Investigation of survival mechanisms of *Pasteurella haemolytica* and *Pasteurella trehalosi* in vivo and in vitro.

HELEN A ROWE

A thesis submitted for the degree of Doctor of Philosophy in the University of Edinburgh.
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DECLARATION

I declare that this thesis has been composed entirely by myself and that the work contained in it, except where clearly stated, was performed by myself.

THE UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS

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Name of Candidate Helen A Rowe

Address Moredun Research Institute, 408 Gilmerton Road, Edinburgh

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Title of Thesis Investigation of survival mechanisms of *Pasteurella haemolytica* and *Pasteurella trehalosi* in vivo and in vitro.

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Pasteurella haemolytica serotypes A1 and A2 and *Pasteurella trehalosi* serotype T10 were cultured in "in-vivo" fluids (ruminant tracheobronchial washings and serum), under defined iron-restrictive conditions, and compared for changes in cellular composition. In analyses by SDS PAGE and Western blotting, envelope and cell contents of the bacteria showed differences in protein content and changes in antigens recognised by convalescent antiserum. Capsule size was not influenced by growth medium. Lipopolysaccharide was detected by PAGE in all the fluids except bovine tracheobronchial washings. However, a Limulus amoebocyte lysate assay did detect trace amounts of endotoxin. Leukotoxin activity was only detected in broth and iron-restricted cultures and not in any "in vivo" fluids. Neutralisation of the toxin with homologous and heterologous convalescent antiserum showed little cross-reactivity in the neutralisation capacity against A1 leukotoxin but heterologous antiserum was effective with leukotoxin of the other serotypes.

Using the same fluids to monitor long term culture, all serotypes showed the capacity for extended survival and resucitation with the addition of nutrents. All three strains survived better in ruminant than in other species' fluids. In non-ruminant fluids and natural water viability was detected only at low temperatures. Morphological changes in both colonial and microscopic appearances were apparent during long term survival. These results imply that this organism possesses some starvation survival mechanisms.

Immunomagnetic beads with bound antibody specific for the three serotypes proved a useful reagent for the isolation of target organisms from host tissues and fluids. Differing protein profiles from those seen in bacteria grown in-vitro were demonstrated. Western blotting unexpectedly failed to identify many antigens when recovered whole cells were compared to those grown in laboratory culture, indicating that perhaps mechanisms were present which helped to evade an immune response.

Superoxide dismutases were detected in all three strains. Levels of enzyme activity, electrophoretic mobility on native PAGE, and metal-active sites were shown to differ. Specific inhibition of activity by potassium cyanide identified a copper/zinc superoxide dismutase only in serotype A2 and this was confirmed by polymerase chain reaction on DNA using specific primers for a fragment of the *sodC* gene. Analysis of cytoplasmic and periplasmic fractions showed superoxide dismutase activity in A1 and A2 to be periplasmic whereas the activity in T10 was cytoplasmic. Under iron-restricted conditions the overall activity of the enzyme was lower and T10 showed presence of superoxide dismutase in the periplasm. In the presence of extraneous superoxide the viability of A1 declined to zero between 15 and 30 minutes whereas that of A2 and T10 were similar to unexposed controls. No specific serum antibody response towards superoxide dismutase was detected by any of the convalescent antisera.

No significant differences could be demonstrated between the interaction of all three serotypes with ovine or bovine macrophages in an *in vitro* phagocytic assay. A2 survived best in ovine and bovine macrophages over a 2 hour period. The effect of various opsonins on phagocytosis and subsequent bacterial survival was investigated. Differences were revealed between serotypes but there was no significant difference within each serotype. Killing of macrophages by T10 was high (~50% killing) and opsonisation increased the killing of macrophages. Using cell inhibitors cytochalasin D and monodansylcadaverine, in the absence of opsonins, the mechanism of entry into the macrophage was shown to be by phagocytosis with serotype A2 in ovine macrophages but was inconclusive with the other serotypes. Differential fixation, using formalin and methanol, of macrophages infected with bacteria revealed that the percentage of macrophages with associated bacteria was lowest when macrophages were incubated with A1, and higher with the other two serotypes. Macrophages infected with serotype A2 had significantly more intracellular bacteria than those infected with the other two serotypes, where they remained predominantly surface associated.

P. haemolytica serotype A2 was more robust throughout the survival experiments than the other two serotypes both in duration and cell morphology. This serotype also possessed a different superoxide dismutase enzyme whose activity was higher than the other serotypes tested and was continually predominantly located in the periplasm. The capacity for A2 to survive in macrophages was superior. This was particularly unusual since the majority of A2 bacteria were located intracellularly in comparison to the other serotypes. Serotype A2 differs markedly with respect to pathogenic mechanisms from A1 and T10 and this may explain why it is the predominant cause of ovine pasteurellosis in the U.K.

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ABBREVIATIONS

BAM - Bovine alveolar macrophages

BCA - Bicinchoninic acid

BHIB - Brain heart infusion broth

BL-3 - Bovine lymphoma cells

BSA - bovine serum albumin

btbw - bovine tracheobronchial washings

BWB - Blot wash buffer

CD - cytochalasin D

CDM - Chemically defined media

cfu - colony forming units

CHAPS - (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate)

Cu/Zn - Copper/Zinc

DNA - Deoxyribonucleic acid

$\alpha\alpha$dp - 2,2, dipyridyl

EDDA - Ethylenediamine Di (o-Hydroxyphenylacetic acid)

EDTA - Diaminoethanetetra-acetic acid disodium salt

E.M. - Electron Microscopy

FBS - Foetal bovine serum

Fe - Iron

FPBS - Formyl saline

GBSS - Gey's balanced salt solution

HAP - *Haemophilus, Actinobacillus* and *Pasteurella*

HCl - Hydrochloric acid

H_2O_2 - Hydrogen peroxide

IgG - Immunoglobulin G

IgA - Immunoglobulin A

IHA - Indirect haemagglutination assay

IMS - Immunomagnetic separation

INT - p-Iodonitrotetrazolium violet

IRP - Iron restricted protein

KCN - Potassium cyanide

kDa - Kilodalton

LAL - Limulus amoebocyte lysate

Lkt - Leukotoxin

LPS - Lipopolysaccharide

LS - Lamb serum

Mab - Monoclonal antibody

MDC - Monodansylcadaverine

MDH - Malic dehydrogenase

Mn - Manganese

MRI - Moredun Research Institute

MTS - (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethooxphenyl)-2-(4-sulphenyl)-2H-tetrazolium,inner salt.

NADP - Nicotinamide adenine dinucleotide phosphate

NBT - Nitroblue tetrazolium

NCS - Newborn calf serum

OAM - Ovine alveolar macrophages

otbw - ovine tracheobronchial washings

PAGE - Polyacrylamide gel electrophoresis

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PMS - Phenazine methosulphate

RBC - Red blood cells

SDS - Sodium dodecyl sulphate

SOD - Superoxide dismutase

SPF - Specific pathogen free

TEMED - N,N,N',N'- tetramethylethylenediamine

TRIS - (TRIS [hydroxymethyl] amino-methane)

TSB - Tryptic soy broth

TSB/YE - Tryptic soy broth / yeast extract

CHAPTER 1

INTRODUCTION

1.1. General introduction

Bacterial diseases are a source of great economic loss to the farming industry and respiratory infections are amongst the most important. In the U.K. during the years 1987-1994 *Pasteurella haemolytica* was the largest cause of bacterial respiratory mortality in cattle, sheep and goats (Central Veterinary Laboratory Report, 1994; Veterinary Laboratories Agency, 1995). Pasteurellosis was also identified as the major systemic disease in sheep. The actual number of deaths is thought to be much higher due to non-reporting or non-diagnosis. In the United States cattle losses from respiratory disease due to pasteurellosis are estimated at upwards of one billion dollars per annum which is greater than all other diseases combined (cited Whiteley *et al.*, 1992). A review by Yates (1982) cited a report in 1972 that 350,000 cattle were lost at a cost of 76 million dollars. Hidden costs including treatment and decreased productivity was estimated at 300 million dollars for that year. The surviving "pulmonary cripples" may also represent a greater loss financially than do the acutely sick animals.

Globally, pasteurellosis has been reported from many countries including South Africa (Cameron, 1966), Ethiopia (Pegram *et al.,* 1979), Sri Lanka (Horadagoda *et al.,* 1981), New Zealand (Prince *et al.,* 1985), Mexico (Colin *et al.*, 1987), Hungary (Fodor *et al.,* 1988), Germany and Syria (Younan *et al.*, 1988) and Malaysia (Mustafa, 1995).

Treatments for Pasteurellosis appear largely ineffective. The use of antibiotics during outbreaks has been reported to be useful (Gilmour & Gilmour, 1989), but the potential for resistant strains to occur is high and animals can be impaired if treatment is delayed (Chang & Carter, 1976; Zimmerman & Hirsh, 1980). The sporadic nature of the disease, occurrence of outbreaks and short clinical manifestation especially with systemic disease point to prevention with vaccination as the best option of protection. Although vaccination has been available it has done little to reduce the impact of disease (Gilmour, 1980; Mosier *et al.,* 1989). The problems associated with the ineffectivness of vaccines include the variety of strains and serotypes involved. There is also an apparent specificity of serotypes for a "host" with A2 and A1 being the most common isolate from ovine and bovine pasteurellosis cases respectively (Frank, 1989; Gilmour & Gilmour, 1989). In the case of cattle, so called "shipping fever" is exacerbated by the bovine respiratory disease complex in which numerous viral potentiators are also present in infection. The specificity of the pathogen for ruminants, and high carriage rates in apparently healthy animals, poses problems in the reproduction of experimental pasteurellosis in conventional animals and laboratory animal models which is problematic for successful vaccine efficacy testing (Gilmour, 1980; Donachie, 1994). The historic methodology of vaccine development in cattle has been criticised by Mosier *et al.* (1989), who says that it is difficult to compare the success of vaccine trials between different groups when standardisation of strains, serotypes, animals and other conditions are not met and reporting in depth is lacking.

All factors have prompted further research into the improvement of vaccines and the exploration of novel treatments by understanding the pathogen-host interactions.

Most important in this is which components or situations may effectively produce protection from disease.

1.2. The Respiratory Tract

In ruminants the mucous membrane at the nasal cavity has different types of epithelium. The membrane within the nostril is stratified squamous epithelium and consists of numerous serous glands. Pseudostratified columnar ciliated epithelium is present and contains numerous mucus-secreting goblet cells. Further down, the mucous and serous cells become mixed (Hare, 1975). Lining the nose down the tracheobronchial tree to the terminal bronchioles is ciliated epithelium. The respiratory bronchioles and alveolar ducts are covered with unciliated cuboidal cells. Throughout the ciliated epithelium goblet cells are found as the major cell type and they become sparser as the airways narrow. Underlying the tissue are the acinar glands comprising glycoprotein and serous and mucous cells. The mucous cells are important in that they produce mucus glycoproteins. The mucus in the tracheobronchioles consists of about 97% water in which inorganic salts, proteins, glycoproteins, proteoglycans and lipids are present (Richardson, 1988). Mucinous glycoproteins in submandibular saliva have been reported to occur in at least two different forms. MG1 is a high molecular weight protein whilst MG2 is of lower molecular weight, though they share common properties (Pruitt *et al.,* 1994). Thornton (1994) also reported two glycoproteins which differ in molecular mass at the protein core level where the two proteins are glycosylated differently and so differ slightly. These then are the building blocks for distinct larger mucins and the relative concentrations of the glycoproteins varies in different secretions. The bovine mucins

contain highly repetitive amino acid sequences dominated by threonine and serine at 45 and 50 % respectively. Proline, alanine and glycine are also present and are 75-80 % of the total amino acid content where they act to link the peptide and carbohydrate of the glycoprotein (Kent, 1978; Schrager & Cumming, 1978). Gottschalk (1960) reported that bovine submaxilliary mucoprotein contained equimolar amounts of sialic acid, (22.4% expressed as N-acetylneuraminic acid) N-acetylglucosamine and small amounts of other sugars such as fucose and galactose. In the sheep the mucoprotein carbohydrate (42%) consists only of N-acetylneuraminic acid and N-acetylglucosamine in the ratio 1:1. These glands are the main mucus secreting salivary glands in ruminants. The mucins are acidic in humans due to the sialic acid properties afforded by N-acetylneuraminic acids (in other mammals N-glycol- and O-acetylated sialic acids are responsible) 2-3% is also due to ester sulphate residues (Kent, 1978).

Underneath the mucous cell lined epithelium lie the acinar glands made up of lymphoid tissues. Present here are follicles of B cells and defined T cell areas adjacent to them. The epithelium overlying the follicles is made up of M cells. These are termed bronchus associated lymphoid tissue (BALT) in a similar manner to the term gut associated lymphoid tissue (GALT). Due to the similarities and functions of the two lymphoid tissues they are collectively referred to as mucosa associated lymphoid tissue (MALT). In the lower respiratory tract the alveolar epithelium contains two types of cells. Type I is the larger squamous cell type for gas exchange whilst type II is a granular pneumocyte and is secretory. This area is where pulmonary surfactant is produced to reduce surface tension in the alveoli and to prevent them from sticking together. The lipid extracts of surfactant contain 74% phospholipid, plus cholesterol, triglycerides and fatty acids (Scarpelli, 1986). In sheep, surfactant consists of 73-79% phosphatidyl choline, 11-12% phosphatidyl glycerol and 8-9% other (Brogden *et al.*,

1986). The lung environment contains other cells connected mainly with defence and includes mast cells, lymphocytes and alveolar macrophages.

1.3. Respiratory Defence Mechanisms

The lung anatomy provides both mechanical and humoral defence against invasion by particles inhaled and more importantly from microorganisms. The ciliated epithelium supports around 200 cilia per cell. They beat in perciliary fluid and are distinct from the viscoelastic layer of mucus (Richardson, 1988). The co-ordinated beating of cilia propels an overlying layer of mucus at a rate of 1cm/minute toward the pharynx where it is swallowed (Kaliner, 1991). The attachment of strongly negatively charged, multiple disaccharide to the side chains of the protein core of the mucus gives a high degree of viscosity to the molecule. The distribution of neuraminic acid, the strongest stable organic acid produced in the metabolism of the animal cell, is especially suited for the functions of lubrication and protection (Gottschalk, 1960). The glycoproteins produced by salivary and mucus-secreting cells have an affinity for micro-organisms and agglutinate and prevent bacteria from adhering based on calcium binding (Rundegren, 1986). These carbohydrate components are also important in that mucus carbohydrates are similar to those on mucous cells and bacteria that adhere to mucosal cell surface carbohydrates will also adhere to mucus and can be removed (Salyers & Whitt, 1994).

The products produced by the serous cells are the innate humoral factors and include lactoferrin. This molecule chelates iron and is bactericidal, bacteriostatic and growth limiting for many species of micro-organisms. It kills and inhibits growth in its apo (iron free) form rather than when it is saturated with iron (Kalmar & Arnold, 1988;

Masson & Heremans, 1966). The action of lactoferrin binding to membranes has been shown to damage the outer membrane of gram negative bacteria, in a manner similar to EDTA, resulting in the subsequent release of LPS, increased cell permeability and increased susceptibility to hydrophobic antibiotics (Ellison *et al*, 1988; Ellison *et al,* 1990; Visca *et al*, 1989). Some organisms are resistant to lactoferrin which has been suggested to correlate with organisms which produce siderophores. There are clinical isolates of *E. coli* that do not bind lactoferrin and are also not inhibited by it (Visca, *et al*, 1990).

The presence of other secretory products can overcome this resistance. In humans lysozyme exerts its anti bacterial effect by hydrolysing the peptidoglycans of the bacterial cell wall by cleaving the β(1-4) glycosidic bond between N-acetyl muramic acid and N-acetylglucosamine. It also activates bacterial autolysins, aggregates bacteria, inhibits bacterial adherence and in the case of oral streptococci inhibits acid production (Iacocno *et al*, 1980; Laible & Germaine, 1985; Rundegren, 1986).

The respiratory mucous membrane is constantly exposed to oxygen on its luminal surface. To restrict opportunities for oxygen-induced injuries, secretions include antioxidants. These are in the form of peroxidases such as lactoperoxidase which protect the mucosal surface from micro-organisms by catalysing the peroxidation of halides and thiocyanate ion to generate reactive oxygen products with antibacterial properties. The catalase and peroxidase activities of these enzymes also protect the mucosal surface in tandem by preventing the accumulation of toxic products of oxygen reduction (Pruitt *et al.,* 1994; Kaliner, 1991).

The geographic distribution of BALT identified in sheep is consistent with the pattern of localisation of gut IgA secreting B cells in the sheep respiratory tract (Scicchaitamo *et al.,* 1984). BALT is made up of both locally derived as well as recirculating cells

which form the pool of thymus-derived lymphocytes which are in the region of 18% T cells and 40% B cells. Of 90% lymphocytes present, approximately 1/3 do not possess either a T or B cell marker. Few immunoglobulin-containing cells are present in tissue sections but those that are contain IgA, and differentiation into IgA producing cells can occur (Bienenstock *et al*, 1976). The collection of follicles in the BALT are called Peyers patches and the M cells (microfold) which line these follicles aid in the presentation of antigens to underlying cells of the immune system. Bacteria are passed to the M cells by secretory (S) IgA which binds bacteria and the Fc portion binds mucin components trapping the bacteria. They are then ingested by the M cells by phagocytosis and passed to the underlying macrophages. The bacterial antigen is processed and presented and MALT T cells are activated which in turn stimulate B cells to produce IgA (Salyers & Whitt, 1994).

IgA is the dominant immunoglobulin in the upper respiratory tract in contrast to the domination of IgG in the lower respiratory tract. Watson & Lascelles (1971) detected IgA in most mucous secretions of sheep and cattle. Antigenic stimulus has been shown to increase IgA secretion. Smith,1975 & Smith *et al,*1975 showed IgA as the major immunoglobulin in saliva, lung, lachrymal fluid and nasal secretion, IgG was present as 40% of antibody in nasal secretions but dominated in colostrum, milk, bile and serum. In another experiment he showed that nasal antibody was only detected after intra-nasal inoculation. Wells *et al.* (1977) using injected, radioactively labelled IgG found that 95% was in the serum whereas in nasal secretions the amount varied from 5-47%. The concentrations in nasal secretions of IgG_1 and IgG_2 were similar and were approximately 2% of the serum concentration, which indicates a lack of transfer from the blood to the nasal secretions in sheep. This finding was similar to that of lachrymal secretion in calves. Scicchitano *et al.* (1986) reported that 81% of IgA was

of local origin in respiratory tract secretions (RTS) and saliva. IgM, IgG 1 and IgG 2 were wholly derived from plasma. IgA and IgM can be transported by secretory component (Sc) which does not bind IgGs. Serum IgA is of gut origin and can be transported selectively into the RTS and saliva depending upon the availability of Sc. In lung lymph 35% of IgA is of plasma origin and its transport is inversely proportional to the extent of local production of IgA and this is reflected by Sc availability.

Secretory component binds dimeric IgA and is an epithelial glycoprotein of ~ 80kDa. Pentameric IgM is also associated with Sc but not in a covalently stabilised complex. The binding of poly IgA and pentameric IgM into the polymer structure depends upon the J chain. IgG producing cells constitute 3-5% of the population in normal human intestinal mucosae but a considerably larger percentage are found in nasal and gastric mucosae. IgD producing cells are 2-10% of glandular immunocytes in the URT. Bégin *et al.* (1981) showed that sheep bronchoalveolar lavage yielded an effluent that was 60% of the affluent volume. The effluent contained an average of $9x10^6$ cells which comprised >70% macrophages, 11% lymphocytes, 4% neutrophils 7% eosinophils and 4% epithelial cells. This is similar to the situation in humans (Reynolds & Newball, 1976).

