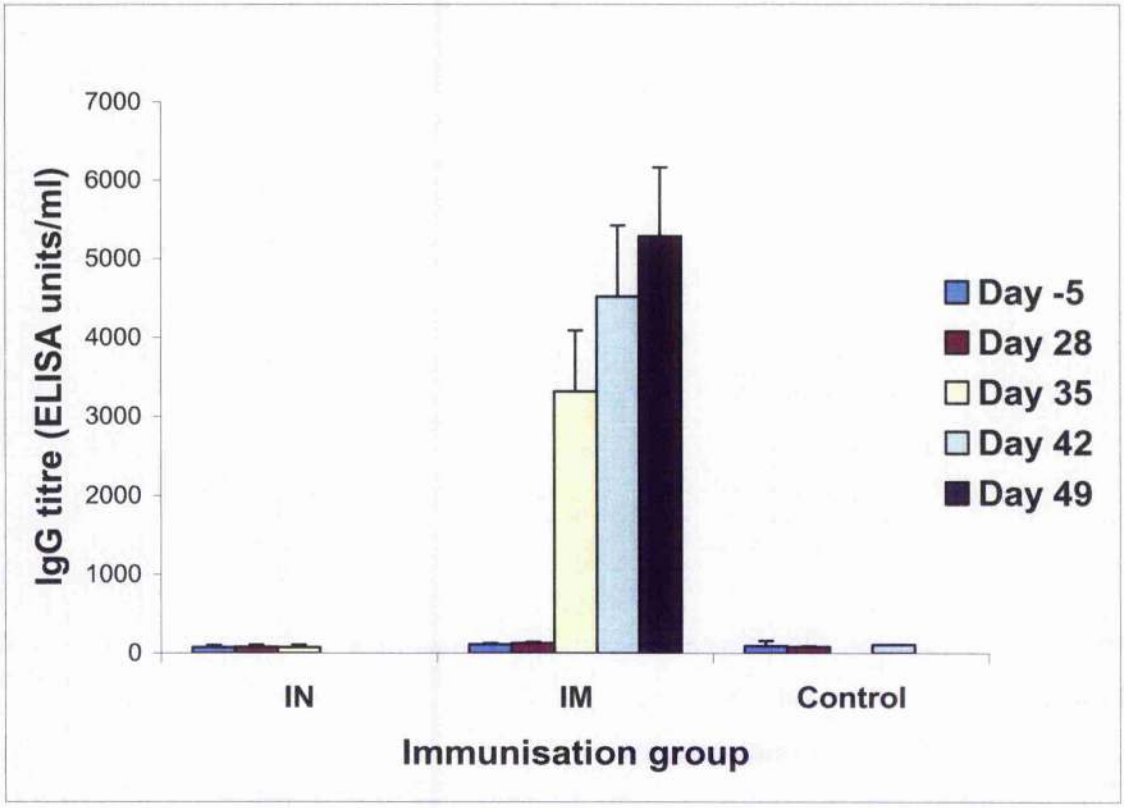
IgM titres in IM-vaccinated calves had not altered at 7 days post-challenge (795 ± 172.2 units) compared to the values obtained on the day of challenge (783 ± 90.1 units). IgM titres in the surviving calves in the IN-vaccinated and control groups (data are not plotted in figure) were 617 and 631 ELISA units/ml, respectively.

3.1.4 Survivors after challenge

All of the four IM-vaccinated animals survived the challenge, up to one week after challenge (Figure 9). Three of four IN-vaccinated animals and two of three non-vaccinated calves did not survive the challenge. Calves that were depressed, with rectal temperatures of 40.5°C or above were put down humanely.

Figure 7 ***P. multocida*-specific IgG titres in sera from vaccinated and control calves (First experiment)**

Titres were assessed by ELISA using *P. multocida* CFE as coating antigen from sera collected 5 days before the first vaccination (day –5), 28 days after the first vaccination and the day of the second vaccination (day 28), 7 days after the second vaccination (day 35), the day of the challenge (day 42), and 1 week post-challenge (day 49). Results are mean values with error bars indicating SEM.


7000
6000
5000
4000
3000
2000
1000
0
IgG titre (ELISA units/ml)
IN
IM
Control
Immunisation group
Day -5
Day 28
Day 35
Day 42
Day 49

3.1.5 Bacteriology

3.1.5.1 Blood samples

Direct culturing of calf blood samples onto SBA at different times after challenge, including immediately prior to euthanasia, provided no evidence of bacteraemia in any of the animals.

3.1.5.2 Post-mortem bacteriology

Bacteriological examination post-mortem (Table 2) found *P. multocida* in the tonsilar tissue of all IN-, IM-vaccinated and two of the control calves (range, 3.6×10^5 to 2×10^7 CFU/g). *P. multocida* was isolated from prescapular lymph node of three of the IN-vaccinated and two of the control animals but only in one of the IM-vaccinated calves. *P. multocida* was found in kidney and lung of one and also in the pleural fluid of one other calf in IN-vaccinated group. *P. multocida* was not found in any of the tested organs of IN- and IM-vaccinated calves. *P. multocida* was also found in prescapular and/or retropharyngeal lymph nodes in one control and three IN-vaccinated calves (range, 1×10^3 to 1.4×10^5 CFU/g) that developed early signs of disease and were killed for humane reasons.

Figure 8 ***P. multocida*-specific IgM titres in sera from vaccinated and control calves (First experiment)**

Titres were assessed by ELISA using *P. multocida* CFE as coating antigen from sera collected 5 days before the first vaccination (day –5), 14 days after the first vaccination (day 14), 28 days after the first vaccination and the day of second vaccination (day 28), 7 days after the second vaccination (day 35), the day of the challenge (day 42), and 7 days post-challenge (day 49). Results are mean values with error bars indicating SEM.

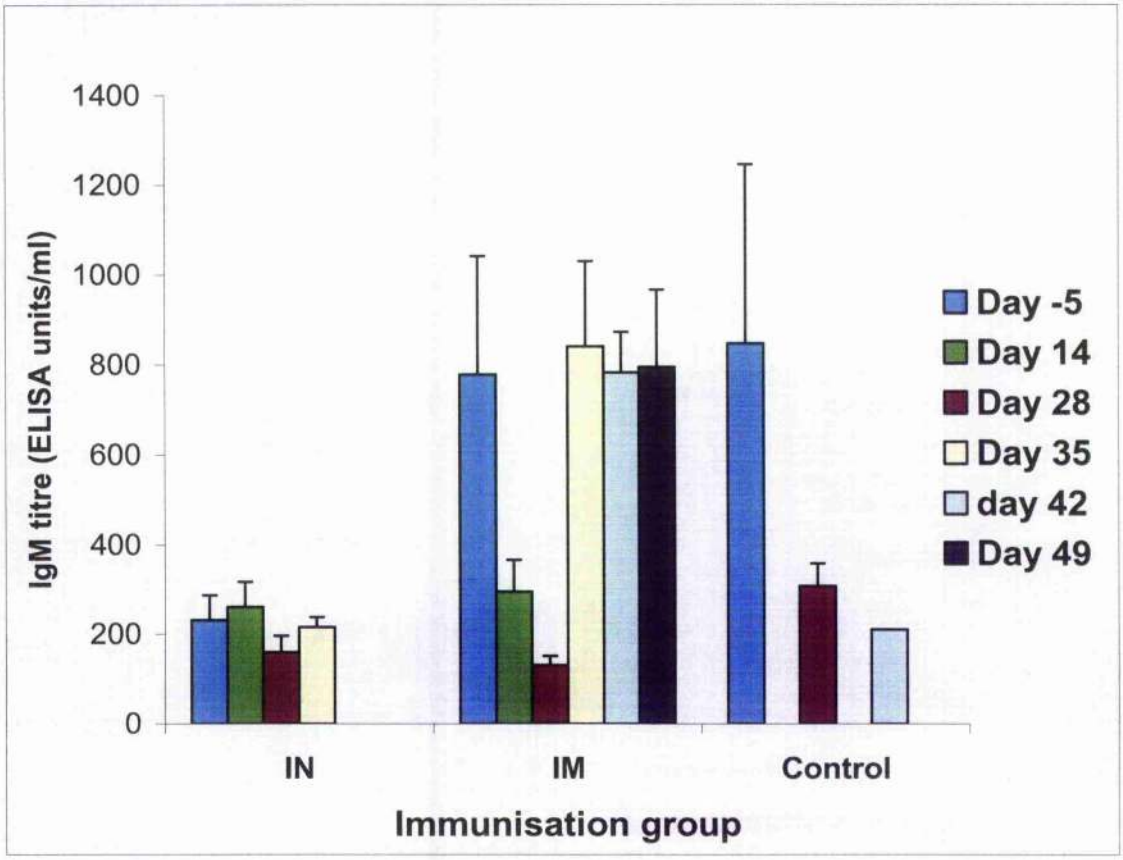

1400
1200
1000
800
600
400
200
0
IgM titre (ELISA units/ml)
IN
IM
Control
Immunisation group
Day -5
Day 14
Day 28
Day 35
day 42
Day 49

Figure 9 Number of survivors after challenge (First experiment)

Number of calves in intramuscularly (IM), intranasally (IN) vaccinated and non-vaccinated (Control) groups at different times after challenge.

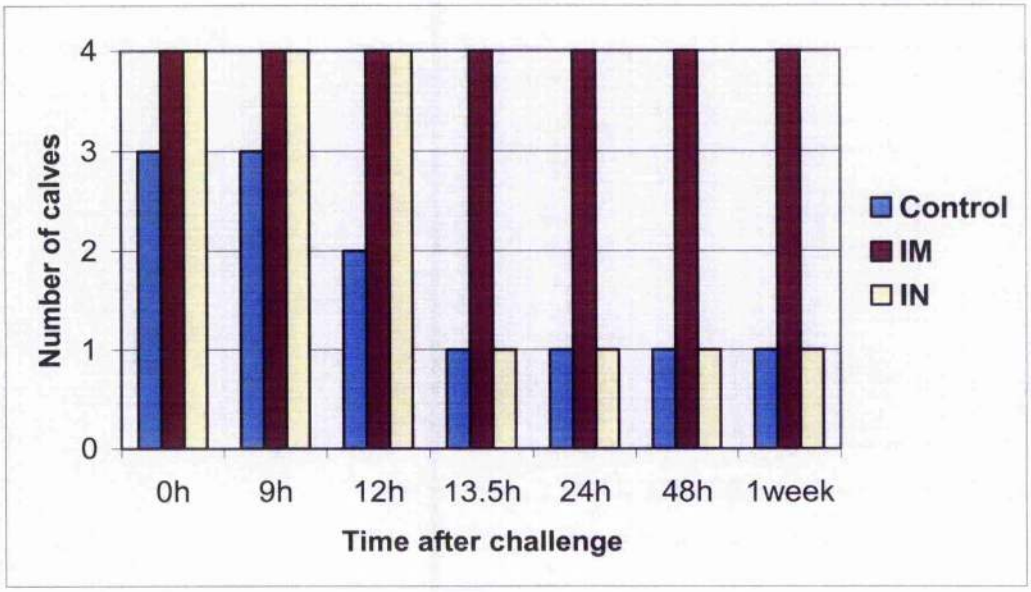


Table 2 Postmortem bacteriology of different organs (First experiment)

Samples of organs and lymph nodes (LN) were homogenised in peptone water (Appendix A4) and cultured on SBA (Appendix A3) containing vancomycin. Swabs from pleural, peritoneal, and pericardial fluids were also cultured. Cultures were incubated overnight and different colonies identified based on the appearance and similarities to *P. multocida* 85020 and JRMT12.

Organ	Immunisation group and animal number										
	Control			IM				IN			
	400778	600780	100414	500772	700034	100035	700781	600766	600033	500779	700767
Brain	-	-	-	-	-	-	-	ND	-	-	-
Spleen	ND	-	ND	-	-	-	-	-	-	-	-
Kidney	ND	-	ND	-	-	-	-	+,*	-	-	-
Lung	ND	-	ND	-	-	-	-	+,*	-	-	-
Liver	ND	-	ND	-	-	-	-	-	-	-	-
Pleural fluid	ND	-	ND	-	-	-	-	-	+,*	-	-
Peritoneal fluid	ND	-	ND	-	-	-	-	+	+	-	-
Pericardial fluid	ND	-	ND	-	-	-	-	ND	+*	-	-
Brain ventricular fluid	ND	-	ND	-	-	-	-	ND	ND	-	-
R. Tonsil	ND	+,*	+,!	+,!	+!	+!	+!	+*	+,*	+,*	+,*
L. Tonsil	ND	+,*		+,*	+!		+!		+,*	ND	+,*
R. Prescapular LN	+	-	+	-	-	-	-	+*	+,!	ND	-
L. Prescapular LN	-	-	+	+,*	-	-	-	+*	+,!	+	-
R. Retropharynge-al LN	-	-	+,*	-	+,*	+,*	-	ND	+,!	-	-
L. Retropharynge-al LN	ND	-	+,*	-	+,*	+,*	-	+,*	+,!	ND	-

+ Bacteria with the appearance of *P. multocida* B:2, * Mucoid bacteria (Possibly other Pasteurellae), ! Other bacteria, ND Not Done

3.2 Response of calves to different doses of JRMT12 after IM vaccination (Second experiment)

In this experiment (Section 2.2.3.2), 4-week-old calves were vaccinated twice intramuscularly with doses of c. 10^9, 10^8, 10^7 CFU of the JRMT12 *aroA* strain, at a 4 week interval. Two weeks after the second vaccination all of the vaccinated and non-vaccinated control calves were challenged subcutaneously with c. 10^7 CFU of *P. multocida* B:2 wild strain 85020 (Section 2.2.3.2).

3.2.1 Clinical responses to vaccination and challenge

3.2.1.1 Mean rectal temperature

At 2 and 5.5 h after the first vaccination, the mean rectal temperatures (± pooled standard errors of the mean [SEM]) of all three vaccinated groups had risen (Figure 10A) and mean temperatures reached the highest level at time 5.5 h in all vaccinated groups. Average temperatures at 5.5 h, for calves vaccinated with 10^9, 10^8 and 10^7 CFU increased from time zero values of 38.76 ± 0.32, 38.72 ± 0.26 and 38.9 ± 0.31 respectively, to highest levels of 40.28±0.18, 40.16±0.34 and 39.72±0.18, respectively. These differences in rectal temperatures for calves vaccinated with 10^9, 10^8 and 10^7 CFU were 1.52, 1.44 and 0.82 °C respectively at 5.5 h after first vaccination, which was significant ($p = 0.002$). The patterns of increase in rectal temperature over time were very similar in calves vaccinated with 10^9 and 10^8 CFU, with a peak at 5.5 h and returning to baseline by 18 h after vaccination. This was despite a single treatment invention with NSAID (Section 2.2.4) for each calf in the 10^9 CFU vaccinated group at either 2 h (n = 1) or 5.5 h (n = 4) after vaccination. However, the mean rectal temperature in calves vaccinated with 10^7 CFU plateaued after 2 h and remained above the baseline up to 24 h after vaccination.

By 7.5 h after the second vaccination, the mean rectal temperatures (± pooled SEM) of all three groups of vaccinated animals had again shown an average rise (Figure 10B). At 7.5 h after the second vaccination dose using 10^9, 10^8 and 10^7 CFU, the mean temperatures reached the highest levels of 40.64 ± 0.13, 40.44 ± 0.19 and 39.76 ± 0.26 respectively, compared to the baseline values of 38.74 ± 0.09, 38.58 ± 0.13 and 39.08 ± 0.2 respectively. Average temperatures at 7.5 h for calves vaccinated with 10^9, 10^8 and 10^7 CFU had therefore increased by 1.9, 1.86 and 0.68 °C respectively. The increase in mean rectal temperature values of 10^7 CFU group had a lower peak and was significantly different

from other groups (p = 0.001). By 24 h after the second vaccination, the mean rectal temperatures of all vaccinated calves had returned to baseline values.

The mean temperatures in all vaccinated and control groups increased between 4.5 and 11.5 h after challenge and reached the highest level at 11.5 h but had returned to baseline values by 28 h (Figure 10C), with the 10^9 CFU vaccinated group returning significantly more quickly. Average temperatures at 11.5 h, for calves vaccinated with 10^9, 10^8 and 10^7 CFU increased 0.66, 0.92 and 1.22 °C, respectively and for the control group it was 1.23 °C. The pattern of responses between the three vaccinated groups were significantly different ($p < 0.001$), with the 10^9 CFU vaccinated group showing only a slight increase in mean temperature after challenge. In the control group, two of the three calves required treatment with NSAID (one at 11.5 h and the other at 23 h after challenge) due to severe dullness and pyrexia. As this treatment resulted in variable results, the data of this group was not statistically analysed.

Figure 10 Changes in rectal temperatures after different vaccine doses given intramuscularly and after challenge (Second experiment)

Rectal temperatures of three groups of calves vaccinated intramuscularly with three different doses of vaccine (10^9, 10^8, and 10^7 CFU), were measured at different intervals after the first vaccination (12A), second vaccination (12B), and challenge with the wild type (12C). Results are the mean rectal temperatures of animals in each group plus a non-vaccinated control group (12C) with SEM as error bars.

Figure 10A Rectal temperature changes after first vaccination

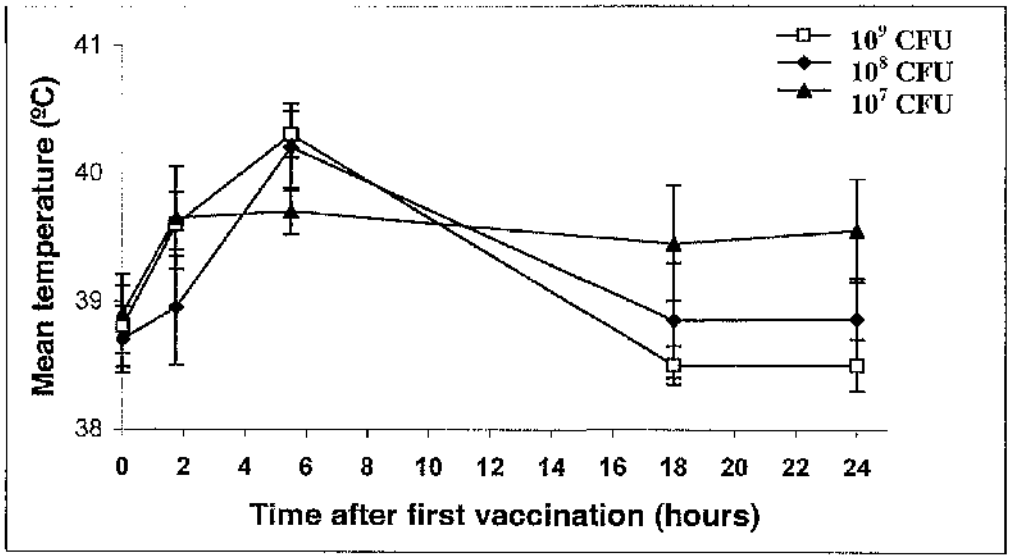


Figure 10B Rectal temperature changes after second vaccination

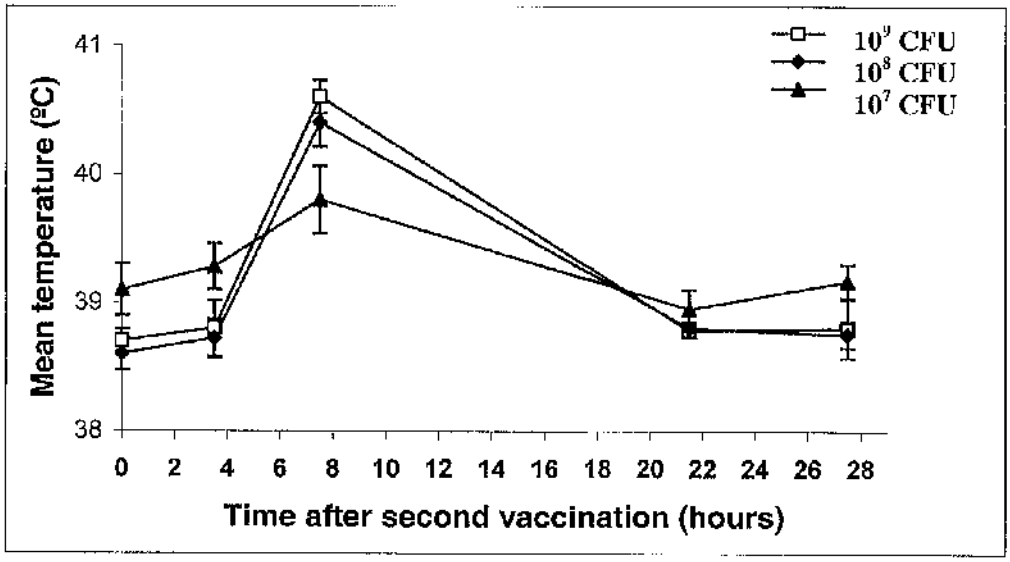
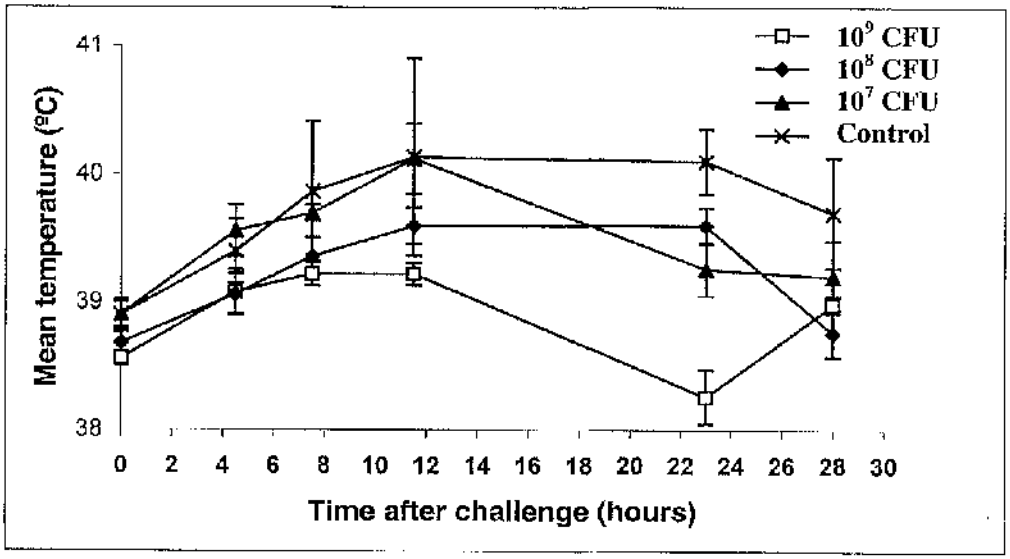


Figure 10C Rectal temperature changes after challenge



3.2.2. Changes in Serum Amyloid A (SAA)

The SAA concentrations after the first vaccination were measured in serum from calves vaccinated with 10^8 CFU as a representative of all vaccinated animals to study the SAA response to vaccination. Concentrations of SAA varied widely between individual calves. The mean SAA level had increased from 29.97 (11.8 – 85.5) μg/ml to 88.3 (61.5 – 122.75) μg/ml by 24 h after vaccination and remained at this level in four calves but the level rose exceptionally high in one of the calves where it increased to a 262.5 μg/ml. The mean SAA concentration at 36 h was high because of this calf. The increase in the mean SAA level after vaccination with 10^8 CFU was remarkably lower than the SAA response to vaccination with 10^9 CFU that was obtained in first experiment which had reached a mean value of 392.5 ± 110.09 μg/ml by 24 h. Vaccination with 10^9 CFU dose caused an 8-fold increase in mean SAA levels with the highest measured level of 715 μg/ml while vaccination with 10^8 CFU dose caused only a 3-fold increase with the highest measured level of 122.75 μg/ml.

The concentrations of SAA varied in individual calves. The mean values before challenge were low in all of the vaccinated and control animals (Figure 11A). After challenge on day 42, all vaccinated groups showed increasing mean concentrations of SAA until 23 h post-challenge. The mean SAA levels appeared to be dropping in all vaccinated groups by 72 h post-challenge In the control group, the mean SAA concentration increased over the whole time period. There was marked variability in SAA levels in different animals The SAA concentrations increased slightly by 11.5 h after challenge in the control group but had increased 26-fold overall by 48 h after challenge, (Figure 11A), whereas only a 3-fold increase was seen in all of the vaccinated groups at this time. Over the period until 72 h post-challenge, mean concentrations of SAA were significantly ($p < 0.001$) higher in the control group than in the vaccinated groups. There was only one calf alive in control group, 72 h after challenge with SAA of 716 μg/ml (data are not plotted in Figure 11A).

3.2.3. Changes in haptoglobin (Hp) concentrations

The serum concentration of haptoglobin (Hp) was also measured. Hp changes after challenge in vaccinated and control animals showed a similar pattern to those observed for SAA (Figure 11B). The mean values before challenge were undetectable in all of the vaccinated and control animals until 7.5 h post-challenge, after which the Hp concentrations increased and, in all but one calf in 10^7 CFU group, reached a maximum value at 48 h post-challenge. At 48 h post-challenge the mean concentration of Hp was

highest in control animals (607±474 μg/ml), followed by the group vaccinated with 10^7 CFU (210±149 μg/ml). Once an animal showed a positive reading it generally had positive readings for the rest of the experiment period. Vaccinated groups (10^9, 10^8, and 10^7CFU) had low median maximum Hp concentrations over the whole period of 10, 10 and 220 μg/ml, respectively, whereas the control group had a median maximum of 860 μg/ml. There was only one calf alive in control group, 72 h after challenge with Hp of 1510 μg/ml (data are not plotted in Figure 11B).. These observed differences were not statistically significant using a Kruska-Wallis test. Larger samples would be needed to determine if the observed lower levels in vaccinated groups are a consequence of vaccination.

Figure 11 Changes in SAA and haptoglobin after challenge (Second experiment)

Calves were vaccinated twice intramuscularly using three different doses of vaccine (10^9, 10^8, and 10^7 CFU). Two weeks after the second vaccination, the vaccinated and control animals were challenged with the wild-type strain. SAA (A) and haptoglobin (B) levels were measured in sera collected on the day of challenge (0 h) and at intervals thereafter. Results represent the mean value of concentrations in each group with SEM as error bars.

Figure 11A Changes in SAA levels after challenge in vaccinated and control calves

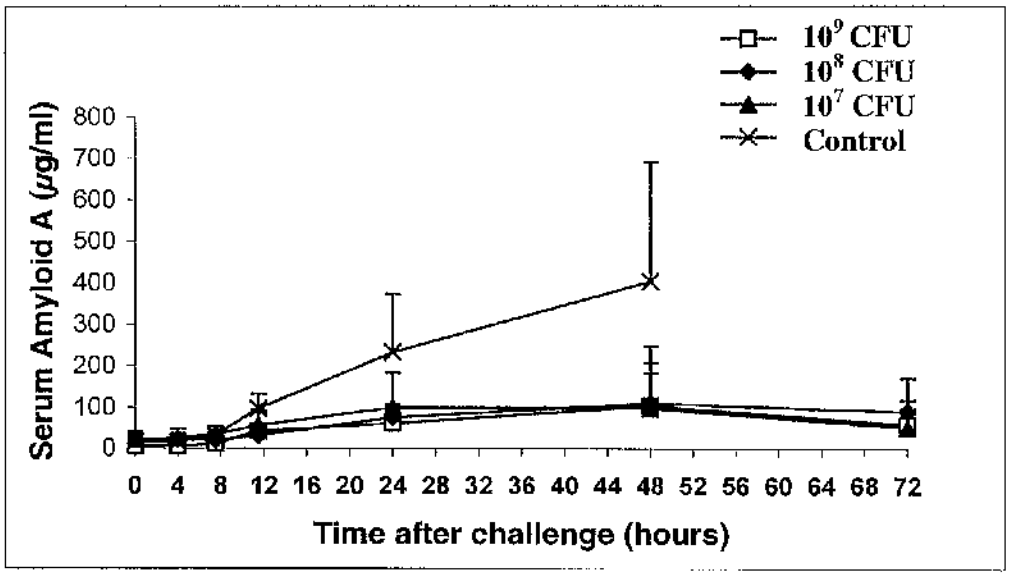
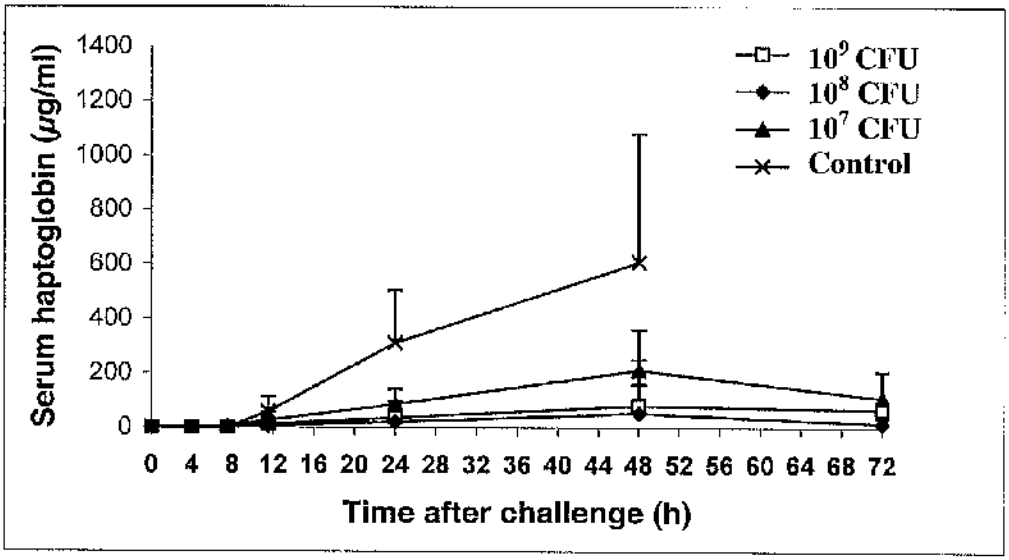


Figure 11B Changes in serum haptoglobin after challenge in vaccinated and control calves



3.2.4 Antibody responses to vaccination and challenge

IgG and IgM levels were measured by ELISA, with CFE as antigen, in sera taken at different stages before and after vaccination and challenge.

3.2.4.1 Serum IgG levels

ELISA data for IgG showed a similar pattern to the IgG titres from first experiment (Section 3.1.3.1). IgG titres showed little change at 28 days after the first vaccination (Figure 12), but increased rapidly 7 days after the second vaccination in calves given 10^9 and 10^8 CFU. For calves given 10^7 CFU, only a slight increase in IgG titres was detected up to the day of challenge and mean IgG titres continued to increase slowly from second vaccination (Day 28) to 3 days after challenge. There was a clear relationship between dose of vaccine and IgG levels, the higher the dose of vaccination, the higher the IgG titre. In the control group, the IgG titres remained fairly static during the experiment and 7 days after second vaccination this was significantly different from 10^9 ($p = 0.018$), 10^8 ($p = 0.001$), and 10^7 ($p = 0.03$) CFU vaccinated groups.

3.2.4.2 Serum IgM levels

ELISA data for IgM showed a similar pattern to the IgM titres from first experiment (Section 3.1.3.2). In pre-vaccinated calves, IgM titres were again high but, by 14 and 28 days after the first vaccination, the IgM titres had decreased and continued at a low level until the second vaccination (Day 28) (Figure 13). In all three vaccination groups, the IgM titres had increased by 7 days after the second vaccination (Day 35) but remained low in control calves and at this point the antibody levels in 10^9 CFU group was significantly ($p = 0.002$) higher than the control group. The graded response seen for IgG titres (Figure 12) was not apparent for the IgM titres and all vaccinated groups achieved similar levels of IgM by the day of challenge. The mean IgM titre in the control group remained fairly constant over the course of the experiment.

Figure 12 IgG titres in sera from vaccinated and control calves (Second experiment)

Titres were assessed by ELISA using *P. multocida* CFE as coating antigen from sera collected before the first vaccination (Day 0), 14 days after the first vaccination (Day 14), 28 days after the first vaccination (Day 28), 7 days after the second vaccination (Day 35), the day of the challenge (Day 42), 2 days post-challenge (Day 44) and 3 days post-challenge (day 45). Results are mean values with error bars indicating SEM.

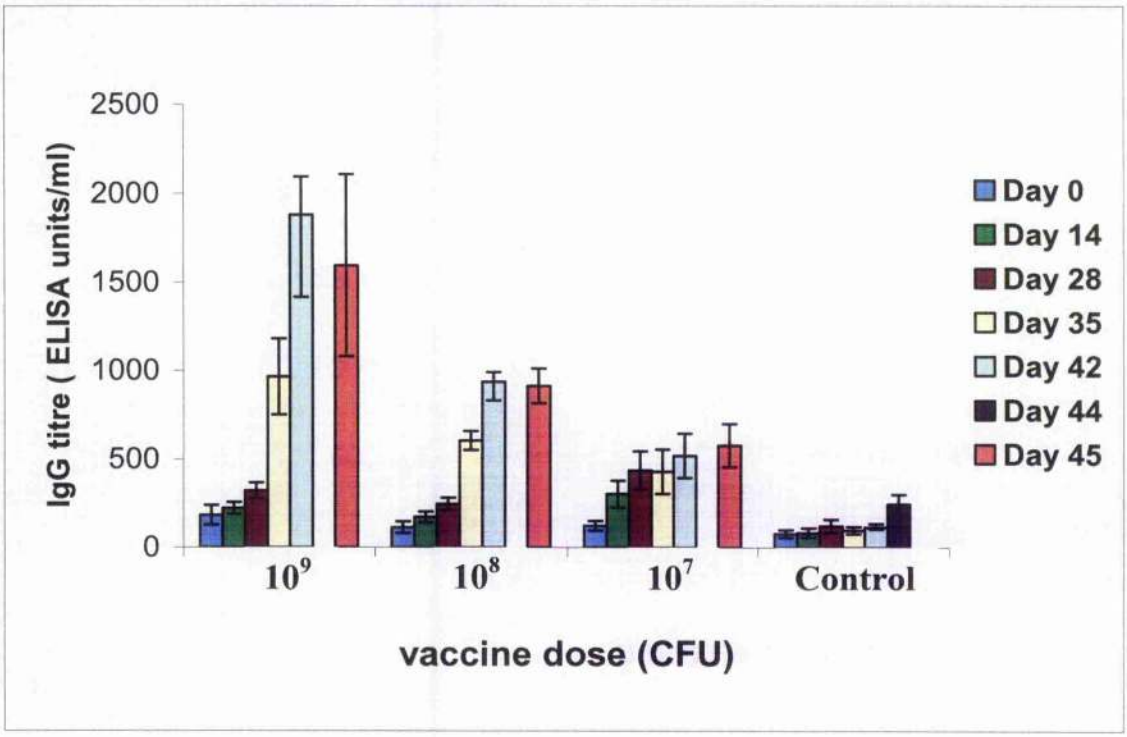
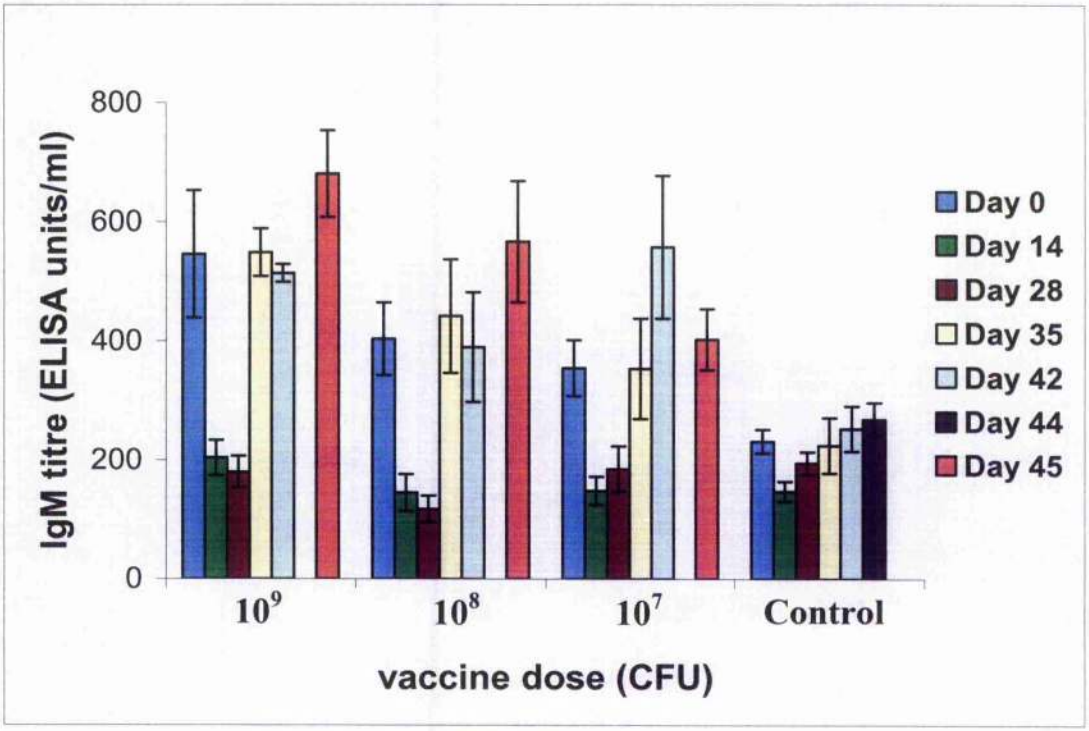

2500
2000
1500
1000
500
0
IgG titre (ELISA units/ml)
Day 0
Day 14
Day 28
Day 35
Day 42
Day 44
Day 45
10^9
10^8
10^7
Control
vaccine dose (CFU)

Figure 13 IgM titres in sera from vaccinated and control calves (Second experiment)

Titres were assessed by ELISA using *P. multocida* CFE as coating antigen from sera collected before the first vaccination (Day 0), 14 days after the first vaccination (Day 14), 28 days after the first vaccination (Day 28), 7 days after the second vaccination (Day 35), the day of the challenge (day 42), 2 days post-challenge (Day 44) and 3 days post-challenge (Day 45). Results are mean values with error bars indicating SEM.


800
600
400
200
0
IgM titre (ELISA units/ml)
Day 0
Day 14
Day 28
Day 35
Day 42
Day 44
Day 45
10^9
10^8
10^7
Control
vaccine dose (CFU)

3.2.5 Survivors after challenge

All of the vaccinated calves survived the challenge. All of the non-vaccinated animals in control group became clinically ill (as shown by dullness and high temperature) between 7.5-11 h after challenge. Two of them were in critical condition and unresponsive to treatment with NSAID and were eventually put down at 48 and 55 h after challenge. Three calves in 10^7 CFU vaccinated group, and one in the 10^9 CFU vaccinated group, were dull but none of them required administration of NSAID and all recovered prior to scheduled euthanasia on the 7th day after challenge.