Macrophages are the first line of phagocytic defence in the LRT. Ryter & DeChastellier (cited, 1983) discussed the lectin like receptors on phagocyte membranes and how the authors postulated on the binding of bacterial surface ligands. Complement, however, is the major product that initiates phagocytic uptake. There are two methods by which complement acts: the classical pathway is triggered by the binding of antibody (mainly IgG) to antigen, the antibody having a receptor on the phagocyte membrane; the alternative pathway involves direct interaction of

bacterial surface components and complement component C3 (present in serum and lung fluids). Phagocytes have receptors for C3b and iC3b (which is C3b with a portion enzymatically removed). Once the macrophage has engulfed the bacterium it is encased in a vacuole called a phagosome where the pH rapidly decreases. A lysosome fuses with the phagosome to produce a phagolysosome (Cross & Kelley, 1990).

Toxic substances are released such as hydrolytic enzymes (lysozyme, proteases), small cationic peptides called defensins are oxygen-independent killing mechanisms (Lehrer, 1992). The respiratory burst which is an oxygen-dependent killing mechanism is one of the major methods by which phagocytes kill bacteria.

The phagocytes do this by using reactive forms of oxygen and of nitrogen created by enzymes. Respiratory burst is accompanied by increased oxygen consumption and increased metabolism of glucose via the hexose monophosphate shunt to form CO_2 and a pentose sugar, in the process generating NADPH and H_2O_2. Reactive oxygen species are produced by myeloperoxidase from lysosomes and the NADPH oxidase system located in the membrane to produce the superoxide radicals as shown below (Klebanoff, 1992);

Superoxide anion (O^-_2) - $2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H$

Singlet oxygen ($^1O_2$) - $Cl + H_2O_2 \rightarrow OCl^- + H_2O$

$$OCl^- + H_2O_2 \rightarrow {}^1O_2 + Cl^- + H_2O$$

Hydroxyl Radical (OH−) - $O^{-}_2 + H_2O_2 \rightarrow OH- OH^- + {}^{'}O_2$

Phagocytic cells which produce these oxygen radicals and other cells protect themselves by producing the metalloenzyme superoxide dismutase (SOD). This enzyme catalyses the conversion of the oxygen radicals into hydrogen peroxide and

oxygen. There are three types of SOD, each with a different metal at the active site. They are split into two families, the Fe and Mn SODs are found in prokaryotic organisms and mainly deal with respiratory oxygen that spontaneously produces an oxygen radical. The Cu/Zn SOD is present in eukaryotic cells and more recently has been discovered in bacteria. Bacterial Cu/Zn SODs are still fairly rare and due to their periplasmic location are implicated in the protection of the bacteria from the effects of oxygen-dependent killing mechanisms in phagocytes (Hassan, 1989; Kroll *et al.*, 1995)

If the oxygen reacts with chlorine hypochlorous acid is produced. Alternatively nitrogen oxides are recognised as being involved in killing mechanisms. Nitrogen in the form of $NO\cdot$, NO_2^- , NO_3^- , produced from L-arginine is recognised in murine macrophages but is not definitive in other species. Alveolar macrophages typically release lower quantities of oxygen than blood monocytes and elicited or activated macrophages respond by producing more superoxide and hydrogen peroxide than do resident macrophages (Cross & Kelley, 1990; Salyers & Whitt, 1994; Klebanoff, 1992; Lehrer, 1992).

1.4. In-vivo Bacteria

Multiplication of some pathogens in-vivo has been shown to be slow, especially at the beginning of infection. Mean generation times may be lengthened from 30 minutes to 20 hours in experimental infections in-vivo (Dalhoff, 1985; Smith, 1990). This has been interpreted to mean that nutritional conditions may be limiting. Host defences, however, also play a part in this. With many organisms the crucial nutrients or environmental factors which determine the growth rate in-vivo are largely unknown.

Carbon, magnesium and phosphate limitation have been studied though more work has focussed on iron restriction. Iron has been shown to be essential to many organisms and adaptation of bacteria to acquire iron in-vivo has been the subject of many reviews (Weinberg, 1978; Bullen, 1981; Neilands, 1981; Martinez *et al.,* 1990). The localisation of infections has sometimes been shown to be linked to the nutritional environment as in the case of *Corynebacterium renale,* a bacterium infecting the kidney in cattle. When this organism is grown in bovine urine and urea enriched peptone water it produces a urease and uses urea for growth (Lovell & Harvey, 1950). *Brucella abortus* which causes another disease of cattle, preferentially utilises erythritol and is localised in tissues such as the placenta, chorion and foetal fluids. Mammalian species such as man , guinea-pigs, rats and rabbits do not have erythritol and so Brucella do not show the tissue predilection (Smith *et al,* 1962). Bacteria recovered and directly examined after in-vivo growth appear more capsulated and have been shown to be resistant to serum killing and phagocytosis. Common observations of fimbrial and pili variation and changes in LPS components are thought also to be advantageous to the bacterium in-vivo (Dalhoff, 1985; Smith, 1990).

Williams (1988) discussed the adaption of bacterial cell envelope structures in vivo. These are involved in promoting adhesion to and colonisation of host tissues by pathogenic bacteria. They help in the acquisition of essential nutrients and in conferring resistance both to host defence mechanisms and to antibiotics used to treat infections. Temperature, pH, osmolarity and oxygen tension have all been identified in influencing bacterial growth. The question of whether the bacteria are attached to a surface and surrounded by a glycocalyx or found as a single cell in suspension are important. Williams (1988) also pointed out the problems of modelling conditions in-

vivo. The influence of nutrient depletion, restriction or limitation need to be considered carefully. Whether growth is to be carried out in media or body fluids, in batch or continous culture need to be considered also. Changes may take place in the envelope during log phase before the onset of stationary phase due to exhaustion of a specific nutrient. Examination of cells at different stages is important. This kind of situation is common in those bacteria in which in-vivo like conditions are monitored. As an example, growth of *Enterococcus* (*Streptococcus*) *faecalis* in serum showed marked differences in cell surface protein antigens and lectin-receptor profiles to those cells grown in broth (Williams, 1988). Allan & Poxton (1994) studying 12 *Bacteroides* strains found varying degrees of serum resistance when grown in minimal media or heat inactivated sheep serum. Although differences in capsule and LPS were apparent these did not correlate with the serum resistance. *E. coli* LPS was monitored by Nelson *et al.* (1991) after growth in depleted media and serum. Increased core production, depletion of O-polysaccharide and decreased core and increased O-polysaccharide were all observed. Changes in capsule production were again also apparent.

In natural environments bacterial biofilms on mucosal surfaces composed of microcolonies embedded in glycocalyx exopolysaccharide are the preferred mode of growth for most bacteria (Coghlan, 1996).

1.5. Bacterial Starvation and Survival

It is established that nutritional availability is an essential bacterial control mechanism in natural environments. The slow growth rates in-vivo may be linked to survival

mechanisms as the probability of spore formation is inversely related to growth rate. In the non-sporulating bacteria different mechanisms of survival are used. *Vibrio fisheri* uses a cell density mechanism due to the differing environments in which it establishes niches. At high cell density they exist as a symbiont in the light organs of squids and fish and are luminescent. When free swimming they exist at low densities and are not luminescent. The *Vibrio fisheri luxI* gene encodes an enzyme responsible for N-3-(oxohexanoyl) homoserine lactone (HSL) synthesis and the *luxR* gene encodes the HSL receptor. The extracellular HSL concentration reflects the local cell density and is thus used to regulate their bioluminescence. At >10nM HSL triggers the *lux R*-regulated transcription of the luciferase gene (Claiborne fuqua *et al.,* 1994; Gomer, 1994). *Myxococcus xanthus* aggregate together and secrete 8 amino acids (A factor) at 10-50μM which will allow cell division and is proportional to the number of bacteria present (Kaiser & Losick, 1993; Gomer, 1994).

When nutrients are low, however, bacteria have to adapt to starvation in order to survive this transitory period. Sporulation in some gram-positive bacteria is well understood and will not be reviewed here. Many gram-negative bacteria have adapted so that they remain viable and do not divide. Kaprelyants *et al.* (1993) cited the suggestion that bacteria could be reduced to three groups: i)viable, to refer to a cell which can form colonies on agar; ii)vital, to cells which can only do so after resuscitation and iii)non-viable to a cell which cannot do so under any tested condition.

Studies with static cultures limited by various elements show that bacteria in the death phase reach an extended steady state, a so called 8th phase with 2-4% of cells remaining viable. This state being termed cryptic growth. This condition is present when a portion of starved microbial cells die releasing products of lysis which can

support growth of survivors (Roszak & Colwell, 1987a). Postgate & Hunter had shown in 1962 that at least 50 dead bacteria are needed to support the growth of one viable bacterium. When studying parameters of *Aerobacter aerogenes* growth, varied responses to starvation have been observed such as reduced respiration and metabolism, differing oxygen uptake and size reduction which serves to increase the surface to volume ratio (Roszak & Colwell, 1987a).

Many survival studies are carried out on marine bacteria whose environment, nutritionally, is one of change for varying lengths of time. There have been few reports of starvation behaviour with human and animal pathogens but *E. coli, Ps. aeruginosa, Enterobacter cloacae, B. bronchiseptica* are examples of some bacteria which have been identified (Porter & Wardlaw, 1993; Moreira *et al.,* 1994). Those organisms which grow slowly at very low nutrient concentrations (oligotrophs) do not revert when high nutrient levels exist. The eutrophs or copiotrophs differ in that they grow only at higher concentrations of nutrients but may well survive low nutrient conditions. An experiment by Koch (1959) on *E. coli* showed that some bacteria use different strategies, based on oligotrophic and eutrophic behaviour,during periods of their life cycle using sensing and switching mechanisms. He found that only two thirds of chemostat grown cells were actively engaged in protein synthesis at any instant. A substantial part of the population was in a "dormant" state during slow culture (Roszak & Colwell, 1987a; Kjelleberg *et al.,* 1987).

Kondo *et al.,* (1994) looking at cell morphology of *Vibrio cholerae* under starvation conditions, showed that they were short rods or spherical with loss of intracellular structures. The surface can be covered in fibrous layers not seen in growing cells. A thick electron dense area in the periplasmic space was observed and thought to be peptidoglycan and may function to reduce nutrient loss. Undulations in the outer

membrane probably aid in reducing cell volume. Kjelleberg & Hermansson (1984) also monitored the cell surface characteristics of marine isolates in response to starvation. They found that all isolates showed size reduction and most increased in hydrophobicity, indicating a dearth of water binding groups and a low degree of polarity.

A strain of *Vibrio* (S14) was used to identify three adaptive phases to starvation. A stringent control phase between 0-30 minutes where observations were made that macromolecular synthesis decreased, 38 proteins were observed to increase, increases in guanosine 3'-diphosphate 5'diphosphate and protein degradation were also shown. The second phase (0.5-6 hours) where 15 proteins increased with the introduction of eight new ones, shifts in fatty acid composition of membranes (Morita, 1993) from cis monoenotic fatty acids to trans monoenoic acids or modification to cyclopropyl derivatives), chemotactic responses to different solutes, degradation of reserve material and the onset of resistance to a variety of stress conditions. The third phase sees decline in the respiration rate, RNA, protein and peptidoglycan synthesis. The synthesis of starvation proteins in the periplasmic space allows for a shift from low to high affinity uptake. Synthesis of exo enzymes takes place transiently during phases 2 and 3. All these changes serve to increase the potential for efficient nutrient acquisition by increasing membrane fluidity, utilising broad substrate specific uptake systems whilst closing down activities not associated with nutrient acquisition and utilisation (Kjelleberg *et al.,* 1993; Ostling *et al.,* 1993).

Zobell & Grant, 1942 (cited by Morita, 1993) who documented the differences in species requirements for growth. Morita (1993) cited the longest experiment studying survival in which 14 isolates of *Ps syringae* subsp *syringae* survived in distilled water

for 24 years. Viable counts were ~10^8 /ml and dropped only 2-3 logs during this time. They also retained their antigenic properties.

Postgate & Hunter (1962) used the term dead in relation to bacteria, in reference to those bacteria which failed to multiply. Some, however, retained their osmotic barriers and could be considered alive. Since then many studies have been undertaken and a review by Kaprelyants *et al.* (1993) pointed out that some bacteria are not revealed by agar plating techniques and can only be monitored by metabolic activity or vital staining.

The viable, non-culturable state was described when discrepancies were noticed between viable and total counts with marine and soil samples. The identification of this state for pathogens and genetically engineered organisms has greatly increased interest in this area (Colwell *et al.,* 1985). Viable non-culturable cells (VBNC) do not differ from starvation cells and have the same morphologic characteristics (Oliver, 1993). Resuscitation can be achieved in liquid or diluted agar media and may need only a narrow nutrient concentration range (Kaprelyants *et al.,* 1993). Oliver (1993) lists those bacteria for which VBNC has been identified. These include *Aeromonas salmonicida, Campylobacter jejuni, Klebsiella pneumoniae, E. coli, Enterobacter aerogenes, Legionella pneumophila, Salmonella enteritidis*, shigellas and vibrios. These are all human or animal pathogens and as Morita (1985) pointed out the study of microbial ecology involves aspects important to public health, especially when there is the ability of pathogens to survive in an ecosystem.

1.6. Nutritional status of the respiratory system

Bacteria within hosts have to contend with defence mechanisms which serve to keep the numbers low, as does competition between the different species present. Nutritional requirements are also important and although the respiratory environment is not as nutritionally harsh as marine or fresh waters, or soil environments, it is one of nutritional restriction. Restriction is a term that is used to imply that nutrients are present but whether they can be acquired or utilised by the bacteria is probably a matter of adaptation.

The volume of respiratory secretions in normal adult humans is 10-100 ml/day. The secretions are 95% water with 1% of carbohydrate, protein, lipid and inorganic material (Yeager, 1971). Respiratory secretions consist of a mixture of mucus glycoproteins (which is 10-15% of lavage proteins), glandular products and plasma proteins. There are few reports on the constitutive amounts of respiratory secretions which differ slightly between reports. All amounts given here are for human secretions unless otherwise stated. Secretions contain the following: protein has been reported to be 4.2mg/ml or 67.6μg/ml (Low *et al.,* 1978; Kaliner, 1991). Albumin is 15% of the total protein (17.4μg/ml Low *et al.,* 1978; 1.42 mg/ml Reynolds & Newball, 1976), and can be used as a standard reference component to differentiate the amount of complement activity present in washings compared with serum. Low *et al.* (1978) reported carbohydrate to be at 8.28μg/ml (20-50% nondializable, Yeager, 1971), non polar lipids 77.8μg/ml, polar lipids 44.1μg/ml and lipid phosphorus

1.09μg/ml. Protein constituents present according to Kaliner (1991) include IgG at 2-4% of total protein, sIgA - 15%, lactoferrin at 2-4%, lysozyme 15-30% (this is all mainly nasal secretion), non secretory IgA-1%, IgM <1%. Nucleic acid is present in secretions at a concentration of 0.017 mg/gm, probably from disrupted cells, indicating that other cell constituents may be present at undetectable levels (Yeager, 1971). Reynolds & Newball (1976) reported the presence of IgE at 77.2 ng/ml and α1-antitrypsin- 0.04mg/ml while transferrin and α2 macroglobulin are present.

The mucous membranes contain many substances. The nasal membranes may contain glutathione, ceruloplasmin, vitamin C and uric acid (5μm, but can achieve 16μm). Serous cells contain products which include neutral endopeptidase, aminopeptidase and glandulin. Components derived from plasma include albumin, immunoglobulins, carboxypeptidase N, angiotensin converting enzyme, kallikrein, calcitonin gene-related peptide, and urea. Many inflammatory mediators are present such as histamine, TAME esterase, PGD2, bradykinin, LTC4, tryptase, major basic protein eosinophil derived neurotoxin (Kaliner, 1991). Yeager (1971) reported that many fractions can be resolved from mucous gland glycoproteins. The two major fractions are a neutral fraction and an acidic fraction with sulfo- and sialoglycoproteins. He also acknowledged work that has been carried out on the osmolarity of secretions. The pulmonary environment is hyperosmotic with respect to serum with sodium at 211mM, chloride-157mM, potassium- 16.6mM and calcium -2.45mM. Adamson 1969 also pointed out the presence of salts but stated also that phosphates and carbonate were synthesised in the lung and that differences did occur between alveolar fluid, lung lymph and plasma. Low *et al.* (1978) analysed phospholipids from human lavage fluids and the percentages found are as follows;

Phosphatidylcholine	83.8%
Phosphatidylglycerol	12.3%
Diphosphatidylglycerol	1.1%
Phosphatidylinositol	1.2%
phosphatidylethanolamine	0.3%
phosphatidic acid	0.4%
phosphatidylserine	0.7%

The fatty acid composition of lavage phosphatidylcholine, the largest constituent of lavage phospholipid is ;

stearic (carbon chain length - 18:0)	3.9%	palmitic (16:0)	72.5%
oleic (18:1)	10%	palmitoleic (16:1)	5.6%
linoleic (18:2)	4.8%	myristic (14:0)	3.2%

Much of the phospholipid components of lung lavage originate from pulmonary surfactant. Harwood *et al.* (1975) carried out an extensive study on the surfactant of sheep. The yield from lungs was 1.21mg/g fresh weight of which protein was present at 14% w/w and lipid 86% w/w. Though mostly lipid, the amino acids constituents of the protein identified were varied.

Amino acids μM/mg dry weight of surfactant.

Aspartamine - 0.09	Proline - 0.21
Threonine - 0.06	Glycine - 0.10
Serine - 0.08	Alanine - 0.06
Glutamine - 0.12	Valine - 0.08
Isoleucine - 0.06	Leucine - 0.12
Tyrosine - 0.03	Phenylalanine - 0.04

Histidine - 0.02		Lysine - 0.07	
Arginine - 0.09		Methionine - 0.02	
1/2 Cystine - 0.05			

The lipid constituents consisted of;

Lipids % w/w of total lipid

Triacylglycerol	6.5	unesterified fatty acid	9.9
Cholesterol	2.9	Phosphatidylethanolamine	1.5
Phosphatidylcholine	58.4	Phosphatidylglycerol	4.4
Phosphatidylinositol	3.5	Lysophosphatidylcholine	10.9
Sphingomyelin	2.5	Others	0.6

The carbon chain lengths of the fatty acids present exhibited considerable variation. The information here suggests that the respiratory airway systems have an abundance of nutrients available to any organism which has the capability to exploit the environment.

1.7. Characteristics of *Pasteurella haemolytica* and *Pasteurella trehalosi*

Pasteurella are gram-negative coccobacilli which are non-motile, facultatively anaerobic, chemo-organotrophic and have both respiratory and fermentative metabolism. D-glucose and other carbohydrates are catabolised with the production of acid, but not gas, and nitrates are reduced to nitrites. Bipolar staining is observed, especially from freshly prepared clinical isolates and slight pleomorphism can also be seen (Holt *et al.,* 1994).

In 1921 Jones first described the bacterium in a slaughterhouse survey giving it the name *Bacillus boviseptica*. Newson & Cross in 1932 named the bacteria which had been isolated from pneumonic sheep lungs as *Pasteurella haemolytica*. In 1959 Smith observed two morphological types of the organism which were in 1961 designated A and T types with the following criteria (Table1).

Table1. Differentiation of A and T biotypes according to Smith (1961).

	A types	T types
Colony morphology	small grey colonies	large colonies with brownish centres
Arabinose fermentation	positive	negative
Trehalose fermentation	negative	positive
Penicillin and tetracycline sensitivity	sensitive	less sensitive than A types
Disease association	enzootic pneumonia in lambs and sheep	septicaemia in older Lambs

In 1968 Biberstein & Francis employed DNA-RNA hybridisation studies and found a low degree of relationship between A and T type representative strains, but found 100% relationship between the A type strains tested. These differences continued to be of interest and Olmos & Biberstein (1979) investigated penicillin sensitivity. They also showed culture with brain heart infusion broth containing basic fuchsin, brilliant green or methylene blue allowed growth of T biotypes but not A biotypes. Biberstein & Kirkham (1979) extended antibiotic susceptibility testing, showing that inhibition by ampicillin, cephalothin, chloramphenicol, tetracycline, erythromycin and nitrofurantoin which was significantly higher for A biotypes. A numerical taxonomic

study (Sneath & Stevens, 1985) with *Pasteurella* genus created three major clusters, of which A and T biotypes were under the cluster designated A. However, A biotypes were then located into phenon group 7 and T biotypes into phenon group 9 using colonial and morphological features, growth, temperature and heat resistance, growth on special media and inhibitors, biochemical properties and acid from carbohydrates. Table 2 is a summary of the differences found between A and T biotypes.

Table 2. Differentiation of A and T biotypes according to Sneath & Stevens (1985).

	A types	T types
Production of catalase	positive	negative
Precipitation on violet red blue agar	positive	negative
Reduction of methylene blue	positive	negative
Acid production on BMA (Basal Medium Agar)	positive	negative
ONPG (*o*-Nitrophenyl β-D-galactopyranoside)	positive	negative
Levan production	negative	positive
Growth on Hugh and Leifson medium	positive	negative
Fermentation of arabinose	positive	negative
Fermentation of dextrin	positive	negative
Fermentation of galactose	positive	negative
Fermentation of D(+) xylose	positive	negative
Fermentation of arbutin	negative	positive
Fermentation of mannose	negative	positive
Fermentation of trehalose	negative	positive

The phenotypic evidence suggested that the species should be separated. In 1987 Craft *et al.* investigated the ability of *P. haemolytica* to be agglutinated by various lectins. Wheat germ lectin agglutinated T but not A biotypes. In 1990 Sneath & Stevens finally separated T biotypes into a new species designated *Pasteurella trehalosi.*

Although now separate, identification of both species is still carried out by serotyping. The indirect haemagglutination assay (IHA), first developed by Biberstein *et al.* in 1960, definined the typing substance as a freely diffusable surface material which is absorbed to erythrocytes. The IHA complemented serotypes with A and T biogroups (Biberstein & Gills, 1962). The assay has been modified to a microtitre plate format (Shreeve *et al.*, 1972) and a rapid microtitre plate assay using glutaraldehyde fixed cells (Fraser *et al.,* 1983). Adlam *et al.* (1984) showed that the system distinguishes strains on the basis of capsular polysaccharide and to date there are 17 distinguishable serotypes (Younan & Fodor, 1995). *Pasteurella haemolytica* comprises serotypes 1, 2, 5, 6, 7, 8, 9, 11, 12, 13, 14, 16, and 17 and *Pasteurella trehalosi* serotypes 3, 4, 10 and 15.

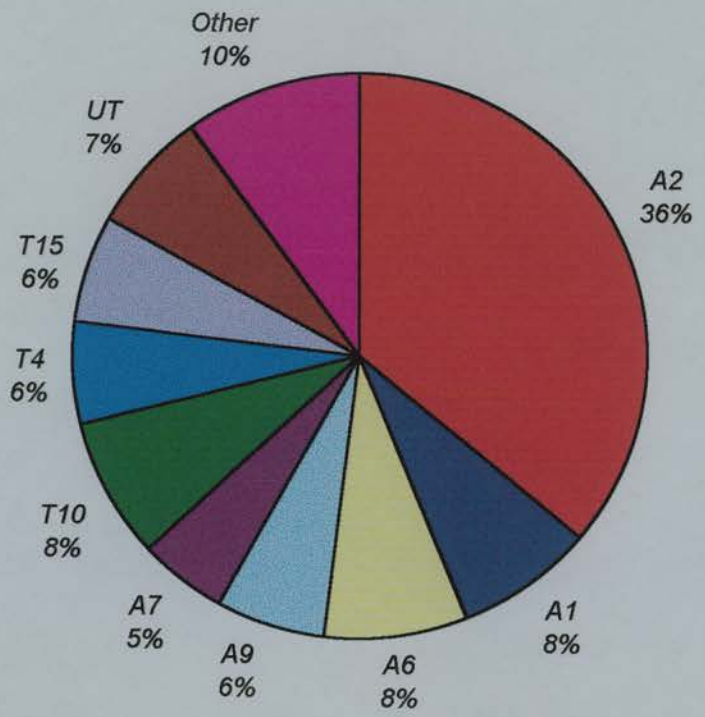
Many isolates cannot be serotyped. In a study by Fraser *et al.* (1982) 6 and 11% (in the years 1974-75 and 1977-81 respectively) of ovine isolates were found to be untypable. With bovine isolates this percentage was higher at 24.2 (Quirie *et al.*, 1986). Most of the untypable isolates were associated with the nasopharynx of healthy animals or the bovine female genital tract, udder and milk. Biberstein (1978) had suggested they were unencapsulated mutants and belonged to the A biotype due to fermentation patterns. Aarsleff *et al.* (1970) showed differences in the production of salicin by untypable strains from classic A types. Techniques using double agar

diffusion and counter current electrophoresis by Donachie in 1984 grouped untypables into nine serogroups as opposed to Frank and Wessman (1978) who used slide agglutination and defined only three groups. Simons *et al.* (1992) identified shared antigens in untypable strains and *P. haemolytica* A1. In 1996 Fodor *et al.* used co-agglutination to serotype strains but this technique was less sensitive than IHA and untypable strains were not investigated. Davies & Donachie (1996) used OMP, LPS and 16S rRNA sequencing profiles to investigate untypables. Five untypable groups (UG 1-5) were created with the first two comprising profiles similar to those of serotypes A1 and A2. UG3 were similar to A11 isolates and UG 4 and 5 were part of distinct groups which were distantly related to *P. haemolytica.*

1.8. Pneumonic Pasteurellosis of Sheep

In temperate climates pneumonia in sheep and lambs is caused primarily by *P. haemolytica*. A wide variety of serotypes are isolated from clinical cases. Fig 1.1

Fig 1.1. Prevalence of *P. haemolytica (A)* and *P. trehalosi* (T) serotypes in pneumonic pasteurellosis in sheep 1982-96.



shows that *P. haemolytica* A2 is the serotype most associated with enzootic pneumonia in lambs and sheep. The disease affects all ages of sheep which are extensively or intensively managed. Lambs under 2 months may develop a generalised septicaemia. Outbreaks in flocks may start suddenly. Obvious respiratory disease is characterised by high temperatures (>40°C), tachypnoea or dyspnoea.

Other animals in the flock may display mild respiratory disease (coughing, oculo-nasal

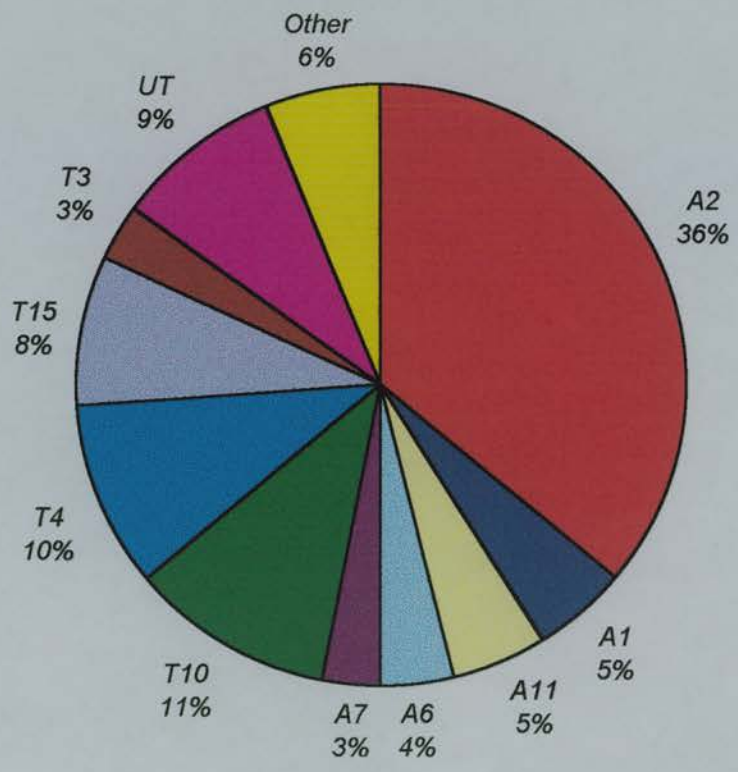
discharges). Morbidity and mortality from acute disease rarely exceeds 10% of the flock. Those which recover may develop chronic lung lesions. Pneumonia outbreaks peak in May and June of each year in the U.K.

Gilmour (1980) reported that pathologically the necropsy findings of animals with acute pasteurellosis are pneumonia with pleurisy and pericarditis. Fibrin clots in fluid are observed in the pleural cavity. The lungs are swollen and are found to be solid, oedematous and haemorrhagic. In lambs which are septicaemic, petechiae are present on the surface of the liver, spleen, kidneys and myocardium. Lymph nodes are swollen and haemorrhagic and fatty degeneration of the liver occurs. Severe pleurisy and pericarditis with restricted lung lesions are observed. Microscopically, alveolar necrosis is seen, with the alveoli filled with fluid containing *P. haemolytica* . The lesion is also filled with large numbers of spindle shaped cells with intensely basophilic nuclei (so called oat cells). In animals which survive disease a collagen capsule infiltrated with neutrophil and lymphoid leukocytes surrounds the oat cells.
Predisposition is an important factor in *P. haemolytica* infections. Climatic changes, flock management, stress, parainfluenza type 3 virus infection, adenovirus, mycoplasma, waning PI3 immunity, reovirus, RSV, pulmonary adenomatosis and some parasitic infections have all been implicated in predisposition. However, this still remains to be well defined epidemiologically. Atypical pneumonia (chronic pasteurellosis) is also observed due to *P. haemolytica* and *Mycoplasma ovipneumoniae. M. arginini, Chlamydia psittaci* and other organisms have also been isolated but their role is thought to be concurrent. In the case of the mycoplasmas they preceed *P. haemolytica* infection (Gilmour & Gilmour, 1989).

1.9. Systemic Pasteurellosis of Sheep

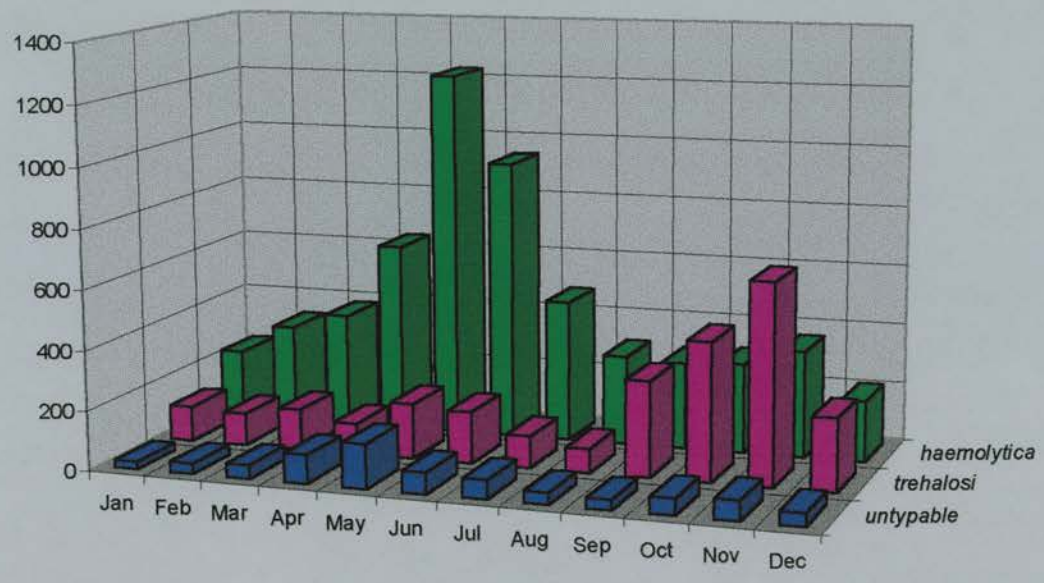
Systemic disease is caused by *P. trehalosi* and *P. haemolytica* serotype A2. It affects older sheep and lambs born earlier the same year. Although most cases are due to *P. haemolytica* serotype A2, *P. trehalosi* pathology has been studied in relation to this syndrome. Four serotypes of *P. trehalosi* exist of which T10 has been shown to be isolated most often from systemic cases. Fig 1.2 (source MRI Pasteurella serotyping service) shows the distribution of serotypes in the disease.

Fig 1.2 Prevalence of *P. haemolytica (A)* and *P. trehalosi* (T) serotypes in systemic pasteurellosis in sheep 1982-96.



Outbreaks are defined usually by the finding of dead sheep. Clinical signs are usually not observed, but include dullness, disinclination to move, pyrexia followed by recumbency, frothy discharge at the mouth, dyspnoea, prostration and finally death (Gilmour, 1980). The course of the disease is usually 6-8 hours. Sporadic deaths occur for a few days with occasional losses over the next few days. The disease due to *P. trehalosi* occurs all year round and cases peak during the autumn. This is in contrast to that seen for *P. haemolytica* infections (Fig 1.3, source MRI Pasteurella serotyping service).

Fig 1.3. Monthly distribution of *P. haemolytica* and *P. trehalosi* isolates from pasteurellosis in sheep 1982-96



Pathologically the carcass is in good condition. Subcutaneous ecchymoses are found over the neck and thorax and haemorrhages on the pleurae, epicardium and diaphragm are seen. Lung lesions are always seen but must be distinguished from apical consolidation due to *P. haemolytica* which may occur concurrently. The air passages contain frothy fluid. The liver is frequently congested with observations of miliary necrotic foci. Lesions also occur in the upper alimentary tract in the form of necrotic

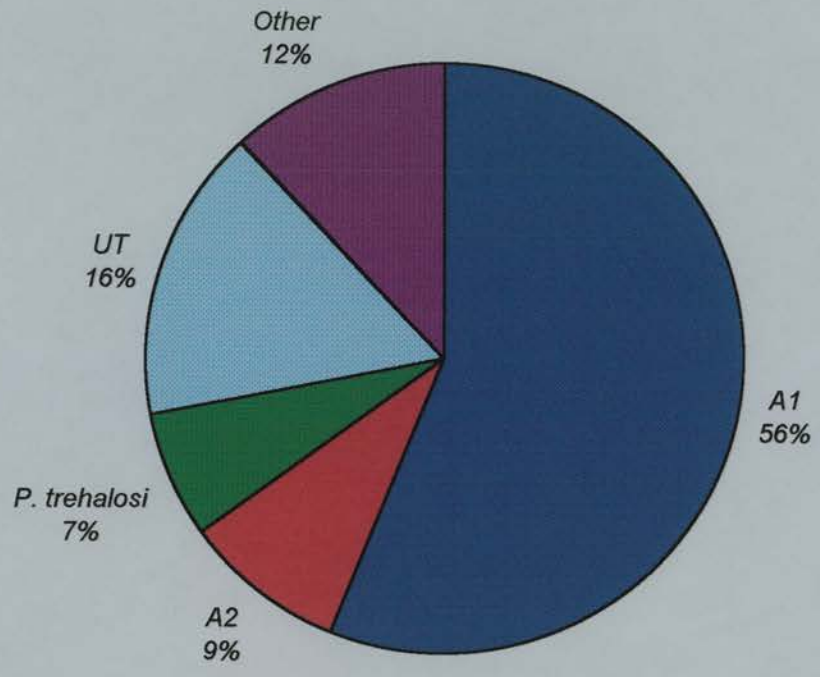
erosion of the posterior pharyngeal epithelium especially in the tonsillar crypts. The necrotic lesions of pharynx and oesophagus consist of necrosis of the epithelium and enlargement of blood vessels with no cellular reaction. Masses of gram negative bacteria adhere to luminal surfaces of ulcers and occlude epithelial vessels and lymphatics. Bacterial emboli are present in the terminal arterial system. The emboli consist of bacteria surrounded by areas of necrosis enclosed by basophilic spindle shaped cells. Large numbers of *P. trehalosi* can be isolated from all areas. Characteristic lesions are found in the lungs, liver, spleen and kidneys. This has been postulated as a systemic disease and not a true septicaemia. The bacteria spread as emboli where they multiply inducing a fatal endotoxaemia. Predisposition to disease is thought to involve climatic changes, transportation and the switch from poor pastures to improved feeding which coincides with observation of peak cases. In lambs tick borne fever has been implicated (Øveras *et al.,* 1993) as well as the mould *Stachybotrys atra* in an outbreak in Hungary. The organism was present in straw used for littering and feeding and it is thought that gastrointestinal mucosal damage and immunosuppression were the possible mechanisms of predisposition (Hajtòs *et al.,* 1983). Antibiotics against systemic disease do work but due to the rapid onset and duration of the infection, vaccination is possibly the best control measure.

1.10. Bovine Pasteurellosis

The disease caused by *P. haemolytica* is a major problem in the large cattle producers of the Americas and in Britain it is also the largest bacterial cause of pneumonia in cattle. Its importance should not be underestimated (CVL, 1994; VDL, 1995). The

terms pneumonic pasteurellosis, shipping fever, bovine respiratory disease complex, bovine fibrinous pneumonia, transit fever, stockyard fever and exposure disease have all been used to describe infection with *P. haemolytica* and *P. multocida* (Yates, 1982). Studies in the U.S. correlate with data at Moredun that A1 is the predominant serotype in cases of pneumonia in cattle (Quirie *et al.*, 1986; Wilkie & Shewen, 1988). This data is observed in Fig 1.4. (source MRI Pasteurella serotyping service).

Fig 1.4. Prevalence of *P. haemolytica (A)* and *P. trehalosi (T)* serotypes in pneumonic pasteurellosis in cattle 1982-96.



In contrast to the findings with sheep, *P. haemolytica* is not readily isolated from healthy unstressed cattle. It is carried but is such a small population of the flora that isolation is sporadic. This may answer the question as to why respiratory disease is not sporadic in the cattle industry but related directly to the gathering of weaned calves,

holding, collection and transportation over long distances to a feed yard. It is postulated that an individual carrier stressed under these conditions and developing respiratory disease will shed and infect other stressed animals in the small confined spaces usually found. This management regime promotes infection throughout the animals present (Frank, 1989).

Clinical disease is similar to that of sheep with observations of fever, depression and nasal discharge. Anorexia is observed, and in some cases increased lacrimation, coughing and diarrhoea (Yates, 1982). At necropsy accumulation of serofibrinous exudate and neutrophils in the air spaces and lymphatics of the lungs is seen. Areas of coagulation necrosis surrounded by inflammatory cells and fibrinous tissue. Acute fibrinous or serofibrinous pleuritis is seen in gross lesion with lobular consolidation and blood stained fibrinous exudate in the air passages. Microscopically the pathology is characterised by acute fibrinous pneumonia with distension of lymphatic vessels with fibrin and neutrophils, and small areas of coagulation necrosis, some of which could be liquefaction necrosis.

A number of viruses ubiquitous to cattle are associated with bovine respiratory disease including PI-3, IBR, RSV, BVDV. Adenoviruses along with, rhinoviruses, herpes viruses, enteroviruses and reoviruses have also been isolated. Although these have been shown to predispose to *P. haemolytica* infection *P. haemolytica* causes pneumonic pasteurellosis independently of viral or other microbial agents and they are not a prerequisite for pneumonia, which is dependent upon the dose and virulence of *P. haemolytica* serotype A1.

1.11. Virulence determinants

The bacterial envelope (Fig 1.5)

The gram-negative envelope consists of multilayered structures giving the bacterium shape and rigidity which protects against osmotic lysis and acts as a permeability barrier. The inner membrane is similar to the gram-positive cytoplasmic membrane with phosphatidyl ethanolamine as the major phospholipid.

Between the inner and outer membrane is the periplasm which contains the peptidoglycan layer. This layer is thinner than that found in gram-positive bacteria but still consists of linear amino sugar chains containing alternating residues of N-acetylglucosamine and N-acetylmuramic acid covalently linked to each other via tetrapeptides. Evidence is emerging that the periplasm contains peptidoglycan in the form of a gel with the volume dependent on the osmolarity of the external medium (Williams, 1988). Also in the periplasm are to be found enzymes such as the β-lactamases, lipoproteins, porin channels, partial flagella structures and chaperone proteins (which are responsible for the tertiary structure of proteins). The outer membrane consists of phospholipid, lipopolysaccharide and protein structures with the most abundant proteins being the porins which are arranged as trimers and allow movement of solutes in and out of the cell. The other outer membrane proteins (OMP) can act as receptors phage and adhesins. The lipopolysaccharide (LPS) is the only lipid in the outer leaflet of the outer membrane and LPS confers on the outer membrane the property of being a rigid structure. LPS can be defined as rough or smooth depending on the presence or absence of O-antigen polysaccharide chain. The exterior of the cell is often covered by the capsule. This is usually polysaccharide in nature and can be homopolysaccharides composed of single sugar long chain

molecules such as cellulose and glucan polymers, or heteropolysaccharides, composed of two or more monosaccharides. Capsules are implicated in the avoidance of immune response mechanisms and are widely used as serotyping antigens due to the diversity of structures.In some organisms, fimbriae, sex pili and flagella protrude outwith the envelope and are implicated in attachment, plasmid transfer and motility respectively (Poxton, 1993).