3.2.6 Serum sensitivity assay

This assay was performed as described in section 2.3.8. Sera with high IgG and IgM titres obtained after the second vaccination on day 42 from IM-vaccinated calves were selected for use in this assay. *P. multocida* wild type was incubated in these sera in the presence of fresh normal calf serum, as a source of complement, to study their bactericidal effects on the organism. A control included the assay with the same conditions in which PBS was used instead of serum. A typical result obtained with one such serum is shown in Figure 14. None of the immune sera obtained from vaccinated calves or fresh calf serum obtained from naïve calves, showed any adverse effects on *P. multocida,* which grew in these sera as shown by a bacteriocidal index > 1, rather than indices < 1 which would represented a killing effect (Figure 14). This is in contrast to results obtained with *E. coli* strain K12 which was not killed in fetal calf serum but was rapidly killed in fresh calf serum and guinea pig serum as complement sources (Figure 15). Heating (56°C for 30 min, for inactivation of complement) fresh calf serum or guinea pig serum had no adverse effects on *E. coli.*

Figure 14 Bactericidal activity of bovine serum on *P. multocida*

Each well of a 96-well microtitre plate (Section 2.3.8) contained 25µl of immune serum from a calf vaccinated twice intramuscularly with high level of IgG and IgM antibody (Immune.Ser), with or without 25µl of fresh normal calf serum (NC.Ser) or 25µl heated Immune Ser. with or without 25µl heated NC.Ser. or 25µl fresh normal calf serum (NC.Ser.) or 50µl heated NC.Ser. or 50µl PBS. A 50µl suspension of *P. multocida* B:2 was added to each well and then incubated for 1 h at 37°C. Sample of 10 µl from undiluted, and dilutions of 10^{-1} and 10^{-2} were taken before and after incubation and were plated out on SBA (Appendix A3) and then incubated overnight.

The bactericidal index is defined as CFU/ml after incubation for 1 h divided by CFU/ml at time zero. Data are the average of two assays ± standard deviation. An index of < 1 would indicate a bactericidal effect.

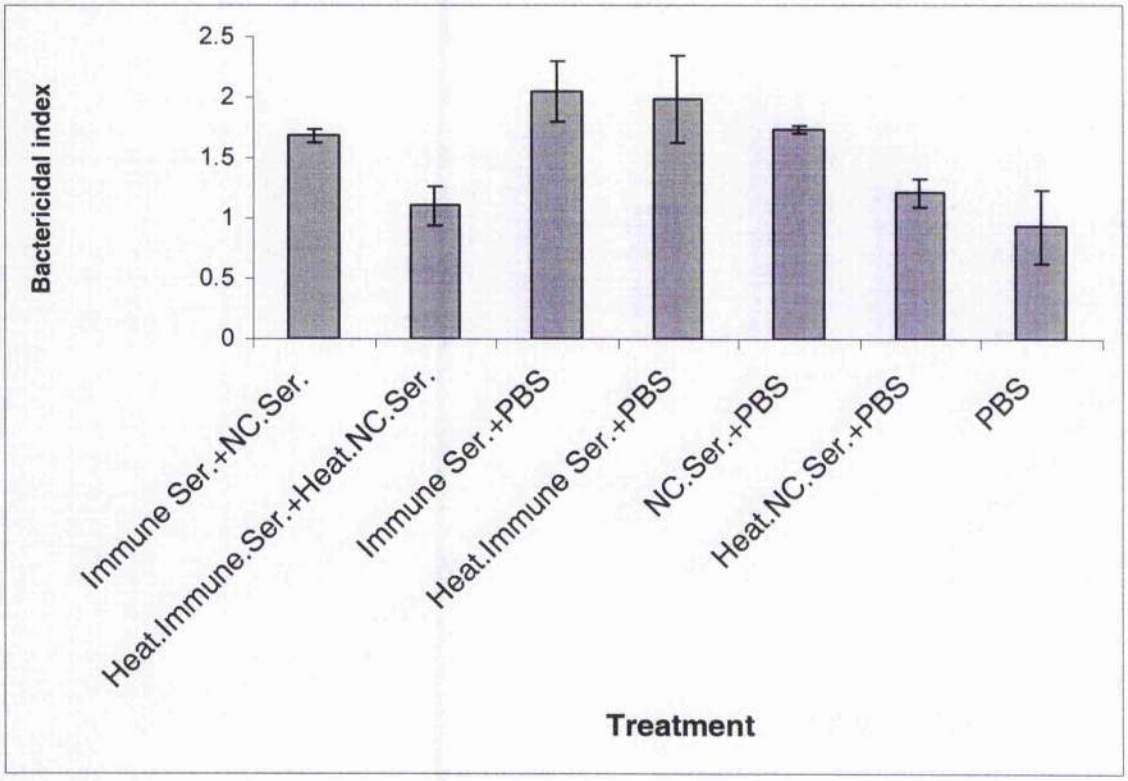
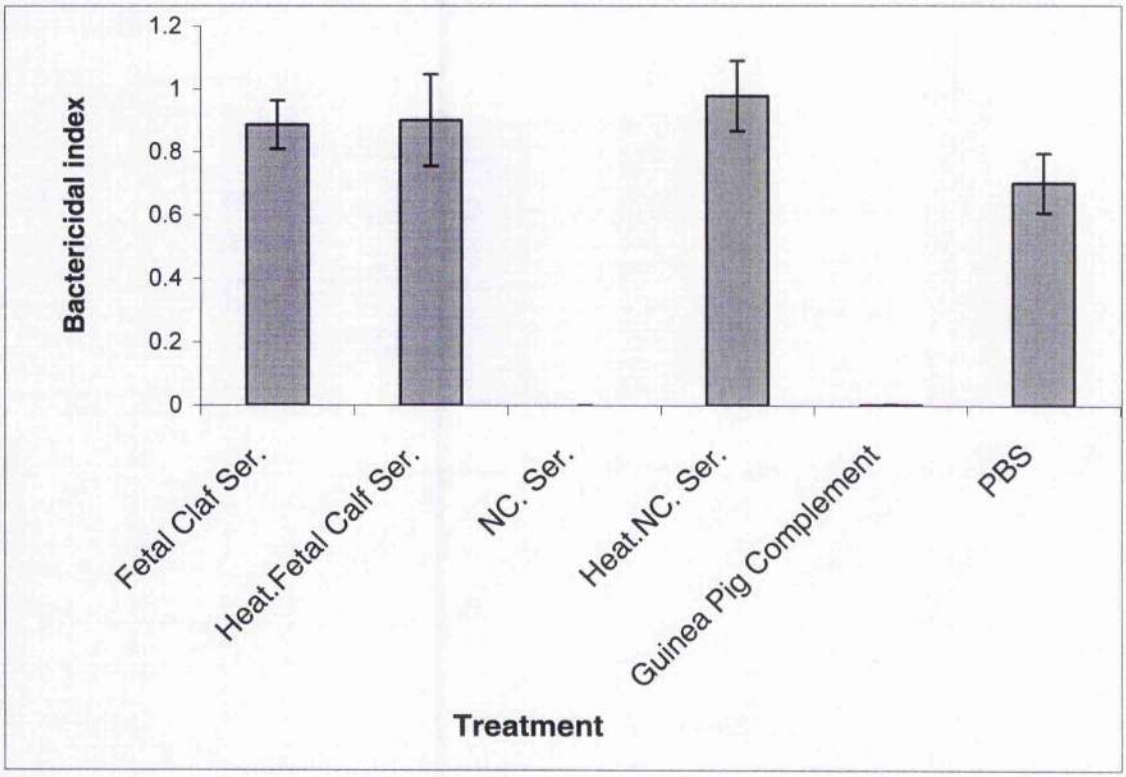

Bactericidal index
2.5
2
1.5
1
0.5
0
Immune Ser.+NC.Ser.
Heat.Immune.Ser.+Heat.NC.Ser.
Immune Ser.+PBS
Heat.Immune Ser.+PBS
NC.Ser.+PBS
Heat.NC.Ser.+PBS
PBS
Treatment

Figure 15 Bactericidal activity of calf serum and guinea pig complement on *E. coli*

Each well of a 96-well microplate (Section 2.3.8) contained 50µl of fetal calf serum (Fetal Calf Ser. Sigma®) heated fetal calf serum (Heat.Fetal Calf Ser.) fresh normal calf serum (N.C.Ser.) or heated calf serum or guinea pig complement (Sigma®) (Guinea Pig Complement) or PBS. A 50µl of a suspension of *E. coli* was added to each well and then incubated for 1 h at 37°C. Sample of 10 µl from undiluted, and dilutions of 10^{-1} and 10^{-2} were taken before (time zero) and after incubation and were plated out on SBA (Appendix A3) and then incubated overnight.

The bactericidal index is defined as CFU/ml after incubation for 1 h divided by CFU/ml at time zero. Data are the average of two assays ± standard deviation. An index of < 1 would indicate a bactericidal effect.


Bactericidal index
1.2
1
0.8
0.6
0.4
0.2
0
Fetal Claf Ser.
Heat.Fetal Calf Ser.
NC. Ser.
Heat.NC. Ser.
Guinea Pig Complement
PBS
Treatment

3.2.7 Investigation of immunogenic components

In this part of the experiment different preparations of *P. multocida* 85020 were prepared and run on SDS-PAGE. Cell components that were separated by SDS-PAGE were then subjected to immunoblotting (Section 2.3.10) with sera obtained from immunised calves. Different components separated by SDS-PAGE and detected by immunoblots were identified by estimation of their molecular weights by comparing with standard MW markers run in parallel with the samples. Some of the immunogenic components were then subjected to mass spectrometry (MS) for identification.

3.2.7.1 SDS-PAGE of *P. multocida* proteins

The SDS-PAGE profiles of a crude CFE (Section 2.3.1) and the sarkosyl-extracted OMP preparation (Section 2.3.3) of *P. multocida* strain 85020 were analysed (Figure 16 and Figure 17). In SDS-PAGE of the CFE, up to 32 bands were observed. On the basis of stain intensity, two bands at 30 and 37 kDa were identified as prominent bands (Figure 16). SDS-PAGE of the OMP preparation revealed up to ten major and 4 minor bands Figure 17) and again the 30 and 37 kDa bands were the predominant bands.

The OMP was also extracted from *P. multocida* strain 85020 grown under iron-depleted conditions by adding 2,2'-dipyridyl 150 μM/ml to BHI (Appendix A.7). Based on intensity of bands on SDS-PAGE gels, 4 bands with molecular weights of 97, 95, 82 and 24 kDa were upregulated and a protein of 26 kDa was downregulated (Figure 18).

3.2.7.2 Immunoblots

To investigate the immunogenic components of *P. multocida,* to which IgG antibody was raised, a CFE of *P. multocida* was blotted against calf sera collected at different stages of vaccination and challenge. The Western blots revealed five major immunogenic bands (51, 37, 30, 26 and 16 kDa) which only appeared using serum one week after the second intramuscular vaccination and which were still present in serum from this calf after challenge that has been shown as a representative of calves. Sera obtained from different individual calves showed essentially the same pattern of blots (Figure 19). A 44 kDa band appeared after first vaccination and remained in all other sera. This band was not taken for identification because however, further studies using a single IM vaccination regime (Section 2.2.3.3) showed the appearance of this band after primary vaccination was not

associated with protection (Section 3.3.1). Sera obtained from different individual calves showed essentially the same pattern of blots.

For clearer separation and subsequent identification of immunogenic bands, immunobloting was also carried out on an OMP preparation. The immunoblot of the OMP preparation against calf immune serum (serum obtained one week after second intramuscular vaccination) revealed two strong bands of 50 and 37 kDa (Figure 20A). The 37 kDa band was the second dominant band in the Coomassie blue stained (Figure 20B) OMP preparation, but appeared as the strongest band in corresponding immunoblot. The 50 kDa band was a faint band by Coomassie blue staining, but showed also a fairly strong band in the immunoblot. The most dominant band in Coomassie blue stained OMP preparation of 30 kDa showed little or no reaction in the corresponding blot. However, the 30 kDa band was visible in Figure 19, the immunoblot of the CFE. This band appeared as a result of vaccination, along with a 16, 26 and 44 kDa bands, but none of these three bands that showed a strong reaction in immunoblot against sera obtained after second vaccination was prominent in the OMP preparation. It is possible that the 50 kDa band identified in OMP immunoblot (Figure 20) may be the same as 51 kDa band identified in CFE immunoblot (Figure 19).

No additional band was observed in immunoblotts of OMP prepared from *P. multocida* grown under iron-depleted conditions (data not shown).

3.2.7.4 Protein identification using mass spectrometry

Two of the strong immunogenic bands (37 and 50 kDa) and the most dominant band (30 kDa) from SDS-PAGE of the OMP preparation were chosen for protein identification. Bands were excised from a SDS-PAGE gel and sent for mass spectrometry (Section 2.3.11). Appendices G1, G2, and G3 show the MASCOT output for the studied bands. The 30 kDa band from SDS-PAGE gel was identified as major outer membrane protein precursor; OmpH of *Pasteurella multocida* (Appendix G.1.2.1). This protein identification has a score of 569, a score of > 63 is significant. The 37 kDa band was identified as an unknown Omp or OmpA of *Pasteurella multocida* with a scores of 759 and 750 respectively (Appendix G.2.2. and G.2.2.2). The 50 kDa band was identified most likely as OmpH *Pasteurella multocida* with a score of 218 (Appendix G.3.2.1).

Figure 16 SDS-PAGE of CFE

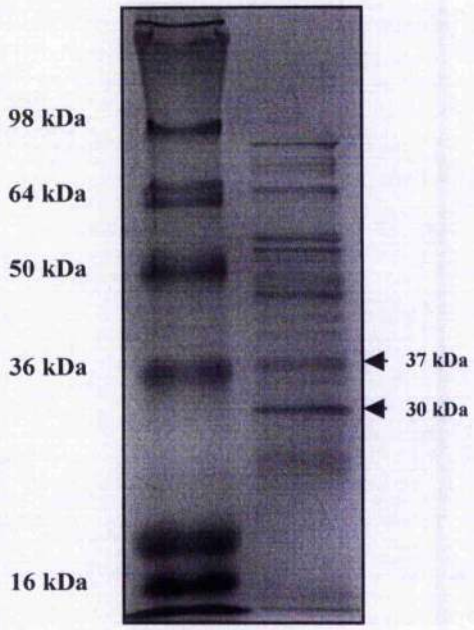


Figure 17 SDS-PAGE of OMP preparation

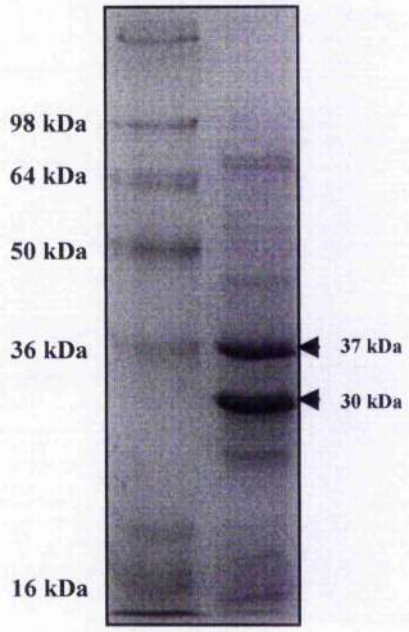
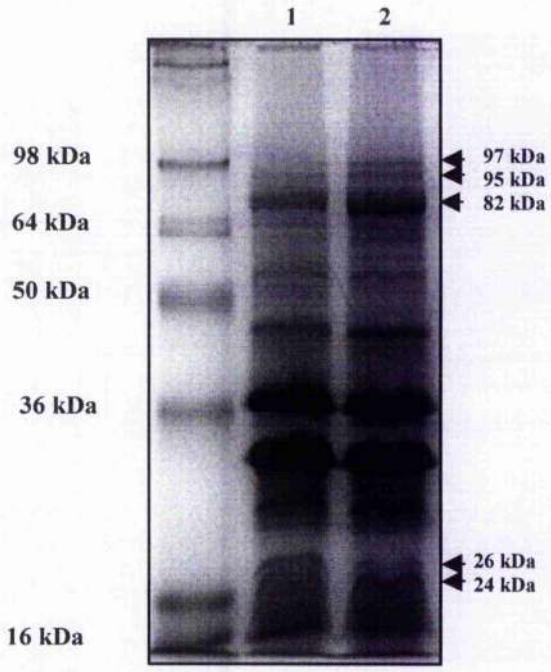
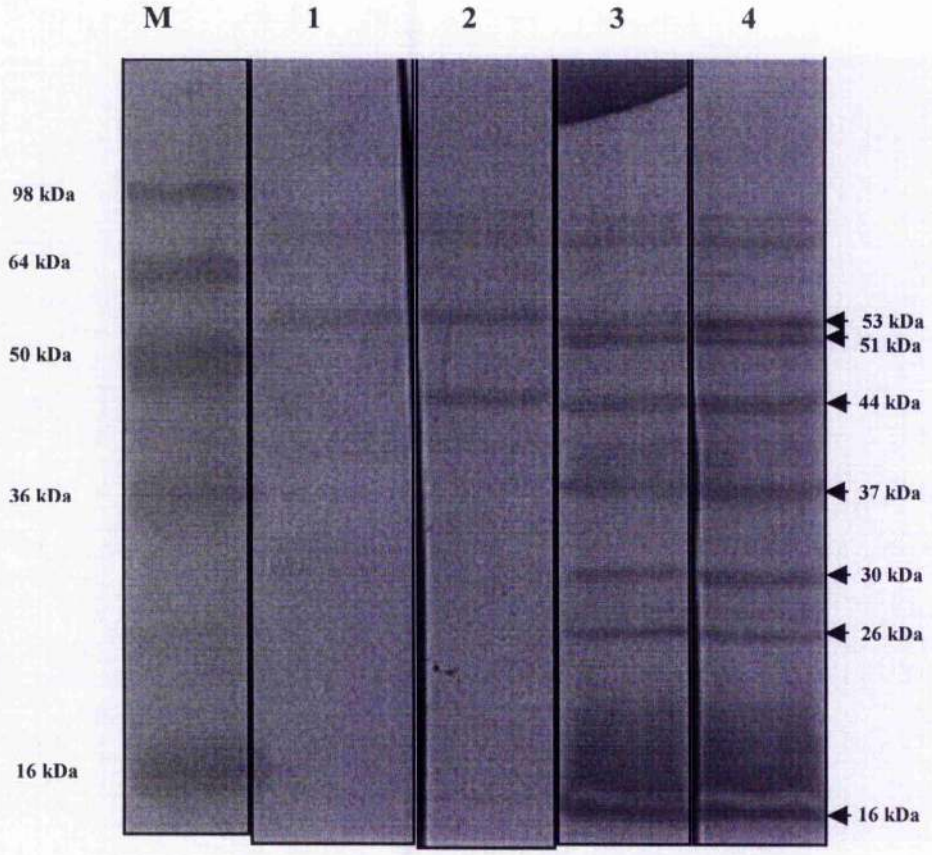


Figure 18 SDS-PAGE of OMP prepared under normal and iron-restricted conditions



Coomassie Blue stained SDS-PAGE (10% slab gel) profiles of CFE (Fig. 16) and OMP preparation (Fig. 17) from *P. multocida* 85020 (right lanes). Left lanes on both gels show the molecular weight standard markers. Samples of 20 µg protein content were used for loading lanes. Figure 18 shows OMP prepared from cells obtained in BHI (lane 1) and BHI+ Dipyridyl (lane 2). Samples of 40µg protein content were used for OMP prepared under iron-limited conditions.

Figure 19 **Immunoblot of CFE of *P. multocida* 85020 against calf sera collected at different stages of vaccination and challenge.**

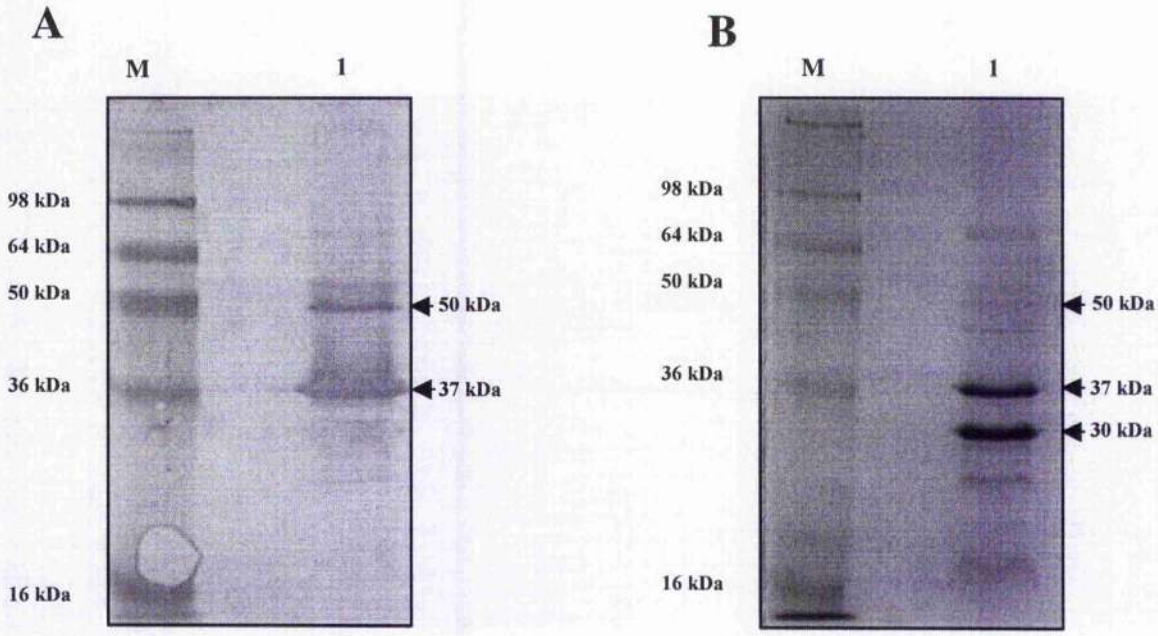


Immunoblots using sera from one of the vaccinated calves taken at intervals after vaccination and challenge (Section 2.2.3.1), as a representative of the IM-vaccinated and challenged animals.

M; Marker, 1; Before vaccination, 2; Four weeks after first vaccination, 3; One week after second vaccination, 4; One week after challenge.

Figure 20 Immunoblot of OMP preparation of *P. multocida* 85020 against serum from a calf one week after second intramuscular vaccination.

SDS-PAGE of OMP preparation (B) and corresponding immunoblot (A) using serum from the calf obtained one week after second IM vaccination (serum number 3 used in Figure 19).



A; Immunoblot of OMP preparation against calf immune serum. B; SDS-PAGE of OMP preparation. M; Molecular weight standards, 1; OMP preparation

3.3 Responses of calves to a single dose of JRMT12 after IM vaccination (Third experiment)

In this experiment, 4-week-old calves were vaccinated once IM using 10^8 CFU JRMT12 strain. Four weeks after vaccination, all of the vaccinated and non-vaccinated control calves were challenged subcutaneously with 10^7 CFU *P. multocida* B:2 wild strain 85020 (Section 2.2.3.3).

3.3.1 Survivors after challenge

In the vaccinated group, only two of the four vaccinated calves survived the challenge. The calves in vaccinated group were five but one calf died and four calves were used in this group. In the non-vaccinated control group, only two of the five calves survived the challenge. These data indicated that a single vaccination of calves does not confer protection against subcutaneous challenge. Therefore, serum antibody responses and APP responses were not investigated.

3.3.2 Cellular responses after vaccination and challenge

Although no protection was afforded by a single vaccination, advantage was taken of the vaccinated and control calves to examine the possible cellular response and to develop assays for this study. Peripheral blood mononuclear cells (PBMCs) were obtained from vaccinated or control calves at different stages before and after challenge (Section 2.3.13), to study their response to CFEs of *P. multocida* B:2 or *P. multocida* A3 or concanavalin-A (ConA) as a known stimulator of the lymphocyte proliferative response.

3.3.2.1 Stimulation of lymphocytes from calves challenged with *P. multocida* B:2 85020 by CFE of *P. multocida* B:2 85020

CFE of *P. multocida* 85020 did not show any stimulatory effects on proliferation of PBMCs isolated from calves at different stages of vaccination (data are not shown). Little stimulatory effect of CFE was observed on PBMCs obtained from calves before and after challenge (Figure 21A). PBMCs from the vaccinated and non-vaccinated control groups showed similar responses to CFE.

3.3.2.2 Stimulation of lymphocytes by concanavalin A (ConA) from calves challenged by *P. multocida* B:2 85020

PBMCs from vaccinated and non-vaccinated control calves, sampled prior to challenge, proliferated in response to ConA. The mean of stimulation index [SI] was 7.8 ± 3.1 in the control and 10.45 ± 4.52 in the vaccinated group (Figure 21B). In both vaccinated and control groups, the mean SI decreased markedly at 12 h after challenge and reached an even lower level 3 days after challenge. In control animals, 7 days after challenge the mean SI had increased to around the pre-challenge value (mean SI=8.1 ± 3.25) but, in vaccinated animals, it had increased only slightly (mean SI=2.05 ± 0.92).

3.3.3 Stimulation of lymphocytes from calves challenged with *P. multocida* A3

A comparison was made between the lymphocyte response to *P. multocida* A3 challenge (pulmonary pasteurellosis) and *P. multocida* B:2 challenge (septicaemic pasteurellosis). The lymphocyte stimulation assay was used to assess the response of PBMCs obtained from calves at different times after intratracheal challenge with *P. multocida* A3 (Section 2.1.4). On the second day after intratracheal challenge with *P. multocida* A3 (experiment performed by Dr. Hodgson at Moredun Research Institute), PBMCs responded to stimulation with the CFE of *P. multocida* A3 (mean SI=10.45 ± 2.79) while PBMCs obtained from control calves that were injected intratracheally with PBS showed no response to stimulation (mean SI=0.18 ± 0.02) (Figure 22A). As indicated by this experiment, PBMCs of challenged calves were able to proliferate by stimulation with CFE, in contrast to the unresponsiveness of PBMCs to CFE of *P. multocida* B:2 after challenge with *P. multocida* serotype B:2 (Section 3.3.2.1).

To investigate any cross reaction, the stimulatory effects of the CFE of *P. multocida* B:2 was also assessed on PBMCs obtained from *P. multocida* A3 challenged calves. The same pattern of response was observed, on the second day of challenge. PBMCs responded to stimulation with sonicated extract of *P. multocida* B:2 (Figure 22B). The similarity in pattern of response, in both of the stimulation assays using different CFEs of A3 and B:2 serotypes of *P. multocida*, indicated a cross reaction between the extracts these serotypes in stimulating the PBMCs. However, the fact that PBMCs from calves challenged with *P. multocida* B:2 did not respond to extract of *P. multocida* B:2 suggested that challenge with this strain was exerting a suppressive effect on the capacity of the lymphocytes to proliferate.

Figure 21 PBMCs response to CFE and ConA before and after challenge with *P. multocida* 85020 (Third experiment)

PBMCs were prepared from vaccinated and non-vaccinated groups of calves on the day of challenge and at different times after challenge with *P. multocida* 85020. In vaccinated group, calves were vaccinated once (third experiment) and all of the animals were challenged after 28 days. For the stimulation assay (21A), 10 µg/well of CFE was added to the cells. For the proliferation assay (21B), 1 µg/well of concanavalin A (ConA) was added to the cells. All of the treated cells along with untreated cells as controls were incubated at 37 °C (Section 2.3.15). On the 3rd day, cells were pulsed with [methyl-^{3}H] thymidine and incubated for 18 h and the amount of incorporated thymidine was measured by scintillation. Proliferation of cells was detected by the level of thymidine incorporation and is shown as bars, which represent the stimulation index (SI). SI is the ratio of treated (CFE or ConA added) compared to untreated cells. Results are the mean of the stimulation indeces for different calves in each vaccinated and non-vaccinated group, error bars showing SEM or SDM (for 3 and 7 days post-challenge data as only 2 calves in each group survived).

Figure 21A **Stimulation of lymphocytes from calves challenged with *P. multocida* B:2 85020 by CFE of the same strain**

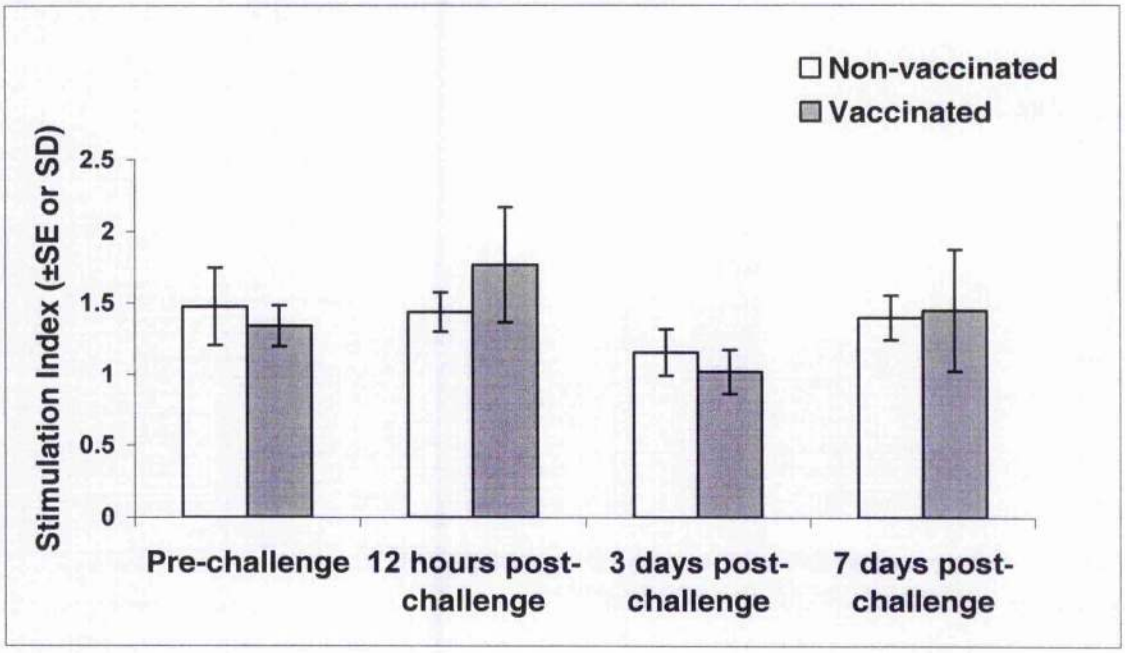


Figure 21B **Proliferative response of lymphocytes to ConA after challenge with *P. multocida* B:2**

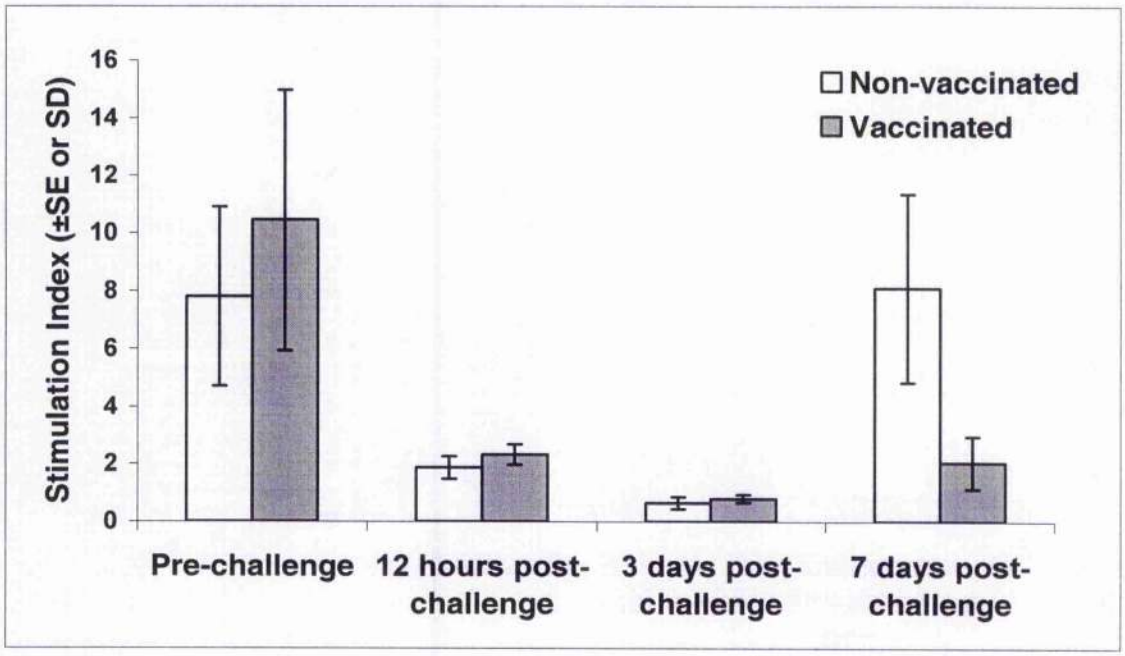


Figure 22 PBMCs response to CFE before and after challenge with *P. multocida* A3

PBMCs were prepared from calves before and at different times after challenge. In the challenged group, *P. multocida* serotype A3 was inoculated intratracheally and the control group received PBS by the same route. For stimulation assay, a CFE of *P. multocida* serotype A3 (Figure 22A) was added to the cells and the proliferation was assessed by thymidine incorporation (see legend to Figure 21). Stimulation assay was also carried out with CFE of *P. multocida* 85020 (Figure 22B).

Bars indicate the proliferative response of PBMCs after stimulation with CFE, measured by thymidine incorporation and shown as stimulation index (SI), which shows the ratio of radiolabel incorporation in treated (CFE was added) compared to untreated cells. Results are the mean values for two calves in each group and error bars indicate the standard deviation.

Figure 22A **Stimulation of lymphocytes from calves challenged with *P. multocida* A3 by *P. multocida* A3 CFE**

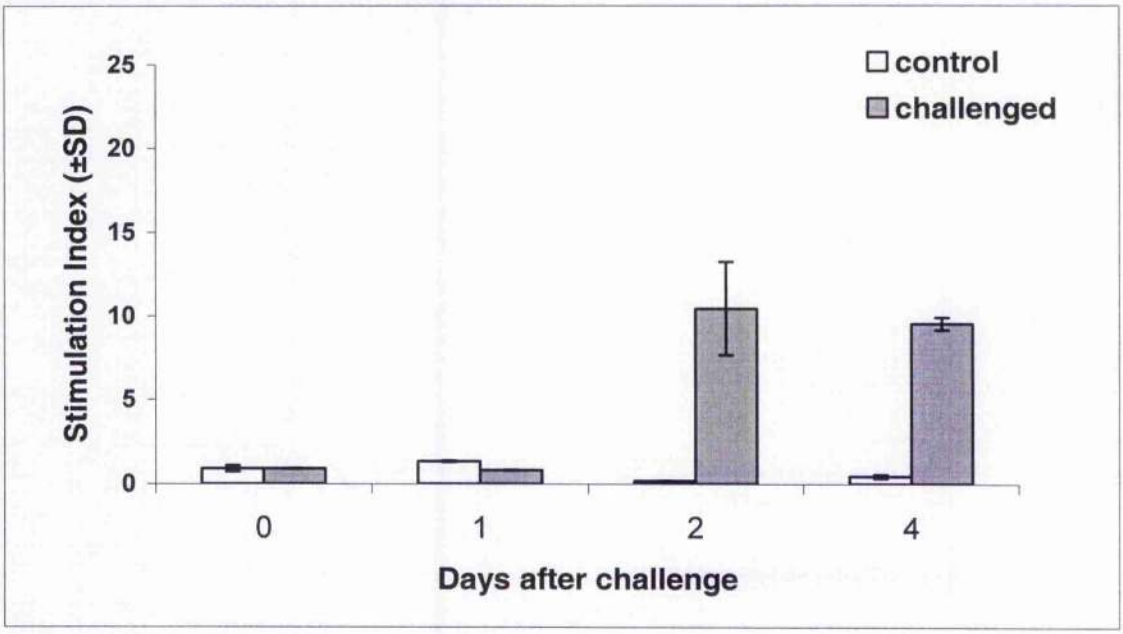
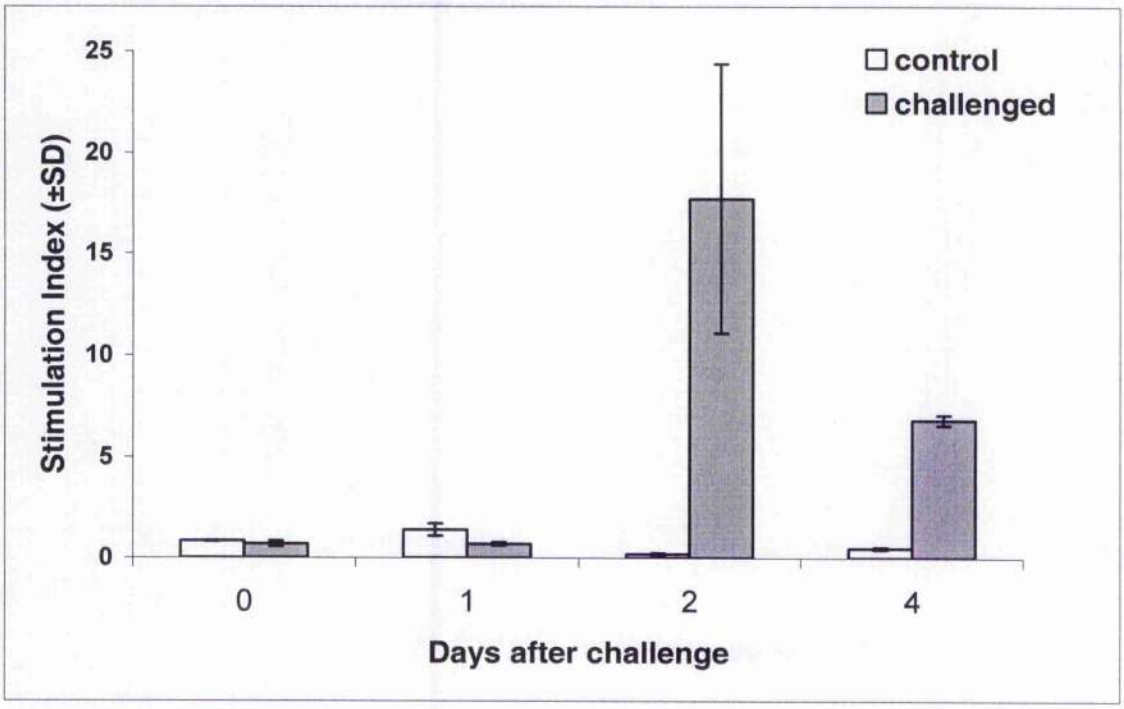


Figure 22B **Stimulation of lymphocytes from calves challenged with *P. multocida* A3 by *P. multocida* B:2 85020 CFE**



3.4 In vitro responses of PBMC

As calves challenged with *P. multocida* B:2 were not readily available, it was decided to investigate the possible suppressive effects of this pathogen on lymphocyte proliferation by using cultured PBMCs obtained from normal calves and treated in vitro with CFE of *P. multocida* B:2.

3.4.1 In vitro suppression of PBMC proliferation

An in vitro system was set up in which the PBMCs obtained from normal calves were treated with CFE, before adding ConA (Section 2.3.15).

Preliminary experiments showed that 10μg protein/well was the optimal concentration of CFE in which PBMCs grew in a similar manner to untreated control cells (Figure 23A). Lower concentrations of CFE promoted increased proliferation presumably due to the extra nutrient components that it contained. CFE at higher concentrations showed adverse effects on growth of PBMC.