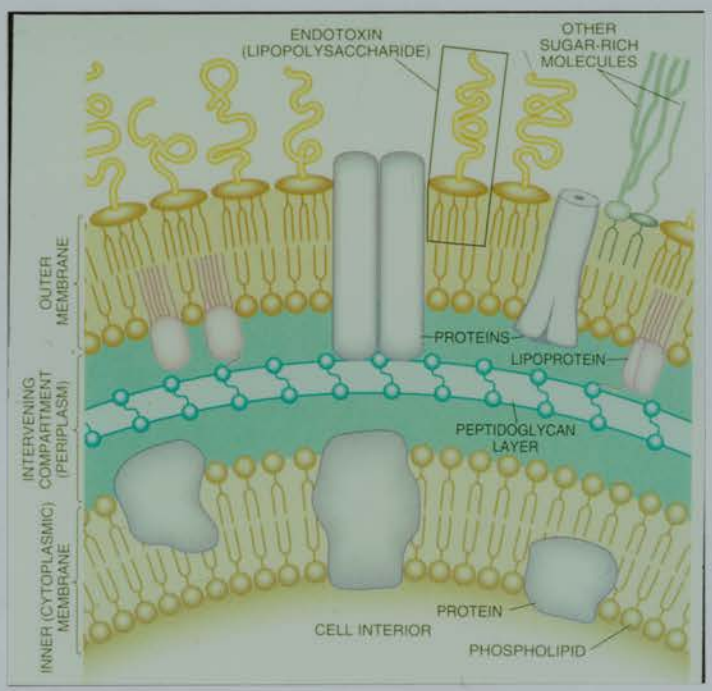


Fig 1.5. The Gram-negative bacterial envelope (Rietschel & Brade, 1992).

Envelope Proteins.

Investigations of the envelope proteins of *P. haemolytica* and *P. trehalosi* have concentrated mainly on those which elicit an immune response. Durham *et al.* (1986) used various extraction procedures on serotype A1 to identify protein bands and antigens by immunoblotting. The envelope proteins of all serotypes were compared (Knights *et al,* 1990) and it was concluded that A and T types could be differentiated. However, different isolates of the same strain, or within A or T types showed very similar profiles, thus not allowing differentiation outside of a biotype separation.

A specific protein of 116 kDa has been identified for serotype A1. The gene for this protein has been cloned and expressed in *E. coli*. The DNA fragment is present in serotypes 2, 5, 6, 7, 9 and 12 but is not expressed. The 116 kDa protein is recognised by immune serum from calves and increased levels of the transcript are observed in iron-rich media (Gonzalez-Rayos *et al.,* 1986, 1991, 1995; Lo *et al.,* 1991). A segment of DNA from A1 was shown to code for three proteins of 28, 30 and 32 kDa proteins of which 28 and 30 reacted with antiserum and were present in serotypes 1-15 when reacted with antiserum against the recombinant protein. This showed that high antibody responses against the 30 kDa protein correlated with resistance to challenge (Craven *et al.,* 1991). A 94 kDa immunogen of the outer membrane was identified in A1 (Nelson & Frank, 1989a; 1989b). Also from A1 a 44 kDa protein was recognised by a monoclonal antibody raised against the 35kDa major OMP of *P. multocida*. The protein in *P. haemolytica* was proposed to act as a monomer with pore-forming ability as it was not detected as a trimer (Lubke *et al.,* 1994). Gatewood *et al.* (1994) reported the expression of unique proteins which were expressed in 46-69 kDa region expressed when cultured in RPMI with and without FCS. These

proteins were also recognised by immune calf serum. Serotype A1 lipoproteins in the 28-30 kDa region have also been shown to be antigenic and the recognition of recombinant lipoprotein in serum has been shown to correlate with resistance in calves to infection (Dabo *et al.,* 1994). Sutherland *et al* (1990) identified proteins expressed in-vivo from A2 organisms grown in chamber implants in sheep. Some differed from in-vitro grown organisms and the identification of host proteins bound to the bacterial surface was suggested. A1 was also investigated for in-vivo expressed proteins using chamber implants in calves and bacteria from infected lungs and compared them with in-vitro iron-deficient and iron-sufficient media grown bacteria and bacteria grown in newborn calf serum (NCS). All conditions showed differences with increases, decreases and induction of various proteins with the conclusion that growth in NCS best represented the protein profile of A1 grown in-vivo (Davies *et al.,* 1994a).

The different growth conditions, extraction procedures and isolates used suggest that the proteins of *P. haemolytica* and *P. trehalosi* still remain incompletely characterised.

Iron-Regulated Proteins (IRPs)

One area which has received consistent extensive investigation is that of iron-regulated proteins (IRPs). Schade & Caroline (1944; 1946) first discovered that a specific iron binding protein in avian blood and egg white inhibited bacterial growth. This was later identified as ovotransferrin (Alderton *et al.,* 1946). Ten years later it was discovered that animals injected with various iron compounds were more susceptible to infection (Jackson & Burrows, 1956a). Iron is an essential requirement for bacterial growth usually in concentrations of 0.4-4μM, but free iron is only

available at 10^{-18} M in the mammalian host environment. This is due to binding proteins with association constants of 10^{36} for the element and only partial saturation of between 30-40%. Most iron is found intracellularly as ferritin, haemosiderin or haem, the haem proteins being the most abundant iron proteins in nature. The transferrins are glycoproteins which bind free iron in the blood while lactoferrin is abundant in mucosal secretions, milk and neutrophils. In an infection the host responds by making any iron even more unavailable. The amount of iron bound to serum transferrin is reduced and this has been suggested to be due to lactoferrin from polymorphonuclear leukocytes which are present at sites of inflammation and can withstand the acidity of that environment. Haemoglobin is sequestered by binding to haptoglobin in the ratio of 1:1. Free haem is bound by another acute phase protein haemopexin and/or serum albumin.

Bacteria, however, have also created elaborate systems for acquiring the iron from these proteins. Siderophores secreted from the bacterial cell are the best characterised of the iron-acquisition mechanisms and are usually of two main types. Organisms such as *E. coli, Salmonella* spp and *Klebsiella* spp produce a hydroxamate type of siderophore while catechol siderophores are produced by organisms such as *Agrobacterium tumefaciens* and *Paracoccus dentrificans.* Siderophores are produced by the organisms and secreted into the surroundings where they chelate iron. This iron is transported back to the cell where attachment to a specific receptor initiates release of the iron into the cell.

Bacteria which do not produce siderophores can acquire iron by direct binding of host iron containing molecules where again iron is released into the cell. There are extensive reviews with *Haemophilus* and *Neisseria* two of the most studied bacterial species (Otto *et al,* 1992; Williams & Griffiths, 1992; Wooldridge & Williams,

1993). The importance of iron in pasteurella infection was first implicated by Chengappa *et al.* (1983) who showed enhanced infection of mice when bovine haemoglobin was mixed and administered with serotypes A1, A2, A8, and A9. Disease in mice was also enhanced in the presence of ferric ammonium citrate administered intravenously immediately before the intraperitoneal administration of T biotypes (Al-Sultan & Aitken, 1984). Studies with *P. haemolytica* A1 grown in RPMI and substituting FBS with transferrin, lactoferrin or conalbumin at 60-70% iron saturation increased the toxicity of the bacteria against bovine peripheral blood leukocytes. In these series of experiments ferritin was not stimulatory whereas haemoglobin and other haem containing molecules did increase toxicity but not to the levels of the transferrins (Gentry *et al.,* 1986). Reissbrodt *et al.* (1994) showed that cross-feeding tests allowed growth of iron-limited *P. haemolytica* in the presence of siderophores ferrioxamine B, E,G, rhizoferrin and 2,3-dihydroxybenzoic acid. Growth was also induced when alpha- ketoacids and alpha-hydroxyacids, acting as primary metabolites, were added indicating a more complex iron acquisition system.

P. haemolytica A2 iron-restricted cells were compared with homologous bacteria recovered from the pleural fluid of experimental lambs. The envelope preparations showed 70 and 100 kDa proteins induced under iron restriction which were not apparent in cells from iron-replete media, but were present in pleural fluid cells (Donachie & Gilmour, 1988). Using convalescent serum on Western blots two proteins were shown to be highly immunogenic only in the pleural fluid cells (70 kDa and 55 kDa) but the 100 kDa protein was not recognised. The 55 kDa protein which was recognised in pleural fluid cells by Western blot but could not be induced in-vitro nor could it be seen on PAGE gels and was discounted as being an IRP. However. *P.*

haemolytica A2 from chamber implants in sheep showed decreased amounts of 70 and 100 kDa proteins (Sutherland *et al.,* 1990).

Similar in-vivo experiments were carried out by Morck *et al.,* (1991) who compared *P. haemolytica* A1 grown in iron-depleted media with those grown in rabbit chamber implants. Proteins were induced at 71, 77 and 100 kDa in iron-restricted media and corresponded to bands from chamber implant cells. The bands were confirmed to be the same using Western blots probed with bovine convalescent sera and rabbit antisera. The bands were not recognised in iron replete grown cells.

Growing serotypes 1-12 under iron restricted conditions using the chelators EDDA and α,α, dipyridyl Deneer & Potter (1989) identified a 77 kDa protein present only in iron restricted cells. Proteins at 71 and 100 kDa were produced at increased amounts under iron restriction. These IRPs were detected by convalescent serum and the 71 and 77 kDa proteins were present in all serotypes and the 100 kDa protein was present in all but serotypes 3 and 9. A 60 kDa protein in iron restricted cells was also present in serotypes 2, 3, 4, 5, 6, 7, 8, 9 and 12 with a 95 kDa present in serotypes 3-7 and 43 kDa only in serotype T10. In a separate study an iron-regulated 35 kDa protein was identified in *P. haemolytica* A2 and shown to be present primarily in the periplasm and was proposed to be important in transportation of iron into the cell (Lainson *et al.,* 1991). Differentiation of iron restricted proteins in A and T biotypes was reported by Murray *et al.,* (1992). A biotypes had proteins at 35 and 70 kDa whilst T biotypes expressed IRPs of 37 and 78 kDa. A monoclonal antibody against the 35 kDa IRP reacted only with A serotypes as did a polyclonal antiserum against the 70 kDa protein.

When *P. haemolytica* A1 isolates from a pneumonic pasteurellosis case and nasal passage isolate were compared for IRPs, 71 and 100 kDa were present in both but 77

kDa protein increased only in the pneumonic isolate. This was the same when the isolates were grown in the presence of ovo and bovine transferrin with the exception that the 77 kDa IRP was not present in either isolate (Davies *et al.,* 1992). The OMPs of in-vitro and in-vivo grown cells were again compared (Davies *et al.,* 1994a) 71, 77 and 100 kDa IRPs were identified in cells retrieved from the lungs. Western blotting identified a 100 kDa protein in in-vitro iron restricted cells but not those from the lungs while the opposite reaction was seen with the 71 kDa IRP. Chamber grown cells showed no enhancement of the proteins which were seen with those isolated from the lungs.

More detailed characterisation of these proteins began when Ogunnariwo & Schryvers (1990) showed that cells would grow in EDDA excess with iron saturated transferrin. This growth, however, was dependent upon the transferrin present. *P. haemolytica* would not grow in the presence of porcine, human, equine or avian transferrins but would grow in the presence of bovine transferrin. This was confirmed with a dot blot assay and indicated a specific receptor. Using affinity purification the receptor was found to be a 100 kDa protein and was similar to that already identified in *Neisseria* and *Haemophilus* spp. No binding was apparent after SDS PAGE and immunoblotting and two other proteins of 71 and 77 kDa were also isolated. This study was extended by Yu *et al.* (1992) who grew *P. haemolytica* in the presence of bovine, ovine or caprine but not human transferrin. This was confirmed again with the dot blot assay, which also showed the proteins were capable of competitively binding bovine, ovine and caprine transferrin only in the presence of unlabelled equine, porcine and human transferrin and not ovine, bovine and caprine. The transferrin receptors of *P. haemolytica* were shown to be composed of two receptor proteins designated Tbp1 and Tbp2 (Yu & Schryvers, 1994). Affinity isolation of Tbp1 by immobilised bovine

transferrin was inhibited when excess bovine transferrin or C-lobe domain of the transferrin protein but not the N-lobe domain were added to soluble membranes. This confirmed that C-lobe is the binding domain for Tbp1 receptor. Tbp2, however, was also not inhibited by excess N-lobe. Thus regions of the C-lobe are involved in binding both Tbp1 and 2 and /or the association is so strong that blocking of one receptor interferes with binding of the other.

Although the proteins are still being fully characterised, the importance of IRPs were shown when Gilmour *et al.* (1991) vaccinated SPF lambs with SSE extracts of cells grown under iron restriction. Significant protection was observed in lambs given the IRP/SSE extract upon homologous challenge with *P.haemolytica* A2. The sera from immune animals which recovered recognised on Western blot proteins of 35, 70 and 100 kDa, the reported IRPs for this serotype. Protection was also reported when a *P. haemolytica* A1 iron regulated bacterin vaccine was used in calves. The sera from protected animals also showed a strong response to IRPs on Western blots (Houghton *et al.,* 1994).

Lipopolysaccharide.

Bacterial lipopolysaccharide (LPS) is found as part of the outer membrane. The LPS consists of three covalently linked regions: lipid A, core oligosaccharide and O-antigen polysaccharide. Lipid A is integrated into the membrane and consists of 6 or 7 short chain fatty acids linked to a glucosamine disaccharide backbone. Lipid A is one of the most potent bacterial non-protein toxins known. The core extends out from this and in enteric bacteria, this short oligosaccharide chain is linked to lipid A via an

unusual 8 carbon sugar called 3-deoxy-D-*manno*-octulosonic acid (KDO). The O-antigen is the outer and serodominant part of the LPS. The LPS which possesses O-antigen side chains is termed smooth LPS whereas those without an O-antigen are termed rough LPS. Pasteurella LPS is similar to that of other gram negative bacteria and the endotoxin is reported to comprise 12-25% of the cell wall (dry weight), it is the major surface antigen of formalin killed cells (cited Confer *et al.,* 1990) and has received much attention both structurally and biologically. Perry & Babiuk (1984) reported the structure of *Pasteurella trehalosi* T4 O- polysaccharide to be different from other smooth type LPS by having a linear polymer composed of a repeating disaccharide unit with the structure;

[-3(-α-D-Gal*p*-(1-3)-β-D-Gal*f*-(1-]$_n$

Leitch & Richards (1988) reported the structure for T3 polysaccharide O- antigen;

[3)-β-D-Glc*p*NAc-(1→4)-α-L-Rha*p*-(1→]$_n$

Richards & Leitch (1989) elucidated the O-antigen of T10;

[→3)-α-D-Gal*p*-(1→3)-β-D-Gal*f*-(1→]$_n$.

As Richards pointed out although T3 and T4 both have repeating disaccharide units their sugar residues differ and so observed immunological cross reactivity between T4 and T10 can be related to structural similarity. This is very different from the structure of the O-polysaccharide of *P.haemolytica* A1 which is a repeating trisaccharide;

→4).β.D-Gal*p*.(1→3).β.D.Gal*p*.(1→3).β.D.Gal*p*Nac.(1→. The core oligosaccharide also contained L-*glycero*-D-*manno* heptose, D-*glycero*-D-*manno* heptose, D-glucose, D-galactose and KDO (Severn & Richards, 1993). Durham *et al.* (1988) probed various serotypes with a monoclonal antibody against A1 LPS and found it cross reacted with serotypes 5, 6, 7, 8 and 12 but less with serotypes 4 and 14 which suggests similar O-

antigens amongst some serotypes. A1 and A2 LPS showed differences in the core oligosaccharides and only A1 possessed an O-side chain. When A1 isolates from pneumonic cases and nasal isolates were compared, low molecular mass components differed (M^{c}Cluskey *et al.,* 1994). Ali *et al.* (1992) went further in characterising LPS from *Pasteurella* spp separating into 10 types with various attributes. The majority of A1 isolates had LPS types 1 or 2 which were smooth with apparently identical O-antigen side chains but different core regions. A2 had types 3 and 4, type 3 being rough with core similar to type 1 and type 4 rough LPS which was different from the rest. Three ovine A1 isolates had type 3 LPS and this was thought to be involved in host specificity vs LPS type. Lacroix *et al.* (1993) identified serotypes A2 and A8 as having rough LPS with other serotypes having smooth types. NMR showed the O-polysaccharide of serotypes A1, 6 and 9 to be identical. Western blots confirmed this with cross reactions between an A1 specific monoclonal antibody and the core oligosaccharide of several A biotypes. A1, 6, 8, 9, and 12 cores were similar but distinct from T biotypes. PAGE profiles of A1, T3 and A5 were shown to be smooth with typical O chain heterogeneity. T3 O chain and core fractions revealed the presence of rhamnose only which was also present in the core of A2. Sialic acid was identified in the O chain of A1 and A5 (Utley *et al.,* 1992). M^{c}Cluskey *et al.* (1994) used the LPS types of Ali and showed A1 isolates from pneumonic and healthy cattle to be smooth while A2 isolates were rough types 3 and 5. The core regions of LPS types 1 and 3 found in A1 and A2 are identical.

In 1981 Rimsay *et al.* used a sequential extraction procedure to separate endotoxic fractions. Smooth LPS precipitate was the most endotoxic and pyrogenic in chick lethality tests and limulus amoebocyte lysate (LAL) assays. The rough LPS was equal in toxicity and Shwartzman reaction to standard *Salmonella* and *Serratia* strains. The

supernatant from smooth LPS was least endotoxic but equally pyrogenic with standard endotoxin preparations. The rough polysaccharide caused no pyrogenicity and had low reactions with other tests.

P. haemolytica A1 endotoxin was infused into sheep using the soluble supernatant fraction of Rimsay *et al.* (1981). The prostaglandin family have been shown to mediate some of the detrimental effects of endotoxin. $PGF_{2\alpha}$, TxB_2, PGI_2 and serotonin were all measured in plasma (Emau *et al.,* 1984). Investigating the differences between rough and smooth LPS in calves, Emau *et al.* (1987) identified a rise in cAMP which was prolonged for smooth LPS. cGMP rose slowly and these observations coincided with the presence of several free fatty acids. The increase appears to result from adenylate cyclase stimulation either by endotoxin or indirectly. Preliminary characterisation of lung surfactant, which binds A1 LPS and shows bactericidal activity towards whole cells, pointed to a proteinaceous component being responsible for the effects. The LPS was investigated in-vivo when *P. haemolytica* A1 ovine and bovine strains were given to calves along with 500μg of LPS associated protein (LAP) and 500μg of LPS (Brogden *et al.,* 1986; 1992 and 1995). The effects were varing degrees of bronchopneumonia. Severity of lesions was less with LAP, which was less than LPS, which was less than whole bacteria. LAP resulted in the largest number of macrophages and lowest number of neutrophils in the lungs which was similar with LPS but the opposite to whole bacteria, however, lesions were more severe. Slocombe *et al.* (1989) investigated *P. haemolytica* A1 deposited in the lungs of calves with or without *E. coli* endotoxin. They showed that *E. coli* endotoxin alone failed to cause any physical derangement. *P.haemolytica* alone however caused severe hypoxaemia, with increased alveolar arterial oxygen difference and total

pulmonary resistance. The animals became hypercarbic with decreased functional residual capacity and developed systemic hypotension without change in arterial pressure. Necropsy showed extensive multifocal areas of necrohaemorrhagic and purulent pneumonia. When *E. coli* was added in combination none of the effects of *P. haemolytica* were changed. Using immunohistochemistry A1 LPS deposition was located in the cytoplasm of neutrophils of the alveolus and alveolar wall, alveolar macrophages, endothelial cells, pulmonary vascular macrophages and epithelial surfaces of calves (Whiteley *et al.,* 1990).