Different concentrations of ConA showed that concentrations between 0.5 and 2 μg/well were optimal for strong proliferation of PBMCs (Figure 23B). Figure 23 also shows that addition of CFE at all concentrations (5, 10, and 20μg/well) for 1 h before addition of ConA suppressed the proliferative effect of ConA at the concentration of (1μg/well).

A further in vitro experiment (Figure 24) again showed that the proliferative response of calf PBMCs to ConA (SI=101.9 ± 3.6) was markedly suppressed (SI=29.3 ± 3.1) when the cells were incubated with the optimal concentration (10 μg/well) of *P. multocida* B:2 CFE for one hour before adding the ConA (a 3-5 fold decrease in response). Incubation of PBMCs with CFE alone (10 μg/well) produced a SI=1.5 which indicated that the extract alone had no suppressive effect on growth of PBMCs. The 10μg/well concentration of CFE and the 1 μg/well concentration of ConA together with the 1 h incubation of cells with extract before addition of ConA, as performed in this in vitro assay, were designated as optimal conditions and were followed in all other in vitro assays.

Figure 23 Effect of different concentrations of *P. multocida* CFE and ConA on proliferation of calf PBMCs

PBMCs obtained from normal calves (Section 2.3.13) were cultured in the presence of different concentrations of CFE of *P. multocida* B:2 (Figure 23A). The proliferative effect of different concentrations of ConA was also measured after addition of three different concentrations of CFE (Figure 23B). Cells were incubated for 1 h with extract before adding the ConA. PBMC proliferation was assessed by measurement of thymidine incorporation and is expressed as scintillation counts per minute (cpm). Bars show the mean values of triple wells, and error bars indicate the SD.

Figure 23A Effect of different concentrations of *P. multocida* CFE on calf PBMCs

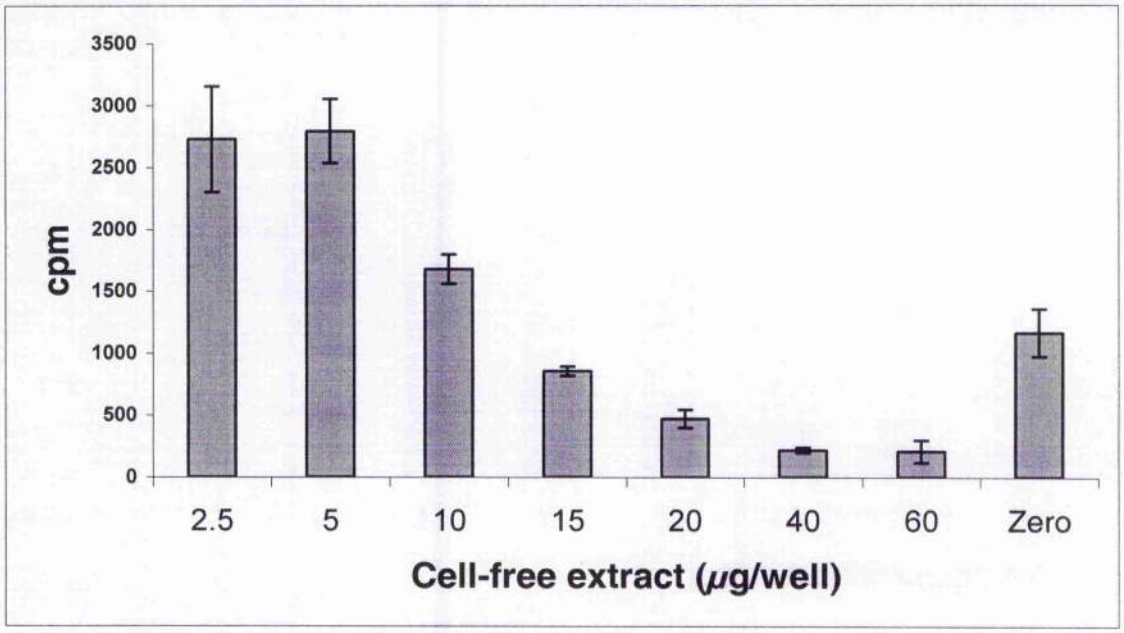


Figure 23B Suppressive effect of different concentrations of *P. multocida* CFE on proliferative effect of different concentrations of ConA on calf PBMCs

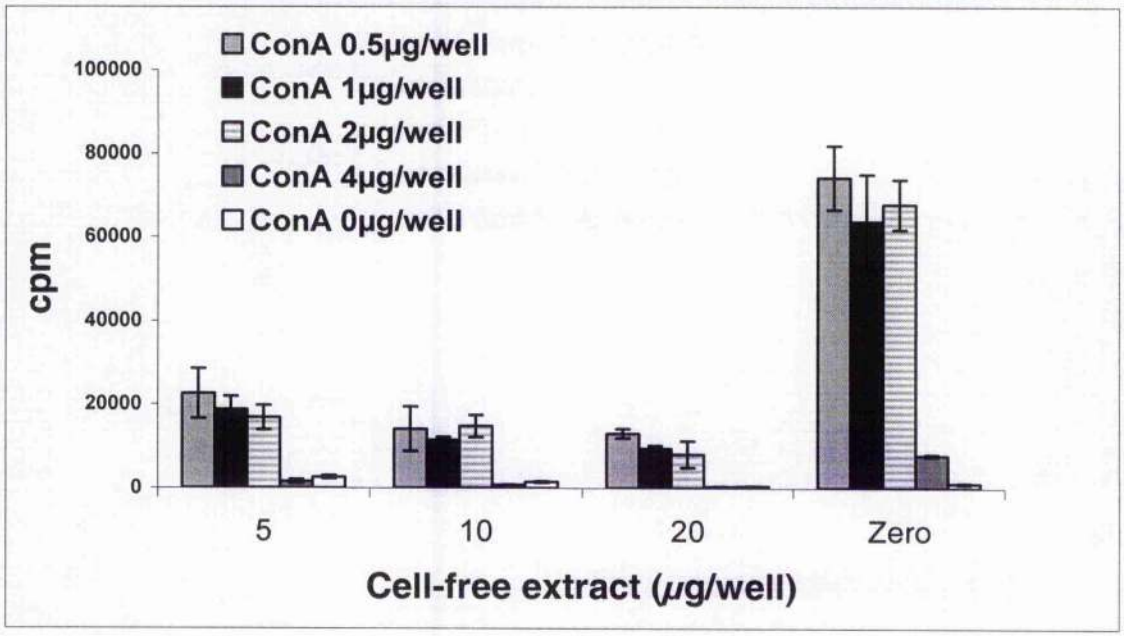
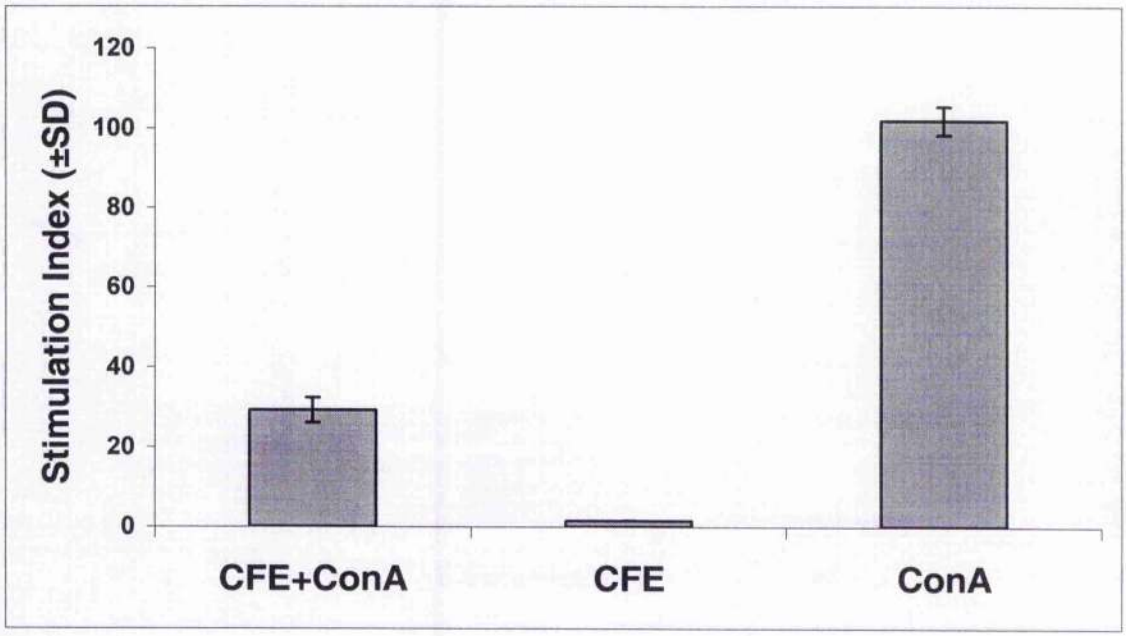


Figure 24 The effect of CFE of *P. multocida* B:2 on proliferative response of PBMCs to ConA

Bars represent the proliferative response of normal calf PBMCs after incubation for 1 h with 10µg/well CFE before addition of 1µg/well ConA. Proliferation was detected by measurement of thymidine incorporation and expressed as stimulation index, which shows the ratio of incorporation in treated (CFE was added) compared to untreated cells. PBMCs were cultured in triplicate and results are mean values, and error bars indicate the standard deviation.



3.4.2 Specificity of the suppressive effects of *P. multocida* B:2 CFE on PBMC proliferation

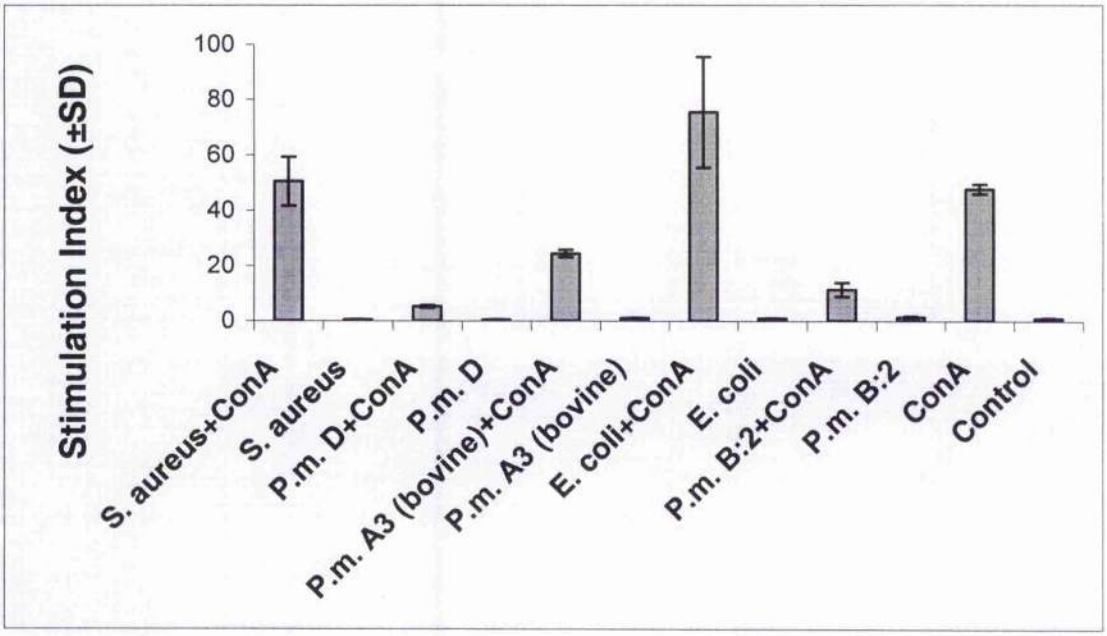
To study if the suppressive effects of *P. multocida* B:2 CFE were specific for this serotype, CFE of a number of other bacteria were prepared and used in the optimised in vitro assay (Figure 25). All of the extracts were prepared in the same way as that of *P. multocida* B:2 (Section 2.3.1) and the protein concentrations, measured by modified Lowry assay (Section 2.3.6.2), were adjusted to 10µg/well. CFE prepared from *Staphylococcus aureus* (Figure 25A) or *Mannheimia haemolytica* serotypes A1 or A2 (Figure 25B) did not affect the proliferative response of PBMCs to ConA. CFE prepared from *Escherichia coli* (Figure 25A) slightly increased the proliferative response to ConA. CFE of *P. multocida* serotype D (pig isolate) (Figure 25A) strongly suppressed the proliferative response of calf PBMCs to ConA. *P. multocida* serotype A3 (bovine pulmonary) (Figure 25A) and *P. multocida* serotype A (ovine pulmonary) (Figure 25B) extracts had a slightly less effect on the proliferative response than *P. multocida* B:2 but appeared to have some suppressive effect. An avian serotype A strain of *P. multocida* (Figure 25B) had no effect. Overall, various degrees of suppression were observed but only with some of the *P. multocida* extracts. None of the other species tested showed suppressive properties.

Figure 25 Effect of CFE of different bacteria on PBMCs and on the proliferative response to ConA.

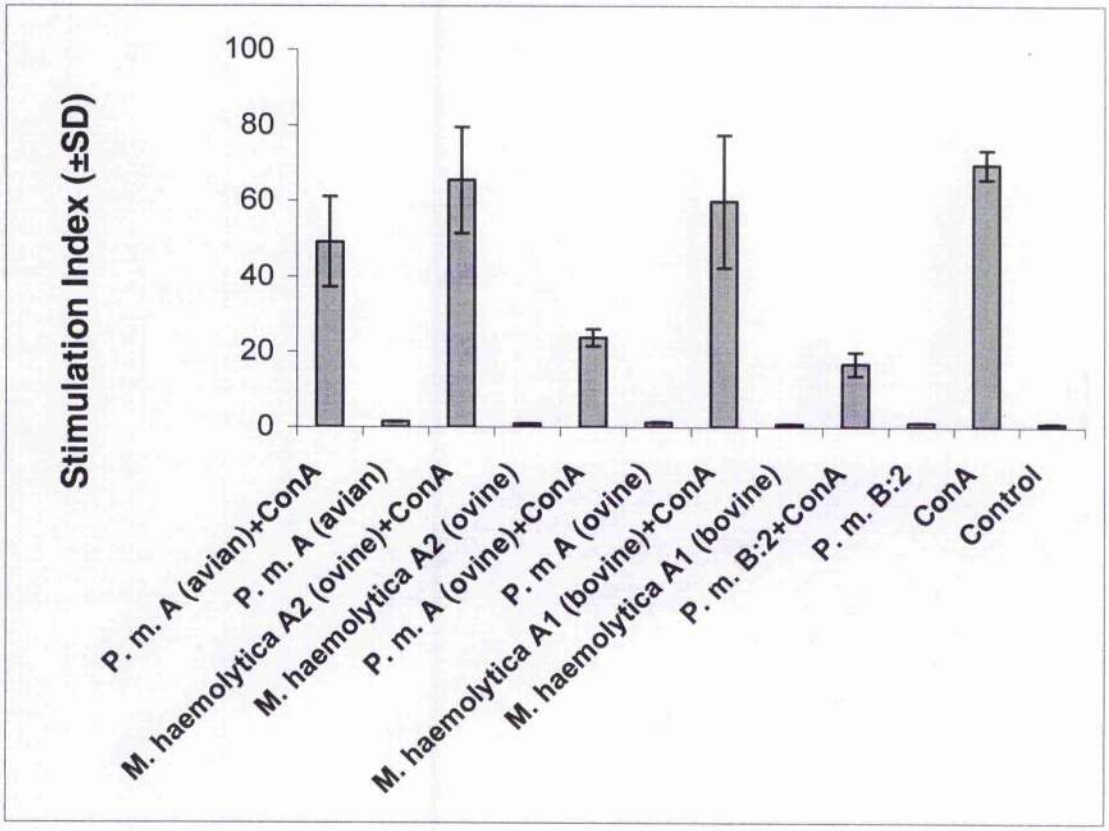
CFE prepared from different bacteria (10µg protein /well) was added to PBMCs prepared from normal calves and incubated for 1 h before addition of ConA (1 µg/well).

Bars represent the proliferative response in the presence (treated cells) and absence (untreated cells) of CFE of different bacterial species. Proliferation measured by thymidine incorporation and shown as stimulation index, which shows the ratio of incorporation in treated to untreated cells (control). The column named as ConA shows the stimulation index of untreated cells to ConA. Cells were cultured in triplicate and results are the mean values of them, error bars indicate the standard deviation. Results shown in the graph A and B were obtained in the same experiment.

A


Stimulation Index (±SD)
100
80
60
40
20
0
S. aureus+ConA
S. aureus
P.m. D+ConA
P.m. D
P.m. A3 (bovine)+ConA
P.m. A3 (bovine)
E. coli+ConA
E. coli
P.m. B:2+ConA
P.m. B:2
ConA
Control

B


Stimulation Index (±SD)
100
80
60
40
20
0
P. m. A (avian)+ConA
P. m. A (avian)
M. haemolytica A2 (ovine)+ConA
M. haemolytica A2 (ovine)
P. m. A (ovine)+ConA
P. m A (ovine)
M. haemolytica A1 (bovine)+ConA
M. haemolytica A1 (bovine)
P. m. B:2+ConA
P. m. B:2
ConA
Control

3.4.3 Characterisation of the suppressive component(s) of *P. multocida* B:2

To begin to characterise the suppressive agent(s) of *P. multocida* B:2 CFE, different treatments were performed on the CFE and fractions were prepared and studied for their suppressive activity (Figure 26). Heating at 80°C for 5 min completely destroyed the suppressive effects of CFE. An OMP preparation of *P. multocida* B:2 (Section 2.3.2) markedly suppressed the proliferative response of PBMCs to ConA (Figure 26A). The suppressive activity of the OMP preparation was also partially destroyed by heating (Figure 26A). CFE dialysed overnight against PBS with a cut off 10,000 Da was still suppressive for calf PBMCs (Figure 26A). A polysaccharide capsule preparation (Section 2.3.4) of *P. multocida* showed no effect on the PBMCs proliferative response to ConA (Figure 26A). The supernate of an 18 h *P. multocida* broth culture increased the PBMC proliferative response to ConA (Figure 26A).

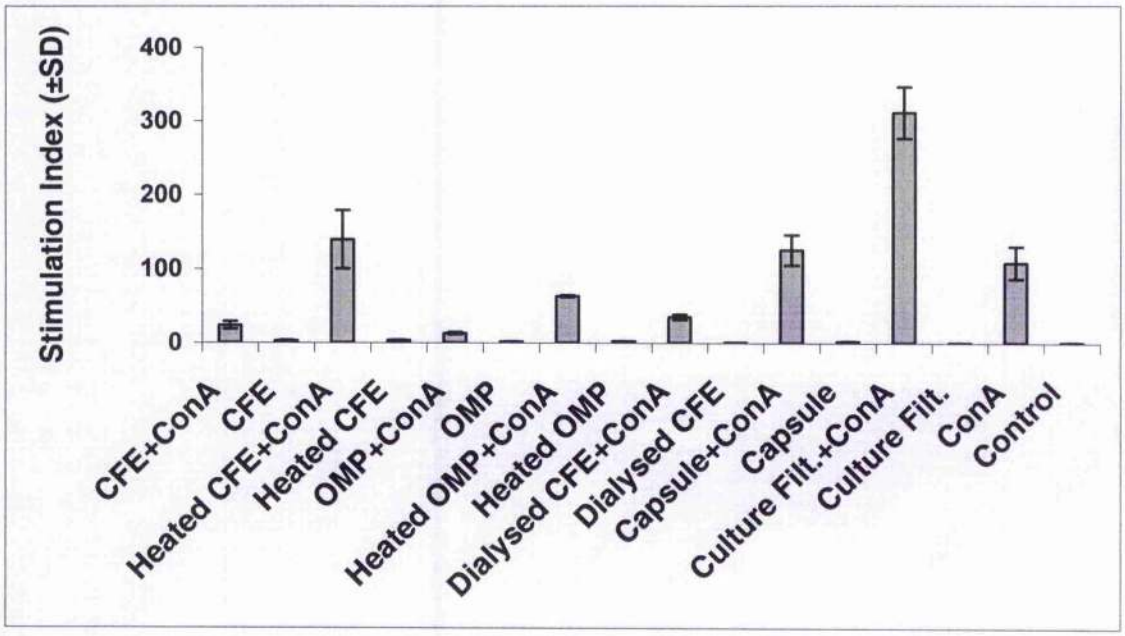
3.4.4 Effect of LPS on PBMC proliferative response to ConA

It was very likely that the CFE prepared from *P. multocida* B:2 contained some traces of LPS. LPS is known as a B cell proliferative agent. As endotoxin, it also could have some adverse effects on different cells. To study the effect of LPS, calf PBMCs were treated with LPS purified from *E. coli* (Section 2.3.5). LPS showed a synergism with ConA in proliferative effects on PBMCs at certain concentrations (Figure 26B). LPS at 2×10^2 EU/well did not cause any changes in proliferative response of PBMCs to ConA. At the higher concentrations, LPS increased the proliferative response of PBMCs to ConA with the highest effect at 2×10^5 EU/well in which the stimulation index increased to 267.07 ± 15.39 (a 2.8-fold increase). At this concentration, LPS alone also caused a 2.8-fold proliferation on PBMCs, but lower concentrations of LPS showed no significant effect on PBMCs. It was concluded that LPS, at least that from *E. coli*, was not having a detrimental effect on the proliferation of PBMCs in response to ConA.

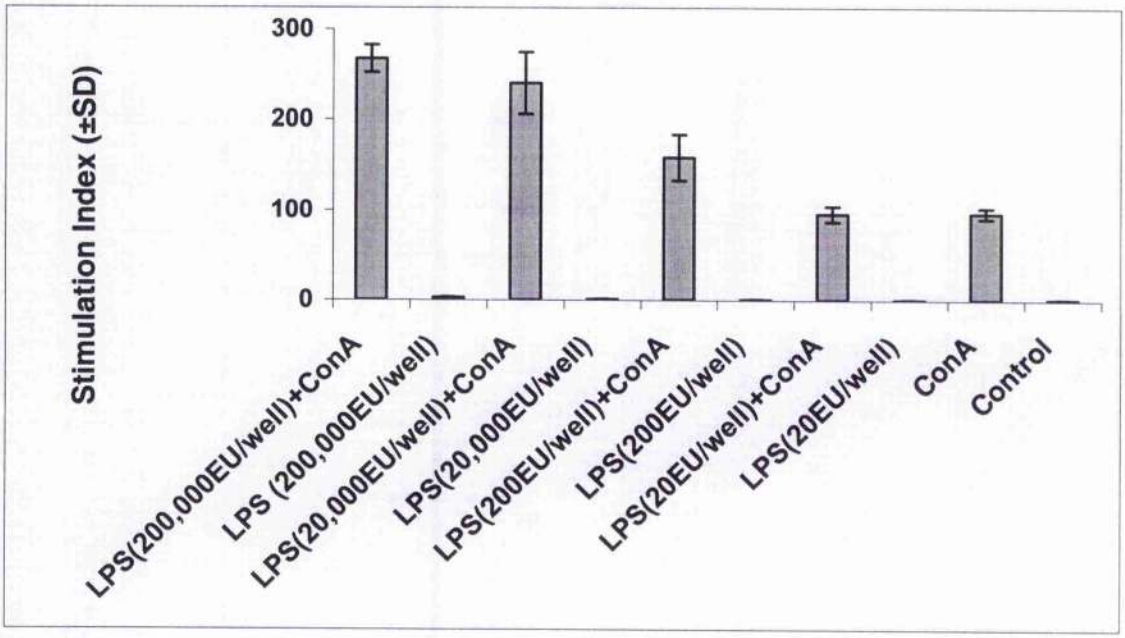
Figure 26 Effect of different preparations of *P. multocida* B:2 and LPS on the proliferative response of PBMCs to ConA

PBMCs were prepared from normal calves and incubated for 1 h with 10μg protein/well of different preparations (Figure 26A) before addition of 1 μg/well ConA. CFE of *P. multocida* B:2 after heating at 80° C for 5 min, overnight dialysis with 10,000 Da cut off against PBS. An OMP preparation of *P. multocida* B:2 (Section 2.3.3), and used at 10 μg/well before and after heat-treatment. A capsular polysaccharide preparation and also a culture filtrate (0.20 μm) of an overnight broth culture of *P. multocida* B:2 (Section 2.3.4) were also assessed. Different concentrations of LPS (purified from *E. coli*, section 2.3.5) were used in another experiment (Figure 26B). Bars show the proliferative response of calf PBMCs to ConA in the presence of these different fractions of *P. multocida* B:2 and *E. coli* LPS. Proliferation was detected by measurement of thymidine incorporation and shown as stimulation index, which shows the ratio of incorporation in treated to untreated cells (control). Bars are the mean values of triplicate cells cultures and error bars indicate the standard deviation.

A


Stimulation Index (±SD)
400
300
200
100
0
CFE+ConA
CFE
Heated CFE+ConA
Heated CFE
OMP+ConA
OMP
Heated OMP+ConA
Heated OMP
Dialysed CFE+ConA
Dialysed CFE
Capsule+ConA
Capsule
Culture Filt.+ConA
Culture Filt.
ConA
Control

B


Stimulation Index (±SD)
300
200
100
0
LPS(200,000EU/well)+ConA
LPS (200,000EU/well)
LPS(20,000EU/well)+ConA
LPS(20,000EU/well)
LPS(200EU/well)+ConA
LPS(200EU/well)
LPS(20EU/well)+ConA
LPS(20EU/well)
ConA
Control

3.4.5 Identification of the population of lymphocytes affected by CFE of *P. multocida*

To determine which population of lymphocytic cells was affected by the presence of *P. multocida* components, FACS (fluorescence-activated cell sorter) analysis was conducted on PBMCs at different stages of proliferation and suppression (Section 2.3.18), for identification of B cell and T cell subpopulations ($CD4^+$, $CD8^+$ and $\gamma\delta^+$ T cells). At the same time, FACS analysis on CFSE-loaded (carboxyfluorescein diacetate succinimidyl ester) cells was also carried out on the cells of the same experiment, to differentiate the different generations of each cell population that had been affected by the bacterial extract (Section 2.3.16). The FACS analysis assay on CFSE unloaded and loaded cells was done in parallel to the lymphocyte proliferation assay and samples for FACS analysis assays were taken at different stages of the in vitro proliferation assay.

3.4.5.1 FACS analysis of PBMCs

The ratio of different cell populations was identified in PBMCs before and after different treatments (Table 3A). Four different cell populations (B cell and $CD4^+$, $CD8^+$, $\gamma\delta^+$,T cells) were identified by indirect immunofluorescence staining of corresponding cell markers using mouse monoclonal antibodies against them as primary antibody, and FITC-conjugated goat antibodies specific for the mouse Ig isotype as the secondary antibody (Section 2.3.17). FACS analysis (using CellQuest®) was carried out on the samples taken at the beginning of the experiment (time zero) and after 72 h (Section 2.3.18). In each population the proliferative and non-proliferative cells were also identified based on their size and the total cells in each population was expressed as sum. A sample of each population was also stained by Ig isotype to assess the negative control (expressed as Isotype) in which the cells were stained only with secondary antibody.

In the Table 3A it can be seen that the percentage of B cells in the control culture increased over the course of the experiment from 11.9 to 28.34 and although the percentage of B cells in the ConA-treated culture (27.81) was similar to the control (28.34), an increase was seen in cultures treated with CFE+ConA (54.31) or CFE (53.25) in which the percentage roughly doubled. Although the percentage of B cells in total showed an increase, the percentage of proliferating B cells, which was differentiated from non-proliferating cells by their smaller size, was at approximately the same level in all samples at the time of harvest at 72 h (between 3.54 and 5.28). The percentage of B cells in ConA and control is about the same level (27.81 and 28.34 respectively). The overall percentage of B cells

increased by 1.92 times in the cells treated with CFE+ConA which was similar to CFE-treated cells which had an increase of 1.88 times compared to control cells. This indicated that the increase in overall percentage of B cells was due to the presence of CFE. It seems that the proliferative response of the B cells to CFE happened in the early stages of culture because, although the overall percentage of B cells increased, the proliferating fraction of B cells was estimated to be low at the time of harvest. It can be concluded that B cells do not play a direct role in lymphocyte proliferation caused by ConA and consequently would not be the target of any suppressive effect by the *P. multocida* extract.

The percentage of $CD4^+$ T cells decreased during the course of the experiment with all treatments including control cells. Comparison between control cells and ConA-treated cells revealed that the proliferating fraction of $CD4^+$ cells increased due to the presence of ConA by a factor of 10-fold. In the presence of CFE, however, ConA stimulated only a 3-fold increase. ConA appeared to stimulate $CD4^+$ cells in a delayed manner because, on the day of harvest (72 h), the proliferating fraction had apparently increased although the overall percentage had not yet increased. Comparison between the percentage of control (15.37) and CFE-treated (15.78) cells revealed that $CD4^+$ T cells were not affected markedly by CFE, and there was no toxic effect of CFE on these cells, so the suppression of the proliferative response was unlikely to be due to toxicity. It can be concluded that, $CD4^+$ T cells are at least one of the populations that responded to ConA in terms of proliferation, as measured by thymidine incorporation, and that this response was suppressed due to the presence of CFE.

Total overall percentage of $CD8^+$ T cells showed only slight changes during the course of the experiment with the exception of decrease in cells treated with CFE. The $CD8^+$ proliferating T cell percentage increased markedly in response to ConA (5.78 –fold compared to control cells) but remained the same in CFE+ConA-treated cells. Comparison between control cells and CFE-treated cells revealed that both the overall percentage of $CD8^+$ T cells (from 11.85 to 8.14) and also the proliferating fraction of these cells was decreased in the presence of CFE (from 1.35 to 0.33). It can be concluded that CFE had some adverse effects on this cell population and that the suppression in the proliferative response of $CD8^+$ T cells to ConA by CFE might be partly accounted for this effect.

The overall percentage of $\gamma\delta^+$ T cells increased slightly during the experiment. The proliferating $\gamma\delta^+$ fraction increased in response to ConA by 10.48-fold compared to control cells and this fraction was only slightly lowered to a 8.42-fold increase in CFE+ConA-treated cells. Comparison between control cells and CFE-treated cells showed that the

percentage of the proliferative fraction of $\gamma\delta^+$ T cells was not affected by CFE (from 1.75 to 1.49) although the overall percentage of this population slightly decreased compared to control cells (from 21.48 to 16.09), indicating that the slight suppression in proliferative response of $\gamma\delta^+$ T cells to ConA in the presence of CFE may have been due to a slight adverse effect of CFE on these cells.

3.4.5.2 FACS analysis of CFSE-loaded PBMCs

CFSE loading of PBMCs was used to more accurately determine the number of cell divisions that the different cell populations had undergone during the 72 h culture period with the different treatments. CFSE-loaded PBMCs were counter-stained in parallel to CFSE-unloaded cells, by indirect immunofluorescence, to detect $CD4^+$, $CD8^+$, $\gamma\delta^+$ T cells, and B cells (Table 3B).

The ratio of different populations of CFSE-loaded cells and proliferative and non-proliferative cells were similar to those shown in Table 3A exept that the percentage of the B cell population was decreased markedly under all treatments. The percentage of B cells at time zero was less (3.01) compared to the value (11.9) for the CFSE-unloaded cells and, in the control, the CFSE-loaded B cells did not increase in 72 h (9.56) cultures as much as it increased in CFSE-unloaded cells (28.34). In fact, the total B cell population did not increase in the presence of CFE, as was seen in (Table 3A).

The percentage of CFSE-loaded $CD4^+$, $CD8^+$ T cells was apparently decreased at the time zero compared to CFSE-unloaded cells (from 43.53 to 28.15 and from 12.56 to 8.59 respectively). However, at the time of harvest under all treatments, the ratio of CFSE-loaded $CD4^+$, $CD8^+$ T cells was the same as CFSE-unloaded cells. The noticeable difference was the increase in the percentage of the proliferating CFSE-loaded $CD4^+$, $CD8^+$ T cells under CFE+ConA treatment (from 5.11 to 9.56 and from 1.29 to 5.69 respectively). It was again clear, however, that CFE affected the proliferative response of $CD4^+$ and $CD8^+$ T cells with a slight effect on $\gamma\delta^+$ T cells.

The pattern of changes of CFSE-loaded $\gamma\delta^+$ T cells was the same as CFSE-unloaded $\gamma\delta^+$ T cells. The only notable difference was the increase in proliferating fraction of control CFSE-loaded $\gamma\delta^+$ T cells (from 1.75 to 5.46).

Table 3 The percentages of different cell types in PBMC populations assessed by FACS analysis, before and after different treatments

A. PBMCs were prepared from a normal calf CFSE-unloaded were treated with ConA (1μg/well), CFE (10μg protein/well), CFE+ConA (CFE was added and after1 h incubation ConA was added) or without any treatment (Control). In B, CFSE-loaded cells were loaded with CFSE before being used in the experiment (Section 2.3.16). Different populations of lymphocytes (B cell and $CD4^+$, $CD8^+$, $\gamma\delta^+$ T cells) were identified by indirect immunofluorescence staining (Section 2.3.17) and FACS analysis was carried out using CellQuest® (Section 2.3.18). The numbers show the percentage of cell types in each preparation. Values at time zero represent FACS analysis at the beginning of the experiment. Other values represent FACS analysis after 72 h of treatment. Proliferating means the fraction of cells in each population that are proliferating. Non-proliferating means the fraction of cells in each population that are not proliferating at the time of harvest. Sum means the overall of proliferating and non-proliferating cells in each population. These were determined by size difference (small cells considered proliferative and large cells non-proliferative). Isotype shows the negative control in which the cells were stained only with secondary antibody. For the CFSE-loaded cells, division and population of different generations of CFSE-loaded cells has been shown by elaboration through ModFit® software in Figure 28.

A (CFSE unloaded)

	B cell			CD4$^+$			CD8$^+$			γδ$^+$			Isotype	Total
	Sum	Non-Prolif.	Prolif.	Sum	Non-Prolif.	Prolif.	Sum	Non-Prolif.	Prolif.	Sum	Non-Prolif.	Prolif.		
Time zero	11.9	9.2	2.7	43.53	25.23	18.3	12.56	11.81	0.75	16.19	15.5	0.69	0.15	84.33
Control	28.34	24.8	3.54	15.37	13.68	1.69	11.85	10.5	1.35	21.48	19.73	1.75	1.26	78.3
ConA	27.81	23.3	4.51	20.54	3.54	17	11.24	4.16	7.08	23.7	5.36	18.34	1.06	84.35
CFE+ConA	54.31	49.3	5.28	20.63	15.52	5.11	9.97	8.68	1.29	25.98	11.24	14.74	1.74	112.63
CFE	53.25	49.69	3.56	15.78	14.56	1.22	8.14	7.81	0.33	16.09	14.6	1.49	2.31	95.57

B (CFSE loaded)

	B cell			CD4$^+$			CD8$^+$			γδ$^+$			Isotype	Total
	Sum	Non-Prolif.	Prolif.	Sum	Non-Prolif.	Prolif.	Sum	Non-Prolif.	Prolif.	Sum	Non-Prolif.	Prolif.		
Time zero	3.01	2.79	0.22	28.15	18.62	9.53	8.59	7.87	0.72	18.25	16.15	2.1	2.76	58
Control	9.56	6.74	2.82	15.71	13.43	2.28	11.09	9.7	1.39	26.2	20.74	5.46	2.71	65.27
ConA	7.85	7.29	0.56	23.93	4.71	19.22	15.47	3.25	12.22	29.9	4.88	25.02	2.73	79.88
CFE+ConA	22.06	17.44	4.62	19.48	9.92	9.56	11.85	6.16	5.69	26.22	8.65	17.57	4.98	84.59
CFE	20.39	17.92	2.47	14.7	13.07	1.63	10.13	9.26	0.87	19.15	17.76	1.39	8.4	72.77

3.4.5.3 Prolifcrative responses evaluated by CFSE staining

Different generations of each cell population were evaluated with CFSE after counter-staining of PBMCs. The results which has been shown in Table 3B for percentages of different populations of T cells ($CD4^+$, $CD8^+$, $\gamma\delta^+$) and this was elaborated by software ModFit® (Section 2.3.18), to generate the frequency of different generations in each population, after treatment with ConA or CFE+ConA (Figure 27).

The $CD4^+$ T cells proliferated (Proliferation Index=2.54) in response to ConA (Figure 27A1), but the proliferative response reduced (Proliferation Index=1.92) in CFE+ConA-treated cells (Figure 27A2). The nonproliferative fraction of $CD4^+$ T cells increased by 1.55-fold (from 0.2 to 0.31) in response to CFE treatment (Figure 27A). The decrease in proliferative response of CFE-treated $CD4^+$ T cells to ConA started from generation 2 and was quite marked in later generations (Figure 27A2).

$CD8^+$ T cells proliferated (Proliferation Index=3.26) strongly in response to ConA (Figure 27B1). However, the proliferative response (Proliferation Index=1.77) of the $CD8^+$ cells to ConA was suppressed by CFE (Figure 27B2). The nonproliferative fraction showed a 3.35-fold increase (from 0.17 to 0.57) in CFE+ConA-treated $CD8^+$ T cells, which indicated a more than 3-fold increase in the fraction of cells that, had not proliferated in response to ConA. The parent generation increased by two fold in CFE-treated $CD8^+$ cells although all of the daughter generations decreased were noticeably less proliferative compared to CFE-untreated cells (Figure 27B2).

The $\gamma\delta^+$ T cells showed proliferation (Proliferation Index=2.56) in response to ConA in a manner similar to $CD4^+$ T cells in which the Proliferation Index was 2.54. The proliferative response of CFE-treated $\gamma\delta^+$ T cells to ConA was only slightly reduced from 2.56 to 2.22 (Figure 27C). The nonproliferative fraction of $\gamma\delta^+$ T cells only increased by 1.46-fold (from 0.13 to 0.19) in CFE+ConA-treated cells, but progress through the generations was not as adversely affected as with $CD4^+$ and $CD8^+$ T cells.

B cells were not included in Figure 27 because they did not show a similar response as that observed in CFSE-unloaded assay (Table 3) due to apparent adverse effects of CFSE on them.

Figure 27 The CFSE fluorescence histograms of different T cell subpopulations ($CD4^+$, $CD8^+$ and, $\gamma\delta^+$)

PBMCs were prepared from a normal calf and were loaded with CFSE and treated as indicated in Table 3. Data for CFSE-loaded PBMCs in Table 3B was elaborated through software ModFit® to visualise different generations of T cells sub-populations including: $CD4^+$ T cells (Figure 27A), $CD8^+$ T cells (Figure 27B), and $\gamma\delta^+$ T cells (Figure 27C). Each time the parental cell population divides, the fluorescence of the next generation halves. Proliferation Index is the sum of the cells in all generations divided by the computed number of original parent cells and represents the increase in cell number over the course of the experiment. The Nonproliferative Fraction is the number of cells in the parent generation at the time of data collection divided by the computed number of cells present in the original culture and represents the fraction in the original culture of cells that have not proliferated during the course of the experiment.

Figure 27A The CFSE fluorescence histogram of $CD4^+$ T cells after treatment with ConA (Figure A1) and CFE+ConA (Figure A2)

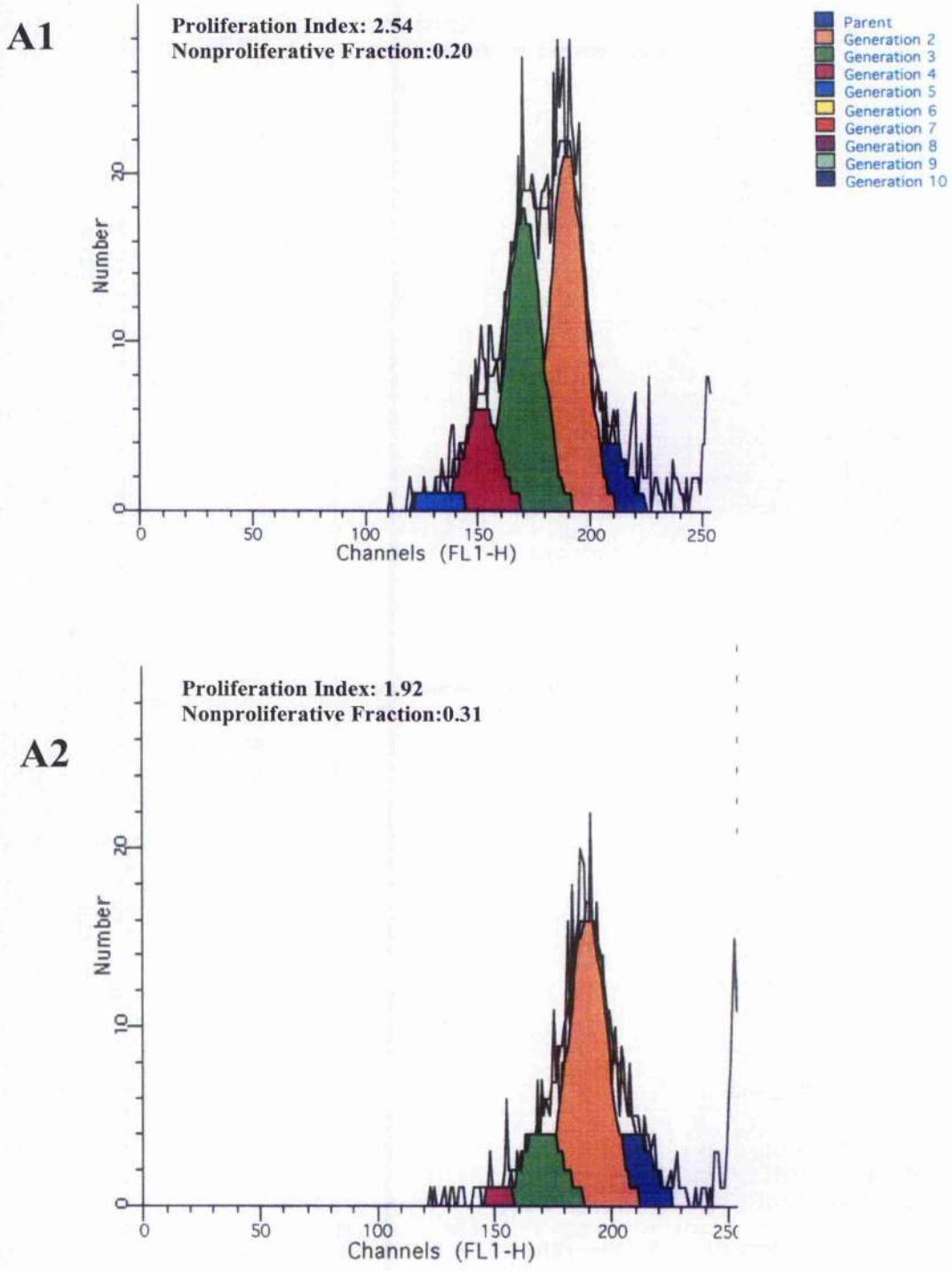


Figure 27B The CFSE fluorescence histogram of $CD8^+$ T cells after treatment with ConA (Figure B1) and CFE+ConA (Figure B2)

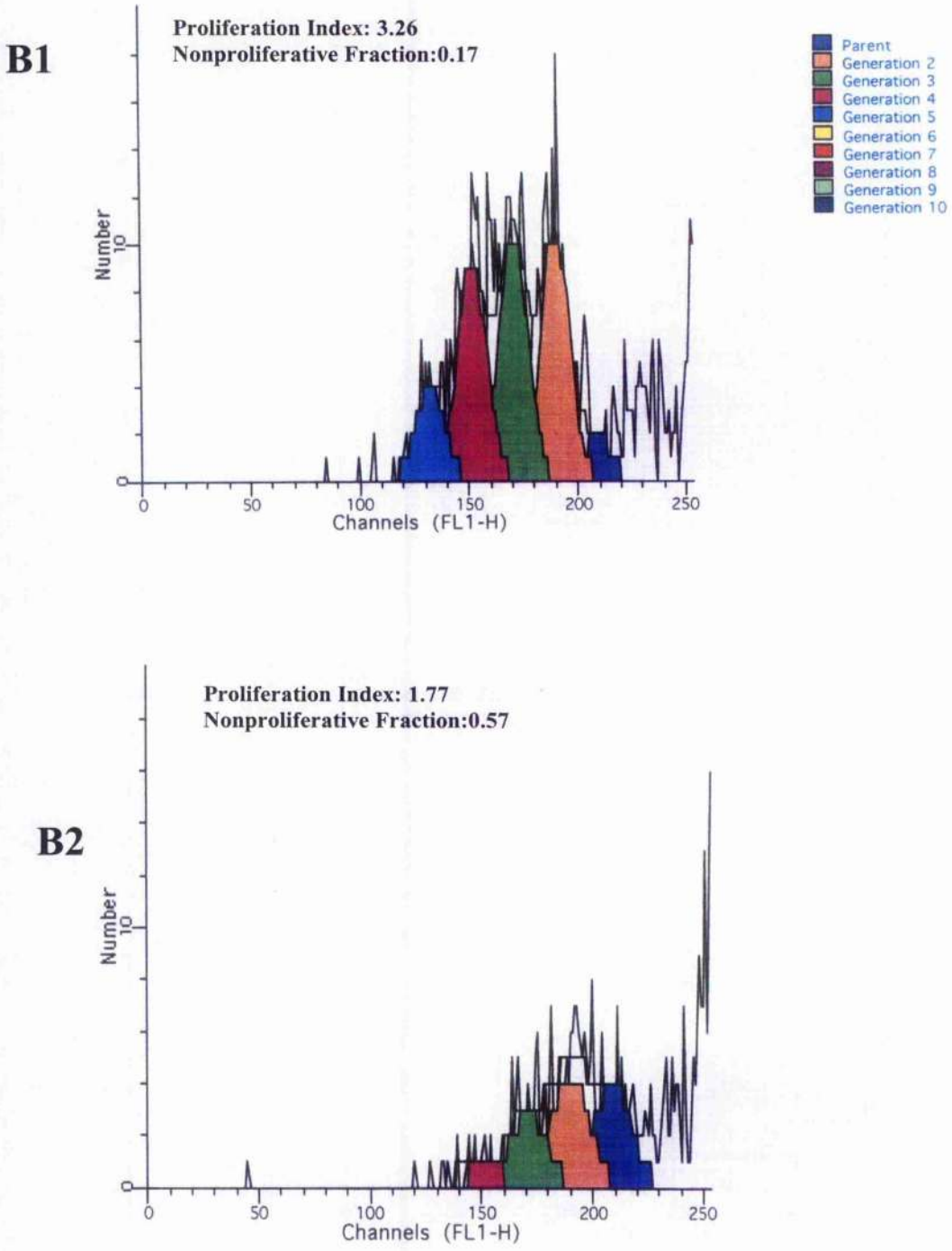
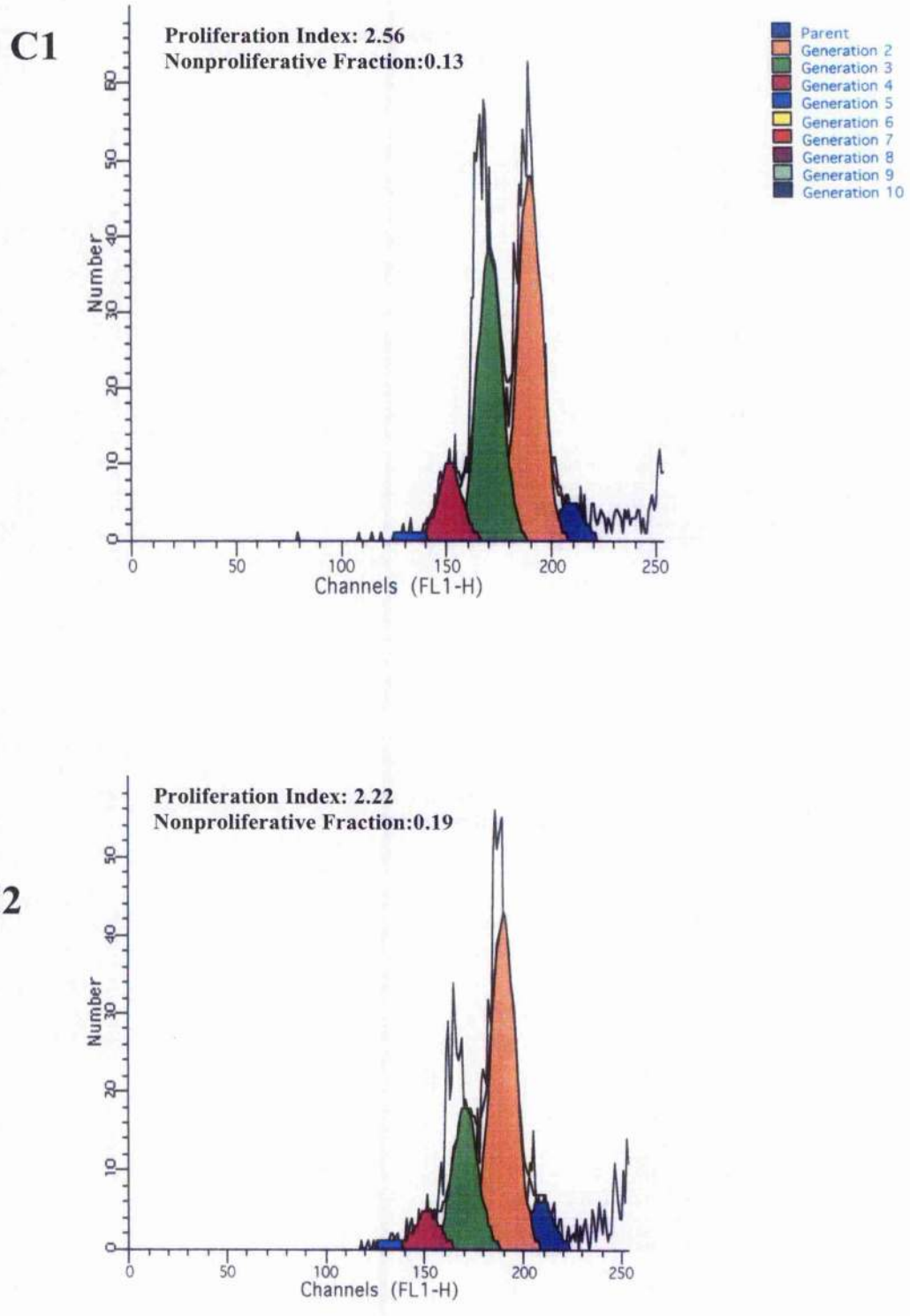


Figure 27C The CFSE fluorescence histogram of $\gamma\delta^+$ T cells after treatment with ConA (Figure C1) and CFE+ConA (Figure C2)



Chapter 4

DISCUSSION

4.1 Prevention of HS

Haemorrhagic septicaemia (HS) has been ranked as a primary fatal disease affecting cattle and buffaloes. HS is economically very important in tropical areas of South and Southeast Asia, Near and Middle East where a noticeably large proportion of the world's population of buffalo (95%) and cattle (30%) exists (FAO, 2003). In these areas, climate conditions are ideal for development and high incidence of the disease.

Treatment is of little value after the appearance of visible clinical signs. Early detection of HS is a crucial element in the success of treatment (Radostits *et al.*, 2000). In primitive husbandry conditions, which are the dominant system in endemic areas, the conditions for early detection of the disease are lacking. Consequently, in all countries where HS occurs, vaccination has been adopted as the practical method of control. Vaccination has been accepted as an effective means of HS control, because the rapid onset and short course of the disease leaves little opportunity for treatment (De Alwis, 1999).

For prevention and control of HS, there are three categories of measures. Measures to be followed in endemic countries on a prophylactic basis, measures to be adopted in an outbreak and measures needed for prevention of spread across regional or national borders. Vaccination is the most important element in all of these measures (Benkirane, 2002).

Currently there are four different types of vaccine commercially available for HS; plain bacterins, alum-precipitated, aluminium hydroxide gel and oil adjuvant vaccines. These vaccines, all of which are inactivated whole-cell vaccines, are being used in different countries and under different regimens (OIE, 2004; Verma & Jaiswal, 1998).

Although the achievements in controlling HS by vaccination have proved the importance of this strategy, there are some deficiencies in current vaccines. Plain bacterins confer only an immunity of very short duration. The alum-precipitated and aluminium hydroxide gel vaccines are believed to confer immunity for 6 months (Bain *et al.*, 1982) and even shorter durations for only about 3-4 months are also reported (De Alwis, 1999). Thus, in some

endemic areas the recommended administration of these vaccines is twice a year. Oil adjuvant vaccine, which is able to confer longer immunity, suffers from difficulty of injection because of the high viscosity of the vaccine. This has made the vaccine unpopular among field users (Shah & de Graaf, 1997). Reactogenicity is another important obstacle for the use of current vaccines. There are different reports of the rate of postvaccination shock which vary from 0.1% (Bain *et al.*, 1982) to 10% (De Alwis, 1999). The high LPS content of these vaccines is identified as the cause of adverse reactions, due to the requirement for high numbers of cells to be present for adequate potency (De Alwis, 1999).

Vaccine development has become an important field of investigation to overcome the deficiencies of current vaccines. The importance of vaccine development was highlighted at the International Workshop on HS (Proceedings of the FAO/APHCA Workshop on Haemorrhagic Septicaemia, February 1991, Colombo, Sri Lanka). It is generally believed that exposure to live organisms is able to confer more solid immunity than immunisation through inactivated vaccines. This has been shown in HS where naturally-acquired immunity, resulting from arrested infection of animals, was superior to immunity caused by vaccination (de Alwis & Sumanadasa, 1982). Thus, a live attenuated vaccine, which has the ability to infect the animal, might also be able to cause stronger and long-term protective immunity against disease.

The efforts towards the introduction of new HS vaccines have focused mainly on the development of live vaccines (involving the production of suitable avirulent, protective mutants by genetic manipulation). Such studies, have resulted in the introduction of three different live vaccine candidates namely; streptomycin-dependent (Str^D) mutant (Wei & Carter, 1978), deer strain (Myint *et al.*, 1987) and acapsular mutants (Boyce & Adler, 2001). Only the deer strain, which is *P. multocida* serotype B:3,4 isolated from a fallow deer in England after an outbreak of septicaemic disease (Jones & Hussaini, 1982), has been subjected to pilot field study (Myint & Carter, 1989). However, the field studies revealed that, by subcutaneous administration, the deer strain was pathogenic in buffalo calves (Myint & Carter, 1989; Myint, 1990). Although aerosol vaccination with this strain is used in Myanmar (Myint *et al.*, 2005), it has not been accepted in other countries (Benkirane, 2002). One reason for this is that the vaccine strain has occasionally been associated with sporadic outbreaks of disease due to unknown reasons (Aalbaek *et al.*, 1999; OIE, 2000; Rimler & Wilson, 1994). Furthermore, because the vaccine contains a potential pathogen, vaccination could be done only under controlled conditions, to prevent the spread of the organism (De Alwis, 1999).

A marker-free *aroA* deletion derivative (strain JRMT12) was constructed in our laboratory from a HS-associated *P. multocida* serotype B:2 (strain 85020) obtained from Sri Lanka. Preliminary experiments confirmed that JRMT12 was highly attenuated for virulence in a mouse model of HS. In the mouse model it was also shown that JRMT12 conferred a high degree of protection against challenge with the wild-type parent strain, when it was injected intraperitoneally or intranasally as a live vaccine (Tabatabaei *et al.*, 2002). These promising results suggested that further studies, on attenuation and the ability of JRMT12 to promote protection, should be done in the natural host.

In the present study, three sets of experiments were carried out to determine the pathogenicity and immunogenicity of the JRMT12 strain in calves as natural hosts of *P. multocida* B:2. Investigation of immune mechanisms involved in possible protection was also a major part of our study.

4.2 Calf responses to JRMT12

4.2.1 Safety of JRMT12

Our results showed that the virulence of the JRMT12 mutant decreased for calves. Intranasal (IN) injection of calves with c. 10^9 CFU of the mutant did not cause the clinical signs of HS. The rectal temperature and SAA levels of animals, taken as an indication of the progress and severity of infection, in the IN vaccinated group remained at normal levels up to 24 h after administration. This indicated that the mutant did not mount an infection in this group via the intranasal route. One possible reason could be the low virulence of the strain. To cause systemic disease like HS, the bacteria at first step would need to cross the respiratory epithelium in sufficient numbers to cause infection and then in next step, the disease. JRMT12 may be too attenuated to cause the infection by this route. There is also the problem of animals swallowing a large part of the vaccine dose given intranasally. In future studies, it would be of interest to challenge the animals intranasally with larger doses of JRMT12, or to deliver the vaccine as a spray or aerosol, and to monitor signs of infection, such as temperature rise and SAA levels. It would also be of interest to swab those animals at regular intervals after vaccination, to try to determine the length of presence of the vaccine strain by this route.

Intramuscular (IM) injection of c. 10^9 CFU of the mutant caused some typical clinical signs of the disease such as dullness and there was a clinical response manifested by a rise in rectal temperature and SAA levels. SAA levels increased significantly after each dose of

IM vaccine and, in each case, reached the highest level in 24 h. The transient febrile response of these animals that, for humane reasons, was controlled by injection of NSAID, was possibly caused by associated bacterial endotoxin. This was considered in setting up the second experiment to study lower doses of vaccine to overcome this reactogenicity. IM injection of calves with three different doses (c.10^9 CFU, c.10^8 CFU, c.10^7 CFU) of JRMT12 showed some typical clinical signs of HS. Rectal temperatures of all vaccinated groups were raised after each dose of vaccine. The patterns of temperature increase over time were very similar for the 10^9 and 10^8 CFU groups despite the intervention with NSAID treatment in the 10^9 CFU group. In calves that received ~10^7 CFU the febrile response was less than in the other two groups ($p=0.002$) as it plateaued after 2 h and remained higher than the baseline up to 24 h after vaccination. In this experiment only SAA level of the 10^8 CFU group was measured after the first vaccination, as a representative response. The SAA levels after vaccination in this group were lower than the SAA responses in the 10^9 CFU group determined in the first experiment. Because of this lower response, and a generally lower reactogenicity, 10^8 CFU was considered a safe dose for IM vaccination. The SAA response to the IM vaccination paralleled the increased rectal temperature, indicating that the attenuated bacteria persisted for some time in the animals, perhaps for long enough to promote an immunogenic response.

In the first experiment, clinical signs of HS after challenge with the wild type strain were prevented by IM vaccination of calves with two doses of JRMT12 at 10^9 CFU. Vaccination by the IN route was not able to prevent clinical signs of the disease after challenge. There was a clear difference between the temperature responses of calves to challenge: IM-vaccinated animals did not show any changes but, in control and IN-vaccinated animals, rectal temperature increased after challenge. SAA levels showed the same pattern of changes in this experiment. IM vaccination with two doses of the mutant prevented dramatic changes in SAA response upon challenge, but IN vaccination of calves did not prevent this rise. In the control and IN-vaccinated groups, SAA levels increased dramatically after challenge. A lack of clinical reaction in IM-vaccinated calves was an indication of the protection conferred in these animals by vaccination.

In this experiment one calf from each of the IN-vaccinated and control groups did not develop the clinical signs of HS after challenge with wild type. Generally, the exact reasons why not all calves develop the disease after challenge has not been understood. As possible reasons, it has been suggested by a study on buffalo calves that the reduced endotoxaemia and a gradual but sustained rise in APP may contribute in helping to control disease (Horadagoda *et al.*, 2001). In this way, the high levels of SAA observed in these

particular calves due to challenge may have played a role in their survival. On the other hand the increase in SAA levels have been used as an indication of severity of infection (Horadagoda *et al.*, 1999). Thus, the large increase in SAA levels recorded in IN-vaccinated calves after challenge, compared with the moderate response of the IM-vaccinated group, was considered the result of a less well-developed immune protection in the IN-vaccinated group. However, there was no clear relation between prechallenge concentrations of SAA and survival after challenge, as the IN-vaccinated calf that survived the challenge had a low SAA level (4.3 μg/ml) while the only survivor calf in control group had a relatively high level of SAA (128 μg/ml).

In the second experiment (IM vaccination using different doses), rectal temperature of calves in the 10^8 and 10^7 CFU groups increased in response to challenge but returned to normal levels by 28 h. At 12 h after challenge the recorded rises in temperatures were dose-dependent: the higher the dose of vaccine the lower the temperature rise after challenge. The temperature response of the 10^7 CFU vaccinated group was about the same as that in the control group after challenge. Because of this observation the 10^7 CFU dose of vaccination could be considered as inadequate for prevention of clinical signs. However, all the calves given 10^7 CFU survived challenge and did not need NSAID asministration, whereas 2 of 3 control calves were given NSAID treatment, but were unresponsive and had to be put down early. In this experiment, haptoglobin (Hp) levels were measured along with SAA levels. In all vaccinated groups SAA and Hp responses to challenge were prevented. Hp showed similar pattern of changes to SAA. The pattern of Hp response that was observed in our experiments supports the studies that identified SAA and Hp as a reliable index to show the level of inflammation (Horadagoda *et al.*, 1993; Horadagoda *et al.*, 1994; Horadagoda *et al.*, 1999). A study on calves challenged intratracheally with *P. multocida* A3 also showed that plasma concentrations of SAA and Hp increased rapidly between 5 and 23 hours in response to challenge and decreased dramatically between 72 and 96 hours after challenge (Dowling *et al.*, 2002). All together, our data and the results of these studies indicate the role of SAA and Hp in inflammatory response of calves to infection which can be used as markers of the severity and progress of the disease.

4.2.2 Potency of JRMT12

Our results indicated that JRMT12 at doses of 10^9, 10^8 and 10^7 CFU injected IM could protect calves against challenge with the wild type 85020 strain. A dose of 10^8 CFU was considered optimal as this produced little reactogenicity either on vaccination or subsequently as a result of challenge. Our results indicated that JRMT12 was able to

induce a strong antibody response only after second IM vaccination. Concentrations of serum IgG rose 50-fold within 7 days after the second IM vaccination using the 10^9 CFU dose and had risen further by the day of challenge. There was no increase in IgG levels of these animals 28 days after the first IM vaccination. On the other hand, IN-vaccinated calves showed no detectable IgG response to vaccination. The lack of an IgG response to IN vaccination dose not preclude the induction of mucosal IgA antibody which was not assessed in our study, although even if this occured, it did not contribute to protection as IN-vaccinated animals were not resistant to challenge. In the second experiment, using three different doses delivered IM, IgG again increased only after the second vaccination and in a dose dependent manner: the higher the dose of vaccine the higher the titre of serum IgG. The reason for the lack of IgG response after primary vaccination is not clear but could be due to a rapid clearance of the JRMT12 vaccine strain. A persistent or repeated exposure to antigen is a key requirement for a strong IgG response. Our data are in agreement with other studies that reported only a gradual increase in serum IgG titres during 6-10 weeks after vaccination with an oil-adjuvant vaccine, which provides a slow release of antigen and prolonged exposure to it (Chandrasekaran *et al.*, 1994a; Chandrasekaran *et al.*, 1994b; Horadagoda *et al.*, 1994; Shah & de Graaf, 1997). The rapid IgG response after a second IM vaccination was also noted in another study which showed a rapid and significant rise in IgG levels 14 days after a booster injection (Horadagoda *et al.*, 1994). However, it appeared that the first dose of IM-vaccination in our experiments acted as priming dose, although it did not persist long enough to raise antibody and the second dose of IM-vaccination acted as a booster that raised high levels of IgG.

A single IM vaccination of calves with 10^8 CFU of JRMT12 did not confer protection against subcutaneous challenge. As discussed before, IM vaccination twice with 10^8 CFU was considered an optimum dose that fully protected calves but showed no obvious reactogenicity. It was of interest, therefore to check if a single vaccination was protective. In the third experiment the efficacy of single IM vaccination regime was studied. Two of the four vaccinated calves and two of the five non-vaccinated calves survived the challenge. The antibody and APP responses of the animals were not investigated, because our results showed that the single vaccination regime was not able to cause protection, as there was no difference between the survival rate of vaccinated and non-vaccinated animals. These data were consistent with the pattern of antibody response observed in previous experiments using two IM vaccinations, which indicated a lack of antibody response after the first vaccination. The results are suggestive of a strong correlation between the induction of humoral responses and active protection.

One possible explanation for the rapid clearance of the vaccine strain could be this *aroA* mutant strain is too attenuated and might have lost the ability to persist long enough for stimulation of a clear primary humoral response in the host. Given that a second vaccination was able to induce a strong IgG response, a possible explanation for rapid clearance of the mutant by the host might be the presence of non-specific cross-reacting antibodies which are able to inactivate the mutant. This theory was supported by our experiments, which indicated the presence of maternal IgM antibodies in the 2 week old calves before vaccination which progressively declined. Maternal IgM may have cross-reacted with and neutralised the live vaccine and minimised any humoral response. This phenomenon has been noted previously in young animals (Black *et al.*, 1985). It was shown by a study that calves vaccinated too young may succumb to HS up to the age of 5 to 6 months due to the presence of maternal immunity (Sheikh *et al.*, 1995), which presumably interferes with the development of an active immune response to the vaccine. It was also found by another study that when calves in the age range of 2 to 5 months were vaccinated, the number of calves under 3.5 months that developed detectable antibody titres was low in comparison with 72% of calves over 3.5 months that responded (De Alwis, 1992). It was recommended commencing the HS vaccination of calves at the age between 4 to 6 month of age to avoid antibodies interfering with vaccination (el-Eragi *et al.*, 2001).

Concentrations of serum IgM rose markedly by 7 days following the second IM vaccination, in a similar manner to IgG, and the concentration achieved at that time was maintained until termination of the experiment. In IN-vaccinated calves there was no increase in serum IgM levels during the experiment. IgM levels were high before vaccination in some groups of animals, including the IM-vaccinated calves. The IgM levels declined following first vaccination and rose again after second dose of vaccine. As indicated earlier, the earlier IgM antibodies were possibly maternally-derived due to infection of the original herd with organisms showing antigenic similarities with *P. multocida* B:2. Because HS has not been reported from UK, these maternal antibodies could not have been due to infection with *P. multocida* B:2. Infection of the upper respiratory system of cattle with *P. multocida* serogroup A is common in the United Kingdom (Dowling *et al.*, 2004). My work has shown cross-reaction of the *P. multocida* B:2 CFE, which was used for coating ELISA plates, with antiserum raised to *P. multocida* A3 strain after using anti-bovine IgM HRP conjugate as secondary antibody, a high titre (3981 U/ml) was obtained, whereas cross-reaction when anti-bovine IgG HRP conjugate was used was minimal (87 U/ml). Therefore, it seems likely, that the presence of IgM antibodies in some of the calves used in our study before vaccination was due to cross-

reacting maternal antibodies determined from infection with *P. multocida* A serogroup in the mother.

The calves used in our study were checked for the presence of *P. multocida* infection by nasal swabbing and any calf showed infection was not used in the experiments. To avoid the problem of cross-reacting antibodies in future studies, the animals should also be monitored for the presence of the cross-reacting antibodies by serological tests such as ELISA. Antigens obtained from *P. multocida* serotype B:2 can be used for coating ELISA microplates. Antigens obtained from other serotypes of *P. multocida* including serotype A3 can also be used for detection of maternal antibodies in animals to be used in experiments to check the presence of infection with other serotypes. Another possible solution to avoid the cross-reacting antibodies would be the selection of animals of higher ages (4-5 months old) or animals that have not consumed colostrum.

In a study on the transfer of colostrum-derived antibodies in newborn calves from HS-vaccinated dams, it was shown that maternal anti-HS IgG antibodies increased rapidly after suckling and then declined over a period between 2 and 4 weeks (el-Eragi *et al.*, 2001). This is different from our results which showed that the IgM declined 2 weeks after first vaccination and reached the lowest level 2 weeks later in both the first and second experiments. In ruminants the intestine is unselectively permeable and all immunoglobulins are absorbed. The passively acquired antibodies decline through normal catabolic processes. The rate of decline differs among immunoglobulin classes and the time taken for them to decline depends on their initial concentration (Tizard, 2000). The initial concentration of colostrum-derived antibodies depends on the amount of colostrum consumed by the newborn calves and the maternal antibody titre (el-Eragi *et al.*, 2001). Exposure of colostrum-derived antibodies to reacting antigens can also accelerate the clearance of maternal antibodies and this would be a possible factor in their decline (el-Eragi *et al.*, 2001). In our experiment this could also have had an impact on clearance of antibodies and would explain the similarity of clearance patterns, despite the difference in age of calves used in first and second experiments. This could also be a possible reason for the differences between our results and the study on kinetics of decline of maternal antibodies in calves.

4.2.3 Serum sensitivity

Our experiments on assessment of the bactericidal activity of the serum showed that, *P. multocida* B:2 strain 85020 is highly resistant to positive serum (containing high levels of

IgG and IgM obtained from calves one week after second IM vaccination) and complement activity in normal fresh calf serum. The wild type strain was incubated in sera obtained from IM-vaccinated calves with high titres of IgG and IgM, in the presence or absence of normal fresh calf serum as a source of complement, to study its sensitivity to complement-mediated killing. *P. multocida* B:2 strain 85020 was resistant and grew in the positive and heated positive serum. The strain also grew in the normal fresh calf serum and the mixture of positive serum and normal fresh calf serum. Heated normal fresh calf serum and the mixture of heated positive serum and heated normal calf serum had no effect on the strain. Each of the fresh and positive sera had stimulatory effects on growth of the strain allowing the number of organisms to double during incubation. On the other hand, after heating of these sera, growth was not apparent, maybe because of the destruction of the growth stimulatory agent(s) due to heating. In order to check the complement activity, the bactericidal effect of these sera was also assessed on an *E. coli* K12 strain. Fresh normal calf serum completely killed all the organisms within one hour but the heated calf serum had no effect. Guinea pig complement was also able to kill the *E. coli* K12 strain.

Fetal calf serum (used as an additive for mammalian cell cultures) had no effect on the organism. The rapid progress of HS disease in naïve animals is indicative of resistance of the bacterium to innate immunity elements such as the complement system. These results support another study which showed that *P. multocida* B:2 was highly resistant to complement activity of normal bovine and murine serum and grew rapidly in either fresh or heat-treated calf serum (Boyce & Adler, 2000). Our results would also support studies which showed that different monoclonal antibodies reacting with the LPS of the bacterium were not bactericidal in the presence of complement, although these antibodies were able to opsonise the bacterium for phagocytosis by mouse macrophages (Adler *et al.*, 1996; Ramdani & Adler, 1991). It would be predicted that, anti-LPS antibodies would be raised in calves after vaccination with the *aroA* strain. Although it is generally believed that immunity to *P. multocida* B:2 is antibody-mediated, the exact mechanisms involved in protection conferred by antibodies is not clear yet. Our results showed that in vitro exposure of the organism to sera with high levels of antibody in the presence and absence of complement does not have an adverse effect on the organism. As also shown by our experiments, challenge of vaccinated animals, which had high levels of antibody, prevented the progress of disease. Thus, it can be assumed either that these antibodies promoted clearance by opsonophagocytosis or neutralised the activity of important virulence factor(s), but the nature of these remains elusive. It would be of interest to test the opsonising effect of these antibodies in vitro using isolated macrophages or a cell line. It should be noted that protection did not prevent the localisation of the *P. multocida* 85020

in tonsilar lymph nodes (Table 2). This indicated that antibodies against the organism may contribute to protection by inhibiting the rapid spread of the organism through the body, a possible role of protective antibodies suggested for infection with *P. multocida* serotype A3 (Ryu & Kim, 2000). In addition, for HS-causing *P. multocida* B:2, latent carrier animals have high levels of antibody, it has also been suggested that these antibodies protect the carrier animals by inhibiting the rapid spread of the organism through the body (De Alwis *et al.*, 1990).

4.3 Immunogenic components

Immunogenic components of *P. multocida* to which antibody were raised during different stages of vaccination and challenge were detected by SDS-PAGE and immunoblotting. About 32 visible protein bands, with approximate molecular weights ranging from 16 to 90 kDa, were detected by SDS-PAGE in CFE of *P. multocida* B:2 strain 85020. The molecular weight of protein bands was estimated by comparison with standard MW markers run in parallel with the samples on the same gel. The SDS-PAGE of the CFE also revealed that two protein bands of approximate sizes of 30 and 37 kDa were predominant, on the basis of the stain intensity and thickness of the bands. Immunoblotting of the CFE, against sera collected from individual calves one week after the second IM vaccination with 10^9 CFU and after challenge, showed the same pattern. In individual sera, seven antigenic components of MW 16, 26, 30, 37, 44, 51 and 53 kDa were detectable. The 53 kDa band, which was visible as a weak band in blots using sera before the first vaccination and was visible for all other sera was not considered as a protective component. The 44 kDa band that appeared 4 weeks after first vaccination, and was also detectable in later sera, was also not considered as a protective antigen because a further experiment using a single vaccination regime showed no protection against challenge implying that this protein was not associated with protection. A protein of 44 kDa along with 37 and 30 kDa proteins were suggested as major immunogens in another study using rabbit hyperimmune serum raised to whole *P. multocida* cells (Pati *et al.*, 1996). Our results are indicative of the presence of antibodies to this 44 kDa protein in unprotected calves. The bands with approximate MW of 51, 37, 30, 26 and 16 kDa, which only appeared one week after the second vaccination and remained after challenge were identified as immunogenic components with possible roles in protection against challenge. As with other Gram-negative bacteria, OMPs of *P. multocida* B:2 have been suggested as possible immunogens (Dawkins *et al.*, 1991a; Pati *et al.*, 1996; Srivastava, 1998). Thus it was considered important to show the ability of JRMT12 to induce antibodies against OMPs. For further

identification of immunogenic components, OMPs of the bacterium were isolated and blotted against calf sera collected one week after second vaccination with 10^9 CFU. Sera obtained from different individual calves showed reasonably similar patterns of blots. SDS-PAGE of the OMP preparation revealed up to ten major and four minor bands. Two strong bands with MW of 37 and 50 kDa, were detected in immunoblots of the OMP preparation. One drawback of the immunogen investigation by immune sera is that antibodies that react only with conformational epitopes, that would be denatured in SDS-PAGE, will not be detected.

For protein identification by mass spectrometry (MS), the 37 and 50 kDa (Figure 20B) bands, as immunogenic components, were excised form the corresponding SDS-PAGE gel. The 30 kDa band, which was the most predominant band of the OMP preparation, on the basis of the stain intensity and thickness of the band, was also chosen for identification by MS. The 30 kDa was identified as OmpH of *P. multocida* with a significant score of 521 (a score of > 63 considered as significant) by MASCOT analysis of the data. The molecular weight of OmpH that the MASCOT search identified was 35 kDa, from *P. multocida* serotype A (strain PM 70, an avian isolate). One reason for this difference in molecular weight of proteins could the unpredictability of the behaviour of membrane proteins in SDS-PAGE. Differences between different serotypes might also be a cause of this difference. The 30 kDa band showed no detectable antigenic reaction on the Western blot although it was the most predominant band in SDS-PAGE of the OMP preparation. Different studies have suggested different degrees of antigenicity for a 30 kDa OMP of *P. multocida* serotype B:2. In a study of an OMP of 31 kDa in *P. multocida* serotype B:2, which was the major band of the OMP preparation, only a faint reaction was found using rabbit hyperimmune serum and this protein was totally unreactive using buffalo immune sera, from animals vaccinated with alum-precipitated vaccine. It was concluded that this protein was only weakly antigenic in nature (Tomer *et al.*, 2002). On the other hand, in another study using rabbit hyperimmune serum, the 30 kDa was designated as one of the three major immunogens of the OMP of *P. multocida* serotype B:2 (Pati *et al.*, 1996). This controversy was also prominent in our study where a 30 kDa band in the CFE showed a strong antigenic reaction (Figure 19) but in an OMP preparation a protein of this size was not able to cause the reaction (Figure 20A). This implies that the 30 kDa protein detected by sera in the CFE was not an OMP and a different protein to that present in the OMP preparation. Surprisingly, the 50 kDa band of the OMP preparation which showed a strong reaction on immunoblot, was also identified as OmpH (see below).

The 37 kDa band was identified as OmpA of *P. multocida* with a significant score of 750. This band on SDS-PAGE was the second major band after 30 kDa and, on the Western blot, revealed the strongest immunogenic reaction, based on the intensity of colour development and thickness of the band (Figure 20A). A 37 kDa band detected as a strong immunogenic antigen in CFE (Figure 19) could have been the same protein present in the OMP preparation. Our results support other studies that have identified a protein of similar size as a main antigenic protein (Johnson *et al.*, 1989; Pati *et al.*, 1996; Tomer *et al.*, 2002). One study showed that an OMP of 37 kDa was one of the chief immunogens that reacted with immune sera from buffalos vaccinated against HS (Tomer *et al.*, 2002). In another study, using rabbit hyperimmune serum, an OMP of 37 kDa was found to be the main antigenic protein in different isolates of HS-related *Pasteurella* (Pati *et al.*, 1996). It can be postulated that the 37 kDa protein identified in our study is the corresponding protein detected in those studies. In those studies, the 37 kDa protein was not subjected to molecular identification, but our results from mass spectrometry showed that this protein was OmpA.

The OmpA protein, which is also called heat-modifiable protein, is an integral component of the OMPs of Gram-negative bacteria. Different functions related to pathogenicity have been attributed to OmpA in different bacteria. In *E. coli,* the OmpA contributes to serum resistance and pathogenicity (Weiser & Gotschlich, 1991). In *P. multocida* serotype A:3 it has recently been shown that the protein is involved in adherence to host cells (Dabo *et al.*, 2003). It has also been suggested that the OmpA may play a role in adaptation of *M. haemolytica* strains to different hosts (Davies *et al.*, 2004).

Our results are supported by recent study on *P. multocida* serotype A (FC-associated) that identified OMPs as immunogenic elements. Studies using genomic and proteomics approaches. In a study using bioinformatic analysis of whole genome of *P. multocida* serotype A, 129 proteins were predicted as outer-membrane proteins, secreted proteins or lipoproteins (Al-Hasani *et al.*, 2007). 105 of the corresponding genes were cloned and recombinant proteins expressed in *E. coli*. To evaluate the range of proteins recognized by the antibody response these recombinant proteins were blotted against pooled serum samples obtained from chickens that had been repeatedly infected and then treated with antibiotics. By database similarity, this study identified 12 immunogenic determinants and 8 of them were recognized as OMPs (including 2 lipoproteins PlpB and Lpp), 3 as hypothetical proteins, and 1 as SurA protein from *Mannheimia*. OmpH was identified as one of the OMPs that reacted with chicken immune serum. OmpA was identified as an OMP reacted with covalescent serum but it also reacted with serum obtained from control

(uninfected) chickens. The use of the same approach, which has been termed reverse vaccinology or reverse immunology, on *P. multocida* serotype B:2 could be informative for identification of immunogenic components, but this method of immunogen discovery requires the whole genome data of this serotype which has not been sequenced yet. However, one drawback of this assay is that it is limited only to the recombinant proteins that can be expressed in vector. Furthermore, a lack of antibody response to the recombinant proteins may be the result of conformational differences after synthesis in *E. coli* compared with native proteins.