Looking at the direct effect on PMNs Confer & Simons (1986) used phenol water extracted LPS from A1. Trypan blue exclusion showed the LPS was not toxic for the PMNs. Phagocytosis and NBT reduction by PMNs was enhanced in the presence of LPS and a 2.5-3 fold increase in migration of cells was observed in a whole peripheral blood leukocyte preparation. Lipopolysaccharide binding protein (LBP), a liver derived acute phase protein, was shown to complex LPS and bind the CD14 receptor of monocytes and neutrophils (Horadagoda *et al.,* 1995). Cytokine release from bovine alveolar macrophages was monitored after incubation with A1 LPS by Yoo *et al.* (1995a). Interleukin 1 (IL-1) was released and tumor necrosis factor (TNFα) peaked at 4 hours and then decreased steadily. Specific mRNA was first detected at 30 minutes and were present at maximum levels at 1-2 hours.

The vast majority of LPS/ cell interaction work has been carried out using bovine pulmonary arterial endothelial cells (BPAEC). Breider *et al.* (1991) added live *P. haemolytica* to monolayers and recorded Cr release and cytotoxic changes by microscopy. Both live and killed *P. haemolytica* produced extensive damage which was prevented by the addition of polymyxin B and so implicated LPS as the major factor in the damage. A1 LPS prepared by the hot phenol water method was incubated

with thymidine labelled BPAEC. The release of radioactivity was time and dose dependent. Neutrophil adherence to BPAEC was increased after exposure of either cell type to LPS. Neutrophil adherence to endothelial cells was inhibited by polymyxin B, actinomycin D and cyclohexamide, the latter two compounds inhibit expression of ELAM-1 one of the adhesion molecules found on endothelial cells and points to an association between LPS and ELAM-1 (Paulsen *et al.,* 1990). The addition of A1 LPS caused expression of tissue factor and an additive effect was observed when recombinant IL-1 β was also added. IL-1 receptor antagonist reduced expression but did not inhibit it. It was postulated that the secretion of IL-1 in-vivo by macrophages may be detrimental by enhancing the effect of LPS on endothelial cells (Breider & Yang, 1994).

Capsular Polysaccharide.

The bacterial capsule, sometimes referred to as glycocalyx, is thought to be anchored to the outer membrane non-covalently but this is poorly understood. Capsules have been shown to function as shields in the absence of anti-capsular antibodies from the bactericidal action of complement and phagocytes. The structural similarities between the same types of capsular polysaccharide and host tissue materials also serve to protect the bacterium from the immune system (Williams, 1988). The structure of some of *P. haemolytica* and *P. trehalosi* capsules have been elucidated by Adlam *et al.* (1986):

A1- →3)-β-N- acetylaminomannuronic -(1→4)-β-N- acetylmannosamine-(1→

the position 4 of the uronic acid is O-acetylated.

A2- →2)-α-D-N acetylneuraminc acid - (8→

A7- →3-O-(2-N-acetyl-6-O-acetylα-D-glucosamine-1)→(phosphate)→3-O-(2-N-acetyl β-D-galactosamine-1)→

T4- →(2-glycerol-1)→(phosphate)→(6-α-D-galactose-1)→
(2-O-acetyl, 3-O-acetyl)

T15- →(2-glycerol-1)→(phosphate)→(4-α-D-gal-1)→
(2-O-acetyl,3-O-acetyl)

T3 - →4)-α-D-gal *p* -(1→1)-L-glycerol-(3→

(T3, repeating unit, Richards & Leitch, 1990)

The capsule of A1 is similar to enterobacterial common antigen. The polymer was purified and was immunogenic in sheep but not in rabbits. Passive haemagglutination showed it to be specific for A1 (Adlam *et al.,* 1984). A2 capsule is identical to the capsular polysaccharide of *Neisseria meningitidis* group B, *E. coli* K1 and *Moraxella nonliquefaciens* in that they contain colominic acid. These are well known as poor immunogens and were implicated in the lack of immunity observed in producing effective bacterin vaccines against A2 strains (Adlam *et al.,* 1987). The capsule of A7 has been shown to be similar to *N. meningitidis* group L and *H. influenzae* type f. The importance of the similarity between pasteurella capsules and capsules of other species was shown when monoclonal antibodies against *N. meningitidis* group B capsule reacted strongly in IHA test and were protective against a septicaemic A2 infection in mice showing the importance in protection of anti-capsule antibodies (Mustafa, 1995). Adlam *et al.* (1985) investigating the capsule of T4 showed it to be a glycerol, galactose phosphate teichoic acid, unusual for a gram-negative. T15 is similar in structure to T4 but the phosphate was linked to C6 of the galactose moeity

rather than C4. This slight difference in structure was enough to make the two serotypes non-cross reacting. T15 is identical to *N. meningitidis* group H and *E. coli* K62 and K2 (Adlam *et al.,* 1985; Richards & Leitch, 1990). Using ELISAs, low level cross reactivity between A2 and antisera against heterologous serotypes and strong cross reactivity between A1 and A6 serotypes was abolished by absorption and suggested shared capsular antigens (Fodor & Donachie, 1988).

During culture the capsule of A1 was observed in early log phase and was greatest in size between 2 and 6 hours and showed diminished presence with time (Corstvet *et al.,* 1982). The observation that CPS (capsular polysaccharide) production occurs in early log phase by Gatewood *et al.* (1994) agrees with this but only when the bacteria were grown in RPMI with FCS (Foetal Calf Serum) and $FeSO_4$ compared with RPMI alone, RPMI + FCS or BHI. Gilmour *et al.* (1985) studying the surface of *P. haemolytica* and *P. trehalosi* with ferritin labelling and maneval stain showed thinner capsular material produced with T biotypes. Using EM, irregular halos were observed with polycationic ferritin, suggesting an incomplete layer but in contrast to Corstvet *et al.* (1982) there was no degradation of CPS in 18 hour cultures observed under SEM. Gentry *et al.* (1987) showed that 128 serial passages of A1 did not diminish the presence of capsule.

The capsule polysaccharide of *P. haemolytica* A1 are important structures in virulence and immunity. When *P. haemolytica* A1 purified capsular polysaccharide was incubated with bovine neutrophils it dramatically reduced their ability to ingest and kill whole bacteria even though they were still able to undergo a respiratory burst. This effect was the same when the bacteria were opsonised so that capsule was not competing for opsonising antibodies. The same experiment was carried out with bovine alveolar macrophages and showed reduced phagocytosis even when the

number of macrophages was greater than that of the bacteria (Czuprynski *et al.,* 1989; 1991a). Czuprynski (1991b) reported that capsular polysaccharide (CPS) induced the release of IL-1 from bovine blood monocytes but not from alveolar macrophages. The addition of polymyxin B, abrogated this effect, probably as a result of binding LPS (estimated at 5pg per μg CPS). TNF (Tumor Necrosis Factor) activity was undetectable using blood monocytes even after 18 hours incubation. The capsule of serotype A1 was located using immunohistochemistry after inoculation of whole bacteria intratracheally in calves. Capsule was identified in the alveolus and alveolar macrophages but not cells of the alveolar wall (Whiteley *et al.,* 1990). Chamber implants in sheep containing serotype A2 showed no difference in the capsule size of bacteria collected compared to A2 cultured in laboratory media, but did show a high degree of aggregation (Sutherland *et al.,* 1990). Brogden *et al.* (1989) deposited A1 capsule into lungs and showed that, in itself, capsule was not a major factor in lung damage and lesions were minimal. When capsule was incubated with lung surfactant the density of the surfactant increased but did not alter the surface tension. A hypothesis was put forward that *P. haemolytica* may attach to a lectin in the surfactant which itself binds alveolar epithelial cells or that the capsule binds surfactant in order that the bacteria masks itself.

Resistance to *P. haemolytica* infection has been shown to correlate with high antibody response to capsule (Confer *et al.,* 1989). Townsend *et al.* (1987) also found that systemic and humoral response to vaccination was inversely related to the magnitude of antibody against capsule found in secretions before vaccination. Using ELISA an adjuvant-treated culture supernatant from log phase growth induced a large titre of capsule specific IgG antibodies in lung lavage. Inoculation of calves and mature cows with purified capsule of serotype A1 showed differing responses (Tigges *et al.,* 1993).

In calves specific IgM, IgG1 and IgG2 were present whereas in cows only IgM and IgG1 were detected. An oil adjuvant mixture produced the highest levels of IgG1 and IgG2 whereas capsule mixed with aluminium hydroxide stimulated the highest IgM specific response. A second inoculation did not increase titres in cows or calves. M^{c}Vey *et al.* (1990) followed serum antibody against capsule from calves in transit. The antibody titres rose by day 15 and stayed at high levels until day 56. Those animals which did not contract bovine respiratory disease complex had higher initial antibody titres. IgG1 anticapsular antibody increased from days 8-29 as evidenced by a decrease in serotype A2 absorption inhibition from 60% on day 8 to 15% on day 29. IgA, IgG2 and IgM were more serotype specific. Both in-vitro complement dependent bacteriolysis and C_3 deposition on the surface of the bacteria increased in a serotype-specific fashion. It appears a humoral response to carbohydrate antigens of capsule increase specifically during the course of a response.

Leukotoxin.

The leukotoxin of *P. haemolytica* is an exotoxin specific for ruminant leukocytes which exerts a variety of detrimental effects on these cells and is probably the best characterised virulence factor of the organism. Since *P. haemolytica* culture supernatants were found to be toxic for bovine bronchoalveolar macrophages (Benson *et al.,* 1978; Markham & Wilkie, 1980a) much work has been carried out. Shewen *et al.* (1982) demonstrated *P. haemolytica* A1 culture supernatant caused damage to bovine leukocytes. Sutherland *et al.,* (1983) showed the same effect for ovine bronchoalveolar macrophages and in 1985 for peripheral blood leukocytes and gastric lymph cells. Shewen & Wilkie, (1985) showed that the cytotoxicity was present in actively growing cells and was typical of an exotoxin.

The use of the bovine lymphoma cell line (BL-3) has advanced the knowledge of the mechanism by which leukotoxin acts. In these cells rapid release of intracellular potassium and uptake of calcium are found. The release of potassium is accompanied by cell swelling, aggregation and adherence to the reaction vessel. Chromium^{51} release increased after 20 minutes and cells showed variable membrane defects. After 1 hour cells were shrunken and 100% of cell LDH was released. The addition of carbohydrate molecules indicated that the pore size in the membrane is 0.9nm (Clinkenbeard *et al.,* 1989 a, b). Cytotoxicity for BL-3 cells was influenced by calcium, as introduction of verampil, a calcium channel blocker, prevented death (Gerbig *et al.,* 1989). Styrt *et al.* (1990) looking at the effects of leukotoxin on bovine granulocytes found the release of LDH at 94% was higher than the lysosomal enzyme arylsulphatase (38%). It appears leukotoxin may selectively disrupt the membrane.

Chang *et al.* (1987) identified leukotoxin as a 105kDa polypeptide which could be neutralised by antibodies. Bovine platelets also release LDH in the presence of *P. haemolytica* A1 culture supernatant (Clinkenbeard & Upton, 1991). Majury & Shewen (1991a & 1991b) reported that leukotoxin caused inhibition of mitogen and antigen proliferative lymphocyte responses. When B and T cell populations were compared, the B enriched populations were more susceptible to the effects of leukotoxin. The effects on bovine polymorphonuclear leukocytes by leukotoxin were measured in terms of viability and chemiluminescence. Inhibition of the latter was observed with the non selective β-adrenoreceptor antagonists propanolol and alprenolol but not oxprenol. Cell receptors were not thought to be blocked or membrane stabilising carried out by the antagonists (Henricks *et al.,* 1990).

Conlon *et al.* (1990) using bovine bronchoalveolar macrophages, induced oxygen radical generation with opsonised zymosan but A1 culture supernatant delayed the

time taken to reach maximum oxygen radical production although total production was increased and the presence of supernatant diminished the effects of cycloxygenase inhibitors. Proliferation of peripheral blood mononuclear cells was inhibited by leukotoxin, the effect being lifted in the presence of anti-leukotoxin monoclonal antibody (Czuprynski & Ortiz-Carranza, 1992). Rabbit anti-leukotoxin serum also prevented the release of histamine from bovine pulmonary mast cells which was induced in the presence of leukotoxin (Adusu *et al.,* 1994).

In immunohistochemical studies Whiteley *et al.* (1990) showed leukotoxin associated with the membranes of degenerating inflammatory cells located in the alveolus. In 1991 Whiteley *et al.* also showed the cytotoxic changes in macrophages but not parenchymal cells of the lungs. Serotype A1 supernatant incubated with bovine tracheal smooth muscle produced contractions in the muscle similar to those induced by the agonists tested but was not dose related and had decreased responsiveness not seen with the agonists. Antagonists abolished or diminished the supernatant effects on the muscle. The supernatant did not act as in a drug receptor manner and showed an all or none response (Belanger *et al.,* 1993). Leukotoxin directly effected the expression of MHC class II molecules on bovine macrophages and proliferation in a mixed lymphocyte assay. Marked downregulation was observed and when cells were activated with interferon (IFNγ), leukotoxin had little or no effect. This reaction, however, is limited only to cells from naive animals (Hughes *et al.,* 1994).

Henricks *et al.* (1992) investigated the effect of leukotoxin on PMN's with respect to the 5-lipooxygenase products arachidonic acid, leukotriene B4 and 5-hydroxyeicosatetraenoic acid which are known to recruit PMN's, exacerbate inflammatory events and microvascular injury. When cells were exposed to arachidonic acid 5-, 12- and 15-hydroxyeicosatraenoic acids and LTB4 were released.

Leukotoxin-dose-dependently enhanced release of 5-HETE and LTB4 in arachidonic acid stimulated cells or in the absence of exogenous arachidonic acid.

Czuprynski & Noel (1990) and Czuprynski *et al.* (1991a) monitored the effects of leukotoxin on bovine neutrophils. Cells were monitored for oxidative activity using chemiluminescence stimulated by opsonised zymosan. Leukotoxin was shown to diminish the chemiluminescence response rapidly but only in cells which were stimulated. Sucrose was shown to neither prevent nor reverse the effect which is the opposite to that shown with BL-3 cells. High concentrations of leukotoxin were inhibitory or lethal to bovine neutrophils. Exposure to leukotoxin in this study resulted in significant neutrophil activation and stimulation of an oxidative burst which is in contrast to the previous study. A possible explanation for this is the use of purified leukotoxin in this study in contrast to the crude leukotoxin preparation of the previous experiment which may have contained an inhibitory factor. Dilute leukotoxin stimulated cytoskeletal alterations indicated by significant shape change. Preferential release of secondary granule constituents (lactoferrin) took place and primary granule release was measured only at low dilutions of leukotoxin and resulted in significant release of cytosolic constituents. Bovine neutrophils were labelled with the fluorescent calcium probe fluo-3 by Ortiz-Carranza & Czuprinski (1992). Incubation with leukotoxin produced an increase in fluorescence of the neutrophils indicating an influx of calcium and a vigorous chemiluminescence response (the latter dependent on extracellular calcium). These effects were blocked effectively with the calcium channel blockers verapamil and propanolol along with a neutralising, anti-leukotoxin monoclonal antibody.

Using bovine neutrophils, exposure to leukotoxin produced increased synthesis of LTB_4 but not thromboxane B_2. An anti-leukotoxin antibody neutralised neutrophil

lysis and LTB_4 synthesis. The effects were calcium dependent. The leukotoxin induced LTB_4 synthesis from endogenous arachidonic acid required leukotoxin induced plasma membrane damage, whereas exogenous arachidonic acid occurs in the absence of plasma membrane damage (Clinkenbeard *et al.,* 1994).

Breider *et al.* (1990) using bovine pulmonary arterial endothelial cells, showed that leukotoxin had little direct effect on the cells. The effects have been put down to LPS although when an anti-leukotoxin monoclonal antibody was incorporated no effect was observed. Using the same cell type Maheshweran *et al.* (1993) confirmed minimal injury to the endothelial cells by leukotoxin. In the presence of neutrophils, however, the endothelial cells were killed. The proposed mechanism of this damage is the release of O_2^- (superoxide), H_2O_2 (hydrogen peroxide) and lysosomal proteases. Hydrogen peroxide enters the endothelial cells and produces a reactive hydroxyl radical (HO−) which initiates lipid peroxidation and cell membrane damage. Catalase offers a low degree of protection against this as it can only scavenge extracellular hydrogen peroxide. 1,3-dimethyl-2 thiourea (DMTU) which can scavenge intracellular peroxide caused a high degree of protection. The elastase inhibitor pentoxifylline, N-methoxy-succinyl-ala-ala-pro-val-chloromethyl ketone (CMK) also gave protection indicating that elastase from degranulated lysosomal of polymorphonuclear leukocytes also contributes to damage. DMTU and CMK together had an additive protective effect.

Leukotoxin mutants have been produced using non-molecular techniques (Chidambaram *et al.,* 1995; Murphy *et al.,* 1995) and used to study haemolysis with the mutant showing no greater release of haemoglobin than the uninoculated media concentrate. Also, lack of toxic activity to BL-3 cells and polymorphonuclear leukocytes has been shown. In animals reduced virulence in both goats and cattle were

observed along with a reduction in lung lesions. Stevens & Czuprynski (1996) has observed the induction of apoptosis in bovine mononuclear cells and granulocytes in-vitro by leukotoxin.

The genes for leukotoxin have been cloned, sequenced and expressed in *E. coli* K12. The gene shows extensive homology ranging from 50-90% with the α-haemolysin of *E. coli* (Lo *et al.,* 1985; 1987; Strathdee & Lo, 1987). Four open reading frames have been identified and designated *lkt C,A,B* and *D* in order of their genetic organisation. *lkt A* codes for the structural polypeptide of the toxin and is activated by the intracellular component encoded by 167 amino acid *lktC* protein (Strathdee & Lo, 1989). From the deduced sequence leukotoxin consists of 953 amino acids with a molecular size of 101.9 kDa. Non denaturing conditions for the *lkt A* protein and activated toxin are identical. *Lkt A* is slightly negatively charged which suggested that activation involved the addition of small charged moieties. Strathdee & Lo (1987) found *lktA* contained conserved hydrophobic regions as well as a set of tandemly repeated domains (6 consisting of L-X-GGXG(N/D)DX) also found in the *HlyA* gene of α-haemolysin. In *hlyA* this has been found to be important in calcium binding of α-haemolysin to target cells (Ludwig *et al.,* 1988). Due to these tandemly repeated domains identified in the *E.coli* haemolysin and and *P. haemolytica* leukotoxin Welch and Lo designated these toxins RTX toxins (Repeats in ToXin). More toxins have been identified and the list to date comprises *Actinobacillus actinomycetemcomitans* leukotoxin, *A. pleuropneumoniae* (3 different haemolysins or leukotoxins), *A. suis* haemolysin, *Proteus vulgaris* haemolysin, *Morganella morganii* haemolysin, Enterohaemorrhagic *E. coli* haemolysin, *Bordetella pertussis* adenylate cyclase toxin (cited Czuprynski & Welch, 1995). Not all members of the family have haemolytic

activity but are potent cytotoxins for various types of nucleated cells. Some, as in the case of *Pasteurella haemolytica* are very specific (ruminants only) and some in the case of *E. coli* which lyses erythrocytes from a wide range of species (Welch, 1991). The *lkt B* and *D* genes have been shown to be responsible for the secretion of the toxin from the bacterial cell. This is a type 1 pathway secretion and is signal sequence independent (Salmond & Reeves, 1993). In the *hlyB* gene the c terminal domain contains the ABC (ATP binding cassette) transporter mechanism. Many bacteria possess this type of transporter as do the RTX toxins (Fath & Kolter, 1993). The *lkt D* gene produces a product which facilitates the movement of leukotoxin out of the bacterial cell (Koronakis *et al.,* 1992). Complementation studies show that in the absence of *hly C* or *lkt C, hly A* or *lkt A* were not toxic. When *hly C* was added to *lkt A* the product was toxic. *lkt C* and *hly A* were, however, neither haemolytic nor cytotoxic (Forestier & Welch, 1990). *E. coli hly B* and *D* genes will efficiently secrete *hly A* or *lkt A* in an *E. coli* background but not vice versa (cited Welch, 1991). The genes point to activation being similar but requirements different. The transport products have evolved to accommodate differences in host cells without compromising recognition of the A protein export signal.

Leukotoxin has been implicated in protection of animals with the presence of neutralising antibodies in experimentally infected sheep (Sutherland, 1989) and naturally-occurring neutralising activity in serum of cattle (Frank, 1989). Crude leukotoxin vaccines in sheep and cattle have conferred protection (Shewen & Wilkie, 1988; Sutherland *et al.,* 1989). The economically viable recombinant leukotoxin has however been shown to be ineffective alone in calves and sheep (Conlon *et al.,* 1991; Donachie, 1994) but in calves when added to other soluble components immunity is enhanced.

Neuraminidase.

Neuraminidase is an enzyme responsible for the removal of sialic acid from glycoproteins. Scharmann *et al.* (1970) first reported that 3 out of 5 strains of *P. haemolytica* tested produced neuraminidase, Frank & Tabatabai (1981) described the production of neuraminidase by bovine and ovine isolates of *P. haemolytica*. Type strains of serotypes 2, 3, 4, 10, and 11 showed no measurable activity. No enzyme activity was found in ovine field isolates except serotype A2, but only at low levels. Serotypes 1, 6, and 7 were intermediate in activity with serotypes 5, 9 and 8 showing the highest levels. There was no significance between the origin of the isolate and the production of the enzyme.

Tabatabai & Frank (1981) also assayed for activity in *P. haemolytica* A1. Fetuin acted as the best substrate and calcium was not necessary for catalysis but activity was increased by the presence of magnesium and manganese. Normal serum did not inhibit but increased activity and is thought to be due to proteolysis of the enzyme by serum proteases or reversible aggregation. Rabbit antiserum was able to inhibit activity indicating it could be part of an antigenic complex. Straus *et al.* (1993) investigated all 16 serotypes. The enzyme in *P. haemolytica* A1 was found to be extracellular and produced at maximum levels during stationary phase. The molecular weight was between 150-170 kDa which would make it the largest neuraminidase identified so far. A1 produced the most active enzyme by the measurement of released sialic acid from all the substrates tested. A11 did not produce a detectable enzyme. Rabbit antiserum against A1 neutralised activity but was not as effective against serotypes 10 and 15. This was unusual as A1, A6, A12, T10 and T15 all share an enzyme of the same size where the rest of the serotypes produce a larger enzyme.

In 1994 Straus & Purdy detected specific antibodies against neuraminidase 14 days after a transthoracic challenge of *P. haemolytica* A1 in goats. The antibody levels were significantly higher than those in the control animals. This indicates that the enzyme is produced in-vivo and antibody production against the enzyme may be important in protection.

Sialoglycoprotease.

Another important enzyme produced by *P. haemolytica* was first described by Otulakowski *et al.* (1983). A *P. haemolytica* A1 leukotoxin preparation was shown to release thymidine labelled products from erythrocyte ghosts. The activity was shown to be present in serotypes 1, 2, 3, 5, 6, 7, 8, 9 and 12 but not 4, 10 and 11. The fragments released were a sialic acid containing fragment and sialic acid. The enzyme has a broad pH range around pH7 and was not inhibited by trypsin, chymotrypsin, thermolysin, thio and serine proteases or neuraminidase, but was inhibited by high concentration and prolonged incubation with O-phenanthroline. It hydrolysed glycophorin more than casein by a factor of four. The enzyme was described as a neutral protease possibly metal dependent and specific for sialoglycoproteins.

Abdullah *et al.* (1992) purified the enzyme and confirmed its specificity for glycophorin A, an O-glycosylated erythrocyte membrane glycoprotein. The activity of the enzyme was abolished when the sialic acid residues were removed from the substrate with neuraminidase. The site of hydrolysis of the enzyme does not correspond to the specificity of known bacterial proteases. The molecular mass of the protein is 35kDa .

Investigating a role in-vivo for the protease, Steininger *et al.,* 1992 showed that it removed the ligand for P-selectin from the surface of myeloid cells, HL-60 cells and neutrophils. The protein removed was ~115 kDa which is close to the apparent molecular weight of P-selectin ligand. The effect was inhibited only by the addition of bovine antiserum, EDTA and an alternative substrate. E-selectin binding was not affected. The levels of CD44 and CD45 on the cells remained constant whereas CD43 production was markedly diminished. Sutherland *et al.* (1992) carried on this work by showing that five of the seven epitopes identified by CD34 antibodies were cleaved by the protease. CD34 is important as its expression is restricted to 1-3% of normal bone marrow leukocytes but these cells contain precursors of all blood lineages. Using immunomagnetic beads coated with anti-CD34 antibody these cells could be released from the beads using the protease. This technique produced a purity of CD34 expressing cells of 90-95% with no loss of viability. This was also achieved by Marsh *et al.* (1992) who used the technique to isolate CD34 positive cells from unfractionated normal human bone marrow mononuclear cells.

The importance of the protease in the human field has recently been exploited. Hu *et al.* (1994) cleaved epitectin from H.EP 2 cells (human laryngeal carcinoma cells) and other mucin type sialoglycoproteins implicated in malignancy. This selective degradation of mucin glycoproteins on cancer cell surfaces has importance in the detection and subsequent destruction of malignant cells. A plasmid containing most of the glycoprotease gene was fused with the secretion signal of *hly A* of *E. coli.* The recombinant plasmid expressed a fusion protein GCP-f which was secreted into the medium when secretion functions *hly B* and *Hly D* were supplied. The protease was readily recovered from other cellular material (Lo *et al.,* 1994). A GCP probe hybridises with genomic DNA from serotypes 5, 6, 8, 9, 11, 12 and weakly with A2.

PCR amplification was positive for A13, 14 and 16 but not T15. Southern blot hybridisation was also positive with the standard plasmid product pPH1.1 with A7, 13, 14 and 16. The activity assay gave the same results. T biotypes are negative and none of the 11 A1 nasal isolates showed activity (Lee *et al.,* 1994a).

Lee *et al.* (1994b) also investigated the in-vivo production of the protease using calf sera. Sera which recognised a band of 35.2 kDa on Western blots also neutralised enzyme activity. When the specificity of immunoglobulin was investigated IgG1 and IgG2 both recognised the band. Correlation between neutralising activity of serum and reduction in percentage pneumonic tissue observed, suggest a relationship between the production of antibodies against the protease and protection.

1.12. Pathogen-Host Interactions

Although the use of single, purified virulence factors alone give some indication of their potential in pathogenesis, infection is multifactorial and the effects of whole bacteria whether live or dead are essential in the elucidation of the infectious process.

Systemic Interactions

Systemic interactions were investigated by Hodgson *et al.* (1993) where typical T disease was identified in rapidity of onset of disease post challenge and gross pathology at necropsy. The number of organisms isolated was higher in tissues than blood. Plasma biochemistry described typical endotoxic shock, with glucose levels

initially increasing then decreasing, maintained or increase in urea and lactacidaemia and metabolic acidosis. Further work using *P. trehalosi* T15, treating one group (9 lambs) with antibiotics, showed clinical and biochemical endotoxaemia. PGE_2 concentrations increased in fourteen out of sixteen lambs but significant levels of TNFα occurred only in control lambs (Hodgson *et al.*,1994).

M^c Donald *et al.* (1983) showed that fresh serum from cattle killed *P. haemolytica* A1 log phase organisms. This effect was complement mediated by the classical pathway, though agglutinating antibody was also present. Calf serum after colostrum feeding killed only 30% of bacteria despite high anti *P. haemolytica* antibody. The amount of IgA present in the colostrum was blamed for blocking the effects of IgG and IgM as nasal secretions also had low indirect bactericidal agglutination. Blau *et al.,* (1987) reported that clinical isolates were resistant to serum killing whereas isolates from asymptomatic cattle varied in susceptibility. This is in contrast to M^{c}Donald's study which used mouse-passaged isolates and log phase bacterial cultures. These methodologies have both been reported to decrease serum resistance, however, the complement-mediated killing by the classical pathway was defined. Sutherland, (1988) developed a microtitre plate assay for monitoring bactericidal activity. Sera and lung washings from SPF lambs convalescent from a live *P. haemolytica* challenge were shown to be bactericidal in the presence of complement. When IgG was purified it was as bactericidal as whole serum and absorption with LPS abolished bactericidal activity.

Cell populations in lamb blood were monitored after intratracheal inoculation of *P. haemolytica* A1. After 24 hours a decrease of CD5+ and CD4+ T cells was observed which returned to post inoculation levels after 5 days. Between 5-9 days there was a significant rise in lymphocytes which were CD5+ but not CD4+ or CD8+. A

depressed lymphocyte response to PHA was observed with 24 hour samples (Sharma & Woldehiwet, 1991). Clarke *et al.,* (1994) using chambers in calves measured increases in IL-1, LTB4, TXB2, PGF1α and PGE2 with neutrophils numbers increasing. After 45 days, dexamethazone inoculation inhibited the chemotaxis of neutrophils and the addition of phenylbutazane reduced the inflammatory response as measured by low concentrations of albumin. A similar experiment by Walker *et al.,* (1994) measured the acute phase protein α1-acid glycoprotein from chamber implants containing bacteria. The levels of the glycoprotein were at the same levels as in serum samples. Albumin in the chambers did rise but not in the serum reinforcing evidence of its presence during inflammation response. In-vitro studies on the effects on peripheral blood mononuclear leukocytes by *P. haemolytica* showed a significantly greater bacterial killing than cells incubated with *Staphylococcus epidermidis.* Heat killed bacteria did not cause cell death and the numbers of cells killed increased with the increase in numbers of bacteria.

Lung Interactions.

Killing (31.9%) of *P. haemolytica* was observed when A1 and A2 were incubated with ovine lung surfactant. This was raised to 98.8% when serum was present. The *P. haemolytica* surfactant complex was also deleterious to the bacteria when FCS, gnotobiotic calf serum, rabbit or guinea pig serum were added. The effect was not seen using bovine lung surfactant. Killing did not correlate with the presence of complement, antibody or lysozyme, while the presence of LPS reduced the effect (Brogden, 1992). *P. haemolytica* A1 inoculated intratracheally into sheep showed an increase in bacterial numbers until four hours which coincided with a marked rise in

neutrophils. Macrophage numbers rose slowly but there was failure to clear bacteria even in the presence of many neutrophils (>90% in lavage by 240 min). Davies *et al.,* (1986) showed that prior infection with PI-3 virus increased the number of macrophages but had no effect on bacterial infection and the bactericidal activity of neutrophils was impaired considerably by dual infection. Investigations into the death of pripheral blood mononuclear leukocytes from different species showed killing of bovine cells was 41.7%, ovine 29.3%, goat 33.2%, horse 3.6%, swine 4% and humans 1.4%. Only live bacteria were cytotoxic, with *E. coli* as a control being cytotoxic for all cell types but the ratios were very high at 10^6:1 (Kaehler *et al.,* 1980a; 1980b).

In 1978 Benson *et al.* incubated *P. haemolytica* A1 with bovine bronchoalveolar macrophages and showed severe cytotoxic morphological changes and a low rate of phagocytosis. This effect was the opposite to that of heat killed bacteria. Maheswaran *et al.,* (1980) followed the fate of *P. haemolytica* A1 in bovine bronchoalveolar macrophages. Ninety percent of the bacteria were phagocytosed in the presence of normal or antiserum and were degraded within 60 minutes. Prolonged incubation resulted in cytotoxic changes and destruction of the macrophages. With a lower ratio of bacteria to macrophage at 10:1 or less, phagocytosis and bacterial killing took place with no effects on the macrophages. In 1985 Dyer *et al.,* also reported on *P. haemolytica* and bovine bronchoalveolar macrophages. The viability of macrophages was related to dosage and presence of opsonising antibody. Superoxide varied with the dose of opsonised *P. haemolytica* but indirectly with macrophage viability. Anion responses were dose dependent with all stimuli except unopsonised *P. haemolytica*. Walker *et al.* (1980) isolated bovine bronchoalveolar macrophages from calves aerosolised with *P. haemolytica* A1. These cells failed to phagocytose *P. haemolytica* in vitro. The presence of whole serum, pulmonary IgA and IgG failed to

enhance this, although calves not exposed to *P. haemolytica* by aerosol also failed to clear the bacteria. In contrast, Czuprynski *et al.* (1987) working with bovine neutrophils and *P. haemolytica* A1 showed ingestion was serum dependent and *P. haemolytica* was killed within 1-4 hours incubation depending on the number of neutrophils present. At 100:1 bacteria to neutrophils a reduction in cell viability was observed. After ingestion, a vigorous luminol enhanced chemiluminesance response was observed indicating that oxygen intermediates were contributing to intracellular killing of *P. haemolytica.*

Bovine bronchoalveolar macrophages incubated with normal bronchoalveolar washings (BAW) inhibited uptake of *P. haemolytica*. BAW from calves inoculated with *P. haemolytica* also inhibited uptake in vitro although serum from these calves had pronounced opsonising activity. Supernatants from the cultures of bovine peripheral blood leukocytes, either stimulated or unstimulated, also inhibited phagocytosis (Markham & Wilkie, 1980a). In 1982 Markham *et al.,* also observed the cytotoxic effects by *P. haemolytica* on bovine polymorphonuclear neutrophil leukocytes resulting in 20% killing of PMNs. Pulmonary macrophages were harvested from calves before and after exposure to *P. haemolytica*. In-vitro macrophages were incubated with *P. haemolytica* in log and decline growth phases. Those from decline phase were phagocytosed whereas log phase bacteria were not phagocytosed by lavage cells pre- or post- *P. haemolytica* inoculation. The cells showed altered morphologic features and were cytolytic (Walker *et al.,* 1984).

With ovine bronchoalveolar macrophages Sutherland & Donachie, (1986) showed that serotypes1-15 caused cytotoxic effects while those which fell into the untypable group showed reduced toxicity. Wilkie *et al.,* (1990) reported that *P. haemolytica* A1 caused deterioration of alveolar macrophages within 1 hour but effects on underlying

parenchymal cells were not apparent until after 12 hours. The bacteria were rarely ingested and the addition of culture supernatant accelerated macrophage deterioration without apparent effects on parenchymal cells. This indicates that the severe tissue destruction of fulminant pneumonic pasteurellosis is not a direct result of bacterial infection. Bovine bronchoalveolar macrophages recovered from segmental lavage of *P. haemolytica* affected calves were 30 times more procoagulant than cells from unaffected lobes and 37 times more than controls. Total lung leukocytes (macrophages and polymorphonuclear leukocytes) showed reduced profibrinolytic activity. The imbalance between leukocyte-directed procoagulant and profibrinolytic effects could lead to increased fibrin deposition and retarded removal (Car *et al.*, 1991).

Breider *et al.*, (1991) monitored the effects of *P. haemolytica* A1 on bovine pulmonary arterial endothelial cells. Both live and killed *P. haemolytica* produced extensive damage on the cells after 22 hours. This damage was prevented only by the presence of both neutrophils and immune sera. Polymyxin B prevented the damage suggesting a role for LPS which would account for the effect of killed organisms. Ovine bronchoalveolar macrophages incubated with *P. haemolytica* A1 caused release of TNFα. This was also induced in the presence of killed *P. haemolytica* (Ellis *et al.*, 1991). When *P. haemolytica* was deposited into the lungs of calves and samples taken TNFα and IL-1β mRNA levels were significantly increased in the lavage cells of animals exposed to *P. haemolytica* than in those of the controls. Increased bioactive levels of IL-1 and immunoreactive TNFα in lavage fluids were also demonstrated (Yoo *et al.*, 1995b).

1.13. Carriage and Colonisation

Pasteurella haemolytica is carried in the nasopharynx of apparently healthy sheep. A study of 100 adult sheep heads from an abattoir in Scotland revealed that 64% of the heads were colonised in the nasopharynx. Of these 38% were of the A biotype and 22% not typable (Gilmour *et al.,* 1974). The lambs acquire infection soon after birth, possibly due to contact with the dams (Shreeve & Thompson, 1970). Serotype A2 is most commonly isolated (Gilmour & Gilmour, 1989) and this appears true for sheep flocks in other countries (Frank, 1982; Prince *et al.,* 1985; Mustafa, 1995). Biberstein *et al.,* in 1970 carried out a longitudinal survey and showed that the carrier rates peaked in late spring and early summer and again in late autumn. This coincided with increased incidence of disease, however there was no evidence this was the cause of disease.

Contrary to the colonisation and disease pattern in sheep, cattle pasteurellosis is caused mainly by serotype A1. Serotype A2, however, is more commonly isolated from the nasopharynx in healthy calves. Studies have indicated that the ratio of A2 to A1 changes during the development of respiratory disease (Wray & Thompson, 1973; Frank & Smith, 1983; Gonzalez & Maheswaran, 1993).

The tonsils are also a large reservoir for pasteurellae. In sheep this is mainly *P. trehalosi* (T biotypes). The study by Gilmour *et al.* (1974) showed 95% of pasteurellae were isolated from the tonsils, of which 40% were T biotype. Al-Sultan & Aitken (1985) identified T biotype isolates from 60% of tonsils of young lambs and from 90% by the age of 9 weeks. Of the ewes 78% carried T biotypes and the authors concluded, in agreement with Shreeve & Thompson (1970), that lambs are

colonised soon after birth. Again, in contrast, cattle tonsiliar carriage is not well documented and experimental colonisation has not been successful (Frank & Briggs, 1992).

The mechanisms that pasteurellae possess to allow bacterial colonisation are not well defined. There are many hypothesies presented which include the loss of fibronectin on epithelial cell surfaces, loss of mucociliary apparatus, interactions with viral infections and environmental stress. These links are putative and experimentally unsubstantiated and outwith the scope of this thesis but are reviewed by Frank, 1988; Whiteley *et al.,* 1992; Confer *et al.,* 1994). Those studies which have been carried out include that of Marriassy *et al.* (1987) which showed that in sheep, infection with *P. haemolytica* was associated with increases in airway secretory cells, gland enlargement and that mucus cells showed increased binding to the lectins of *Ulex europus* I (carbohydrate residue α-L-fuc) and *Arachis hypogea* (carbohydrate residue αβ-Gal). Kaissi & Kaissi, 1983 showed ciliostasis of ovine tracheal organ cultures by *P. haemolytica*. Epithelial damage was not observed until 24 hours after inoculation. Ciliated cells were damaged more than non-ciliated. The exfoliation of ciliated epithelial cells was found to occur by a process of extrusion rather than a ciliocytophthonic process as found with damage by irritant chemicals and particles. Cytopathic changes were also observed without intimate association with the organism.

Muller *et al.,* 1988 investigating *Pasteurella multocida* showed that some strains of *P. haemolytica* were adherent to cells but that this was less than with the adherent *P. multocida*. Muller & Mannheim (1995) showed the presence of sialidase and N-acetylneuraminate lyase in *P. trehalosi* and the former enzyme in *P. haemolytica.*

These were considered important as the removal of sialic acid residues from salivary mucoprotein impairs the protective role of mucus against invading organisms. Botcher *et al.,* 1993 showed that untypables, A1, A2 and A11 outer membrane proteins and capsule bound to preparations of epithelial cell wall fractions of trachea and tracheal mucus of cattle. High binding capacity was observed with serovar A1 strains. Uhlich *et al.,* 1993 reported that *P. haemolytica* A1 adhered to bovine nasal mucus in vitro and that this was altered by enzymatic degradation of both protein and carbohydrate components of mucus. Vilela *et al.* (1992) investigating adherence to ovine mammary gland epithelium found adherence and some internalisation in the epithelial cells. A 70 kDa IRP was implicated in adherence. A more controversial result is the production of fimbriae. Morck *et al.,* 1987 using electron microscopy identified a vast glycocalyx and rigid fimbrial structure on *P. haemolytica* A1. Potter *et al.,* (1988) purified fimbriae and showed them to be made up of a 35 kDa protein subunit but did not rule out the possibility that it was conjugative pilus. Morck *et al.,* (1989) also identified the glycocalyx and fimbrial structure on the trachea of feedlot calves. Other workers have reported failure to reproduce these results, although expression in vivo and phase variation have not been ruled out (Confer *et al.,* 1994). The need for definitive studies in this area is urgent.