Different environmental conditions may cause changes in OMPs composition, as these proteins are critical for interaction with the environment. A study on changes in expression of OMPs of FC-associated *P. multocida* serotype A in response to environmental conditions showed that only 4 proteins exhibited different expression in bacteria isolated from the blood stream of infected chicken (the natural host) compared to bacteria grow in vitro (Boyce & Adler, 2006). Three proteins (TufA, TufB and Pm0803) were upregulated and one (OmpW, an OMP in many Gram-negative bacteria with undefined function) downregulated. In this study, analysis of the OMPs obtained from *P. multocida* grown in vitro (in BHI) under iron-depleted conditions showed that only Pm0803 (97 kDa) was upregulated in response to low iron while one protein was downregulated (OmpW with theoretical mass of 22 kDa) (Boyce *et al.*, 2006). It was concluded by this study that differential expression of these two proteins (Pm0803 and OmpW) is similar to that seen in vitro. The presence of TufA and TufB (components of elongation factor Tu) in the outer membrane is intriguing as under normal conditions they are cytoplasmic proteins. This study might suggest that TufA and TufB are preferentially targeted to the outer membrane under in vivo conditions (Boyce & Adler, 2006). Our experiments using iron-depleted conditions (by adding 2,2'-dipyridyl 150 µmol/ml to BHI) showed that 4 proteins with molecular weight of 97, 95, 82 and 24 kDa were upregulated (presented at higher concentrations based on intensity and thickness of bands on SDA-PAGE gels) and a protein of 26 kDa was downregulated. The 97 or 95 kDa protein could be the same as that identified by Boyce *et al.* (2006). The 82 kDa protein showed the highest increase in response to iron depletion. In a study on HS-associated *P. multocida* an 82-kDa OMP was found that specifically binds bovine transferrin (Veken *et al.*, 1996). However, the OMP extracts prepared from iron-depleted cultures were not included in our experiments, since none of these upregulated proteins showed reaction in Western blotting assays.

Although the research on *P. multocida* has focused on identification of virulence factors and immunogenic components, only a few potentially protective antigens including OmpH

(Luo *et al.*, 1999), PlpB (Tabatabai & Zehr, 2004) and iron-reponsive OMPs (Ruffolo *et al.*, 1998) has clearly been characterized. Although a number of OMPs have been identified in other *P. multocida* serotypes, no comparable data are available for *P. multocida* serotype B:2. Our experiments showed that the vaccination of calves using JRMT12 was able to raise high levels of antibody against different components of the wild type organism. We also identified the 37 kDa component as OmpA to which there was a strong IgG response. It can be suggested that this protein may play a role in protection and would be a potential vaccine candidate for further studies, although immunological reactivity to an antigen does not necessarily indicate a role in protective immunity against HS. Because of the relationship of the OmpA with protection of calves against challenge, this protein could be a candidate for the assessment of the immune status of animals by using it as a coating antigen for ELISA. Recombinant OmpA could be expressed, for example for inclusion in an acellular vaccine containing purified antigens. It can also be suggested that the OmpA may play a role in pathogenesis of HS and can be a candidate for further studies on pathogenesis of the disease.

The identification of the 50 kDa immunogenic protein was unsatisfactory. It was recognised by the MASCOT search as the 35 kDa OmpH of *P. multocida* with a significant score of 218. The reason for identification of the 50 kDa band as OmpH is not clear. The occurrence of OMPs on different locations of SDS-PAGE has also been reported by a recent study on FC-associated *P. multocida* serotype A. The study showed that many proteins were identified from more than one gel slice. In that study the whole length of SDS-PAGE gel was sliced into 8 pieces and OmpH and OmpA were identified from 5 and 8 slices of gels respectively (Boyce *et al.*, 2006). No explanation was given for this observation in that study. As an explanation for the appearance of this band at a location not expected from its molecular weight on SDS-PAGE, the isolated band might be contamination due to smearing of the 35 kDa OmpH protein which was present in high concentrations in the OMP preparation. This may have obscured any faint band on SDS-PAGE, which showed a strong immunogenic reaction, and this protein, present in low concentration, was not then identified by MS. Another possible reason, for the appearance of OmpH as a 50 kDa band on SDS-PAGE of the OMP preparation, is the suggestion that this band was a complex of the OmpH with another, possibly periplasmic, protein which functions in transportation of OMPs. This was shown in *E. coli* where a 17 kDa periplasmic protein bound with high selectivity to the OMPs during SDS-PAGE. This protein is believed to function as a periplasmic molecular chaperone which assists OMPs on their way from cytoplasm to the outer membrane (Chen & Henning, 1996). However, further experiments are needed to identify these OMP chaperones and to prove this

hypothesis, especially as it might be expected that this protein would be present in equimolar amounts to OmpH and therefore detectable by MS. The strong immunogenicity of the band of 37 kDa, deserves further investigation

4.4 Cellular responses

4.4.1 In vivo experiments

The relative contributory roles of cellular and humoral immunity to long-term protection against IIS have not yet been established. Although it is generally believed that humoral immunity is the main component of immunity, the contribution of cell-mediated immunity cannot be excluded. In the third experiment (single IM vaccination regime) the cellular responses of vaccinated and control groups of calves after challenge were studied. A lymphocyte stimulation assay was used to assess the effects of a CFE of *P. multocida* on peripheral blood mononuclear cells (PBMCs) isolated from calves at different times after challenge.

CFE of *P. multocida* did not show any stimulatory effects on PBMCs isolated from vaccinated or control calves at different stages after challenge. The lack of stimulation of PBMCs by CFE could have been due to the lack of a cellular response. In other words, if in animals infected with *P. multocida* there was a cellular response (cell priming) during the week after challenge, the isolated PBMCs should have proliferated in the presence of CFE (as antigen). It might also have been expected that PBMCs from calves vaccinated with JRMT12 would repond to the presence of CFE but this was not observed. In further experiments on *P. multocida* serotype A:3, our results showed that the PBMCs obtained from calves challenged with serotype A:3, proliferated in the presence of CFE, a response that was not observed in similar experiment using PBMCs from calves challenged with serotype B:2. The lymphocyte proliferation assay was also carried out on PBMCs from calves challenged with *P. multocida* serotype A:3 to compare the cellular responses after *P. multocida* A3 challenge (pulmonary pasteurellosis) with *P. multocida* B:2 challenge (septicaemic pasteurellosis). PBMCs obtained from calves challenged intratracheally with *P. multocida* A3 responded to stimulation with the CFE of *P. multocida* A3, but PBMCs of control animals that were injected intratracheally with PBS, showed no response to stimulation. Time course experiments showed that the cellular response to *P. multocida* A3 extract began on the second day after challenge. The cellular response of the PBMCs is indicative of a role for the induction of cellular response in *P. multocida* A3 infected

calves. The rapid response (second day) in this experiment can be an indicative of sensitisation of the innate immune cells and in vitro re-exposure to CFE caused production of proinflamatory cytokines, which subsequently caused activation of these cells. The activation of macrophages obtained from experimentally infected animals by in vitro treatment using different bacterial components has recently been reported by other studies but the exact mechanism are not clear yet (Mueller *et al.*, 2006; Pestka & Zhou, 2006; Powers *et al.*, 2006). A study on *P. multocida* A3 vaccinated mice suggested that the immunity is not cellular (Collins, 1973; Collins & Woolcock, 1976). However, our results are in accordance with a recent study that showed the stimulatory effect of antigen on splenocytes of mice of 8 weeks age vaccinated subcutaneously with LPS-protein complex extracted from *P. multocida* A3. The authors also showed the DTH (delayed type hypersensitivity) reaction in footpads of vaccinated mice two weeks after vaccination as further evidence of cell-mediated immunity. The authors suggested further investigations to determine the cellular response of cattle (Ryu & Kim, 2000). Our results clearly indicated a difference between the two different serotypes of *P. multocida*, which are causative agents of two different diseases, in terms of cellular responses. Interestingly, further experiments showed that the PBMCs obtained from *P. multocida* A3 challenged calves, were also able to respond to CFE of *P. multocida* B:2. This is clearly suggesting antigenic cross-reaction between these two serotypes, a phenomenon that had previously been shown in our ELISA experiments using sera positive for the A3 serotype which also showed that.

Our results are indicative of an immunosuppressive effect of *P. multocida* B:2. Unresponsiveness of lymphocytes after challenge may have a role in the pathogenesis of HS. The lack of response could be a result of a direct effect of the bacterium on the cells of the immune system. The injected bacteria in the challenge dose are likely to be taken to the regional lymph nodes by macrophages. It was observed a short time after subcutaneous injection of the challenge dose in the right shoulder area, that the right prescapular lymph node became swollen. Bacteriological investigations post-mortem also showed that tonsils and retropharyngeal lymph nodes were the main areas where of the organism lodged after challenge. In these lymphatic organs, the organism would be in close contact with lymphatic cells. The natural route of infection is obviously different, which is by inhalation and/or ingestion, and the initial site of multiplication is the tonsilar region. Artificial intranasal and oral infections result in a longer course of the disease and more profound lesions while subcutaneous injection results in a rapid onset, shorter course and fewer pathological lesions (De Alwis, 1999). The differences in route of infection are likely to have an impact on the immunosuppressive effect we observed.

In parallel to the assessment of the stimulatory effects of CFE of *P. multocida* B:2, the proliferative response of isolated PBMCs to ConA was also assessed (lymphocyte proliferation assay), as a control to measure the proliferative potency of PBMCs. Proliferation of lymphocyte populations after stimulation with non-specific mitogens is used for assessment of cell-mediated immunity (Karol, 1998), and inhibition of the proliferative response caused by different parasites (Onah *et al.*, 1998; Scott *et al.*, 1999), bacteria (Majury & Shewen, 1991a; Majury & Shewen, 1991b; Park *et al.*, 1993), bacterial components (Chen *et al.*, 2000; Vanden Bush & Rosenbusch, 2004) or chemical agents (Franklin *et al.*, 1991; Gummert *et al.*, 1999; Mehrotra *et al.*, 2002; Peltier *et al.*, 2000) has also been studied. Our experiments showed that PBMCs of animals challenged with *P. multocida* B:2, in both vaccinated and non-vaccinated groups, transiently lost their ability to proliferate in response to ConA. In both vaccinated and control groups, the mean SI (stimulation index) as a response to ConA had decreased significantly at 12 h after challenge and reached the lowest level at 3 days after challenge. In control animals, 7 days after challenge the mean SI increased to around the pre-challenge value. The results were indicative of a possible suppressive effect of challenge with *P. multocida* B:2 on calf PBMCs. This observation, which does not appear to have been reported previously, is in keeping with the lack of response of PBMCs from challenged animals to repond to CFE. More experiments were undertaken to investigate the suppressive agent(s) and the identification of the suppressed cells.

It has been shown that high levels of acetate, which happens in a metabolic disorder of cattle called ketosis, were able to suppress the in vitro proliferative response of PBMCs to ConA, and it was suggested this may alter the immune response in vivo (Franklin *et al.*, 1991). High levels of steroids in the serum of diseased calves could also be a possible cause of suppression in PBMCs as it has been suggested as the reason for temporarily unresponsivness of calf lymphocytes to mitogens around the time of birth (Tizard, 2000). Unfortunately little data are available for pathophysiological changes caused by HS. In one study, LPS was administrated to buffalo calves to produce an HS clinical syndrome. The study showed that endotoxic shock, similar to that seen in other septicaemic clinical conditions, constituted the main clinical changes, which were similar to HS (Horadagoda *et al.*, 2001). It was shown that LPS promoted an acute phase response, including production of tumor necrosis factor-α (TNF-α) and leucopenia (Horadagoda *et al.*, 2002). Leucopenia as a consequence of the disease could result in the absence of a proliferative response in PBMCs obtained from diseased calves. Leucopenia may result in the absence or reduction of the fraction(s) of cells proliferating in response to ConA or play critical role in the

proliferative response. Further experiments using FACS analysis on identification of changes in population of different cells fractions after onset of HS could be informative.

4.4.2 In vitro experiments

As it was not possible to do further in vivo experiments, but as it appeared that *P. multocida* B:2 was having a suppressive effect on PBMC response, it was decided to test the CFE of *P. multocida* 85020 in vitro to see if this contained components that might cause the in vivo effect. To do this, an in vitro system was set up in which calf PBMCs obtained from a normal calf were treated with CFE before adding ConA. Preliminary experiments, using different concentrations of CFE, showed that 10µg/well was the optimal concentration of CFE in which PBMCs (2×10^5/well) grew normally but where the proliferative response to ConA was suppressed. To find out the optimum concentration of ConA for in vitro assay, further experiments using different concentrations of ConA showed that the 1 µg/well was the optimum concentration with which PBMCs were able to proliferate strongly and the suppressive effect of CFE was also observed. In the in vitro experiments, it was shown that the proliferative response of normal PBMCs to ConA was 60-100 times that of non-stimulated control cells and that, was markedly suppressed when PBMCs were incubated with the optimal concentration of CFE of *P. multocida* B:2 for one hour before adding the ConA. Different experiments showed that the addition of CFE caused a 3-5 fold decrease in the proliferative response to ConA. The conditions developed for the in vitro assay were taken as the standard method and the same protocol was followed in all other in vitro experiments.

To avoid difficulties of obtaining calf blood and also difficulties with PBMCs primary cell culture, it would have been convenient to substitute the PBMCs primary culture with a cell line. To be used as a substitute of PBMCs, the cell line should be able to proliferate in reponse to ConA and also this prolferative response should be suppressed after treatment with *P. multocida* B:2 CFE. A number of cell lines were available and were tested for their ability to proliferate in response to ConA. Three different cell lines including J774 (a mouse macrophage), BL-3 (a bovine B cell), and Jurkat (a human T cell) were tested. It was unfortunate that none of these three cell lines proliferated in response to ConA. Further experiments, testing different cell lines, could be helpful. Another possible substitute for calf PBMCs could be the PBMCs obtained from other small laboratory animals, as these are more accessible than calf PBMCs. Our preliminary experiments using rabbit PBMCs showed that they proliferate strongly in response to ConA. Our further experiments also showed that the proliferative response of rabbit PBMCs was suppressed

after adding *P. multocida* B:2 to them. Although rabbit PBMCs showed promising results as a potential substitute for calf PBMCs, it was decided to continue the experiments on calf PBMCs as it was not clear to us that the same mechanisms are involved in the suppression of these two different PBMCs. Further experiments are needed to show that the same mechanism are involved in suppression of these two different PBMCs.

To study if the suppressive effect of *P. multocida* B:2 CFE was specific for this strain, CFEs of a number of other bacteria were prepared and used in the in vitro assay on calf PBMCs. CFEs obtained from *Staphylococcus aureus* or *Manheimia haemolytica* serotypes A1 (bovine isolate) or A2 (ovine isolate) did not affect the proliferative response of PBMCs to ConA. CFE of *Escherichia coli* slightly increased the proliferative response. *P. multocida* serotype A3 CFE had a slightly less effect on the proliferative response than *P. multocida* B:2 CFE. A serotype A (chicken isolate) strain of *P. multocida* had no effect. CFE of *P. multocida* serotype D (pig isolate) strongly suppressed the proliferative response of calf PBMCs to ConA. Overall, the results indicated that the suppressive effect was only observed in two other *P. multocida* strains, one *P. multocida* serotype D (the causative agent of atrophic rhinitis in pigs) and the other, with less suppressive properties, *P. multocida* serotype A3 (the causative agent of pneumonia in cattle). *P. multocida* D produces a dermonecrotic toxin which is the main cause of atrophy in the nasal turbinate bones, the most important consequence of the disease (Jordan *et al.*, 2003). A contributory role of the toxin in the suppression of proliferation of PBMCs could be investigated. *P. multocida* serotype A3 is associated with pneumonic pasteurellosis of cattle. The pathogenesis of the disease caused by this organism, like that of HS caused by *P. multocida* B:2 strains, is not well understood.

4.4.2.1 Characterisation of suppressive agent(s)

After the observation of in vitro suppressive activity of CFE of *P. multocida* B:2, further investigations were focused on two main subjects; preliminary characterisation of the suppressive agent(s) and identification of the suppressed cells. For characterisation of the suppressive component(s), *P. multocida* B:2 CFE was investigated for its suppressive activity after different treatments. Heating at 80°C for 5 min completely destroyed the suppressive effects. In vitro experiments also showed that an OMP preparation of *P. multocida* B:2 markedly suppressed the proliferative response of PBMCs to ConA. The suppressive activity of OMP was again partially destroyed by heating. Dialysis of CFE with a cut off 10,000 Da was still suppressive for calf PBMCs. Capsule of *Pasteurella*, which is known to be polysaccharide, was possibly one of the constituents of CFE, but a

polysaccharide capsule fraction, or the supernatant of an 18-hour broth culture of *P. multocida* B:2, was not suppressive and increased the proliferative response of PBMCs to ConA. The lack of suppressive activity in broth culture medium indicated that the suppressive agent(s) had not been secreted and/or released during growth of the bacterium. All together, these data suggested that the suppressive agent(s) of CFE, or at least an active part of the agent, which was heat-sensitive, was made of protein. The suppressive properties of the OMP preparation indicated the presence of the agent in this cell fraction. The suppressive effect of OMP was only partially destroyed by heating; one possible reason for this could be the contamination of the OMP with some traces of sarkosyl used during the process of extraction. Sarkosyl is a strong detergent and resistant to heat, and has detrimental effects on cells. Although the OMP was washed several times after sarkosyl treatment, it is quite possible that there were still some traces left in the OMP preparation. The suppressive activity of dialysed CFE showed that the suppressive agent(s) had a molecular weight higher than 10,000 Da, which was the dialysis membrane cut-off.

The effect of LPS on calf PBMCs was also investigated because it was quite likely that the CFE prepared from *P. multocida* B:2 would contain LPS. To do this, PBMCs were treated with LPS purified from *E. coli* before adding ConA. LPS at the concentration of 200 EU/well was not suppressive and did not cause any changes in proliferative response of PBMCs but, at higher concentrations, LPS showed a synergism with ConA in promoting proliferative effects of PBMCs. LPS is known as a B cell proliferative agent and, as endotoxin, it also could have some adverse effects on different cells. It was unlikely that the LPS content of CFE had adverse effects on PBMCs because the CFE alone did not cause any decrease in growth rate of PBMCs at the concentration of 10µg/well used. At lower concentrations a slightly increased proliferative response of PBMCs (2-3 times) to CFE was observed which may have been the result of proliferation of the B cell fraction of PBMCs in response to the LPS content of cel-free exract, a phenomenon that was also supported by the FACS analysis results (see below). To exclude the possibility of proliferation of B cells at the expense of other types of cell in PBMCs, further experiments using FACS analysis showed that the CFE did not cause any changes to other cell populations (see below). Although using LPS purified from *P. multocida* could have been more relevant to the possible LPS effects of CFE, the data obtained using LPS of different origin excluded the possibility of adverse effects of LPS on PBMCs. In another study it was shown that *E. coli* LPS alone at 10-0.1 ug/ml induced proliferation of cattle PBMCs (Stevens *et al.*, 1997). It has also been shown that LPS purified from *P. multocida* A3 was proliferative for mouse B cells (Ryu & Kim, 2000).

4.4.2.2 Identification of proliferated and suppressed cells

FACS analysis was used to determine which population of lymphocytic cells was affected over the course of the in vitro experiments. B cell and T cell subpopulations ($CD4^+$, $CD8^+$ and $\gamma\delta^+$) in normal calf PBMCs were identified and the changes in their relative proportions after different treatments was investigated. The ratio of proliferating and non-proliferating fractions was also determined, to assess the proliferative status of each population. The proportion of cells in different generations of each population was also determined using FACS analysis on CFSE loaded cells. FACS experiments on CFSE-loaded and unloaded cells was conducted in parallel to the lymphocyte proliferation assay (standard in vitro procedure) and at different stages of the in vitro proliferation assay.

FACS analysis of PBMCs showed that the overall percentage of B cells increased over the course of in vitro experiment on suppressive effects of *P. multocida* B:2 CFE and the increase was at highest levels in cultures treated with CFE+ConA or CFE alone in which the percentage was double that of the control cells. Comparing the B cell response in control cells with ConA-treated cells revealed that ConA did not cause any B cell responses. The overall percentage of B cells increased by 1.92 times in CFE+ConA – treated cells and by 1.88 times in CFE-treated cells compared to control cells. This indicated that the increase in overall percentage of B cells was due to the presence of CFE. It seemed that the proliferative response of B cells to CFE happened in the early stages of culture because the overall percentage of B cells increased but the proliferating fraction of B cells was low at the time of harvest. This idea is supported by a study indicating the early proliferative response of B cells to synthetic oligodeoxynucleotides (ODN, a B cell proliferative agent). The study also showed that the B cells of bovine PBMCs did not proliferate in response to ConA (Pontarollo *et al.*, 2002). It can be concluded that B cells do not respond to ConA and consequently are not involved in the suppression of ConA-induced proliferation of PBMCs by CFE.

FACS analysis results of $CD4^+$ T cells indicated that the overall percentage of $CD4^+$ cells decreased during the experiment under all treatments. Comparison between ConA-treated and CFE+ConA-treated cells revealed that the proliferating fraction of $CD4^+$ cells was markedly decreased due to the presence of CFE. ConA increased the percentage of proliferating $CD4^+$ compared to the control cells by 10-fold, but by only 3-fold with CFE+ConA. ConA apparently stimulated $CD4^+$ cells in a delayed manner because on the day of harvest (3^{rd} day) the proliferating fraction had increased, although the overall percentage had increased only slightly. The delay in response may be the consequence of

the indirect response of T cells to ConA as it has been shown that the mitogenic response of T cells is through the production of IL-2 by monocytes (see section 10.3.2) and the expression of the mature IL-2 receptor (CD25) by T cells (Yamanaka *et al.*, 2003). Comparison between control and CFE-treated cells revealed that the percentage of $CD4^+$ cells was not affected by CFE indicating that there was no toxic effect of CFE on these cells, which meant that the suppression of the proliferative response to ConA could not be accounted for by toxicity. It can be concluded that $CD4^+$ cells responded to ConA in terms of proliferation and that this response was suppressed in the presence of CFE.

The overall percentage of $CD8^+$ T cells showed only slight changes during the experiment under all treatments. However, the proliferating fraction of $CD8^+$ cells increased markedly in response to ConA (6 fold compared to control cells) but this was not so apparent in CFE+ConA-treated cells. Comparison between control and CFE-treated cells revealed that the overall percentage of $CD8^+$ cells and also the proliferating fraction of these cells was decreased by CFE. It can be concluded that CFE had some adverse effects on this cell population and the suppression in the proliferative response to ConA could have been partly due to this effect on $CD8^+$ cells.

FACS results for $\gamma\delta^+$ T cells indicated that, in contrast to other T cell subpopulations, the overall percentage of this population increased slightly during the experiment except in CFE-treated cells. The proliferating $\gamma\delta^+$ fraction increased in response to ConA by up to 10 fold compared to control cells and this fraction was slightly lowered (to an 8.42 fold increase) in CFE+ConA-treated cells. Comparison between control and CFE-treated cells showed that the proliferative fraction of $\gamma\delta^+$ cells was not affected by CFE although the overall percentage of this population was slightly decreased indicating that the decrease happened in the early stages of culture. These data are indicative of a slight suppression by CFE of the proliferative response of $\gamma\delta^+$ T cells to ConA, perhaps due to a slight toxicity of CFE on these cells.

CFSE-loaded PBMCs were also analysed by FACS experiments to follow the changes in the different generations of each population during in vitro assay. The changes in the percentages of different cell populations showed the same pattern as observed for CFSE-unloaded cells with the exception of the B cells that were low at time zero compared to the previous experiment. It was believed that this dramatic decrease in the percentage of B cells may be due to the high sensitivity of these cells to the toxic effects of CFSE, at the concentration used in our experiment. The data obtained for B cells were not analysed, but data obtained for other cell populations were considered acceptable because the results of

the previous experiments showed that B cells did not play a direct role in ConA proliferation and suppression by CFE. Comparison of the data for the other populations showed that the results of CFSE-loaded T cell subpopulations are almost at the same ratios, with the exception of ratios for the control $CD4^+$ cells at time zero. The exact reason for this difference was not clear but the effects of CFSE could be the cause of this difference. Because the data for $CD4^+$ cells were similar to the previous experiment, these data were taken as acceptable for further analysis. Data obtained for CFSE-loaded PBMCs were elaborated by software ModFit® to study the frequency of different generations in each population.

Analysed results showed that, $CD8^+$ T cells proliferated strongly in response to ConA (Proliferation Index= 3.26). The proliferative response of the $CD8^+$ T cells to ConA cells was suppressed by CFE (Proliferation Index=1.77). The nonproliferative fraction showed a 3.35-fold increase in CFE-treated $CD8^+$ T cells, which indicated more than a three-fold increase in the fraction of cells that have not proliferated in response to ConA. The parent generation increased by two times in CFE treated $CD8^+$ T cells although all of the daughter generations decreased rationally compare to CFE untreated cells (Figure 27B). Analysis of data for $CD4^+$ T cells indicated that this subpopulation of T cells proliferated in response to ConA (Proliferation Index=2.54), and the nonproliferative fraction of $CD4^+$ T cells increased by 1.55 in response to CFE+ConA treatment. The decrease in proliferation of CFE+ConA-treated $CD4^+$ cells started from generation 2 but was most marked in generation 3 which constituted 17.28 % of the whole $CD4^+$ population. $\gamma\delta^+$ T cells showed proliferation in response to ConA similar to $CD4^+$ T cells (Proliferation Index=2.56) and, although the nonproliferative fraction of $\gamma\delta^+$ T cells increased by 1.46 fold in CFE+ConA-treated cells, progress through the generations was not as adversely affected as with $CD4^+$ and $CD8^+$ T cells.

4.4.3 Conclusions from cellular responses

The in vitro experiments showed that CFE of *P. multocida* had suppressive effects on the proliferative response of normal calf PBMCs to Con-A. It was also shown by FACS analysis experiments that $CD4^+$ and $CD8^+$ T cells were the principal cells which proliferated in response to Con-A, and were those suppressed by a CFE of *P. multocida*. The mechanism of the immunosuppression described here remains unclear and demands further study. The exact mechanism involved in in vitro T cell proliferation by ConA is poorly understood. Concanavalin A (ConA) belongs to the family of lectins (carbohydrate-binding proteins derived from plants and bacteria). It has potent T-cell mitogenic activity

and has been extensively used to study the in vitro lymphocyte response (Mukherjee *et al.*, 2005). Mitogen stimulation of lymphocytes in vitro is believed to mimic stimulation by specific antigens fairly closely. Stimulation of T cell receptor with ConA induces a signal transduction pathway which stimulates IL-2 secretion and upregulation of the α subunit of the IL-2 receptor, CD25 (the β and γ subunits are constitutively expressed). Secreted IL-2 functions in an autocrine and paracrine manner by binding to the full receptor (α, β, and γ subunits) to initiate proliferation of stimulated lymphocytes (Peltier *et al.*, 2000). IL-2 is a crucial element in cell-mediated immune responses both in vivo and in vitro and, in cattle, IL-2 levels can be used to measure specific cellular immune responses (Miller-Edge & Splitter, 1986). Stimulation of bovine lymph node cells with ConA causes a biphasic IL-2 secretion. The first phase of IL-2 production occurs after 3 hours and the second phase between 10 and 16 hours. This study also showed the accumulation of IL-2 mRNA before each phase of IL-2 secretion (Weinberg *et al.*, 1988). In the 2-signal model of mitogen activation of T cells, the first signal occurs with mitogen and monocytes (another population of cells in PBMCs) which cause the production of second signal that is IL-2 (Gelfand *et al.*, 1985). IL-1 which is secreted by monocytes acts as the first signal and induces the expression of IL-2 receptors in a subpopulation of T cells which, after activation by IL-2, culminates in DNA synthesis and proliferation by these cells (Conti *et al.*, 1991a; Conti *et al.*, 1991b). In vitro experiments on bovine PBMCs have indicated that monocytes are necessary for optimum stimulation of T cells (Pontarollo *et al.*, 2002). According to the 2-signal model of T cell activation, suppression could be at the point of IL-2 secretion and/or induction of CD25. The mechanism of suppression by ovine uterin serpin (OvUs; an immunosuppressive protein secreted by the ewe uterus during pregnancy) on proliferative response of PBMCs has been investigated. This study showed that OvUs inhibited ConA-induced expression of CD25 in $\gamma\delta^-$ T cells but not $\gamma\delta^+$ T cells. The study also revealed that OvUs was also inhibitory to lymphocyte proliferation induced by human IL-2. It was concluded that OvUs suppressed lymphocyte proliferation by blocking CD25 expression. It was also suggested that the lack of suppressive effect of OvUs on ConA-stimulated CD25 expression in $\gamma\delta^+$ T cells may indicate a different mechanism of activation of these cells or insensitivity to inhibition by OvUs (Peltier *et al.*, 2000). This study supports a role for CD25 in the suppression observed here, since the in vitro experiments showed that *P. multocida* CFE inhibited the proliferative response in $\gamma\delta^-$ T cells (including $CD4^+$ and $CD8^+$ T cells) but not $\gamma\delta^+$ T cells. More experiments are needed to find out the exact mechanism of suppression. Experiments to study the changes in IL-2 concentrations during suppression and investigation of CD25 expression in different T cell subpopulations would be helpful in this regard.

The relevance of the in vitro suppressive effects of *P. multocida* CFE and in vivo immunosuppression, observed in challenged calves, is not clear as these observations may be due to two different phenomena. In vitro suppressive properties of CFE are more likely to be clinically reflected as a chronic manifestation but the progress of disease in HS is usually acute. Although *P. multocida* B:2 is known as the causative agent of HS, the organism exists in the tonsils and nasopharynx of some apparently healthy carrier cattle and buffaloes (De Alwis, 1999). Carrier status is actually a chronic infection with the bacterium but how the organism survives in carrier host tonsils, which are in close contact with lymphatic organs, is not yet understood. Our in vitro experiments showed that *P. multocida* contains a component(s) with the potency to inhibit the activity of T cells. The suppressive properties of the organism would be a possible explanation of its survival in carrier animals (see below). Other possible consequences of immunosuppression can be in converting a latent carrier to an active carrier. In the active carriers, the bacterium presents in nasopharynx and, from this site, is shed in nasal secretions. Latent carriers harbour the organism in tonsils in which the bacterium is believed to multiply and intermittently spill over into nasopharynx (De Alwis *et al.*, 1990). The mechanisms involved in converting a latent carrier to an active carrier have not been revealed yet. It was shown that stressful conditions and even administration of immunosuppressive preparations (dexamethazone and methylprednisolone acetate) were not able to cause latent carriers to shed the organism in their nasal secretions (De Alwis, 1999).

Bacterial pathogens have acquired the ability to overcome host defence mechanisms (Brunham *et al.*, 1993). Different bacterial pathogens have adopted different strategies to interfere with host innate and adaptive immune mechanisms (Hornef *et al.*, 2002). Different bacterial components and products play different roles in modulating host immune reactions, most of them stimulatory but a minority are inhibitory. It has been suggested that every component of bacteria with adjuvant activity can also impose suppressive effects on the immune system by appropriate manipulation of timing, dose, and selection of antigen (Schwab, 1975). Virulent Gram-negative bacteria (e.g. *Salmonella, Shigella, Pseudomonas aeruginosa*), which are able to cause septicaemia and endotoxaemia, affect the immune system by the suppression of phagocytosis and cell-mediated immunity (Vorob'ev *et al.*, 2001). The in vitro immunomodulatory effects of *P. multocida* serotype D (the causative agent of atrophic rhinitis) was investigated in a study on mouse splenocytes. This study showed treatment of splenocytes with each of purified porin or LPS stimulated IL-1α, IL-6, TNF-α, IFN-γ and IL-12 mRNA while no effect was detected on IL-4 and IL-10. It was concluded that *P. multocida* porin was able to modulate immunological responses by affecting the release of different cytokines (Iovane *et al.*,

1998b). The immunosuppressive properties of *P. multocida* D was also shown in another study where intranasal challenge of pigs and mice with a toxigenic strain or its CFE reduced the IgG response of these animals (Jordan *et al.*, 2003).

The immunomodulatory effects of *P. multocida* B:2 have not been well studied. It has been shown that OMP of *P. multocida* B:2 is antiphagocytic as OMP was able to interfere with phagocytosis of opsonised *Candida albicans* by murine peritoneal cells in vivo. It was suggested that the antiphagocytic mechanisms induced by OMP enhanced the virulence of the organism (Srivastava, 1998). The immunosuppressive effects of *Yersinia*, which is close taxonomically to *Pasteurella*, have been extensively studied and the mechanisms involved in T cell suppression have been recently revealed. *Yersinia pseudotuberclosis* (the causative agent of gastroenteritis and lymphadenitis in humans) is likely to be in contact with lymphocytes in the intestinal Peyer's patches, lymph nodes, spleen and liver in which the organism colonises and multiplies. Preliminary studies reported that T cells transiently exposed to *Yersinia* were suppressed for TCR (T cell receptor) stimulation (Yao *et al.*, 1999). Further in vitro studies showed that YopH (*Yersinia* outer protein H), which was known as being antiphagocytic and had protein tyrosine kinase activity (Sing *et al.*, 2002), specifically suppressed T cells by targeting the adaptor molecules LAT (linker for activation of T cells) and SLP-76 (SH2-domain-containing leukocyte protein of 76 kDa) (Gerke *et al.*, 2005). As mentioned earlier, *P. multocida* B:2 exists in the tonsils of carrier animals without being affected by the destructive effects of the immune system. Our in vitro experiments indicated suppressive effects of a protein component(s) of CFE which also existed in an OMP preparation of *P. multocida* B:2. Our results indicate suppressive effect on T cells which, if they are similar to the immunosuppressive effects of *Yersinia* OMP on T cells, could account for the resistance of the bacterium against immunity elements in the tonsils. This observation may be applicable to understanding the pathogenicity of *P. multocida* B:2 and the development of the immune response during infection. Interactions between host cells and pathogens are extremely complex and the involvement of multiple inter-dependent pathways in signalling and regulatory systems has made it very difficult to study these interactions. Therefore, it is difficult to predict the combined behaviour by just investigating the interactions between bacteria and individual components of the immune system. However, these investigations can be informative and helpful in understanding the process of disease development. They can also provide information on mechanisms that the pathogen uses to overcome or escape the host immune system.

APPENDICES

Appendix A: Medium and solution composition for bacterial growth

A.1 Brain heart infusion (BHI broth)

Brain heart infusion, dehydrated (Oxoid)	37 g
Distilled water	1000 ml

The medium was dissolved, dispensed and autoclaved at 121°C for 15 min and, after overnight incubation at 37°C, kept at 4°C.

A.2 Nutrient broth (NB)

Nutrient broth, dehydrated (Oxoid)	13 g
Distilled water	1000 ml

The medium was dissolved, dispensed and autoclaved at 121°C for 15 min and, after overnight incubation at 37°C, kept at 4°C.

A.3 Sheep blood agar (SBA)

Blood agar base, dehydrated (Oxoid)	40 g
Distilled water	1000 ml

The medium was suspended in water and brought to the boil to dissolve completely, then sterilised by autoclaving at 121°C for 15 min. It was cooled to 45-50°C and 5% sterile defibrinated sheep blood (B&E laboratories) added. The medium was mixed and poured into sterile plates.

A.4 Peptone water

Peptone water dehydrated (Oxoid)	15 g
Distilled water	1000 ml

The medium was dissolved and autoclaved at 121°C for 15 min and, after overnight incubation at 37°C, kept at 4°C.

A.5 Vancomycin stock solution

Vancomycin	1 g
Distilled water	10 ml

The stock solution was filtered through a 0.2 µm-pore-size membrane (Sartorius) and stored at –20°C. It was added to medium to a final concentration of 1 mg/ml.

A.6 10 × Phosphate-buffered saline (PBS)

NaCl	1.7 M
KCl	100 mM
$Na_2HPO_4.12H_2O$	40 mM
KH_2PO_4	10 mM

Distilled water to 1000 ml. The solution was autoclaved at 121°C for 15 min and kept at 4°C. It was diluted 1/10 and adjusted to pH to 7.2 before use.

A.7 Dipyridyl stock solution

2,2'-Dipyridyl (Sigma)	20 mM

The stock solution was filtered through a 0.2 µm-pore-size membrane (Sartorius) and stored at 4°C. It was added to BIII broth to a final concentration of 150 µM. The medium was kept overnight at 4°C before use.

Appendix B: Protein analysis solutions

B.1 5 × loading buffer

Glycerol	5ml
20% SDS	2.5 ml
2-mercapto-ethanol	0.5 ml
1 M Tris-HCl (pH 6.8)	2.5 ml
Bromophenol blue	0.25% (w/v)

B:2 Stacking solution

30% Acrylamide/Bis (37.5:1) (BioRad)	9 ml
1 M Tris-HCl (pH 6.8)	7.5 ml
distilled water	42 ml
10% (w/v) SDS	50 µl

B:3 Resolving gel (10%)

30% Acrylamide/Bis (37.5:1) (BioRad)	3.3 ml
1 M Tris-HCl (pH 8.8)	3.75 ml
distilled water	3 ml
10% (w/v) SDS	100 µl
10% (w/v) Ammonium persulphate	100 µl
TEMED (add last)	10 µl

Enough for casting 4 small gels.

B:4 10 × Running buffer

Tris-Base	0.25 M
Glycine	1.92 M
SDS	0.035 M

Distilled water to 2000 ml. Diluted 1 in 10 before use.

B:5 Coomassie Blue stain/Destain solution

Coomassie Blue	0.05% (w/v)
Methanol	500 ml
Glacial acetic acid	100 ml
Distilled water	400 ml

For making destain solution, Coomassie Blue was omitted.

B.6 Solutions for Modified Lowry's protein assay

B.6.1 Reagent A

Na_2CO_3	2 g
NaOH	0.4 g
Sodium tartrate	0.16 g
SDS	1 g
Distilled water	100 ml

B.6.2 Reagent B

$CuSO_4.5H_2O$	4 g
Distilled water	100 ml

B.6.3 Reagent C

Reagent A	100 ml
Reagent B	1 ml

Soultions A and B are stable indefinitely at room temperature. On day of use reagent C is prepared and, Folin-Ciocalteu phenol reagent is dilute 1 to 1 with distilled water.

B.7 Standard protein solution (BSA in PBS)

Bovine serum albumin (BSA, Sigma)	20 mg
PBS (Appendix A.6)	10 ml

Aliquots of solution were kept in –70°C. Different concentrations were prepared with PBS.

B:8 Sarkosyl solution

N-lauroylsarcosine sodium salt (Sigma)	500 mg
Distilled water	100 ml

Appendix C: Solutions for immunoblotting

C. 1 10 × Transfer buffer

Tris-Base	0.25 M
Glycine	1.92 M

Distilled water to 2000 ml. Diluted 1 in 10 before use and made up with 20% (v/v) methanol.

C.2 PBST

1 × PBS	Appendix A.6
Tween 20	0.2% (v/v)
pH 7.4	

C.3 Blocking buffer

PBST	Appendix C.2
Skimmed milk (Marvel)	10% (w/v)

C.4 PBST diluent

PBST	Appendix C.2
Skimmed milk (Marvel)	5% (w/v)

C.5 Substrate solution

3'3-Diaminobenzidine (DAB) (Sigma)	0.05 g
PBS	98 ml
1% (w/v) $CoCl_2$	2 ml
30% (v/v) H_2O_2	100 µl

Appendix D: ELISA solutions

D.1 Carbonate-bicarbonate coating buffer (pH 9.6)

Carbonate-bicarbonate (Sigma)	1 capsule
Distilled water	100 ml

Freshly prepared before use (pH 9.6).

D.2 Washing buffer (PBS-Tween)

1 × PBS	Appendis A.6
Tween 20	0.05% (v/v)

D.3 ELISA Blocking buffer

Gelatin (Sigma)	100 µg
PBS	100 ml

Made up to 100 ml and warmed in water bath with stirring 60°C for 1 h.

D.4 Stop solution

Sulphuric acid	0.25 M

Appendix E: Cell culture solutions

E.1 RPMI complete

Heat-inactivated FBS* (BioWest)	10% (v/v)
L-Glutamine (Gibco)	2 mM
Penicillin (Moredun Research Institute)	50,000 U
Streptomycin (Moredun Research Institute)	50 mg
Nystatin (Moredun Research Institute)	0.5% (v/v)
Gentamicin (Sigma)	1% (w/v)
HEPES (Sigma)	30 mM

* Foetal Bovine Serum

Added to 410 ml RPMI 1640 medium (Life Technologies).

E.2 Concanavalin A (ConA)

ConA (Sigma)	5 mg
RPMI 1640	10 ml

Aliquot of 200 µl kept in –20°C. For use, mixed with 800 µml RPMI 1640, remainder on each occasion was discarded.

E.3 10 × RBC lysis buffer

NH_4Cl	1.5 M
$NaHCO_3$	0.1 M
diNaEDTA	1 mM
distilled water	900 ml

Adjusted to pH 7.4 with 1N HCl or 1N NaOH. Made up to 1000 ml and stored up to 6 month at 4°C. Diluted 1 to 10 for use, kept on ice, unused diluted buffer discarded.

Appendix F: FACS solutions

F.1 CFSE (carboxyfluorescein diacetate succinimidyl ester) stock solution

CFSE in PBS	20mM

Aliquots of 20 µl kept in –20°C. For use, each vial was mixed with 40 µl of prewarmed (37°C) PBS to reach to working concentration of 10 µM.

F.2 Wash buffer

FBS	2 ml
Penicillin	10,000 U
Streptomycin	10 mg
HBSS*	98 ml

*Hanks Balance Solution (Sigma)

F.3 FACS buffer

FBS	5 ml
10% (w/v) NaN_3	2 ml
PBS	93 ml

F.4 FACS Fix

40% (v/v) Paraformaldehyde	2.5 ml
PBS	97.5 ml

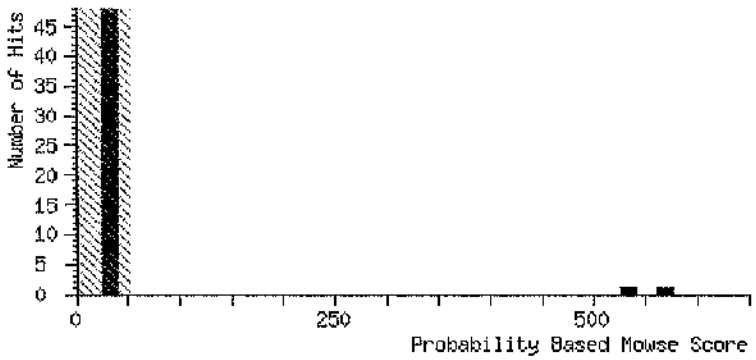
Aliquots of paraformaldehyde kept at –20°C. PBS warmed to 60°C before addition and thorough mixing.

Appendix G: Mascot and BLAST search results

G.1 Mass Spectrometry results used to determine the peak list for the 30 kDa OMP band.

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 52 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



G.1.1 Peptide Summary Report for 30 kDa OMP

1. gi|32363216 **Mass:** 37084 **Score:** 569 **Queries matched:** 23
   Major outer membrane protein precursor (MOMP) (Outer membrane protein H)
2. gi|2853242 **Mass:** 34935 **Score:** 521 **Queries matched:** 20
   Outer membrane protein; OmpH [*Pasteurella multocida*]

G.1.2 Amino acid sequence coverage for 30 kDa OMP

G.1.2.1 Match to: gi|32363216 Score: 569

1. gi|32363216 **Mass:** 37084 **Score:** 569 **Queries matched:** 23
   Major outer membrane protein precursor (MOMP) (Outer membrane protein H)

Sequence Coverage: **38%**

1 MKKTIVALAV AAVAATSANA ATVYNQDGTK VDVNGSVRLI LKKEKNERGD
51 LVDNGSRVSF KASHDLGEGL SALAYAELRF SKNEKVEVKD AQNQQVVRKY
101 EVERIGNDVH VKRLYAGFAY EGLGTLTFGN QLTIGDDVGV SDYTYFLGGI
151 NNLLSSGEKA INFKSAEFNG FTFGGAYVFS ADADKQAPRD GRGFVVAGLY
201 NRKMGDVGFA LEAGYSQKYV TAAAKQEKEK AFMVGTELSY AGLALGVDYA
251 QSKVTNVEGK KRALEVGLNY DINDKAKVYT DLIWAKEGPK GATTRDRSII
301 LGAGYKLHKQ VETFVEGGWG REKDANGVTT KDNKVGVGLR VHF

NCBI BLAST search of gi|32363216 against nr

Taxonomy: *Pasteurella multocida*

Links to retrieve other entries containing this sequence from NCBI Entrez:

gi|2853238 from *Pasteurella multocida*

gi|92918682 from *Pasteurella multocida*

gi|92918684 from *Pasteurella multocida*
gi|92918686 from *Pasteurella multocida*
gi|92918688 from *Pasteurella multocida*
gi|124262427 from *Pasteurella multocida*

G.1.2.2 Match to: gi|2853242 Score: 521

2. gi|2853242 Mass: 34935 Score: 521 Queries matched: 20

Outer membrane protein; OmpH [*Pasteurella multocida*]

outer membrane protein; OmpH [*Pasteurella multocida*]
Nominal mass (M_r): 34935; Calculated pI value: 9.17
NCBI BLAST search of gi|2853242 against nr
Taxonomy: *Pasteurella multocida*

Sequence Coverage: 38%

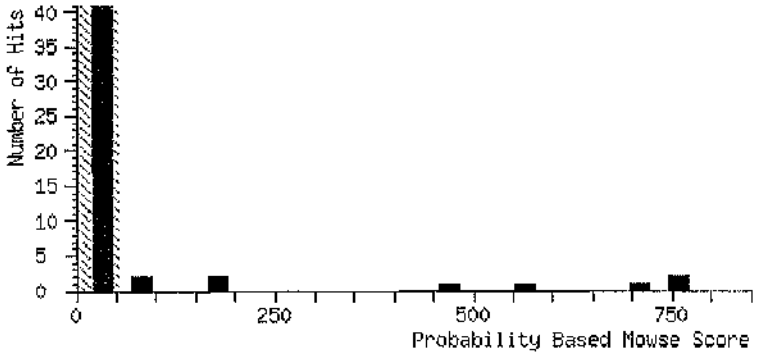
1 ATVYNQDGTK VDVNGSVRLI LKKEKDKRGD LVDNGSRVSF KASHDLGEGL
51 SALAYAELRF STKEEVEVTQ NQQVVRKYKV ERIGNDVHVK RLYAGFAYEG
101 LGTLTFGNQL TIGDDVGVSD YTYFLGGINN LLSSGEKAIN FKSAEFNGFT
151 FGGAYVFSAD ADKQAARDGR GFVVAGLYNR KMGDVGFALE AGYSQKYVTE
201 TAKQEKEKAF MVGTELSYAG LALGVDYAQS KVTNVDGKKR ALEVGLNYDL
251 NDKAKVYTDL IWAKKGPKGA TTRDRAIILG AGYKLHKQVE TFVEGGWGRT
301 KNAAGVTTKD NKVGVGLRVH F

G.2 Mass spectrometry results used to determine the peak list for the 37kDa OMP band.

Significant hits: gi|12721089 unknown [*Pasteurella multocida* subsp. multocida str. Pm70]
gi|49182350 OmpA [*Pasteurella multocida*]
gi|49182354 OmpA [*Pasteurella multocida*]
gi|49182352 OmpA [*Pasteurella multocida*]
gi|17861366 OmpA [*Pasteurella multocida*]
gi|19881365 hemagglutinin antigen [Haemophilus paragallinarum]
gi|32363216 Major outer membrane protein precursor (MOMP) (Outer membrane protein H)
gi|75431565 outer membrane protein and related peptidoglycan-associated (lipo)proteins [Actinobacillus succinog
gi|23124869 COG2199: FOG: GGDEF domain [Nostoc punctiforme PCC 73102]
gi|32030015 COG2885: Outer membrane protein and related peptidoglycan-associated (lipo)proteins [Haemophilus somnus 2336]
gi|1332435 outer membrane protein [Aeromonas salmonicida]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 52 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



G.2.1 Peptide Summary Report for 37 kDa OMP

1. gi|12721089 **Mass:** 38122 **Score:** 759 **Queries matched:** 13
   unknown [*Pasteurella multocida* subsp. multocida str. Pm70]
2. gi|49182350 **Mass:** 37785 **Score:** 750 **Queries matched:** 12
   OmpA [*Pasteurella multocida*]
3. gi|49182354 **Mass:** 38126 **Score:** 727 **Queries matched:** 12
   OmpA [*Pasteurella multocida*]
4. gi|49182352 **Mass:** 37620 **Score:** 542 **Queries matched:** 9
   OmpA [*Pasteurella multocida*]
5. gi|17861366 **Mass:** 37708 **Score:** 475 **Queries matched:** 8
   OmpA [*Pasteurella multocida*]
6. gi|19881365 **Mass:** 36785 **Score:** 171 **Queries matched:** 3
   hemagglutinin antigen [Haemophilus paragallinarum]
7. gi|32363216 **Mass:** 37084 **Score:** 168 **Queries matched:** 5
   Major outer membrane protein precursor (MOMP) (Outer membrane protein H)
8. gi|75431565 **Mass:** 33452 **Score:** 77 **Queries matched:** 1
   outer membrane protein and related peptidoglycan-associated (lipo)proteins [Actinobacillus succinog
9. gi|23124869 **Mass:** 66430 **Score:** 72 **Queries matched:** 1
   COG2199: FOG: GGDEF domain [Nostoc punctiforme PCC 73102]
10. gi|32030015 **Score:** 55 **Queries matched:** 1
    COG2885: Outer membrane protein and related peptidoglycan-associated (lipo)proteins [Haemophilus somnus 2336]
11. gi|1332435 **Mass:** 35003 **Score:** 54 **Queries matched:** 1
    outer membrane protein [Aeromonas salmonicida]

G.2.2 Amin acid sequence coverage for 37 kDa OMP

G.2.2.1 Match to: gi|12721089 Score: 759

1. gi|12721089 **Mass:** 38122 **Score:** 759 **Queries matched:** 13
   unknown [*Pasteurella multocida* subsp. multocida str. Pm70]

unknown [*Pasteurella multocida* subsp. multocida str. Pm70]
Nominal mass (M_r): **38122**; Calculated pI value: **9.09**
NCBI BLAST search of gi|12721089 against nr
Unformatted sequence string for pasting into other applications
Taxonomy: *Pasteurella multocida* subsp. multocida str. Pm70
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|12721089 from *Pasteurella multocida* subsp. multocida str. Pm70

Sequence Coverage: 52%

1 MKKTAIALTI AALAAASVAQ AAPQPNTFYV GAKAGWASFH DGLNQAKYLE
51 APEATFGFKR NSVTYGVFGG YQITDNFAVE LGYDDFGRAK LRMAEKDQKA
101 KDAAKHTNHG AHLSLKASYP VLDGLDIYAR VGAALIRSDY KVYDHSDPAK
151 LPQFKRTHST QVSPVFAGGL EYAFMPELAL RVEYQWVNNV GKVKDVLGER
201 VDYRPDIGSV TAGLSYRFGQ SVYVPEVVSK TFTLNSDVTF GFDKADLKPA
251 AQNVLDGIYG EIAQLKSASV AVAGYTDRLG SDAYNLKLSQ RRADTVANYL
301 VAKGVAQNAI SATGHGEANP VTGNKCDSVK GRKALIACLA DDRRVEIAVK
351 GNK

G.2.2.2 Match to: gi|49182350 Score: 750

2. gi|49182350 **Mass:** 37785 **Score:** 750 **Queries matched:** 12
OmpA [*Pasteurella multocida*]

OmpA [*Pasteurella multocida*]
Nominal mass (M_r): **37785**; Calculated pI value: **8.89**
NCBI BLAST search of gi|49182350 against nr
Taxonomy: *Pasteurella multocida*

Sequence Coverage: 48%

1 MKKTAIALTI AALAAASVAQ AAPQPNTFYV GAKAGWASFH DSLNQFNDIT
51 EPATGFKRNS VTYGVFGGYQ ITDNFAVELG YDDFGRAKAR ATDPKTKETV
101 DAAKHTNHGA HLSLKASYPV LDGLDVYARV GAALVRSDYK VYDKEPADLS
151 FLKRTHSTQV SPVFAGGLEY AFMPELALRV EYQWLNNVGK LKDAKGERVD
201 YRPDIGSVTA GLSYRFGQSV YVPEVVSKTF TLNSDVTFGF DKADLKPAAQ
251 NVLDGIYGEI AQLKSASVAV AGYTDRLGSD AYNLKLSQRR ADTVANYLVA
301 KGVAQNAISA TGHGEANPVT GNKCDSVKGR KALIACLADD RRVEIAVKGN
351 K

G.2.2.3 Match to: gi|49182354 Score: 727

3. gi|49182354 **Mass:** 38126 **Score:** 727 **Queries matched:** 12
OmpA [*Pasteurella multocida*]

OmpA [*Pasteurella multocida*]

Nominal mass (M_r): **38126**; Calculated pI value: **8.76**

NCBI BLAST search of gi|49182354 against nr

Taxonomy: *Pasteurella multocida*

Sequence Coverage: **49%**

1 MKKTAIALTI VALAVASVAQ AAPQPNTFYV GAKAGWASFH DGLNQAKYLE
51 APEATFGFKR NSVTYGVFGG YQITDDFAVE LGYDDFGRAK LRIAETDQKA
101 RDVAKHTNHG AHLSLKASYP VLDGLDVYAR VGAALIRSDY KVYDHSDPAK
151 LPQLKRTHST QVSPVFAGGL EYAFMPELAL RVEYQWVNNV GKLKDVDGNR
201 VDYRPDIGSV TAGLSYRFGQ SVYVPEVVSK TFTLNSDVTF GFDKADLKPA
251 AQNVLDGIYG EIAQLKSASV AVAGYTDRLG SDAYNLKLSQ RRADTVANYL
301 VAKGVAQNAI SATGHGEANP VTGNKCDSVK GRKALIACLA DDRRVEIAVX
351 GNK

G.2.2.4 Match to: gi|49182352 Score: 542

4. gi|49182352 **Mass:** 37620 **Score:** 542 **Queries matched:** 9

OmpA [*Pasteurella multocida*]

OmpA [*Pasteurella multocida*]

Nominal mass (M_r): **37620**; Calculated pI value: **9.26**

NCBI BLAST search of gi|49182352 against nr

Taxonomy: *Pasteurella multocida*

Sequence Coverage: **34%**

1 MKKTAIALTI VALAVASVAQ AAPQPNTFYV GAKAGWASFH DGLNQIEYVS
51 KTSFGSKRNS VTYGVFGGYQ ITDNFAVELG YDDFGRAKVR ANSKTTAFDA
101 AKHTNHGTHL SLKASYPLLD GLDVYARVGA ALIRSDYKLY APLINKRLSP
151 HFKITQVSPV FAGGLEYAFI PELTLRVEYQ WVNNVGKFEY ADGQYADFRP
201 DIGSVTAGLS YRFGQSVYVP EVVSKTFTLN TDVTFGFDKA DLKPAAQNVL
251 DGIYGEIAQL KSASVAVAGY TDRLGSDAYN LKLSQRRADT VANYLVAKGV
301 AQNAISATGR GEANPVTGNK CDSVKGRKAL IACLADDRRV EIAVKGNK

G.2.2.5 Match to: gi|17861366 Score: 475

5. gi|17861366 **Mass:** 37708 **Score:** 475 **Queries matched:** 8

OmpA [*Pasteurella multocida*]

OmpA [*Pasteurella multocida*]

Nominal mass (M_r): **37708**; Calculated pI value: **9.01**

NCBI BLAST search of gi|17861366 against nr

Taxonomy: *Pasteurella multocida*

Sequence Coverage: **34%**

1 MKKTAIALTI VALAVASVAQ AAPQPNTFYV GAKAGWASFH DGLNQIEYVS
51 KTSFGSKRNS VTYGVFGGYQ ITDNFAVELG YDDFGRAKVR ANSKTTAFDA
101 AKHTNHGTHL SLKASYPLLD GLDVYARVGA ALIRSDYKLY APLINKRLSP
151 HFKITQVSPV FAGGLEYAFI PELTLRVEYQ WVNNVCKFEY ADGQYADFRP

201 DIGSVTAGLS YRFGQSVYVP EVVSKTFTLN TDVTFGFDKA DLKPAAQNVL
251 DGIYGEIAQL KSASVAVAGY TDCLGSDAYN LKLSQRRADT VANYLVAKGV
301 AQNAISATGH GEANPVTGNK CDSVKGRKAL IACLADDRRV EIAVKGNK

G.2.2.6 Match to: gi|19881365 Score: 171

6. gi|19881365 Mass: 36785 Score: 171 Queries matched: 3

hemagglutinin antigen [Haemophilus paragallinarum]

hemagglutinin antigen [Haemophilus paragallinarum]

Nominal mass (M_r): **36785**; Calculated pI value: **8.62**

NCBI BLAST search of gi|19881365 against nr

Taxonomy: Avibacterium paragallinarum

Sequence Coverage: **15%**

1 MKKTAIALAI AGLTAASVAQ AAPQANTFYA GAKAGWASFH DGLNQFENSQ
51 NADGTLRNSV TYGVFGGYQI TDNFAVELGY DDFGRAKSRQ GGETVIKHTN
101 HGAHLSLKAS YPVLEGLDVY ARVGAALIRS DYKPTKRAAP NQTHEHSLKV
151 SPVFAGGLEY NLPSLPELAL RVEYQWVNKV GRVEKDGSRV DYTPSIGSVT
201 AGLSYRFGQS APVVEPKVVA KTFALNSDVT FAFGKANLRP EAQNVLDGIY
251 GEIAQLKSVQ VDVAGYTDRI GSEAANLKLS QRRADTVANY LVSKGVAQEV
301 ISSTGYGEAN PVTGAKCDTV KGRKALIACL ADDRRVEISV KGEE

G.2.2.7 Match to: gi|32363216 Score: 168

7. gi|32363216 Mass: 37084 Score: 168 Queries matched: 5

Major outer membrane protein precursor (MOMP) (Outer membrane protein H)

Major outer membrane protein precursor (MOMP) (Outer membrane protein H)

Nominal mass (M_r): **37084**; Calculated pI value: **8.96**

NCBI BLAST search of gi|32363216 against nr

Taxonomy: *Pasteurella multocida*

Links to retrieve other entries containing this sequence from NCBI Entrez:

gi|2853238 from *Pasteurella multocida*

gi|92918682 from *Pasteurella multocida*

gi|92918684 from *Pasteurella multocida*

gi|92918686 from *Pasteurella multocida*

gi|92918688 from *Pasteurella multocida*

gi|124262427 from *Pasteurella multocida*

Sequence Coverage: **23%**

1 MKKTIVALAV AAVAATSANA ATVYNQDGTK VDVNGSVRLI LKKEKNERGD
51 LVDNGSRVSF KASHDLGEGL SALAYAELRF SKNEKVEVKD AQNQQVVRKY
101 EVERIGNDVH VKRLYAGFAY EGLGTLTFGN QLTIGDDVGV SDYTYFLGGI
151 NNLLSSGEKA INFKSAEFNG FTFGGAYVFS ADADKQAPRD GRGFVVAGLY
201 NRKMGDVGFA LEAGYSQKYV TAAAKQEKEK AFMVGTELSY AGLALGVDYA
251 QSKVTNVEGK KRALEVGLNY DINDKAKVYT DLIWAKEGPK GATTRDRSII

301 LGAGYKLHKQ VETFVEGGWG REKDANGVTT KDNKVGVGLR VHF

G.2.2.8 Match to: gi|75431565 Score: 77

8. gi|75431565 **Mass:** 33452 **Score:** 77 **Queries matched:** 1

outer membrane protein and related peptidoglycan-associated (lipo)proteins [Actinobacillus succinog

outer membrane protein and related peptidoglycan-associated (lipo)proteins [Actinobacillus succinog

Nominal mass (M_r): **33452**; Calculated pI value: **6.53**

NCBI BLAST search of gi|75431565 against nr

Taxonomy: Actinobacillus succinogenes 130Z

Links to retrieve other entries containing this sequence from NCBI Entrez:

gi|74276276 from Actinobacillus succinogenes 130Z

Sequence Coverage: **4%**

1 MKKTAIALAI AGLAAASVAQ AAPQENTFYA GARVGWSAMH HGVDRIADQF
51 VTDGGLNRNS VTYGVFGGYQ ILNQNNFGLA AELGYDFFGK TKADAAGYHA
101 AHGASLALKP SYEIYPDLDV YGKVGVALVR NMYKAETLSA SGDAEKFNKT
151 KASLILGAGV EYAILPELAA RLEYQWLSKA GNLDSALEDA GYNGTNARYS
201 PDIHSVTAGL SYRFGQGAAP VAAPEVVNKT FTLNSDVTFG FNKSTLKPEA
251 ASTLDGIYSE IAQVSSPAVA VNGYADRIGK DAANLKLSQR RAETVANYLV
301 SKGVDSNAIT ADRLRFS

G.2.2.9 Match to: gi|23124869 Score: 72

9. gi|23124869 **Mass:** 66430 **Score:** 72 **Queries matched:** 1

COG2199: FOG: GGDEF domain [Nostoc punctiforme PCC 73102]

COG2199: FOG: GGDEF domain [Nostoc punctiforme PCC 73102]

Nominal mass (M_r): **66430**; Calculated pI value: **7.59**

NCBI BLAST search of gi|23124869 against nr

Taxonomy: Nostoc punctiforme PCC 73102

Sequence Coverage: **2%**

1 MQQSFLGSIR TRLIASFLIV ALIPLLLLAS INKQTTEKAL TDNARQALSA
51 AAKETTNRID AFIDGNLNAV RVEAILPGLA RYLSLTPKQR DDSPEIQLAT
101 ETLIRLSRKD MLNILSYALL DLNGKNILDT HTSNIGEDES VQDYFKNPLK
151 TGFSFVSSMK RSPIISDLVT LFFSSPVRNA KGDIVGVLRV SYNATVVQQL
201 VTRETERAGA KSFAILLDEN NIYLAHSTAS ELLFKSIVPL PADVVTQLQQ
251 ERRLPNYPVK ELATNESKLK QALDNKQSDL ITSLSATGNQ VNLIAIARLK
301 YKPWSVLFAQ PLTVALAPVE KQISDAMFLF AIIASVVTII AFAIGQVLTK
351 PIIYLTNIVF QFTAGHLDIR AKISSKDEIG QLAKSFNNMA LQLQTSLETL
401 EQRVQERTAE LVIAKEKAED ANHKLEQLVN LDGLTQVANR RCFDGRLQEE
451 WKCLAREQRP LSLILLDVDK FKLYNDYYGH LGGDDCLITI AQTMQQVVHR
501 PADLVARYGG EEFSVLLPNT DLLGAIKVAQ SIQQAICDRA IPHTQSDIKD
551 IVTLSLGITS VIPTCDIKPD TIIASADKAL YSAKKKGRDR YCTHLDI

G.2.2.10 Match to: gi|32030015 Score: 55

10. gi|32030015 Score: 55 Queries matched: 1

COG2885: Outer membrane protein and related peptidoglycan-associated (lipo)proteins [Haemophilus somnus 2336]

COG2885: Outer membrane protein and related peptidoglycan-associated (lipo)proteins [Haemophilus somnus 2336]

Nominal mass (M_r): **27412**; Calculated pI value: **9.48**

NCBI BLAST search of gi|32030015 against nr

Taxonomy: Haemophilus somnus 2336

Sequence Coverage: **5%**

1 MKKTIIALSI AGLSLSVQAA PQSKYVLCRC ESRLGIFPDG LNQFENSTEK
51 KGTVRNSVAY GIFGGYQITD HVAVELGSEY FGQAKGRKDK KEAKHTAQGM
101 QLGLKASYPV LEGLDIYGRV GAALIRSNYL NVKNFENSAD GVKNTLKVSP
151 VFAAGVEYSL PSLPELALRL EYQWVKGVGK ARKNDGKRFD YTPSIGAVTL
201 GLSYRFGQKP VMAPEVVNKV FSLNSDVNFA FAKDTLKPEA QQTFGRCLW

G.2.2.11 Match to: gi|1332435 Score: 54

11. gi|1332435 Mass: 35003 Score: 54 Queries matched: 1

outer membrane protein [Aeromonas salmonicida]

outer membrane protein [Aeromonas salmonicida]

Nominal mass (M_r): **35003**; Calculated pI value: **5.08**

NCBI BLAST search of gi|1332435 against nr

Taxonomy: Aeromonas salmonicida

Links to retrieve other entries containing this sequence from NCBI Entrez:

gi|1588799 from Aeromonas salmonicida

Sequence Coverage: **2%**

1 MKMKMAPALI ALAIAAMGTS TAQAADDWYT GIGAGWAYGH DLNDFGKDAD
51 KDATALSLFG GYNFNDYYAA ELGYLYAGKA GVDFKTQGAT LSGLARLPLN
101 DIFSVFAEGG AYFNHVNGNG NSDNGTAPLA GLGLTAKLSD LIDVQARYRY
151 MWNLGDEQKT WETNMSVATL ELVMHPNRTS YVAPVAAPAP EPVPEPVVVD
201 KSFALSSDVL FAFGKSTLKP EGVAALNTLY HQNVDVQPKD GSAVVVGYTD
251 RIGSDAYNLK LSEARARTVA DFLVGKGLPA GKVAIEGRGE ASPVTGTQCD
301 GIKAKAQLIA CLAPDRRVEV RVTGIQEVTQ

G.3 Mass spectrometry results used to determine the peak list for the 50kDa OMP band.

Significant hits: gi|2853240 outer membrane protein; OmpH [*Pasteurella multocida*]
gi|2853242 outer membrane protein; OmpH [*Pasteurella multocida*]
gi|12721089 unknown [*Pasteurella multocida* subsp. multocida str. Pm70]
gi|16751299 OmpH [*Pasteurella multocida*]

gi|75431565 outer membrane protein and related peptidoglycan-associated (lipo)proteins [Actinobacillus succinog

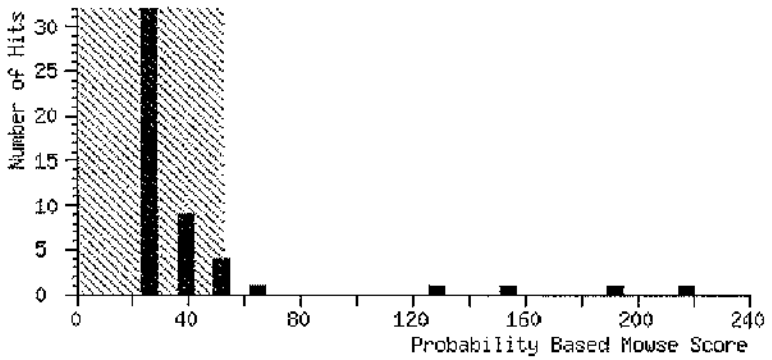
gi|72121591 hypothetical protein Reut_B4426 [Ralstonia eutropha JMP134]

gi|77389887 conserved hypothetical protein [Rhodobacter sphaeroides 2.4.1]

gi|78696479 hypothetical protein BradDRAFT_3909 [Bradyrhizobium sp. BTAi1]

Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 52 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



G.3.1 Peptide Summary Report for 50 kDa OMP

1. gi|2853240 **Mass:** 35143 **Score:** 218 **Queries matched:** 6
   outer membrane protein; OmpH [*Pasteurella multocida*]
2. gi|2853242 **Mass:** 34935 **Score:** 190 **Queries matched:** 5
   outer membrane protein; OmpH [*Pasteurella multocida*]
3. gi|12721089 **Mass:** 38122 **Score:** 149 **Queries matched:** 3
   unknown [*Pasteurella multocida* subsp. multocida str. Pm70]
4. gi|16751299 **Mass:** 34993 **Score:** 123 **Queries matched:** 4
   OmpH [*Pasteurella multocida*]
5. gi|75431565 **Mass:** 33452 **Score:** 69 **Queries matched:** 1
   outer membrane protein and related peptidoglycan-associated (lipo)proteins [Actinobacillus succinog
6. gi|72121591 **Mass:** 13914 **Score:** 55 **Queries matched:** 1
   hypothetical protein Reut_B4426 [Ralstonia eutropha JMP134]
7. gi|77389887 **Mass:** 37427 **Score:** 55 **Queries matched:** 1
   conserved hypothetical protein [Rhodobacter sphaeroides 2.4.1]
8. gi|78696479 **Mass:** 20528 **Score:** 53 **Queries matched:** 1
   hypothetical protein BradDRAFT_3909 [Bradyrhizobium sp. BTAi1]

G.3.2 Amin acid sequence coverage for 50 kDa OMP

G.3.2.1 Match to: gi|2853240 Score: 218

1. gi|2853240 **Mass:** 35143 **Score:** 218 **Queries matched:** 6

outer membrane protein; OmpH [*Pasteurella multocida*]

outer membrane protein; OmpH [*Pasteurella multocida*]

Nominal mass (M_r): **35143**; Calculated pI value: **8.84**

NCBI BLAST search of gi|2853240 against nr

Taxonomy: *Pasteurella multocida*

Sequence Coverage: **24%**

1 ATVYNQDGTK VDVNGSVRLI LKKEKNERGD LVDNGSRVSF KASHDLGEGL
51 SALAYAELRF STKEKVEVKD TQNPPKVVRT YEVEKIGNDV HVKRLYAGFA
101 YEGLGTLTFG NQLTIGDDVG VSDYTYFLGG INNLLSSGEK AINFKSAEFN
151 GFTFGGAYVF SADADKQAPR DGRGFVVAGL YNRKMGDVGF ALEAGYSQKY
201 VTVAKQEKEK AFMVGTELSY AGLALGVDYA QSKVTNVDGK KRALEVGLNY
251 DINDKAKVYT DLIWAKKGPK GATTRDRSII LGAGYKLHKQ VETFVEGGWG
301 REKDANGVTT KDNVVGVGLR VHF

G.3.2.2 Match to: gi|2853242 Score: 190

2. gi|2853242 **Mass:** 34935 **Score:** 190 **Queries matched:** 5

outer membrane protein; OmpH [*Pasteurella multocida*]

outer membrane protein; OmpH [*Pasteurella multocida*]

Nominal mass (M_r): **34935**; Calculated pI value: **9.17**

NCBI BLAST search of gi|2853242 against nr

Taxonomy: *Pasteurella multocida*

Sequence Coverage: **22%**

1 ATVYNQDGTK VDVNGSVRLI LKKEKDKRGD LVDNGSRVSF KASHDLGEGL
51 SALAYAELRF STKEEVEVTQ NQQVVRKYKV ERIGNDVHVK RLYAGFAYEG
101 LGTLTFGNQL TIGDDVGVSD YTYFLGGINN LLSSGEKAIN FKSAEFNGFT
151 FGGAYVFSAD ADKQAARDGR GFVVAGLYNR KMGDVGFALE AGYSQKYVTE
201 TAKQEKEKAF MVGTELSYAG LALGVDYAQS KVTNVDGKKR ALEVGLNYDL
251 NDKAKVYTDL IWAKKGPKGA TTRDRAIILG AGYKLHKQVE TFVEGGWGRT
301 KNAAGVTTKD NKVGVGLRVH F

G.3.2.3 Match to: gi|12721089 Score: 149

3. gi|12721089 **Mass:** 38122 **Score:** 149 **Queries matched:** 3

unknown [*Pasteurella multocida* subsp. multocida str. Pm70]

unknown [*Pasteurella multocida* subsp. multocida str. Pm70]

Nominal mass (M_r): **38122**; Calculated pI value: **9.09**

NCBI BLAST search of gi|12721089 against nr

Taxonomy: *Pasteurella multocida* subsp. multocida str. Pm70

Links to retrieve other entries containing this sequence from NCBI Entrez:

gi|12721089 from *Pasteurella multocida* subsp. multocida str. Pm70

Sequence Coverage: **12%**

1 MKKTAIALTI AALAAASVAQ AAPQPNTFYV GAKAGWASFH DGLNQAKYLE
51 APEATFGFKR NSVTYGVFGG YQITDNFAVE LGYDDFGRAK LRMAEKDQKA
101 KDAAKHTNHG AHLSLKASYP VLDGLDIYAR VGAALIRSDY KVYDHSDPAK
151 LPQFKRTHST QVSPVFAGGL EYAFMPELAL RVEYQWVNNV GKVKDVLGER
201 VDYRPDIGSV TAGLSYRFGQ SVYVPEVVSK TFTLNSDVTF GFDKADLKPA
251 AQNVLDGIYG EIAQLKSASV AVAGYTDRLG SDAYNLKLSQ RRADTVANYL
301 VAKGVAQNAI SATGHGEANP VTGNKCDSVK GRKALIACLA DDRRVEIAVK
351 GNK

G.3.2.4 Match to: gi|16751299 Score: 123

4. gi|16751299 Mass: 34993 Score: 123 Queries matched: 4

OmpH [*Pasteurella multocida*]

OmpH [*Pasteurella multocida*]

Nominal mass (M_r): **34993**; Calculated pI value: **9.17**

NCBI BLAST search of gi|16751299 against nr

Taxonomy: *Pasteurella multocida*

Sequence Coverage: **18%**

1 SNLANAATVY NQDGTKVDVN GSVRLILKKE KNERGDLVDN GSRVSFKASH
51 DLGEGLSALA YAELRFSKNE KVEVKDAQNQ QVVRKYEVER IGNDVHVKRL
101 YAGFAYEGLG TLTFGNQLTI GDDVGVSDYT YFLGGINNLL SSGEKAINFK
151 SAEFNGFTFG GAYVFSADAD KQAPRDGRGF VVAGLYNRKM GDVGFALEAG
201 YSQKYVTAAA KQEKEKAFMV GTELSYAGLA LGVDYHNLKS NVEGKNVLLK
251 WLNLCIMTSK VNCLFGKKGQ RALQNRLYVG GSNLQHEPCE GLAKKFMLHK
301 NKWVVRPFIV IKTGTCL

G.3.2.5 Match to: gi|75431565 Score: 69

5. gi|75431565 Mass: 33452 Score: 69 Queries matched: 1

outer membrane protein and related peptidoglycan-associated (lipo)proteins [Actinobacillus succinog

outer membrane protein and related peptidoglycan-associated (lipo)proteins [Actinobacillus succinog

Nominal mass (M_r): **33452**; Calculated pI value: **6.53**

NCBI BLAST search of gi|75431565 against nr

Taxonomy: Actinobacillus succinogenes 130Z

Links to retrieve other entries containing this sequence from NCBI Entrez:

gi|74276276 from Actinobacillus succinogenes 130Z

Sequence Coverage: **4%**

1 MKKTAIALAI AGLAAASVAQ AAPQENTFYA GARVGWSAMH HGVDRIADQF
51 VTDGGLNRNS VTYGVFGGYQ ILNQNNFGLA AELGYDFFGK TKADAAGYHA
101 AHGASLALKP SYEIYPDLDV YGKVGVALVR NMYKAETLSA SGDAEKFNKT

151 KASLILGAGV EYAILPELAA RLEYQWLSKA GNLDSALEDA GYNGTNARYS
201 PDIHSVTAGL SYRFGQGAAP VAAPEVVNKT FTLNSDVTFG FNKSTLKPEA
251 ASTLDGIYSE IAQVSSPAVA VNGYADRIGK DAANLKLSQR RAETVANYLV
301 SKGVDSNAIT ADRLRFS

G.3.2.6 Match to: gi|72121591 Score: 55

6. gi|72121591 **Mass:** 13914 **Score:** 55 **Queries matched:** 1

hypothetical protein Reut_B4426 [Ralstonia eutropha JMP134]

hypothetical protein Reut_B4426 [Ralstonia eutropha JMP134]

Nominal mass (M_r): **13914**; Calculated pI value: **8.39**
NCBI BLAST search of gi|72121591 against nr
Taxonomy: Ralstonia eutropha JMP134
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|72121591 from Ralstonia eutropha JMP134

Sequence Coverage: **6%**

1 MVRNRSFPYN GLAPSFCHAE SLQDHSGRFI VSAIAVVPCL LCGELARRWS
51 DRHDHVLEGR IYRCARCGGR FSVTGEAYGE IIHGNWDTTE LMTAVRQRIA
101 TGELPRIEGI AGLPSVIAVG RQAS

G.3.2.7 Match to: gi|77389887 Score: 55

7. gi|77389887 **Mass:** 37427 **Score:** 55 **Queries matched:** 1

conserved hypothetical protein [Rhodobacter sphaeroides 2.4.1]

conserved hypothetical protein [Rhodobacter sphaeroides 2.4.1]

Nominal mass (M_r): **37427**; Calculated pI value: **6.02**
NCBI BLAST search of gi|77389887 against nr
Taxonomy: Rhodobacter sphaeroides 2.4.1
Links to retrieve other entries containing this sequence from NCBI Entrez:
gi|77389887 from Rhodobacter sphaeroides 2.4.1

Sequence Coverage: **2%**

1 MRIDNATPWP ATFTMAFDAA GHEQVVLVTK ASFALPRGTG PCQPTEPLPL
51 IESDLFGADP AADAPVQEYD FAPHKPFCDV LLHGRAHAPE GRPVTELPVG
101 LRLGGWSKRL TVRGARIWLR AAAGWRVSDP RPFTSQPIGY DHAYGGTDPD
151 PGGGGRAAVF EENPAGLGFY PLRPDRESAP LPHTAEAGAD ATDPRGGLRP
201 MALGPVGRTW LPRRRHAGTY DAAYFESRTP FLPLDFDPRY FQAAAEDQQI
251 PYPKGGEPVE LVNLSPRGRI ATWLPRLQIL ATFERRSGRL TQRIANLDTV
301 LFLPEEEVVT LTFRTRITAE RDIFEFARVL VSARQEATDG

G.3.2.8 Match to: gi|78696479 Score: 53

8. gi|78696479 **Mass:** 20528 **Score:** 53 **Queries matched:** 1

hypothetical protein BradDRAFT_3909 [Bradyrhizobium sp. BTAi1]

hypothetical protein BradDRAFT_3909 [Bradyrhizobium sp. BTAi1]

Nominal mass (M_r): **20528**; Calculated pI value: **7.88**

NCBI BLAST search of gi|78696479 against nr

Links to retrieve other entries containing this sequence from NCBI Entrez:

gi|78516057 from Bradyrhizobium sp. BTAi1

Sequence Coverage: **3%**

1 MMSLLGFDAI GRLATGQLPR SAGVNTLFAA TTSAIALSGR AASFQLVLSV

51 PVANVNCAGL AVSTGITLPA LQAGWLTSGL SARYSFGAAA SRGEYSVAGV

101 SAASLGNMVA AGRSYDVGAP PAVFVGALAG QSAAFQLGGY DVAYGRGFES

151 WYPLAAVAGE WQGSASATQS WLPAPAAIPS WLDGAAPSSR WDPTSSPLPP

201 WKSI

BIBLIOGRAPHY

Aalbaek, B., Eriksen, L., Rimler, R. B., Leifsson, P. S., Basse, A., Christiansen, T. & Eriksen, E. (1999). Typing of *Pasteurella multocida* from haemorrhagic septicaemia in Danish fallow deer (*Dama dama*). *Apmis* **107**, 913-920.