1.14. Disease Models

When investigating models for ovine pasteurellosis to test for the efficacy of vaccines and treatments two main problems are encountered. The specificity of the bacteria for the ruminant species excludes a large number of laboratory animals including cats,

dogs, hens, pigeons, rabbits and guinea pigs (cited Sutherland, 1989). In contrast Nocard (1892) (cited by Shoo, 1989) revealed that fowls, dogs and rats resisted all modes of inoculation but that mice, rabbits, guinea-pigs and pigeons were susceptible to intraperitoneal inoculation resulting in severe purulent peritonitis. Smith 1958 developed a mouse model by injecting mice intraperitoneally with mucin. This was used by Evans & Wells 1979 to test the efficacy of *P. haemolytica* vaccines but this does not resemble the natural infection in the lung and differing immunology between murine and ovine hosts may play an important part in extrapolating efficacy between species. The widespread natural colonisation amongst healthy conventional sheep and the presence of antibodies against *P. haemolytica* causes problems when infecting animals experimentally. This can work but high numbers of *P. haemolytica* are required and is not always reproducible (Gilmour, 1980). Gilmour *et al.,* (1975) produced pasteurellosis in SPF lambs which was indistinguishable from natural disease using aerosol. The percentage of lambs infected increased when PI-3 virus was administered 7 days prior to the *P. haemolytica* aerosol. This system of PI-3 virus/*P. haemolytica* challenge is used frequently at Moredun due to its consistently high reproduction of experimental infection (Sharp *et al.,* 1978). Other combinations to reproduce disease include *M. ovipneumoniae* and *P. haemolytica* which produced a pathology more like atypical pneumonia (Gilmour *et al.,* 1979; Jones *et al.,* 1983). Respiratory syncitial virus (RSV) and *P. haemolytica* produce disease histologically similar to natural cases of acute pneumonia in lambs (Al-Darraji *et al.,* 1982) and this was also seen in conventionally reared lambs (Trigo *et al.,* 1984) and Davies *et al.,* (1982) used adenovirus followed by *P. haemolytica* to produce disease successfully. Systemic pasteurellosis was reproduced in sheep but used high numbers of bacteria at $\sim 10^{10}$ cfu ml^{-1} intravenously administered. However, killed organisms caused the same

effect (Smith, 1960). Using serotype T4 Gilmour *et al.,* (1980) administered the bacteria in agar but this was only partially successful in that low numbers of bacteria were needed and deaths occurred but not all lesions were reproducible. Suarez Guemes *et al.,* (1987) reproduced systemic disease by hydrocortisone induced immunosuppression and dietary changes. Inoculation of *P. haemolytica* was not necessary so the source of infection was therefore endogenous. The hypothesis for this event was that the bacteria invade through the gastrointestinal system. Hodgson *et al.,* (1993) using T10 and T15 showed that subcutaneous injection of doses of approximately 10^9 cfu ml^{-1} into SPF lambs reproduces disease. Endotoxic shock is the main clinical manifestation and the roles of TNFα and PGE2 in disease have now been evaluated (Hodgson *et al.,* 1994).

Mouse and guinea-pig models have been suggested for bovine pneumonic pasteurellosis. Initial experimental reproduction of disease in calves used preparations from sick or dead animals using pleural fluid, blood, pneumonic lung suspension and nasal exudate. Many of these failed to produce disease but some produced signs of respiratory illness though *P. haemolytica* was either not isolated or lesions characteristic of the disease were not produced (cited Shoo, 1989). The involvement of viruses in the bovine respiratory disease complex led to experiments using *P. haemolytica* in combination with viruses. Infectious bovine rhinotracheitis (IBR), PI-3, bovine virus diarrhoea virus (BVD) or bovine herpes virus 1 (BHV1) all produce disease with varying degrees of success. The best reproduction was given by aerosols of BHV1 administered 4 days prior to *P. haemolytica* aerosol. Synergism was shown with *Mycoplasma bovis* and *P. haemolytica* in calves (Houghton & Gourlay, 1983). The main problem cited by Shoo, (1989) for unsuccessful challenge models was the inoculum and state of the culture. Combination infections used low numbers of *P.*

haemolytica whereas *P. haemolytica* alone caused fibrinous pneumonia when 5ml of 10^9 cfu/ml was used (Panciera & Corstvet, 1984) and the culture was log phase (Ames *et al.,* 1985). This combination administered to susceptible calves either intratracheally or intrathoracically consistently produced disease.

1.15 Aims of Thesis

The aims of this thesis were to compare representative strains of the three main serotypes responsible for causing pasteurellosis in ruminants. Ovine and bovine serum, ovine and bovine tracheobronchial washings and media either iron-replete or iron-restricted, were used as culture fluids to investigate the possibility that these fluids would initiate changes in the virulence factors associated with *Pasteurella* spp. Antiserum raised specifically against the serotypes was employed to define the importance of the virulence factors in vivo and to identify any new antigens which may be important for use in vaccine improvement. The same in-vivo fluids were also used to assess the survival capability of the serotypes. Survival mechanisms have been widely investigated in organisms found in the environment (Rozak and Colwell, 1987) but little research has focused on those organisms which are commonly found colonising mucosal surfaces and which can proliferate to cause overwhelming disease. Due to the ability of bacteria to adapt to their environments, many characteristics essential for survival in a particular environment can be lost on sub-culture in laboratory culture medium. As observations made in-vitro must be identified as being present in-vivo there was a requirement for a method to isolate *Pasteurella* spp from in-vivo tissues. These would then be compared observations made in in-vitro studies.

Oxygen radicals cause irreparable damage to DNA, enzymes and other important components of bacteria. They are generated by phagocytes and found in the mucosal environment as a defence against invading micro-organisms. Superoxide dismutase is an enzyme which neutralises the damaging effects of oxygen radicals. The

investigation into the presence of superoxide dismutase in *Pasteurella* spp. is important to define whether this enzyme plays a role in protection of the bacteria from these essential defence mechanisms. This investigation leads onto the analysis of the interaction that the bacteria have with resident alveolar macrophages. These cells are regarded as the first line of defence against proliferation in the lower respiratory tract. There has been substantial research into the interaction of bovine alveolar macrophages and serotype A1. This study endeavours to investigate the interaction of both ovine and bovine macrophages with the three important serotypes and to monitor any apparent differences.

The significant feature of this research was to use, where possible, in-vivo fluids, in-vivo bacteria and in-vivo host cells to attempt to relate any observations in-vitro to the situation in vivo. The requirement to mimic in-vivo environments was necessary firstly to ease the difficult problem of isolating in-vivo organisms. Secondly, to identify components and adaptation mechanisms which may lead to enhanced protection through the production of improved vaccines or other protective treatments for pasteurellosis.

CHAPTER 2

MATERIALS AND METHODS

2.1. General methods

Bacteria and culture conditions

The strains used throughout this thesis are *Pasteurella haemolytica* serotypes A1 (strain M4/1/2, a bovine lung isolate), A2 (strain 124/92, ovine lung isolate) and *Pasteurella trehalosi* serotype T10 (152/94, an isolate from systemic pasteurellosis). These are all standard challenge strains of known virulence.

Other strains of *P. haemolytica* and *P. trehalosi* used where stated are part of the strain collection kept at Moredun Research Institute (MRI) and all were recovered from cases of pasteurellosis at MRI or sent to MRI for serotyping. All strains were stored at -70°C in nutrient broth No 2 (Oxoid) or on beads (Prolab). Strains were recovered from storage, thawed and plated onto 7% sheep blood agar and incubated at 37°C for 18 h. Further culture is as otherwise stated but most broth cultures of *P. haemolytica* were carried out in tryptic soy broth with 1% yeast extract (TSB, YE, Difco) and *P. trehalosi* was cultured in TSB alone. *Escherichia coli* was stored on slopes and a loopful of culture was inoculated onto nutrient agar or into nutrient broth (Oxoid). *Listeria monocytogenes* was also stored on slopes and cultured by inoculation onto 7% sheep blood agar or into nutrient broth. *Neisseria meningitidis* was stored at -70° C. Once thawed the culture was grown on GC medium (Difco) containing 2% haemoglobin and vitox supplement (Oxoid), or in Muller Hinton broth (Mast Diagnostics) with 10% glycerol. *Haemophilus somnus* was provided as a plate

culture and was cultured on BHI agar (Difco) with 0.5% yeast extract and 7% bovine blood or in BHIB with 0.05% sodium-L-aspartate, 0.1% tris and 0.001% thiamine monophosphate. All bacteria mentioned were incubated at 37°C overnight with the exception of *N. meningitidis* and *H. somnus* which on occasion were incubated for a further 24 h.

Collection of lung washings

This was done as described by Sutherland, 1989. Ovine and bovine lungs were collected from local abbatoirs or from animals at MRI. Phosphate buffered saline (PBS pH 7.4, 400- 1200ml), depending on the size of the lungs, was poured into the trachea. The lungs were briefly massaged and the fluid then poured into a container through a gauze covered filter. The washings were centrifuged at 500 x g for 30 min to pellet debris and cells and the supernatant collected. The supernatant was further centrifuged at 2000 x g for 30 min to pellet any bacteria present. Supernatants were then filtered through a 0.45μm filter. Samples were plated onto blood agar and cultured at 37° C overnight to check for purity and stored at -20°C.

Production of convalescent lamb serum

P. haemolytica serotypes A1 and A2 and *P. trehalosi* serotype T10 were taken from a blood agar plate and cultured in nutrient broth for 3 h at 37° C with shaking. The bacteria were washed twice in PBS and resuspended in PBS to approximately 10^6 colony forming units (cfu) ml^{-1}. 2ml of culture was given intratracheally (to SPF lambs approximately 6 months of age) and subcutaneously (1ml) on days 1 and 20. Any animal that showed signs of illness was given 2ml of tetramycin intramuscularly.

Bleeds were taken before, and 10 days after each challenge in order to monitor seroconversion. A final intra-nasal inoculation was given on day 34.

Production of rabbit antiserum

Cultures were grown overnight in TSB, YE at 37°C, washed in PBS and resuspended in 5ml of 0.05% formalin in PBS and stored at 4°C. Innoculation proceedures were as follows,

Day1- 0.5ml subcutaneously

Day3- 1ml subcutaneously

Day4- 2ml subcutaneously

Day7- 1ml intravenously

Day11- 2ml intravenously

Day15- 2ml intravenously

Day22- 2ml intravenously

Day29- 2ml intravenously

Day36- 2ml intravenously

At this stage rabbits given serotypes A1 and T10 seroconverted and showed high IHA titres. The rabbit inoculated with A2 was given three further doses of 1ml of live A2 intravenously 25 days apart.

Indirect Haemagglutination Assay

A loopful of bacterial culture was suspended in 1ml of PBS containing 0.03% formalin (FPBS). The suspension was heated at 56°C in a water bath for 30 min, to release the antigen from the cells. Glutaraldehyde-fixed (5%) ox red blood cell (RBC) suspension (100µl) was added to the bacterial suspension and incubated for another 30 min at 37°C. The RBC complex was pelleted at 2,900 x g for 20 sec and washed twice in 1ml of fresh FPBS. Rabbit antiserum 25µl belonging to each of the 12 *P. haemolytica* and four *P. trehalosi* serotypes was added to a U-bottomed microtitre plate. The RBC complex was then added at an equal volume and left to stand at room temperature for 1-2 h or overnight. A positive result was indicated by an even mat of RBCs over the bottom of the well and negative results were indicated by a small button of RBCs in the centre of the well.

When testing antiserum a series of doubling dilutions from undilute to 1 in 2048 were made in U-well microtitre plates. These were carried out in duplicate for each test antiserum and for the positive control antiserum. The rest of the method was as described except one lane of duplications contained sensitised RBCs (those complexed to bacterial antigen) and the other contained unsensitised RBCs (diluted 1 in 10 in FPBS). The serum titre was the dilution at which the even mat of RBCs was observed before RBC buttons were observed.

2.2. Analysis of Strains Form Different Growth Conditions

Iron Restriction

Iron restriction and depletion was investigated using three methods (see Appendix I) for chemically defined media (Jackson & Burrows, 1956; Wessman *et al.*, 1966; Hu *et al.*, 1986) and TSB with various molar concentrations of αα-dipyridyl and/or EDDA.

Envelope Preparation

Whole cells after culture were pelleted at 2000 x g, washed and resuspended in 250mM Tris (Tris [hydroxymethyl] amine-methane)-HCl. The suspension was then sonicated (ultra sonic disintergrator, MSE) at 20 amp for 30 sec bursts (5-10 repeats) whilst kept on ice. The suspension was then centrifuged at 2000 x g for 30 min to pellet unbroken cells. The pellet was discarded and the supernatant was centrifuged further at 30000 x g in a Ti 70 rotor (Beckman) for 1.5 h at 4°C. The supernatant was stored at -70°C and the pellet washed twice in 250mM Tris-HCL, resuspended in PBS and stored at -20°C.

SDS PAGE

Samples were dissolved in sample buffer (2% w/v sodium dodecyl sulphate (SDS), 5% w/v glycerol, 2% v/v 2-mercaptoethanol, 0.002% bromophenol blue and 125mM Tris/HCl, pH 6.8) and heated in a boiling water bath for 5 min. Samples were then

resolved by SDS-polyacrylamide gel electrophoresis (SDS PAGE) on a 10% (w/v) gel using the discontinous buffer system of Laemmli (1970). Proteins were detected by Coomassie blue and/or silver staining (all procedures detailed in Appendix I).

Western Blotting

SDS-PAGE gels were electroblotted onto nitrocellulose (Schleicher & Schuell) by the method of Towbin *et al.* (1979). Nitrocellulose was blocked using 10mg/ml BSA in blot wash buffer (BWB) for 1 h at 37°C or overnight at room temperature with gentle agitation and was then was washed three times in BWB. For detection, the nitrocellulose was incubated at 37°C for 1 h with a monoclonal antibody or an antiserum diluted in BWB and then washed as described above. Nitrocellulose was then incubated with horseradish-peroxidase conjugated to anti-mouse, anti-rabbit, anti-sheep IgG (Scottish Antibody Production Unit) or anti-pig, anti-horse, anti human and anti-dog IgG (Sigma) and again washed in BWB. Development was carried out at room temperature in 100mM Tris pH7.0, 0.5mg/ml diaminobenzidine and 2μl/ml of 30% hydrogen peroxide. The reaction was terminated by the addition of water (all procedures detailed in Appendix I).

Cytotoxicity Assay (Promega, Cell Titre 96 AQ Assay)

Bovine leukaemia derived cells (BL-3, 50µl) at 4x 10^6 /ml were added to a 96 well flat-bottomed plate (Nunc) in Hanks/PBS (1:1). Toxin (50µl of a 20mg/ml freeze dried preparation) was added and 50µl of Hanks/PBS. If neutralisation of the toxin was being measured the toxin and antiserum were incubated for 20 min at room temperature prior to addition to the cells. Controls consisted of cells alone, dead cells (10µl of Triton X100) and toxin without cells. The toxin and cells were incubated at 37°C for 1 hour. After incubation, 20µl of MTS/PMS (2ml of 2mg/ml MTS + 100 µl of 0.92 mg/ml PMS) was added to all wells except the first row (blank) and incubated for up to 4 h at 37°C. The plate was then read at 490 nm on a MR5000 microplate reader (Dynatech).

Visualisation of Capsule

The staining method of Maneval (1941) was used. Stock solutions of 1% aqueous Congo red stain (BDH) and acid fuchsin (30ml of 5% aqueous phenol, 8ml of aqueous glacial acetic acid and 4ml of 30% aqueous ferric chloride). A loopful of bacterial suspension was mixed on a microscope slide with a loopful of congo red, spread thinly and air dried. The smears were then counter stained with acid fuchsin for 2 min, drained and blotted dry. Bacteria appeared as red stained bodies on a blue background with capsules negatively stained, appearing as clear halos.

Leukotoxin Preparation

A loopfull of culture from a blood agar plate growth was used to inoculate a 10ml starter culture of brain heart infusion broth (BHIB). After incubation overnight at 37° C this was added to 400ml of BHIB and further incubated at 37° C on a shaker for three hours. Bacteria were pelleted by centrifugation (2000x g) and resuspended in 50 ml RPMI containing 7% (V/V) FBS. Incubation was for 30-45 mins at 37° C whilst shaking. The bacteria were pelleted by centrifugation as above and the supernatant collected. 1ml aliqouts were either freeze dried or stored at -70° C.

Lipopolysaccharide (LPS) Preparation (Micro phenol method of Fomsgaard *et al.*, 1993,)

A loopful of bacterial culture was inoculated into 20ml of media and incubated overnight at 37°C. The cells were pelleted at 2000 x g, washed twice and resuspended in 500µl of pyrogen free water and transferred to an Eppendorf tube. Aqueous phenol (500µl of 90% v/v) was added and the tube vortexed for 10 sec followed by placement in a water bath at 70°C for 10 min with vortexing at intervals. The tube was immediately placed on ice for a minimum of 2 min and centrifuged at 2900 x g for 4 min at room temperature. The upper clear layer was removed carefully with a pastette and stored at -20°C.

SDS PAGE for LPS

Using the method of Hancock & Poxton (1988) samples were boiled in sample buffer (0.125M Tris/HCl pH6.8, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol and 0.002% bromophenol blue) for 5 min. The samples were resolved using 14% (w/v) gels using the discontinous buffer system of Laemmli (1970). LPS was visualised using silver stain (detailed proceedures in Appendix I).

LPS Stain

The gel was placed into a container with 200ml of 25% propan-2-ol and 7% acetic acid overnight. The solution was discarded and replaced with 1.05g of periodic acid in 150ml of distilled water and 4 ml of the above solution for 5 min.

On discarding the above solution the gel was washed in at least 4 changes of distilled water for over 4 h. The gel was then incubated for 15 min in a solution consisting of 21ml of 0.36% sodium hydroxide, 1.4ml of ammonia solution (0.88 SG), 4ml of 19.4% silver nitrate solution (which had been added slowly) topped up with 100ml of water. The gel was again washed in 4 changes of distilled water for over 40 min. The water was replaced with fresh 0.005% citric acid in 200ml of 0.019% formaldehyde and left to develop. When the desired staining was reached, the gel was repeatedly rinsed in large volumes of distilled water.

Endotoxin analysis

The biological activity of LPS was determined in the Limulus amoebocyte lysate (LAL) assay (reagents supplied by Associates of Cape Cod International, Inc. Liverpool UK.). This was carried out by G.Moon, MRI.

2.3. Survival

Survival Cultures

Bacteria were grown overnight at 37°C. The culture was washed in PBS and adjusted to 10^5 cfu/ml. Bacterial suspension (20µl) was added per ml of fluid. The fluids were used either undilute or diluted in PBS after it was shown that PBS alone did not support growth or survival after 24 h. These were incubated at the designated temperature and sampled after 24 h and again at various times after that depending upon the experiment. Viable counts were assessed on sheep blood agar. When no detectable colonies were present samples were inoculated into TSB and incubated at 37°C overnight.

Analysis of survival cultures

Samples were stained using the methods of Gram or Maneval. Nutrient analysis of protein, glucose and iron was carried out in The Biochemistry Dept, MRI by J. Small.

Transmission electron microscopy

Bacterial cultures were washed, placed onto copper grids and negatively stained with 0.1% ammonium molybdenum. Grids were observed at the Biochemistry Dept, Edinburgh University and photos were taked by Mr Derek Notman.