Abbas, A. K. & Lichtman, A. H. (2003). *Cellular and Molecular Immunology*, Fifht edn. Philadelphia: Elsevier Science.

Adler, B., Chancellor, R., Homchampa, P., Hunt, M., Ruffolo, C., Strugnell, R. & Wapling, D. (1996). Immunity and vaccine development in *Pasteurella multocida* infections. *J Biotechnol* **44**, 139-144.

Al-Hasani, K., Boyce, J., McCarl, V. P., Bottomley, S., Wilkie, I. & Adler, B. (2007). Identification of novel immunogens in *Pasteurella multocida*. *Microb Cell Fact* **6**, 3.

Ali, H. A., Sawada, T., Hatakeyama, H., Ohtsuki, N. & Itoh, O. (2004). Characterization of a 39kDa capsular protein of avian *Pasteurella multocida* using monoclonal antibodies. *Vet Microbiol* **100**, 43-53.

Amonsin, A., Wellehan, J. F., Li, L. L., Laber, J. & Kapur, V. (2002). DNA fingerprinting of *Pasteurella multocida* recovered from avian sources. *J Clin Microbiol* **40**, 3025-3031.

Angen, O., Mutters, R., Caugant, D. A., Olsen, J. E. & Bisgaard, M. (1999). Taxonomic relationships of the [*Pasteurella*] *haemolytica* complex as evaluated by DNA-DNA hybridizations and 16S rRNA sequencing with proposal of *Mannheimia haemolytica* gen. nov., comb. nov., *Mannheimia granulomatis* comb. nov., *Mannheimia glucosida* sp. nov., *Mannheimia ruminalis* sp. nov. and *Mannheimia varigena* sp. nov. *Int J Syst Bacteriol* **49 Pt 1**, 67-86.

Angen, O., Ahrens, P., Kuhnert, P., Christensen, H. & Mutters, R. (2003). Proposal of *Histophilus somni* gen. nov., sp. nov. for the three species incertae sedis '*Haemophilus* somnus', '*Haemophilus agni*' and '*Histophilus ovis*'. *Int J Syst Evol Microbiol* **53**, 1449-1456.

Athar, M. K., Karim, M. S., Mannam, S. & Ahmed, M. (2003). Fatal *Pasteurella* sepsis and hairy-cell leukemia. *Am J Hematol* **72**, 285.

Baba, T., Ando, T. & Nukina, M. (1978). Effect of bursectomy and thymectomy on *Pasteurella multocida* infection in chickens. *J Med Microbiol* **11**, 281-288.

Baba, T. (1984). Cell-mediated immune protection in chickens against *Pasteurella multocida*. *Res Vet Sci* **36**, 225-230.

Bain, R. V. (1954). Studies on haemorrhagic septicaemia in cattle, naturally acquired immunity in Siamese buffaloes. *Br Vet J*, 481.

Bain, R. V. & Knox, K. W. (1961). The antigens of *Pasteurella multocida* type I. II. Lipopolysaccharides. *Immunology* **4**, 122-129.

Bain, R. V., De Alwis, M. C., Carter, C. & Gupta, B. K. (1982). Harmorrhagic Septicaemia. In *FAO Animal Production and Health Paper 33*. Rome: Food and Agriculture Organisation of the United Nations.

Baldwin, C. L., Sathiyaseelan, T., Naiman, B., White, A. M., Brown, R., Blumerman, S., Rogers, A. & Black, S. J. (2002). Activation of bovine peripheral blood gammadelta T cells for cell division and IFN-gamma production. *Vet Immunol Immunopathol* **87**, 251-259.

Benkirane, A., DE Alwis, M.C.L. (2002). Haemorrhagic septicaemia, its significance, prevention and control in Asia. . *Vet Med - Czech* **47**, 234-240.

Berge, A., Fagergren, A. & Stiernstedt, S. H. (2002). *Pasteurella multocida* septicaemia in 2 Swedish patients. *Scand J Infect Dis* **34**, 138-139.

Bisgaard, M. (1994). Taxonomy of the family *Pasteurellaceae*. In Third International Conference on *Haemophilus, Actinobacillus, and Pasteurella*, pp. 1-7. Edited by F. A. L. W. Donachie, Hodgson J. C. Edinburgh, Scotland, UK.

Black, L., Francis, M. J. & Nicholls, M. J. (1985). Protecting young domesticated animals from infectious disease. *Vet Ann* **25**, 46-61.

Blackall, P. J., Asakawa, T., Graydon, R. J., White, M., Adamson, M., Wade, L. K. & Lowe, L. B. (1995). In-vitro antibacterial properties of tilmicosin against Australian isolates of *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* from pigs. *Aust Vet J* **72**, 35-36.

Blackall, P. J., Christensen, J. P. & Bisgaard, M. (1998). Diversity among isolates of *Actinobacillus equuli* and related organisms as revealed by ribotyping. *Aust Vet J* **76**, 423-425.

Blackall, P. J., Christensen, H., Beckenham, T., Blackall, L. L. & Bisgaard, M. (2005). Reclassification of *Pasteurella* gallinarum, [*Haemophilus*] *paragallinarum*, *Pasteurella avium* and *Pasteurella* volantium as *Avibacterium gallinarum* gen. nov., comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium* comb. nov. and *Avibacterium volantium* comb. nov. *Int J Syst Evol Microbiol* **55**, 353-362.

Blackall, P., Angen, Fegan, N., Blackall, L., Mutters, R. & Bisgaard, M. (2001). Characterisation of a novel *Mannheimia* sp from Australian feedlot cattle. *Aust Vet J* **79**, 634-639.

Boot, R. & Bisgaard, M. (1995). Reclassification of 30 *Pasteurella*ceae strains isolated from rodents. *Lab Anim* **29**, 314-319.

Borrathybay, E., Sawada, T., Kataoka, Y., Ohtsu, N., Takagi, M., Nakamura, S. & Kawamoto, E. (2003a). A 39 kDa protein mediates adhesion of avian *Pasteurella multocida* to chicken embryo fibroblast cells. *Vet Microbiol* **97**, 229-243.

Borrathybay, E., Sawada, T., Kataoka, Y., Okiyama, E., Kawamoto, E. & Amao, H. (2003b). Capsule thickness and amounts of a 39 kDa capsular protein of avian *Pasteurella multocida* type A strains correlate with their pathogenicity for chickens. *Vet Microbiol* **97**, 215-227.

Bosch, M., Tarrago, R., Garrido, M. E., Campoy, S., Fernandez de Henestrosa, A. R., Perez de Rozas, A. M., Badiola, I. & Barbe, J. (2001). Expression of the *Pasteurella multocida ompH* gene is negatively regulated by the Fur protein. *FEMS Microbiol Lett* **203**, 35-40.

Bosch, M., Garrido, E., Llagostera, M., Perez de Rozas, A. M., Badiola, I. & Barbe, J. (2002a). *Pasteurella multocida exbB*, *exbD* and *tonB* genes are physically linked but independently transcribed. *FEMS Microbiol Lett* **210**, 201-208.

Bosch, M., Garrido, M. E., Llagostera, M., Perez De Rozas, A. M., Badiola, I. & Barbe, J. (2002b). Characterization of the *Pasteurella multocida hgbA* gene encoding a hemoglobin-binding protein. *Infect Immun* **70**, 5955-5964.

Bosch, M., Garrido, M. E., Perez de Rozas, A. M., Badiola, I., Barbe, J. & Llagostera, M. (2004). *Pasteurella multocida* contains multiple immunogenic haemin- and haemoglobin-binding proteins. *Vet Microbiol* **99**, 103-112.

Bowe, F., O'Gaora, P., Maskell, D., Cafferkey, M. & Dougan, G. (1989). Virulence, persistence, and immunogenicity of *Yersinia enterocolitica* O:8 aroA mutants. *Infect Immun* **57**, 3234-3236.

Boyce, J. D. & Adler, B. (2000). The capsule is a virulence determinant in the pathogenesis of *Pasteurella multocida* M1404 (B:2). *Infect Immun* **68**, 3463-3468.

Boyce, J. D., Chung, J. Y. & Adler, B. (2000a). Genetic organisation of the capsule biosynthetic locus of *Pasteurella multocida* M1404 (B:2). *Vet Microbiol* **72**, 121-134.

Boyce, J. D., Chung, J. Y. & Adler, B. (2000b). *Pasteurella multocida* capsule: composition, function and genetics. *J Biotechnol* **83**, 153-160.

Boyce, J. D. & Adler, B. (2001). Acapsular *Pasteurella multocida* B:2 can stimulate protective immunity against pasteurellosis. *Infect Immun* **69**, 1943-1946.

Boyce, J. D., Wilkie, I., Harper, M., Paustian, M. L., Kapur, V. & Adler, B. (2002). Genomic scale analysis of *Pasteurella multocida* gene expression during growth within the natural chicken host. *Infect Immun* **70**, 6871-6879.

Boyce, J. D. & Adler, B. (2006). How does *Pasteurella multocida* respond to the host environment? *Curr Opin Microbiol* **9**, 117-122.

Boyce, J. D., Cullen, P. A., Nguyen, V., Wilkie, I. & Adler, B. (2006). Analysis of the *Pasteurella multocida* outer membrane sub-proteome and its response to the in vivo environment of the natural host. *Proteomics* **6**, 870-880.

Brickell, S. K., Thomas, L. M., Long, K. A., Panaccio, M. & Widders, P. R. (1998). Development of a PCR test based on a gene region associated with the pathogenicity of *Pasteurella multocida* serotype B:2, the causal agent of haemorrhagic septicaemia in Asia. *Vet Microbiol* **59**, 295-307.

Brogden, K. A. & Rebers, P. A. (1978). Serologic examination of the Westphal-type lipopolysaccharides of *Pasteurella multocida*. *Am J Vet Res* **39**, 1680-1682.

Brogden, K. A., Rhoades, K. R. & Heddleston, K. L. (1978). A new serotype of *Pasteurella multocida* associated with fowl cholera. *Avian Dis* **22**, 185-190.

Brown, A., Hormaeche, C. E., Demarco de Hormaeche, R., Winther, M., Dougan, G., Maskell, D. J. & Stocker, B. A. (1987). An attenuated *aroA Salmonella typhimurium* vaccine elicits humoral and cellular immunity to cloned beta-galactosidase in mice. *J Infect Dis* **155**, 86-92.

Brown, W. C., Rice-Ficht, A. C. & Estes, D. M. (1998). Bovine type 1 and type 2 responses. *Vet Immunol Immunopathol* **63**, 45-55.

Brunham, R. C., Plummer, F. A. & Stephens, R. S. (1993). Bacterial antigenic variation, host immune response, and pathogen-host coevolution. *Infect Immun* **61**, 2273-2276.

Capitini, C. e. a. (2002). Wound infection with *Neisseria weaveri* and a novel subspecies of *Pasteurella multocida* in a child who sustained a tiger bite. *Clinical Infetious Diseases* **15**, 74-76.

Carter, G. R. (1955). Studies on *Pasteurella multocida*. I. A hemagglutination test for the identification of serological types. *Am J Vet Res* **16**, 481-484.

Carter, G. R. (1962). Animal serotypes of *Pasteurella multocida* from human infections. *Can J Public Health* **53**, 158-161.

Carter, G. R. (1964). Correlation between Hemagglutinating Antibody and Mouse Protection in AntiPasteurella (*Pasteurella multocida*) Sera. *Can J Microbiol* **10**, 753-756.

Carter, G. R. (1967). Pasteurellosis: *Pasteurella multocida* and *Pasteurella hemolytica*. *Adv Vet Sci* **11**, 321-379.

Carter, G. R. & Subronto, P. (1973). Identification of type D strains of *Pasteurella multocida* with acriflavine. *Am J Vet Res* **34**, 293-294.

Carter, G. R. & Rundell, S. W. (1975). Identification of type A strains of P multocida using *staphylococcal hyaluronidase*. *Vet Rec* **96**, 343.

Carter, G. R. & Chengappa, M. M. (1980). Hyaluronidase production by type B *Pasteurella multocida* from cases of hemorrhagic septicemia. *J Clin Microbiol* **11**, 94-96.

Carter, G. R., Myint, A., Van Khar, R. & Khin, A. (1991). Immunisation of cattle and buffaloes with live haemorrhagic septicaemia vaccine. *Vet Rec* **129**, 203.

Ceron, J. J., Eckersall, P. D. & Martynez-Subiela, S. (2005). Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Vet Clin Pathol* **34**, 85-99.

Chamberlain, L. M., Strugnell, R., Dougan, G., Hormaeche, C. E. & Demarco de Hormaeche, R. (1993). *Neisseria gonorrhoeae* strain MS11 harbouring a mutation in gene *aroA* is attenuated and immunogenic. *Microb Pathog* **15**, 51-63.

Chandrasekaran, S., Kennett, L., Yeap, P. C., Muniandy, N., Rani, B. & Mukkur, T. K. (1994a). Relationship between active protection in vaccinated buffaloes against haemorrhagic septicaemia and passive mouse protection test or serum antibody titres. *Vet Microbiol* **41**, 303-309.

Chandrasekaran, S., Kennett, L., Yeap, P. C., Muniandy, N., Rani, B. & Mukkur, T. K. (1994b). Characterization of immune response and duration of protection in buffaloes immunized with haemorrhagic septicaemia vaccines. *Vet Microbiol* **41**, 213-219.

Chanter, N., Magyar, T. & Rutter, J. M. (1989). Interactions between *Bordetella bronchiseptica* and toxigenic *Pasteurella multocida* in atrophic rhinitis of pigs. *Res Vet Sci* **47**, 48-53.

Chaudhuri, P. & Goswami, P. P. (2001). Cloning of 87 kDa outer membrane protein gene of *Pasteurella multocida* P52. *Res Vet Sci* **70**, 255-256.

Chen, L., Paulsen, D. B., Scruggs, D. W., Banes, M. M., Reeks, B. Y. & Lawrence, M. L. (2003). Alteration of DNA adenine methylase (*Dam*) activity in *Pasteurella multocida* causes increased spontaneous mutation frequency and attenuation in mice. *Microbiology* **149**, 2283-2290.

Chen, R. & Henning, U. (1996). A periplasmic protein (Skp) of *Escherichia coli* selectively binds a class of outer membrane proteins. *Mol Microbiol* **19**, 1287-1294.

Chen, T. Y., Lei, M. G., Suzuki, T. & Morrison, D. C. (1992). Lipopolysaccharide receptors and signal transduction pathways in mononuclear phagocytes. *Curr Top Microbiol Immunol* **181**, 169-188.

Chen, W., Shu, D. & Chadwick, V. S. (2000). Inhibition of mitogen-induced murine lymphocyte proliferation by *Helicobacter pylori* cell-free extract. *J Gastroenterol Hepatol* **15**, 1000-1006.

Chengappa, M. M. & Carter, G. R. (1977). Demonstration of bacteriocin activity in bovine and bison strains of *Pasteurella multocida*. *Am J Vet Res* **38**, 1183-1185.

Chengappa, M. M., Myers, R. C. & Carter, G. R. (1982). Capsular and somatic types of *Pasteurella multocida* from rabbits. *Can J Comp Med* **46**, 437-439.

Chevalier, G., Le Henaff, M. & Wroblewski, H. (1992). [Purification, amino acid composition and N-terminal sequence of the major protein (protein H) of the outer membrane of *Pasteurella multocida*]. *C R Acad Sci III* **314**, 253-258.

Christensen, H., Bisgaard, M., Bojesen, A. M., Mutters, R. & Olsen, J. E. (2003). Genetic relationships among avian isolates classified as *Pasteurella haemolytica*, *'Actinobacillus salpingitidis'* or *Pasteurella anatis* with proposal of *Gallibacterium anatis* gen. nov., comb. nov. and description of additional genomospecies within *Gallibacterium* gen. nov. *Int J Syst Evol Microbiol* **53**, 275-287.

Christensen, H., Angen, O., Olsen, J. E. & Bisgaard, M. (2004a). Revised description and classification of atypical isolates of *Pasteurella multocida* from bovine lungs based on genotypic characterization to include variants previously classified as biovar 2 of *Pasteurella canis* and *Pasteurella avium*. *Microbiology* **150**, 1757-1767.

Christensen, H. & Bisgaard, M. (2004). Revised definition of *Actinobacillus sensu stricto* isolated from animals. A review with special emphasis on diagnosis. *Vet Microbiol* **99**, 13-30.

Christensen, H., Bisgaard, M., Aalbaek, B. & Olsen, J. E. (2004b). Reclassification of Bisgaard taxon 33, with proposal of *Volucribacter psittacicida* gen. nov., sp. nov. and

Volucribacter amazonae sp. nov. as new members of the *Pasteurellaceae*. *Int J Syst Evol Microbiol* **54**, 813-818.

Chung, J. Y., Zhang, Y. & Adler, B. (1998). The capsule biosynthetic locus of *Pasteurella multocida* A:1. *FEMS Microbiol Lett* **166**, 289-296.

Collins, F. M. (1973). Growth of *Pasteurella multocida* in vaccinated and normal mice. *Infect Immun* **8**, 868-875.

Collins, F. M. & Woolcock (1976). Immune responses to *Pasteurella multocida* in the mouse. *J Reticuloendothel Soc* **19**, 311-321.

Conti, P., Reale, M., Barbacane, R. C., Panara, M. R., Bongrazio, M., Dempsey, R. A. & Dinarello, C. A. (1991a). Reduced mitogen stimulation of DNA synthesis in human lymphocytes by a human recombinant interleukin-1 receptor antagonist. *Immunol Lett* **28**, 19-25.

Conti, P., Reale, M., Panara, M. R., Barbacane, R. C., Bongrazio, M. & Dempsey, R. A. (1991b). Human recombinant interleukin-1 receptor antagonist inhibits lymphocyte blastogenesis induced by concanavalin A. Restorative effect of hrIL-1. *FEBS Lett* **286**, 137-141.

Cooney, B. J. & Lo, R. Y. (1993). Three contiguous lipoprotein genes in *Pasteurella haemolytica* A1 which are homologous to a lipoprotein gene in *Haemophilus influenzae* type b. *Infect Immun* **61**, 4682-4688.

Cox, A. J., Hunt, M. L., Boyce, J. D. & Adler, B. (2003). Functional characterization of *HgbB*, a new hemoglobin binding protein of *Pasteurella multocida*. *Microb Pathog* **34**, 287-296.

Dabo, S. M., Debey, B. M., Montelongo, M. & Confer, A. W. (1999). Genomic DNA restriction site heterogeneity in bovine *Pasteurella multocida* serogroup A isolates detected with an rRNA probe. *J Med Microbiol* **48**, 279-286.

Dabo, S. M., Confer, A. W. & Quijano-Blas, R. A. (2003). Molecular and immunological characterization of *Pasteurella multocida* serotype A:3 OmpA: evidence of its role in *P. multocida* interaction with extracellular matrix molecules. *Microb Pathog* **35**, 147-157.

Davies, R. L., Wall, R. A. & Borriello, S. P. (1990). Comparison of methods for the analysis of outer membrane antigens of *Neisseria meningitidis* by western blotting. *J Immunol Methods* **134**, 215-225.

Davies, R. L., Parton, R., Coote, J. G., Gibbs, H. A. & Freer, J. H. (1992). Outer-membrane protein and lipopolysaccharide variation in *Pasteurella haemolytica* serotype A1 under different growth conditions. *J Gen Microbiol* **138 (Pt 5)**, 909-922.

Davies, R. L., MacCorquodale, R., Baillie, S. & Caffrey, B. (2003). Characterization and comparison of *Pasteurella multocida* strains associated with porcine pneumonia and atrophic rhinitis. *J Med Microbiol* **52**, 59-67.

Davies, R. L., MacCorquodale, R. & Reilly, S. (2004). Characterisation of bovine strains of *Pasteurella multocida* and comparison with isolates of avian, ovine and porcine origin. *Vet Microbiol* **99**, 145-158.

Dawkins, H. J., Hyatt, A., Johnson, R. B., Spencer, T. L., Ramdani & Adler, B. (1991a). Evidence of phenotypic dichotomy within an individual *Pasteurella multocida* type strain and among some haemorrhagic septicaemia-causing field isolates. *Res Vet Sci* **50**, 368-370.

Dawkins, H. J., Ramdani, Johnson, R. B. & Spencer, T. L. (1991b). Haemorrhagic septicaemia: correlation of vaccinal antibody responses in mice with protection against *Pasteurella multocida* strain M1404. *Vet Microbiol* **27**, 309-326.

De Alwis, M. C. & Carter, G. R. (1980). Preliminary field trails with a streptomycin-dependent vaccine against haemorrhagic septicaemia. *Vet Rec* **106**, 435-437.

De Alwis, M. C., Carter, G. R. & Chengappa, M. M. (1980). Production and characterization of streptomycin dependent mutants of *Pasteurella multocida* from bovine haemorrhagic septicaemia. *Can J Comp Med* **44**, 418-422.

De Alwis, M. C. (1982). Immune status of buffalo calves exposed to natural infection with haemorrhagic septicaemia. *Trop Anim Health Prod* **14**, 29-30.

de Alwis, M. C. & Sumanadasa, M. A. (1982). Naturally acquired immunity to haemorrhagic septicaemia among cattle and buffaloes in Sri Lanka. *Trop Anim Health Prod* **14**, 27-28.

De Alwis, M. C., Wijewardana, T. G., Gomis, A. I. & Vipulasiri, A. A. (1990). Persistence of the carrier status in haemorrhagic septicaemia (*Pasteurella multocida* serotype 6:B infection) in buffaloes. *Trop Anim Health Prod* **22**, 185-194.

De Alwis, M. C. (1992). Haemorrhagic septicaemia--a general review. *Br Vet J* **148**, 99-112.

De Alwis, M. C. (1993). Pasteurellosis in Production Animals: a Review. In *Pasteurellosis in Production Animals*, pp. 11-22. Edited by B. E. e. a. Patten: Australian Centre for International Agricultural Research (ACIAR).

De Alwis, M. C. (1994). Haemorrhagic Septicaemia (*Pasteurella multocida* serotype B:2 and E:2 Infection) in Cattle and Buffaloes. In *Haemophilus, Actinobacillus, and Pasteurella (3rd International Conference)*
Edited by W. Donachie, Lainson, F.A., and Hodgson, J.C. Edinburgh, Scotland.

De Alwis, M. C. L. (1995). Haemorrhagic septicaemia (*Pasteurella multocida* serotype B:2 infection) in cattle and buffaloes. In *Haemophilus, Actinobacillus, and Pasteurella*, pp. 9-24. Edited by W. L. Donachie, F. A.; Hodgson J. C. Moredun Research Institute, Edinburgh, Scotland: Plenum Press.

De Alwis, M. C. L. (1999). Haemorrhagic Septicaemia. In *ACIAR Monograph No 57*, pp. 141.

DeBey, B. M., Roth, J. A., Brogden, K. A., Cutlip, R. C., Stevens, M. G., Jones, T., Briggs, R. E. & Kluge, J. P. (1996). In vitro lymphocyte proliferative responses and gamma-interferon production as measures of cell-mediated immunity of cattle exposed to *Pasteurella haemolytica*. *Can J Vet Res* **60**, 263-270.

Delamarche, C., Manoha, F., Behar, G., Houlgatte, R., Hellman, U. & Wroblewski, H. (1995). Characterization of the *Pasteurella multocida skp* and *firA* genes. *Gene* **161**, 39-43.

Delpy, L. P. & Rastegar, R. (1938). *Bull Acad Vet France* **9**, 256.

Dhanda, M. R. (1959). Immunization of cattle against haemorrhagic septicaemia with purified capsular antigens. *Indian Veterinary Journal* **36**, 6-8.

Diallo, I. S. & Frost, A. J. (2000). Survival of avian strains of *Pasteurella multocida* in chicken serum. *Vet Microbiol* **72**, 153-161.

Dicker, I. B. & Seetharam, S. (1991). Cloning and nucleotide sequence of the *firA* gene and the *firA200*(Ts) allele from *Escherichia coli*. *J Bacteriol* **173**, 334-344.

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. *Res Vet Sci* **73**, 37-44.

Dowling, A., Hodgson, J. C., Daglcish, M. P., Eckersall, P. D. & Sales, J. (2004). Pathophysiological and immune cell responses in calves prior to and following lung challenge with formalin-killed *Pasteurella multocida* biotype A:3 and protection studies involving subsequent homologous live challenge. *Vet Immunol Immunopathol* **100**, 197-207.

Drzeniek, R., Scharmann, W. & Balke, E. (1972). Neuraminidase and N-acetylneuraminate pyruvate-lyase of *Pasteurella multocida*. *J Gen Microbiol* **72**, 357-368.

Dutta, J. K., Rathore, B. S., G., M. S., Singh, R. & Sharma, G. C. (1990). Epidemiological studies and occurence of haemorrhagic septicaemia in India. *Indian Veterinary Journal* **67**, 893-899.

Eckersall, P. D. & Conner, J. G. (1988). Bovine and canine acute phase proteins. *Vet Res Commun* **12**, 169-178.

Ekins, A. & Niven, D. F. (2002). Identification of *fur* and *fldA* homologs and a *Pasteurella multocida tbpA* homolog in *Histophilus ovis* and effects of iron availability on their transcription. *J Bacteriol* **184**, 2539-2542.

el-Eragi, A. M., Mukhtar, M. M. & Babiker, S. H. (2001). Specific antibodies of *Pasteurella multocida* in newborn calves of vaccinated dams. *Trop Anim Health Prod* **33**, 275-283.

FAO (2003). Selected Indicators of Food and Agriculture Development in Asia-Pacific Region 1992-2002: Regional office for Asia and the Pacific, Bangkok, October 2003.

Fernandez de Henestrosa, A. R., Badiola, et al., (1997). Importance of the *galE* gene on the virulence of *Pasteurella multocida*. *FEMS Microbiol Lett* **154**, 311-316.

Foged, N. T. (1991). Detection of stable epitopes on formaldehyde-detoxified *Pasteurella multocida* toxin by monoclonal antibodies. *Vaccine* **9**, 817-824.

Francis, B. K., Schels, H. F. & Carter, O. R. (1980). Type E *Pasteurella multocida* associated with haemorrhagic septicaemia in Zambia. *Vet Rec* **107**, 135.

Franklin, S. T., Young, J. W. & Nonnecke, B. J. (1991). Effects of ketones, acetate, butyrate, and glucose on bovine lymphocyte proliferation. *J Dairy Sci* **74**, 2507-2514.

Frost, A. J. & Adler, B. (2000). *Pasteurella multocida*: the elusive determinants of virulence and immunity. *Vet Microbiol* **72**, 1-2.

Fuller, T. E., Kennedy, M. J. & Lowery, D. E. (2000). Identification of *Pasteurella multocida* virulence genes in a septicemic mouse model using signature-tagged mutagenesis. *Microb Pathog* **29**, 25-38.

Galdiero, M., Palomba, E., De, L., Vitiello, M. & Pagnini, P. (1998). Effects of the major *Pasteurella multocida* porin on bovine neutrophils. *Am J Vet Res* **59**, 1270-1274.

Garrido, M. E., Bosch, M., Medina, R., Bigas, A., Llagostera, M., Perez De Rozas, A. M., Badiola, I. & Barbe, J. (2003a). *fur*-independent regulation of the *Pasteurella multocida hbpA* gene encoding a haemin-binding protein. *Microbiology* **149**, 2273-2281.

Garrido, M. E. b., Bosch, M., Medina, R., Llagostera, M., Perez de Rozas, A. M., Badiola, I. & Barbe, J. (2003b). The high-affinity zinc-uptake system *znuACB* is under control of the iron-uptake regulator (*fur*) gene in the animal pathogen *Pasteurella multocida*. *FEMS Microbiol Lett* **221**, 31-37.

Gatto, N. T., Dabo, S. M., Hancock, R. E. & Confer, A. W. (2002). Characterization of, and immune responses of mice to, the purified OmpA-equivalent outer membrane protein of *Pasteurella multocida* serotype A:3 (Omp28). *Vet Microbiol* **87**, 221-235.

Gebert, B., Fischer, W., Weiss, E., Hoffmann, R. & Haas, R. (2003). *Helicobacter pylori* vacuolating cytotoxin inhibits T lymphocyte activation. *Science* **301**, 1099-1102.

Gelfand, E. W., Cheung, R. K., Mills, G. B. & Grinstein, S. (1985). Mitogens trigger a calcium-independent signal for proliferation in phorbol-ester-treated lymphocytes. *Nature* **315**, 419-420.

Gerke, C., Falkow, S. & Chien, Y. H. (2005). The adaptor molecules LAT and SLP-76 are specifically targeted by *Yersinia* to inhibit T cell activation. *J Exp Med* **201**, 361-371.

Gilmour, N. J., Donachie, W., Sutherland, A. D., Gilmour, J. S., Jones, G. E. & Quirie, M. (1991). Vaccine containing iron-regulated proteins of *Pasteurella haemolytica* A2 enhances protection against experimental pasteurellosis in lambs. *Vaccine* **9**, 137-140.

Goldsby, R. A., Kindt, T. J., Osborne, B. A. & Kuby, J. (2003). *Immunology*, Fifth Edition edn: W. H. Freeman and Company.

Goswami, P. P., Chaudhuri, P., Tiwari, V., Parihar, N. S. & Harbola, P. C. (2004). Cloning and sequencing of a 16 kDa outer membrane protein gene of *Pasteurella multocida* P52. *Vet Res Commun* **28**, 17-25.

Greenwood, D., Slack, R. C. & Peutherer, J. F. (2002). *Medical Microbiology, a guide to microbial infections: pathogenesis, immunity, laboratory diagnosis and control*, Sixteenth edn: Churchill Livingstone.

Grossmann, E., Erler, W. & Schimmel, D. (1998). [Protein patterns of *Pasteurella multocida* strains in relation to the animal host and the geographic site of isolation]. *Berl Munch Tierarztl Wochenschr* **111**, 90-92.

Gummert, J. F., Barten, M. J., Sherwood, S. W., van Gelder, T. & Morris, R. E. (1999). Pharmacodynamics of immunosuppression by mycophenolic acid: inhibition of both lymphocyte proliferation and activation correlates with pharmacokinetics. *J Pharmacol Exp Ther* **291**, 1100-1112.

Hale, T. L. (1995). *Shigella* vaccines. In *Molecular and clinical aspects of bacterial vaccine development*, pp. 179-204. Edited by C. E. Ala'Aldeen: John Wiley & Sons.

Hamilton-Miller, J. M. & Shah, S. (1996). Anomalous but helpful findings from the BBL Crystal ID kit with *Haemophilus* spp. *Lett Appl Microbiol* **23**, 47-48.

Hansen, L. M. & Hirsh, D. C. (1989). Serum resistance is correlated with encapsulation of avian strains of *Pasteurella multocida*. *Vet Microbiol* **21**, 177-184.

Harper, M., Boyce, J. D., Wilkie, I. W. & Adler, B. (2003). Signature-tagged mutagenesis of *Pasteurella multocida* identifies mutants displaying differential virulence characteristics in mice and chickens. *Infect Immun* **71**, 5440-5446.

Harper, M., Cox, A. D., St Michael, F., Wilkie, I. W., Boyce, J. D. & Adler, B. (2004). A heptosyltransferase mutant of *Pasteurella multocida* produces a truncated lipopolysaccharide structure and is attenuated in virulence. *Infect Immun* **72**, 3436-3443.

Hassanin, H. H., Toth, T. E., Eldimerdash, M. M. & Siegel, P. B. (1995). Stimulation of avian respiratory phagocytes by *Pasteurella multocida*: effects of the route of exposure, bacterial dosage and strain, and the age of chickens. *Vet Microbiol* **46**, 401-413.

Heddleston, K. L. (1966). Immunologic and serologic comparison of three strains of *Pasteurella multocida*. *Cornell Vet* **56**, 235-241.

Heddleston, K. L., Rhoades, K. R. & Rebers, P. A. (1967). Experimental pasteurellosis: comparative studies on *Pasteurella multocida* from Asia, Africa, and North America. *Am J Vet Res* **28**, 1003-1012.

Heddleston, K. L., Gallagher, J. E. & Rebers, P. A. (1972). Fowl cholera: gel diffusion precipitin test for serotyping *Pasteurella multocida* from avian species. *Avian Dis* **16**, 925-936.

Heddleston, K. L. & Rebers, P. A. (1975). Properties of free endotoxin from *Pasteurella multocida*. *Am J Vet Res* **36**, 573-574.

Hernanz Moral, C., Flano del Castillo, E., Lopez Fierro, P., Villena Cortes, A., Anguita Castillo, J., Cascon Soriano, A., Sanchez Salazar, M., Razquin Peralta, B. & Naharro Carrasco, G. (1998). Molecular characterization of the *Aeromonas hydrophila aroA* gene and potential use of an auxotrophic *aroA* mutant as a live attenuated vaccine. *Infect Immun* **66**, 1813-1821.

Herzig, C., Blumerman, S., Lefranc, M. P. & Baldwin, C. (2006). Bovine T cell receptor gamma variable and constant genes: combinatorial usage by circulating gammadelta T cells. *Immunogenetics* **58**, 138-151.

Hodgson, J. C. (2001). Immunology - Backgroung: Moredun Research Institute.

Hodgson, J. C., Moon, G. M., Quirie, M. & Donachie, W. (2003). Association of LPS chemotype of *Mannheimia* (*Pasteurella*) *haemolytica* A1 with disease virulence in a model of ovine pneumonic pasteurellosis. *J Endotoxin Res* **9**, 25-32.

Hodgson, J. C., Finucane, A., Dagleish, M. P., Ataei, S., Parton, R. & Coote, J. G. (2005). Efficacy of vaccination of calves against hemorrhagic septicemia with a live *aroA* derivative of *Pasteurella multocida* B:2 by two different routes of administration. *Infect Immun* **73**, 1475-1481.

Hodgson, J. C., Watkins, C. A. & Bayne, C. W. (2006). Contribution of respiratory burst activity to innate immune function and the effects of disease status and agent on chemiluminescence responses by ruminant phagocytes in vitro. *Vet Immunol Immunopathol* **112**, 12-23.

Holland, W. G., My, L. N., Dung, T. V., Thanh, N. G., Tam, P. T., Vercruysse, J. & Goddeeris, B. M. (2001). The influence of T. evansi infection on the immuno-responsiveness of experimentally infected water buffaloes. *Vet Parasitol* **102**, 225-234.

Homchampa, P., Strugnell, R. A. & Adler, B. (1992). Molecular analysis of the *aroA* gene of *Pasteurella multocida* and vaccine potential of a constructed *aroA* mutant. *Mol Microbiol* **6**, 3585-3593.

Homchampa, P., Strugnell, R. A. & Adler, B. (1994). Construction and vaccine potential of an *aroA* mutant of *Pasteurella haemolytica*. *Vet Microbiol* **42**, 35-44.

Homchampa, P., Strugnell, R. A. & Adler, B. (1997). Cross protective immunity conferred by a marker-free *aroA* mutant of *Pasteurella multocida*. *Vaccine* **15**, 203-208.

Horadagoda, A., Eckersall, P. D., Alsemgeest, S. P. & Gibbs, H. A. (1993). Purification and quantitative measurement of bovine serum amyloid-A. *Res Vet Sci* **55**, 317-325.

Horadagoda, A., Eckersall, P. D., Hodgson, J. C., Gibbs, H. A. & Moon, G. M. (1994). Immediate responses in serum TNF alpha and acute phase protein concentrations to infection with *Pasteurella haemolytica* A1 in calves. *Res Vet Sci* **57**, 129-132.

Horadagoda, N. & Belak, K. (1990). Demonstration of *Pasteurella multocida* type 6:B (B:2) in formalin-fixed paraffin-embedded tissues of buffaloes by the peroxidase anti-peroxidase (PAP) technique. *Acta Vet Scand* **31**, 493-495.

Horadagoda, N. U., Knox, K. M., Gibbs, H. A., Reid, S. W., Horadagoda, A., Edwards, S. E. & Eckersall, P. D. (1999). Acute phase proteins in cattle: discrimination between acute and chronic inflammation. *Vet Rec* **144**, 437-441.

Horadagoda, N. U., Hodgson, J. C., Moon, G. M., Wijewardana, T. G. & Eckersall, P. D. (2001). Role of endotoxin in the pathogenesis of haemorrhagic septicaemia in the buffalo. *Microb Pathog* **30**, 171-178.

Horadagoda, N. U., Hodgson, J. C., Moon, G. M., Wijewardana, T. G. & Eckersall, P. D. (2002). Development of a clinical syndrome resembling haemorrhagic septicaemia in the buffalo following intravenous inoculation of *Pasteurella multocida* serotype B:2 endotoxin and the role of tumour necrosis factor-alpha. *Res Vet Sci* **72**, 194-200.

Hormaeche, C. E. (1995). *Salmonella* vaccines. In *Molecular and clinical aspects of bacterial vaccine development*, pp. 119-154. Edited by Ala'Aldenn: John Wiley & Sons.

Hornef, M. W., Wick, M. J., Rhen, M. & Normark, S. (2002). Bacterial strategies for overcoming host innate and adaptive immune responses. *Nat Immunol* **3**, 1033-1040.

Hughes, H. P., Campos, M., Potter, A. A. & Babiuk, L. A. (1992). Molecular chimerization of *Pasteurella haemolytica* leukotoxin to interleukin-2: effects on cytokine and antigen function. *Infect Immun* **60**, 565-570.

Hunt, M. L., Adler, B. & Townsend, K. M. (2000). The molecular biology of *Pasteurella multocida*. *Vet Microbiol* **72**, 3-25.

Ibrahim, R. S., Sawada, T., Shahata, M. & Ibrahim, A. (2000). Cross-protection and antigen expression by chicken embryo-grown *Pasteurella multocida* in chickens. *J Comp Pathol* **123**, 278-284.

Iovane, G., Galdiero, M., Vitiello, M. & De Martino, L. (1998a). Effect of *Pasteurella haemolytica* outer membrane proteins on bovine neutrophils. *FEMS Immunol Med Microbiol* **20**, 29-36.

Iovane, G., Pagnini, P., Galdiero, M., Cipollaro de l'Ero, G., Vitiello, M., D'Isanto, M. & Marcatili, A. (1998b). Role of *Pasteurella multocida* porin on cytokine expression and release by murine splenocytes. *Vet Immunol Immunopathol* **66**, 391-404.

Jablonski, P. E., Jablonski, L. M., Pintado, O., Sriranganathan, N. & Hovde, C. J. (1996). Identification of *Pasteurella multocida* tryptophan synthase beta-subunit by antisera against strain P1059. *Microbiology* **142 (Pt 1)**, 115-121.

Jacques, M. (2002). Virulence factors of *Pasteurellaceae*, formidable animal pathogens. *ASM News* **68**, 175-179.

Johnson, G. (1994). *Pasteurella* tenosynovitis following a dog bite. *Injury* **25**, 690-691.

Johnson, L. B., Busuito, M. J. & Khatib, R. (2000). Breast implant infection in a cat owner due to *Pasteurella multocida*. *J Infect* **41**, 110-111.

Johnson, R. B., Dawkins, H. J., Spencer, T. L., Saharee, A. A., Bahaman, A. R., Ramdani & Patten, B. E. (1989). Evaluation of bovine antibody responses to haemorrhagic septicaemia vaccine. *Res Vet Sci* **47**, 277-279.

Johnson, R. B., Dawkins, H. J. & Spencer, T. L. (1991). Electrophoretic profiles of *Pasteurella multocida* isolates from animals with hemorrhagic septicemia. *Am J Vet Res* **52**, 1644-1648.

Jones, T. O. & Hussaini, S. N. (1982). Outbreaks of *Pasteurella multocida* septicaemia in fallow deer (*Dama dama*). *Vet Rec* **110**, 451-452.

Jordan, R. W., Hamilton, T. D., Hayes, C. M., Patel, D., Jones, P. H., Roe, J. M. & Williams, N. A. (2003). Modulation of the humoral immune response of swine and mice mediated by toxigenic *Pasteurella multocida*. *FEMS Immunol Med Microbiol* **39**, 51-59.

Kabay, M. J., Mercy, A. R., Lloyd, J. M. & Robertson, G. M. (1992). Vaccine efficacy for reducing turbinate atrophy and improving growth rate in piggeries with endemic atrophic rhinitis. *Aust Vet J* **69**, 101-103.

Karol, M. H. (1998). Target organs and systems: methodologies to assess immune system function. *Environ Health Perspect* **106 Suppl 2**, 533-540.

Kasten, R. W., Hansen, L. M., Hinojoza, J., Bieber, D., Ruehl, W. W. & Hirsh, D. C. (1995). *Pasteurella multocida* produces a protein with homology to the P6 outer membrane protein of *Haemophilus influenzae*. *Infect Immun* **63**, 989-993.

Kasten, R. W., Carpenter, T. E., Snipes, K. P. & Hirsh, D. C. (1997a). Detection of *Pasteurella multocida*-specific DNA in turkey flocks by use of the polymerase chain reaction. *Avian Dis* **41**, 676-682.

Kasten, R. W., Wakenell, P. S., Ahmad, S., Yilma, T. D. & Hirsh, D. C. (1997b). Lack of protection against avian cholera by vaccination with recombinant P6-like protein from *Pasteurella multocida*. *Avian Dis* **41**, 972-976.

Kaufmann, S. H. E., Sher, A. & Ahmed, R. (2002). *Immunology of Infectious Diseases.* Mashington DC: American Siciety for Microbiology.

Kawamoto, E., Sawada, T., Suzuki, K. & Maruyama, T. (1990). Serotypes of *Pasteurella multocida* isolates from rabbits and their environment in Japan. *Nippon Juigaku Zasshi* **52**, 1277-1279.

Kaweh, M., Sohrab, V. & Baharsefat, M. (1960). Bovine pasteurellosis in Iran. Methods of vaccination. *Arch Inst Hessarak (Razi Vaccine and Serum Research Institute, Iran)* **12**, 99-100.

Kemper, C. & Atkinson, J. P. (2007). T-cell regulation: with complements from innate immunity. *Nat Rev Immunol* **7**, 9-18.

Knipp, U., Birkholz, S., Kaup, W. & Opferkuch, W. (1993). Immune suppressive effects of *Helicobacter pylori* on human peripheral blood mononuclear cells. *Med Microbiol Immunol (Berl)* **182**, 63-76.

Kotloff, K. L., Noriega, F., Losonsky, G. A., Sztein, M. B., Wasserman, S. S., Nataro, J. P. & Levine, M. M. (1996). Safety, immunogenicity, and transmissibility in humans of CVD 1203, a live oral *Shigella flexneri* 2a vaccine candidate attenuated by deletions in *aroA* and *virG*. *Infect Immun* **64**, 4542-4548.

Krause, T., Kunstyr, I. & Mutters, R. (1989). Characterization of some previously unclassified *Pasteurellaceae* isolated from hamsters. *J Appl Bacteriol* **67**, 171-175.

Kuhnert, P., Korczak, B., Falsen, E., Straub, R., Hoops, A., Boerlin, P., Frey, J. & Mutters, R. (2004). *Nicoletella semolina* gen. nov., sp. nov., a new member of *Pasteurella*ceae isolated from horses with airway disease. *J Clin Microbiol* **42**, 5542-5548.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lee, M. D., Wooley, R. E., Glisson, J. R. & Brown, J. (1988). Comparison of *Pasteurella multocida* serotype 3,4 isolates from turkeys with fowl cholera. *Avian Dis* **32**, 501-508.

Lee, M. D., Wooley, R. E., Brown, J. & Glisson, J. R. (1991). A survey of potential virulence markers from avian strains of *Pasteurella multocida*. *Vet Microbiol* **26**, 213-225.

Li, Z., Nestor, K. E., Saif, Y. M., Bacon, W. L. & Anderson, J. W. (1999). Effect of selection for increased body weight on mitogenic responses in turkeys. *Poult Sci* **78**, 1532-1535.

Liu, D., Lawrence, M. L. & Austin, F. W. (2004). Specific PCR identification of *Pasteurella multocida* based on putative transcriptional regulator genes. *J Microbiol Methods* **58**, 263-267.

Lo, M., Boyce, J. D., Wilkie, I. W. & Adler, B. (2004). Characterization of two lipoproteins in *Pasteurella multocida*. *Microbes Infect* **6**, 58-67.

Loosmore, S. M., Yang, Y. P., Coleman, D. C., Shortreed, J. M., England, D. M. & Klein, M. H. (1997). Outer membrane protein D15 is conserved among *Haemophilus influenzae* species and may represent a universal protective antigen against invasive disease. *Infect Immun* **65**, 3701-3707.

Lu, Y. S., Afendis, S. J. & Pakes, S. P. (1988). Identification of immunogenic outer membrane proteins of *Pasteurella multocida* 3:A in rabbits. *Infect Immun* **56**, 1532-1537.

Lu, Y. S., Lai, W. C., Pakes, S. P. & Nie, L. C. (1991a). A monoclonal antibody against a *Pasteurella multocida* outer membrane protein protects rabbits and mice against pasteurellosis. *Infect Immun* **59**, 172-180.

Lu, Y. S., Lai, W. C., Pakes, S. P. & Stefanu, C. (1991b). The outer membrane of *Pasteurella multocida* 3:A protects rabbits against homologous challenge. *Infect Immun* **59**, 4517-4523.

Lugtenberg, B., van Boxtel, R., Evenberg, D., de Jong, M., Storm, P. & Frik, J. (1986). Biochemical and immunological characterization of cell surface proteins of *Pasteurella multocida* strains causing atrophic rhinitis in swine. *Infect Immun* **52**, 175-182.

Luo, Y., Glisson, J. R., Jackwood, M. W., Hancock, R. E., Bains, M., Cheng, I. H. & Wang, C. (1997). Cloning and characterization of the major outer membrane protein gene (*ompH*) of *Pasteurella multocida* X-73. *J Bacteriol* **179**, 7856-7864.

Luo, Y., Zeng, Q., Glisson, J. R., Jackwood, M. W., Cheng, I. H. & Wang, C. (1999). Sequence analysis of *Pasteurella multocida* major outer membrane protein (OmpH) and application of synthetic peptides in vaccination of chickens against homologous strain challenge. *Vaccine* **17**, 821-831.

Maheswaran, S. K., Thies, E. S. & Dua, S. K. (1976). Studies on *Pasteurella multocida*. III. In vitro assay for cell-mediated immunity. *Avian Dis* **20**, 332-341.

Maheswaran, S. K. & Thies, E. S. (1979). *Pasteurella multocida* antigen-induced in vitro lymphocyte immunostimulation, using whole blood from cattle and turkeys. *Res Vet Sci* **26**, 25-31.

Majury, A. L. & Shewen, P. E. (1991a). The effect of *Pasteurella haemolytica* A1 leukotoxic culture supernate on the in vitro proliferative response of bovine lymphocytes. *Vet Immunol Immunopathol* **29**, 41-56.

Majury, A. L. & Shewen, P. E. (1991b). Preliminary investigation of the mechanism of inhibition of bovine lymphocyte proliferation by *Pasteurella haemolytica* A1 leukotoxin. *Vet Immunol Immunopathol* **29**, 57-68.

Manoha, F., Chevalier, G., Wroblewski, H. & Delamarche, C. (1994). Cloning and expression of two *Pasteurella multocida* genes in Escherichia coli. *Biochimie* **76**, 9-14.

Marandi, M. & Mittal, K. R. (1996). Characterization of an outer membrane protein of *Pasteurella multocida* belonging to the OmpA family. *Vet Microbiol* **53**, 303-314.

Marion, T. N., Dzierzak, E. A., Lee, H. S., Adams, R. L. & Janeway, C. A., Jr. (1984). Non-dinitrophenyl-binding immunoglobulin that bears a dominant idiotype (Id460) associated with antidinitrophenyl antibody is specific for an antigen on *Pasteurella pneumotropica*. *J Exp Med* **159**, 221-233.

Marsden, M. J., Vaughan, L. M., Foster, T. J. & Secombes, C. J. (1996). A live (*delta aroA*) Aeromonas salmonicida vaccine for furunculosis preferentially stimulates T-cell responses relative to B-cell responses in rainbow trout (*Oncorhynchus mykiss*). *Infect Immun* **64**, 3863-3869.

Martrenchar, A. & Njanpop, B. M. (1994). [First case of an outbreak of hemorrhagic septicemia caused by *Pasteurella multocida* serotype B6 in northern Cameroon]. *Rev Elev Med Vet Pays Trop* **47**, 19-20.

May, B. J., Zhang, Q., Li, L. L., Paustian, M. L., Whittam, T. S. & Kapur, V. (2001). Complete genomic sequence of *Pasteurella multocida*, Pm70. *Proc Natl Acad Sci U S A* **98**, 3460-3465.

Mehrotra, S., Singh, V. K., Agarwal, S. S., Maurya, R. & Srimal, R. C. (2002). Antilymphoproliferative activity of ethanolic extract of *Boerhaavia diffusa* roots. *Exp Mol Pathol* **72**, 236-242.

Miller-Edge, M. & Splitter, G. (1986). Detection of impaired T cell-mediated immune responses to herpesvirus (BHV-1) in cattle. *Vet Immunol Immunopathol* **13**, 1-18.

Mock, M., Crasnier, M., Duflot, E., Dumay, V. & Danchin, A. (1991). Structural and functional relationships between *Pasteurella multocida* and enterobacterial adenylate cyclases. *J Bacteriol* **173**, 6265-6269.

Morishita, T. Y., Snipes, K. P. & Carpenter, T. E. (1990). Serum resistance as an indicator of virulence of *Pasteurella multocida* for turkeys. *Avian Dis* **34**, 888-892.

Morton, R. J., Simons, K. R. & Confer, A. W. (1996). Major outer membrane proteins of *Pasteurella haemolytica* serovars 1-15: comparison of separation techniques and surface-exposed proteins on selected serovars. *Vet Microbiol* **51**, 319-330.

Motoi, Y., Itoh, H., Tamura, K., Miyamoto, T., Oohashi, T. & Nagasawa, S. (1992). Correlation of serum concentration of alpha 1-acid glycoprotein with lymphocyte blastogenesis and development of experimentally induced or naturally acquired hepatic abscesses in cattle. *Am J Vet Res* **53**, 574-579.

Mueller, M., Stamme, C., Draing, C., Hartung, T., Seydel, U. & Schromm, A. B. (2006). Cell activation of human macrophages by lipoteichoic acid is strongly attenuated by lipopolysaccharide-binding protein. *J Biol Chem* **281**, 31448-31456.

Muhairwa, A. P., Christensen, J. P. & Bisgaard, M. (2002). Serum resistance of *Pasteurella multocida* in avian and porcine sera, and comparative virulence investigations of selected serum-sensitive and resistant strains in chickens. *Avian Pathol* **31**, 183-191.

Mukherjee, S., Ahmed, A. & Nandi, D. (2005). CTLA4-CD80/CD86 interactions on primary mouse CD4+ T cells integrate signal-strength information to modulate activation with Concanavalin A. *J Leukoc Biol* **78**, 144-157.

Muller, H. E. & Krasemann, C. (1974). [The virulence of *Pasteurella multocida* strains and their neuraminidase production (author's transl)]. *Zentralbl Bakteriol [Orig A]* **229**, 391-400.

Muneer, R., Akhtar, S. & Afzal, M. (1994). Evaluation of three oil-adjuvant vaccines against *Pasteurella multocida* in buffalo calves. *Rev Sci Tech* **13**, 837-843.

Muniandy, N., Love, D. N. & Mukkur, T. K. (1998). Immunogenicity of purified lipopolysaccharide or protein-oligosaccharide conjugates of *Pasteurella multocida* type 6:B in mice. *Comp Immunol Microbiol Infect Dis* **21**, 257-279.

Murata, H., Shimada, N. & Yoshioka, M. (2004). Current research on acute phase proteins in veterinary diagnosis: an overview. *Vet J* **168**, 28-40.

Musa, B. E., Conner, G. H., Carter, G. R., Gupta, B. N. & Keahey, K. K. (1972). Physiologic and pathologic changes in calves given *Escherichia coli* endotoxin or *Pasteurella multocida*. *Am J Vet Res* **33**, 911-916.

Mutapi, F., Burchmore, R., Mduluza, T., Foucher, A., Harcus, Y., Nicoll, G., Midzi, N., Turner, C. M. & Maizels, R. M. (2005). Praziquantel treatment of individuals exposed to *Schistosoma haematobium* enhances serological recognition of defined parasite antigens. *J Infect Dis* **192**, 1108-1118.

Myint, A., Carter, G. R. & Jones, T. O. (1987). Prevention of experimental haemorrhagic septicaemia with a live vaccine. *Vet Rec* **120**, 500-501.

Myint, A. & Carter, G. R. (1989). Prevention of haemorrhagic septicaemia in buffaloes and cattle with a live vaccine. *Vet Rec* **124**, 508-509.

Myint, A., Jones, T. O. & Nyunt, H. H. (2005). Safety, efficacy and cross-protectivity of a live intranasal aerosol haemorrhagic septicaemia vaccine. *Vet Rec* **156**, 41-45.

Myint, A. a. C., G.R. (1990). Field use of live haemorrhagic septicaemia vaccine. *Vet Rec* **126**, 648.

Nagy, L. K. & Penn, C. W. (1976). Protection of cattle against experimental haemorrhagic septicaemia by the capsular antigens of *Pasteurella multocida*, types B and E. *Res Vet Sci* **20**, 249-253.

Nagy, L. K. H., N.; Sudana, T.G. (1979). Vaccination experiments with capsular antigens of *Pasteurella multocida*. Sri Lanka: FAO/APHCA.

Namioka, S. & Murata, M. (1961). Serological studies on *Pasteurella multocida*. III. O antigenic analysis of cultures isolated from various animals. *Cornell Vet* **51**, 522-528.

Namioka, S. & Murata, M. (1964). Serological Studies on *Pasteurella multocida*. V. Some Epizootiological Findings Resulting from O Antigenic Analysis. *Cornell Vet* **54**, 520-534.

Nikaido, H. (2003). Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* **67**, 593-656.

Noriega, F. R., Wang, J. Y., Losonsky, G., Maneval, D. R., Hone, D. M. & Levine, M. M. (1994). Construction and characterization of attenuated *delta aroA delta virG Shigella flexneri* 2a strain CVD 1203, a prototype live oral vaccine. *Infect Immun* **62**, 5168-5172.

O'Gaora, P., Roberts, M., Bowe, F., Hormaeche, C., Demarco de Hormaeche, R., Cafferkey, M., Tite, J. & Dougan, G. (1990). *Yersinia enterocolitica aroA* mutants as carriers of the B subunit of the *Escherichia coli* heat-labile enterotoxin to the murine immune system. *Microb Pathog* **9**, 105-116.

OIE (2000). Haemorrhagic Septicaemia. In *Manual of standards for diagnostic tests and vaccines*, pp. Part 2 Section 2.3. Chapter 2.3.12.: Off. int. Epiz. (OIE).

OIE (2004). HAEMORRHAGIC SEPTICAEMIA. In Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th edition, 2004: OIE.

Olsen, J. E., Bisgaard, M., Kuhnert, P. & Christensen, H. (2005). The Current Status of *Pasteurellaceae* Systematics. . In *ASM Conference on Pasteurellaceae*, pp. 14. Hawaii: American Society for Microbiology.

Olson, N. C., Hellyer, P. W. & Dodam, J. R. (1995). Mediators and vascular effects in response to endotoxin. *Br Vet J* **151**, 489-522.

Onah, D. N., Hopkins, J. & Luckins, A. G. (1998). Proliferative responses of peripheral blood leucocytes of sheep infected with Trypanosoma evansi. *Scand J Immunol* **48**, 170-176.

Oyston, P. C., Russell, P., Williamson, E. D. & Titball, R. W. (1996). An *aroA* mutant of *Yersinia pestis* is attenuated in guinea-pigs, but virulent in mice. *Microbiology* **142 (Pt 7)**, 1847-1853.

Park, Y. H., Fox, L. K., Hamilton, M. J. & Davis, W. C. (1993). Suppression of proliferative response of BoCD4+ T lymphocytes by activated BoCD8 + T lymphocytes in the mammary gland of cows with *Staphylococcus aureus* mastitis. *Vet Immunol Immunopathol* **36**, 137-151.

Pati, U. S., Srivastava, S. K., Roy, S. C. & More, T. (1996). Immunogenicity of outer membrane protein of *Pasteurella multocida* in buffalo calves. *Vet Microbiol* **52**, 301-311.

Paustian, M. L., May, B. J. & Kapur, V. (2001). *Pasteurella multocida* gene expression in response to iron limitation. *Infect Immun* **69**, 4109-4115.

Paustian, M. L., May, B. J. & Kapur, V. (2002). Transcriptional response of *Pasteurella multocida* to nutrient limitation. *J Bacteriol* **184**, 3734-3739.

Peltier, M. R., Liu, W. J. & Hansen, P. J. (2000). Regulation of lymphocyte proliferation by uterine serpin: interleukin-2 mRNA production, CD25 expression and responsiveness to interleukin-2. *Proc Soc Exp Biol Med* **223**, 75-81.

Penn, C. W. & Nagy, L. K. (1976). Isolation of a protective, non-toxic capsular antigen from *Pasteurella multocida*, types B and E. *Res Vet Sci* **20**, 90-96.

Pestka, J. & Zhou, H. R. (2006). Toll-like receptor priming sensitizes macrophages to proinflammatory cytokine gene induction by deoxynivalenol and other toxicants. *Toxicol Sci* **92**, 445-455.

Petersen, S. K., Foged, N. T., Bording, A., Nielsen, J. P., Riemann, H. K. & Frandsen, P. L. (1991). Recombinant derivatives of *Pasteurella multocida* toxin: candidates for a vaccine against progressive atrophic rhinitis. *Infect Immun* **59**, 1387-1393.

Pijoan, C., Trigo, E., Hogg, A. (1988). Atrophic rhinitis in pigs associated with a toxinogenic strain of *Pasteurella multocida* serotype A. In *Proceedings of International Congress on Pig Vet Soc*, pp. 32. Rio de Janeiro.

Pontarollo, R. A., Rankin, R., Babiuk, L. A., Godson, D. L., Griebel, P. J., Hecker, R., Krieg, A. M. & van Drunen Littel-van den Hurk, S. (2002). Monocytes are required for optimum in vitro stimulation of bovine peripheral blood mononuclear cells by non-methylated CpG motifs. *Vet Immunol Immunopathol* **84**, 43-59.

Porter, J. F., Connor, K., Krueger, N., Hodgson, J. C. & Donachie, W. (1995). Predisposition of specific pathogen-free lambs to *Pasteurella haemolytica* pneumonia by *Bordetella parapertussis* infection. *J Comp Pathol* **112**, 381-389.

Powers, K. A., Szaszi, K., Khadaroo, R. G., Tawadros, P. S., Marshall, J. C., Kapus, A. & Rotstein, O. D. (2006). Oxidative stress generated by hemorrhagic shock recruits Toll-like receptor 4 to the plasma membrane in macrophages. *J Exp Med* **203**, 1951-1961.

Priebe, S. & Schwarz, S. (2003). In vitro activities of florfenicol against bovine and porcine respiratory tract pathogens. *Antimicrob Agents Chemother* **47**, 2703-2705.

Prince, G. H. & Smith, J. E. (1966). Antigenic studies on *Pasteurella multocida* using immunodiffusion techniques. II. Relationships with other gram-negative species. *J Comp Pathol* **76**, 315-320.

Radostits, O. M., Gay, C. C., Blood, C. C. & Hinchcliff, K. W. (2000). Veterinary Medicine (9th Ed.), Ninth Edition edn: Harcourt Publishers Ltd.

Rajeev, S., Nair, R. V., Kania, S. A. & Bemis, D. A. (2003). Expression of a truncated *Pasteurella multocida* toxin antigen in *Bordetella bronchiseptica*. *Vet Microbiol* **94**, 313-323.

Ramdani, Dawkins, H. J., Johnson, R. B., Spencer, T. L. & Adler, B. (1990). *Pasteurella multocida* infections in mice with reference to haemorrhagic septicaemia in cattle and buffalo. *Immunol Cell Biol* **68 (Pt 1)**, 57-61.

Ramdani & Adler, B. (1991). Opsonic monoclonal antibodies against lipopolysaccharide (LPS) antigens of *Pasteurella multocida* and the role of LPS in immunity. *Vet Microbiol* **26**, 335-347.

Ramdani & Adler, B. (1994). Serotype and genus specific protein antigens of *Pasteurella multocida*, revealed by monoclonal antibodies. *Vet Microbiol* **41**, 29-40.

Ravaoarinoro, M., Ciurli, C., Toma, E. & Morisset, R. (1994). Rapid method for isolating detergent-insoluble outer membrane proteins from *Pseudomonas aeruginosa*. *Electrophoresis* **15**, 594-596.

Rhoades, K. R. & Rimler, R. B. (1987). Effects of *Pasteurella multocida* endotoxins on turkey poults. *Avian Dis* **31**, 523-526.

Rimler, R. B., Rebers, P. A. & Phillips, M. (1984). Lipopolysaccharides of the Heddleston serotypes of *Pasteurella multocida*. *Am J Vet Res* **45**, 759-763.

Rimler, R. B. & Brogden, K. A. (1986). *Pasteurella multocida* isolated from rabbits and swine: serologic types and toxin production. *Am J Vet Res* **47**, 730-737.

Rimler, R. B. & Rhoades, K. R. (1987). Serogroup F, a new capsule serogroup of *Pasteurella multocida*. *J Clin Microbiol* **25**, 615-618.

Rimler, R. B., Angus, R. D. & Phillips, M. (1989). Evaluation of the specificity of *Pasteurella multocida* somatic antigen-typing antisera prepared in chickens, using ribosome-lipopolysaccharide complexes as inocula. *Am J Vet Res* **50**, 29-31.

Rimler, R. B. & Rhoades, K. R. (1989). Solubilization of membrane-associated cross-protection factor(s) of *Pasteurella multocida*. *Avian Dis* **33**, 258-263.

Rimler, R. B. & Rhoades, K. R. (1994). Hyaluronidase and chondroitinase activity of *Pasteurella multocida* serotype B:2 involved in hemorrhagic septicaemia. *Vet Rec* **134**, 67-68.

Rimler, R. B. & Wilson, M. A. (1994). Re-examination of *Pasteurella multocida* serotypes that caused haemorrhagic septicaemia in North America. *Vet Rec* **134**, 256.

Rimler, R. B. (1996). Passive immune cross-protection in mice produced by rabbit antisera against different serotypes of *Pasteurella multocida*. *J Comp Pathol* **114**, 347-360.

Rimler, R. B. (2000). Restriction endonuclease analysis using HhaI and HpaII to discriminate among group B *Pasteurella multocida* associated with haemorrhagic septicaemia. *J Med Microbiol* **49**, 81-87.

Rimler, R. B. (2001). Purification of a cross-protective antigen from *Pasteurella multocida* grown in vitro and in vivo. *Avian Dis* **45**, 572-580.

Rimler, R. B., Glisson J.R. (1997). Fowl Cholera (in Diseases of Poultry). In *Diseases of Poultry*: Iowa State University Press.

Roitt, I., Brostoff, J. & Male, D. (1998). *Immunology*, Fifth Edition edn: Dianne Zack.

Roy, S. C., More, T., Pati, U. S. & Srivastava, S. K. (1996). Effect of *Pasteurella multocida* vaccination on buffalo polymorphonuclear hydrogen peroxide and nitric oxide production. *Vet Immunol Immunopathol* **51**, 173-178.

Ruffolo, C. G. & Adler, B. (1996). Cloning, sequencing, expression, and protective capacity of the *oma87* gene encoding the *Pasteurella multocida* 87-kilodalton outer membrane antigen. *Infect Immun* **64**, 3161-3167.

Ruffolo, C. G., Jost, B. H. & Adler, B. (1998). Iron-regulated outer membrane proteins of *Pasteurella multocida* and their role in immunity. *Vet Microbiol* **59**, 123-137.

Ryu, H. I. & Kim, C. J. (2000). Immunologic reactivity of a lipopolysaccharide-protein complex of type A *Pasteurella multocida* in mice. *J Vet Sci* **1**, 87-95.

Sathiyaseelan, T. & Baldwin, C. L. (2000). Evaluation of cell replication by bovine T cells in polyclonally activated cultures using carboxyfluorescein succinimidyl ester (CFSE) loading and flow cytometric analysis. *Res Vet Sci* **69**, 275-281.

Sawada, T., Rimler, R. B. & Rhoades, K. R. (1982). Indirect hemagglutination test that uses glutaraldehyde-fixed sheep erythrocytes sensitized with extract antigens for detection of *Pasteurella* antibody. *J Clin Microbiol* **15**, 752-756.

Sawada, T., Rimler, R. B. & Rhoades, K. R. (1985). Hemorrhagic septicemia: naturally acquired antibodies against *Pasteurella multocida* types B and E in calves in the United States. *Am J Vet Res* **46**, 1247-1250.

Scharmann, W. & Blobel, H. (1972). [Serological studies on bacterial neuraminidases with special reference to the neuraminidase of *Pasteurella multocida*]. *Zentralbl Bakteriol [Orig A]* **221**, 322-326.

Schryvers, A. B., Wong, S. S. & Bryan, L. E. (1986). Antigenic relationships among penicillin-binding proteins 1 from members of the families *Pasteurellaceae* and *Enterobacteriaceae*. *Antimicrob Agents Chemother* **30**, 559-564.

Schwab, J. H. (1975). Suppression of the immune response by microorganisms. *Bacteriol Rev* **39**, 121-143.

Scott, P. C., Markham, J. F. & Whithear, K. G. (1999). Safety and efficacy of two live *Pasteurella multocida aro-A* mutant vaccines in chickens. *Avian Dis* **43**, 83-88.

Shah, N. H., Biewenga, J. & de Graaf, F. K. (1996). Vacuolating cytotoxic activity of *Pasteurella multocida* causing haemorrhagic septicaemia in buffalo and cattle. *FEMS Microbiol Lett* **143**, 97-101.

Shah, N. H. & de Graaf, F. K. (1997). Protection against haemorrhagic septicaemia induced by vaccination of buffalo calves with an improved oil adjuvant vaccine. *FEMS Microbiol Lett* **155**, 203-207.

Sheikh, M. A., Tasneem, K., Zafar, M. S., Butt, I. A. & Shakoori, A. R. (1995). Effect of storage on the prevalent alum-precipitated hemorrhagic septicaemia vaccine in Pakistan and preparation of a more efficient oil adjuvant vaccine using dense culture of *Pasteurella multocida* Roberts type 1 on an improved culture medium. *Zentralbl Veterinarmed B* **42**, 28-34.

Sheikh, M. A., Anzam, M. & Shakoori, A. R. (1996). Observations on haemorrhagic septicaemia in Pakistan livestock. *Zentralbl Veterinarmed B* **43**, 293-304.

Shigidi, M. T. & Mustafa, A. A. (1979). Biochemical and serological studies on *Pasteurella multocida* isolated from cattle in the Sudan. *Cornell Vet* **69**, 77-84.

Sing, A., Roggenkamp, A., Geiger, A. M. & Heesemann, J. (2002). *Yersinia enterocolitica* evasion of the host innate immune response by V antigen-induced IL-10

production of macrophages is abrogated in IL-10-deficient mice. *J Immunol* **168**, 1315-1321.

Solano, W., Giambrone, J. J. & Panangala, V. S. (1983). Comparison of enzyme-linked immunosorbent assay and indirect hemagglutination test for quantitating antibody responses in chickens against *Pasteurella multocida*. *Avian Dis* **27**, 1034-1042.

Srivastava, S. K. (1998). Outer membrane protein of *Pasteurella multocida* serotype B:2 is immunogenic and antiphagocytic. *Indian J Exp Biol* **36**, 530-532.

Stevens, M. G., Olsen, S. C. & Cheville, N. F. (1997). Comparative effects of bovine cytokines on cattle and bison peripheral blood mononuclear cell proliferation. *Comp Immunol Microbiol Infect Dis* **20**, 155-162.

Subramaniam, S., Chua, K. L., Tan, H. M., Loh, H., Kuhnert, P. & Frey, J. (1997). Phylogenetic position of *Riemerella anatipestifer* based on 16S rRNA gene sequences. *Int J Syst Bacteriol* **47**, 562-565.

Sundrud, M. S., Torres, V. J., Unutmaz, D. & Cover, T. L. (2004). Inhibition of primary human T cell proliferation by *Helicobacter pylori* vacuolating toxin (VacA) is independent of VacA effects on IL-2 secretion. *Proc Natl Acad Sci U S A* **101**, 7727-7732.

Tabatabaei, M. (2000). Construction and characterisation of attenuated derivatives of *Pasteurella multocida*. In *Division of Infection and Immunity, Facult of Biomedical and Life Sciences*. Glasgow, Scotlan, UK: University of Glagow.

Tabatabaei, M., Liu, Z., Finucane, A., Parton, R. & Coote, J. (2002). Protective immunity conferred by attenuated *aroA* derivatives of *Pasteurella multocida* B:2 strains in a mouse model of hemorrhagic septicemia. *Infect Immun* **70**, 3355-3362.

Tabatabai, L. B. & Zehr, E. S. (2004). Identification of five outer membrane-associated proteins among cross-protective factor proteins of *Pasteurella multocida*. *Infect Immun* **72**, 1195-1198.

Taylor, P. W. (1983). Bactericidal and bacteriolytic activity of serum against gram-negative bacteria. *Microbiol Rev* **47**, 46-83.

Tizard, I., R. (2000). Veterinary Immunology an introduction, Sixth Edition edn: W. B. Saunders Company.

Tomer, P., Chaturvedi, G. C., Minakshi, Malik, P. & Monga, D. P. (2002). Comparative analysis of the outer membrane protein profiles of isolates of the *Pasteurella multocida* (B:2) associated with haemorrhagic septicaemia. *Vet Res Commun* **26**, 513-522.

Townsend, K. M., Dawkins, H. J. & Papadimitriou, J. M. (1997). REP-PCR analysis of *Pasteurella multocida* isolates that cause haemorrhagic septicaemia. *Res Vet Sci* **63**, 151-155.

Townsend, K. M., Frost, A. J., Lee, C. W., Papadimitriou, J. M. & Dawkins, H. J. (1998a). Development of PCR assays for species- and type-specific identification of *Pasteurella multocida* isolates. *J Clin Microbiol* **36**, 1096-1100.

Townsend, K. M., O'Boyle, D., Phan, T. T., Hanh, T. X., Wijewardana, T. G., Wilkie, I., Trung, N. T. & Frost, A. J. (1998b). Acute septicaemic pasteurellosis in Vietnamese pigs. *Vet Microbiol* **63**, 205-215.

Truscott, W. M. & Hirsh, D. C. (1988). Demonstration of an outer membrane protein with antiphagocytic activity from *Pasteurella multocida* of avian origin. *Infect Immun* **56**, 1538-1544.

Vanden Bush, T. J. & Rosenbusch, R. F. (2002). Mycoplasma bovis induces apoptosis of bovine lymphocytes. *FEMS Immunol Med Microbiol* **32**, 97-103.

Vanden Bush, T. J. & Rosenbusch, R. F. (2004). Characterization of a lympho-inhibitory peptide produced by *Mycoplasma bovis*. *Biochem Biophys Res Commun* **315**, 336-341.

Vasfi Marandi, M., Dubreuil, J. D. & Mittal, K. R. (1996). The 32 kDa major outer-membrane protein of *Pasteurella multocida* capsular serotype D. *Microbiology* **142 (Pt 1)**, 199-206.

Vasfi Marandi, M. & Mittal, K. R. (1997). Role of outer membrane protein H (OmpH)- and OmpA-specific monoclonal antibodies from hybridoma tumors in protection of mice against *Pasteurella multocida*. *Infect Immun* **65**, 4502-4508.

Veken, J. W., Shah, N. H., Klaasen, P., Oudega, B. & de Graaf, F. K. (1996). Binding of host iron-binding proteins and expression of iron-regulated membrane proteins by different serotypes of *Pasteurella multocida* causing haemorrhagic septicaemia. *Microb Pathog* **21**, 59-64.

Verma, N. D. (1988). *Pasteurella multocida* B:2 in haemorrhagic septicaemia outbreak in pigs in India. *Vet Rec* **123**, 63.

Verma, N. K. & Lindberg, A. A. (1991). Construction of aromatic dependent *Shigella flexneri* 2a live vaccine candidate strains: deletion mutations in the *aroA* and the *aroD* genes. *Vaccine* **9**, 6-9.

Verma, R. & Jaiswal, T. N. (1997). Protection, humoral and cell-mediated immune responses in calves immunized with multiple emulsion haemorrhagic septicaemia vaccine. *Vaccine* **15**, 1254-1260.

Verma, R. & Jaiswal, T. N. (1998). Haemorrhagic septicaemia vaccines. *Vaccine* **16**, 1184-1192.

Vivas, J., Riano, J., Carracedo, B., Razquin, B. E., Lopez-Fierro, P., Naharro, G. & Villena, A. J. (2004). The auxotrophic *aroA* mutant of *Aeromonas hydrophila* as a live attenuated vaccine against *A. salmonicida* infections in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol* **16**, 193-206.

Vivas, J., Razquin, B., Lopez-Fierro, P. & Villena, A. J. (2005). Modulation of the immune response to an *Aeromonas hydrophila aroA* live vaccine in rainbow trout: effect of culture media on the humoral immune response and complement consumption. *Fish Shellfish Immunol* **18**, 223-233.

Vorob'ev, A. A., Borisova, E. V., Molozhavaia, O. S. & Borisov, V. A. (2001). [Immunosuppressive effects of pathogenic gram-negative bacteria]. *Vestn Ross Akad Med Nauk*, 21-25.

Walker, J. C. & Verma, N. K. (1997). Cloning and characterisation of the *aroA* and *aroD* genes of *Shigella dysenteriae* type 1. *Microbiol Immunol* **41**, 809-813.

Wang, H., Gao, Y., Gong, Y., Chen, X., Liu, C., Zhou, X., Blackall, P. J., Zhang, P. & Yang, H. (2007). Identification and immunogenicity of an immunodominant mimotope of *Avibacterium paragallinarum* from a phage display peptide library. *Vet Microbiol* **119**, 231-239.

Watt, J. M., Swiatlo, E., Wade, M. M. & Champlin, F. R. (2003). Regulation of capsule biosynthesis in serotype A strains of *Pasteurella multocida*. *FEMS Microbiol Lett* **225**, 9-14.

Wei, B. D. & Carter, G. R. (1978). Live streptomycin-dependent *Pasteurella multocida* vaccine for the prevention of hemorrhagic septicemia. *Am J Vet Res* **39**, 1534-1537.

Weinberg, A. D., Magnuson, N. S., Reeves, R., Wyatt, C. R. & Magnuson, J. A. (1988). Evidence for two discrete phases of IL-2 production in bovine lymphocytes. *J Immunol* **141**, 1174-1179.

Weiser, J. N. & Gotschlich, E. C. (1991). Outer membrane protein A (OmpA) contributes to serum resistance and pathogenicity of *Escherichia coli* K-1. *Infect Immun* **59**, 2252-2258.

Wijewardana, T. G., De Alwis M.C.L. and Vipulasiri A.A. (1982). An agar gel diffusion test for rapid identification of *Pasteurella multocida* type B (Carter). *Sri Lanka Vet J* **30**, 12-14.

Wijewardana, T. G. D. A., M. C. L.; Bastianz, H. L. G. (1986). Cultural, biochemical, serological and pathogenecity studies of strains of *Pasteurella multocida* isolated from carrier animals and outbreaks of haemorrhagic septicaemia. *S L Vet J* **34**, 43-57.

Wilson, M. A., Rimler, R. B. & Hoffman, L. J. (1992). Comparison of DNA fingerprints and somatic serotypes of serogroup B and E *Pasteurella multocida* isolates. *J Clin Microbiol* **30**, 1518-1524.

Xia, X., Wei, T., Xie, Z. & Danchin, A. (2002). Genomic changes in nucleotide and dinucleotide frequencies in *Pasteurella multocida* cultured under high temperature. *Genetics* **161**, 1385-1394.

Yamanaka, H., Hagiwara, K., Kirisawa, R. & Iwai, H. (2003). Proinflammatory cytokines in bovine colostrum potentiate the mitogenic response of peripheral blood mononuclear cells from newborn calves through IL-2 and CD25 expression. *Microbiol Immunol* **47**, 461-468.

Yao, T., Mecsas, J., Healy, J. I., Falkow, S. & Chien, Y. (1999). Suppression of T and B lymphocyte activation by a Yersinia pseudotuberculosis virulence factor, yopH. *J Exp Med* **190**, 1343-1350.

Zhao, G., Pijoan, C., Choi, K., Maheswaran, S. K. & Trigo, E. (1995). Expression of iron-regulated outer membrane proteins by porcine strains of *Pasteurella multocida*. *Can J Vet Res* **59**, 46-50.