2.4. Immunomagnetic Separation

Serum Adsorption

Brain Heart Infusion Broth (BHIB Oxoid, 1L) was inoculated with an overnight culture of *P. haemolytica* or *P. trehalosi* grown at 37°C . The resulting cultures were washed in PBS at 5500 x g for 1 h and the pellet resuspended in 0.5% formalin in PBS. This was left overnight in the fridge and centrifuged at 2400 x g for 30 min and the supernatant discarded. The pellet was washed in PBS and resuspended in 20ml of PBS.

A total of 5ml of non-target bacteria were mixed together and centrifuged to obtain a pellet. The pellet was resuspended in 2ml of antiserum raised against the target bacteria and diluted 1 in 4 with sterile 0.9% saline. The suspension was incubated at room temperature, while shaking for 2 h. The suspension was then centrifuged and the

supernatant used to resuspend a fresh 5ml pellet of non target bacteria. This was incubated at 4°C overnight. The room temperature step was repeated twice more and 10% BSA was added to the antiserum which was stored at -20°C. This procedure was repeated for each target antiserum.

Dot Blots

Strips of nitrocellulose were wetted in BWB and allowed to dry at room temperature. Formalin killed cells (2µl) used in the above experimental procedure were spotted onto the nitrocellulose and allowed to dry at room temperature for 1 h. This was then washed in BWB three times for 5 min each wash. The blots were then treated as Western blots.

Immunomagnetic Separation (As described by manufacturer's instructions)

Direct Method

Dynabeads (Dynal) were washed three times in PBS/BSA (0.1%) with retrieval of the beads carried out on a Dynal magnetic particle retriever. Beads (20µl) were added to 1ml of monoclonal antibody hybridoma solution or absorbed antiserum containing 5-20µg of antibody/ mg of dynabeads. This was incubated at 4°C for 2 h with gentle mixing. The beads were then collected with the magnet and washed with PBS/ Tween (0.005%) for 5 min three times. The beads were collected and resuspended in 1ml of target cell suspension (10^4 cfu/ml as determined using M°Farland standards,

(Biomerieux) in PBS/BSA and the mixture incubated for 30 min at 4°C. The beads were again collected and washed as previously described.

Indirect Method

The target bacteria were grown for 4 h at 37°C with shaking, washed and diluted with PBS to 10^4 cfu/ml. The suspension (1ml in PBS/BSA) was incubated with 200µl of antiserum for 30 min at 4°C. This was then centrifuged at 11600 x g for 10 min and the supernatant discarded. The pellet was washed twice more and incubated with 20µl of prewashed dynabeads and incubated in PBS/BSA for 2 h at 4°C. The beads were collected with the magnet and washed three times in PBS/Tween. The beads were plated onto sheep blood agar for detection of bacteria and, where required, an IHA test was carried out on colonies to determine the serotype and hence the specificity of the antibody-coated beads for the target organism. The specificity was also determined by incorporating various mixtures of serotypes and other organisms together at the target cell suspension stage.

Crosslinking

Dynalbeads (200µl) complexed to antibody were washed as normal then equilibrated in 0.2 M triethanolamine pH 9.0. The beads were resuspended in 10ml of triethanolamine and 52mg of dimethyl suberimidate was added to the suspension which was then incubated for 45 min at room temperature with mixing. The beads were recovered and resuspended in 10 ml of triethanolamine and incubated in the same fashion for a further 2 h. The beads were again recovered and washed gently in

PBS/Triton X100 for 10 min. The beads were washed three times in PBS and either used immediately or stored at 4°C in PBS/ 0.02% sodium azide until required.

Isolation from tissues

Approximately 1g of tissue was thawed at 37°C and macerated in a stomacher bag with 10ml of PBS. This was centrifuged at 210 x g for 20 min in order to remove cellular and tissue debris. The supernatant was retained and samples plated onto sheep blood agar. The supernatant was centrifuged at 2400 x g for 30 min and the cell pellet retained. This was resuspended in PBS/BSA with antiserum and the indirect method followed or incubated with beads and the direct method followed. Detection was as previously described.

Isolation from nasal swabs

Nasopharyngeal swabs were taken and the ends added to 3ml of PBS and vortexed until a suspension was visible. The swab end was then removed and centrifuged at 2400 x g for 30 min. The pellet was resuspended in PBS and detection carried out by the direct or indirect method.

2.5. Superoxide dismutase Detection

Preparation of bacterial lysates.

Colonies were scraped from a pure culture on a blood agar plate and suspended in PBS. This was spread plated onto sheep blood agar and incubated overnight at 37°C. The resulting bacterial lawns were scraped off by means of a bent glass rod and suspended in lysate buffer (10mM Tris, 1mM EDTA, 0.5mM NADP, 0.005%DNAse, pH 6.8). The bacterial lysates were prepared by disrupting the cells with 0.1mm diameter zirconium beads in a mini beadbeater (Biospec Products Inc, Bartlesville, Okla) for 2 min. The lysate was clarified by centrifugation (11000 × g) and stored at -70°C. Protein concentration was determined using BCA protein assay (Pierce).

SOD detection using Polyacrylamide gel electrophoresis (PAGE).

This was based upon the method of Beauchamp & Fridovitch (1971). Electrophoresis was performed using Bio-Rad Mini Protean II system. Gels (0.75mm) were prepared using a non-denaturing discontinous Tris-glycine buffer system, with the resolving gel (7.5% acrylamide/bis) containing 1.5M Tris/HCL pH 8.8, and the stacking gel (4% acrylamide/bis) containing 0.5M Tris/HCL pH 6.8. Electrophoresis was carried out using chilled electrode buffer and run on ice at 200v for 45 min (detailed procedure in Appendix I).

Staining for SOD activity.

Enzyme activity was visualised by soaking gels in 5mM nitro-blue tetrazolium (NBT) for 15 min followed by 20 min in a working buffer (10ml 0.1M Tris-Base pH9, 42µl TEMED, 10µl 28mM riboflavin). Gels were illuminated on a light box until the development of colour was achieved and achromatic bands observed.

SOD assay.

This was carried out according to the manufacturer's instructions (RANSOD kit, Randox, Co.Antrim,UK). Samples were mixed with substrate and xanthine oxidase was added to start the reaction. Absorbance of samples and standards were read at a wavelength of 500nm after 30 min incubation at room temperature.

Units of enzyme activity were calculated on the basis that 1 unit of SOD inhibits maximum colour formation at 50% based on the standard curve.

Malate dehydrogenase (MDH) assay.

This was by the method outlined in the Worthington Enzyme Manual. The assay was carried out by mixing 260µl of 0.1M phosphate buffer pH7.4, 20µl 0.00375 M NADH, 10µl 0.006M oxaloacetic acid. Sample (10µl) was added and the decrease in

absorbance at 340nm over 1-5 min recorded. Units of activity were calculated using the formula :

$$\text{Units/ mg}^{-1}\text{ protein} = \frac{\Delta A340/\text{min}}{6.22} \times \frac{1}{\text{mg protein}} \times \frac{1}{\text{ml reaction mixture}}$$

Inhibition studies of SOD.

These experiments were carried out to identify the type of SOD in the bacteria. EDTA inhibits all SOD types whereas hydrogen peroxide inhibits Cu/Zn and Fe SODs and potassium cyanide inhibits only Cu/Zn SOD. Prior to electrophoresis or assay, samples were incubated for 30 min with final concentrations in aqueous solutions of 5mM potassium cyanide, 5mM hydrogen peroxide or 5mM EDTA. During gel staining the inhibitors were also present in the NBT and riboflavin solutions.

Polymerase chain reaction (PCR)

The method of Lainson *et al.* (1996). Genomic DNA was obtained from an overnight broth culture and prepared using the Genome DNA Kit (Bio 101). This was used as a template in PCR. PCR was carried out using Taq polymerase and buffer (Boehringer) and nested primers P6748 (AAGGTGGCAAGCTCACAGCAG) and P6749 (TTCAAGCGAGGGGCTAATAACT). Thermal cycling conditions were 30 cycles of denaturation at 94° C for 1 min; annealing at 51°C for 1 min; extension at 72°C for 1 min followed by a single extension at 72°C for 10 min. Samples were checked for the presence of a product by electrophoresis on a 2% agarose gel.

Preparation of periplasmic and cytoplasmic fractions.

Osmotic shock method (based on the method of Stabel *et al.*, 1994). Freshly harvested bacteria were washed twice in cold TRIS (10mM, pH8), NaCl (20mM). The bacteria were resuspended in 2.5ml TRIS (30mM, pH8), 20% sucrose and a final concentration of 1mM EDTA. The suspension was shaken at room temperature for 10 min and the bacteria were then pelleted by centrifugation. The pellet was resuspended in 2.5ml of cold distilled water and shaken for a further 10 min at 4^0C. The bacteria were pelleted and the supernatant collected.

CHAPS/lysozyme method (based on the method of Stabel *et al.,* 1994). Bacteria were adjusted to 10^{10} cfu ml^{-1} in PBS, using M^{c}Farland standards, and centrifuged to pellet cells. The pellet was resuspended in 0.2M TRIS/HCl and an equal volume of 0.2M TRIS/HCl, 1M sucrose. 0.5% CHAPS and a final concentration of 100g ml^{-1} of lysozyme was added. Mild osmotic shock was carried out using an equal volume of distilled water which was then incubated at room temperature for 2h and followed by centrifugation. The supernatant was collected and concentrated using using Sephadex G-100 coated dialysis tubing.

EDTA/lysozyme method (based on the method of Lainson *et al.*, 1991). Bacteria were adjusted to 10^{10} cfu ml^{-1} washed in PBS and resuspended in 1ml TRIS/HCl (200mM, pH8) and incubated for 10 min at room temperature. The above buffer (1ml) containing 1M sucrose, 0.5mM EDTA and 120g ml^{-1} of lysozyme was added and incubated for 5 min. This was then diluted 1:1 with distilled water, incubated at room

temperature for 30 min with vigorous shaking. The suspension was then centrifuged and supernatant collected and concentrated as described.

Chloroform method (based on the method of Stabel *et al.*, 1994). Bacterial culture (20 ml) was washed in saline and the supernatant discarded. The pellet was resuspended in the residual saline and 0.1 ml chloroform added and incubated at room temperature for 15 min when 5 ml TRIS (10mM pH8) was also added. After centrifugation the upper portion was carefully removed for assaying.

Freeze thaw method. Bacterial cultures (20ml) were washed twice in PBS and resuspended in 500µl of PBS. The cell suspensions were then frozen at -20°C and thawed at 37°C at least 10 times. After centrifugation at 10000 x g the supernatants (periplasmic fraction) were removed for assaying. The remaining bacterial pellets were lysed using the bead-beating method described previously in order to release cytoplasmic contents. All periplasmic and cytoplasmic contents were assayed for SOD activity and malic dehydrogenase.

Bactericidal Assay

Bacterial cultures (a loopful) were grown in nutrient broth at 37°C with shaking for 4.5 h. The bacteria were washed twice in PBS and resuspended in 0.1M phosphate buffer pH 7.5. Using a serowell microtitre plate, control wells contained xanthine (0.05mM), 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025mM) in 0.1M phosphate buffer pH 7.5 and 0.002 units of xanthine oxidase.

Following mixing the production of visual formazan dye was monitored to show that production of superoxide had occurred. INT is toxic to *Pasteurella* spp and was omitted from the test wells. Controls contained xanthine, buffer and bacteria at 10^5 cfu/ml. Experimental wells contained xanthine, xanthine oxidase and the same bacterial inoculum. The plate was incubated at 37^0 C and 10µl samples were removed at times 0, 15 and 30 min. Samples were serially diluted, plated onto 7% sheep blood agar in duplicate and incubated at 37^0 C overnight.

Reactivity with antiserum

This experiment was designed to show any interaction between the SOD and antibodies which may have been raised specifically against the enzyme during infection. The convalescent antiserum was incubated in equal volumes with the sample for 30 min prior to electrophoresis. To determine whether specific IgG bound to and inhibited SOD activity, 10µl of sample was mixed in a total volume of 100 mg/ml^{-1} of purified IgG in 0.1 M Tris/HCl pH8. This was incubated with mild agitation at room temp for 1 h. Protein G (5ml Protein G immobilised on Sepharose 4B, Fast Flow, Sigma) was added and the incubation continued for a further 30 min. Samples were centrifuged and the supernatant removed and assayed using the Ransod kit. Negative control antiserum and purified IgG were obtained from an specific pathogen free (SPF) lamb given orf virus.

2.6. Macrophage Interaction

Collection and culture of alveolar macrophages (As described by Sutherland, 1989).

Collection was as previously described in collection of lung washings but instead of discarding the pellet from the cell debris stage this was kept. If contaminating red blood cells were present they were removed by resuspending the pellet in 10 ml of 1x PBS. Double distilled water (20 ml) was added and mixed quickly for 30 sec. 10x PBS (2 ml) were added and centrifuged at 800 x g for 5 min and the supernatant contained the cells. Cells were washed in Hanks' balanced salt solution (Hanks) and resuspended in RPMI (detailed recipie in Appendix I).

Viability was determined with nigrosin (0.1%) and macrophages were counted using an improved Neubauer haemocytometer. Macrophage concentration was adjusted to $1x10^7$ cells/ml and 100µl added to 96 well tissue culture plates. These were incubated overnight at 39°C in 5% CO_2 until required.

Phagocytosis assay

Based on the method described by M°Neil *et al.* (1994). Bacterial cultures were adjusted to approximately $1x10^6$ cfu ml^{-1}, washed and resuspended in RPMI without antibiotics. The macrophage cultures were washed three times in Hanks, the bacteria added and incubated at 37°C. After incubation macrophages were washed three times in Hanks then RPMI containing 50ug/ml of gentamicin was added (to kill extracellular bacteria) and this was incubated for a further 90 minutes. This medium was then washed three times with Hanks and macrophages were lysed with the

addition of 100µl of 1% saponin in Gey's balanced salt solution (GBSS) and 2% of fetal bovine serum (FBS). From this serial dilutions were carried out on the lysate and plated onto sheep blood agar to determine the numbers of surviving intracellular bacteria.

Opsonisation of bacteria was carried out by incubating washed bacteria with a 1 in 10 dilution of antiserum.

Cytochalasin-D and monodansylcadaverine were used at 2µg/ml and 200µM respectively and incubated for at least 1 hour with macrophages prior to infection.

Intracellular/ Extracellular bacterial Ratio.

This was based on the method described by Hondalus & Mosser (1994). Macrophages ($1x10^7$) and bacteria ($1x10^6$) were prepared as above except that culture was carried out in 2 well glass slides (Chamber slide, Lab-Tek Napenville IL). After 1 h incubation the slides were washed and to one chamber was added 100% methanol and to the other was added 3% formaldehyde. The slides were incubated for 30 min and washed with GBSS. The chamber slides were Gram stained and left to dry. Using 1000x magnification, the number of bacteria associated with 100 macrophages was counted.

Macrophage cytotoxicity Assay (Promega Cytotox Kit).

Macrophages ($1x10^6$) were cultured in 96 well tissue culture plates as previously described. The medium was washed three times with Hanks/PBS (1:1) and bacteria ($1x10^5$) were added in Hanks/PBS. The plate was incubated for 30 min at 37°C with

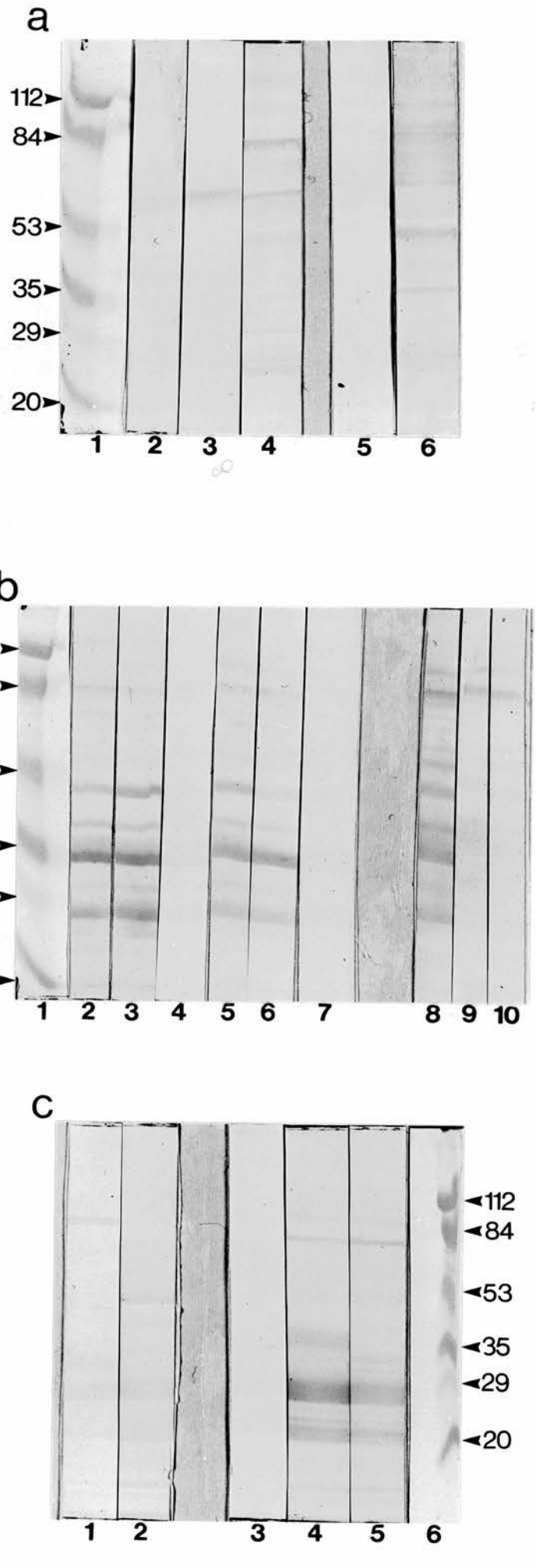
shaking. The plate was then centrifuged at 500 x g for 1 h. A 50µl sample was removed from each well and added to a fresh plate. Substrate (50µl) was added to all wells and the plate incubated for 30 min at room temperature in the dark. Stop solution (50µl) was added and the absorbance read at 490nm on a microplate reader (Dynatech). Controls were set up as per manufacturer's instruction

Fig 3.1. Western blots of serotypes A1 (a), A2 (b) and T10 (c) envelopes probed with sequential bleeds in the production of homologous convalescent lamb serum (used in chapters 3, 6 and 7) and rabbit antiserum (used in chapter 5).

(a) Molecular weight markers (lane 1), lamb 97 pre bleed (lane 2), lamb 97 second bleed (lane 3), lamb 97 final bleed (lane 4), rabbit 439 pre bleed (lane 5), rabbit 439 final bleed (lane 6).

(b) Molecular weight markers (lane 1), lamb 268 final bleed (lane 2), lamb 268 second bleed (lane 3), lamb 268 pre bleed (lane 4), lamb 127 final bleed (lane 5), lamb 127 second bleed (lane 6), lamb 127 pre bleed (lane 7), rabbit 0010 final bleed (lane 8), rabbit 0010 second bleed (lane 9), rabbit 0010 pre bleed (lane 10).

(c) Rabbit 440 pre bleed (lane 1), rabbit 440 final bleed (lane 2), lamb 112 pre bleed (lane 3), lamb 112 second bleed (lane 4), lamb 112 final bleed (lane 5), molecular weight markers (lane 6).


a
112
84
53
35
29
20
1 2 3 4 5 6
b
112
84
53
35
29
20
1 2 3 4 5 6 7 8 9 10
c
112
84
53
35
29
20
1 2 3 4 5 6

Western blots probed with rabbit antiserum (Fig 3.1a-b) showed an equally strong IgG response against bacterial envelopes of the serotypes when compared to the pre-bleed serum. The IHA results, which can be seen in Table 3.1, did show higher titres than the ovine antiserum except for serotype A2 which only agglutinated when the antiserum was neat. The standard A2 antiserum used in the test was also of low titre.

SEROTYPE	ANTISERUM	SPECIES	ANTIBODY TITRE
A1	STD	RABBIT	1/16
A2	STD	RABBIT	NEAT
T10	STD	RABBIT	1/16
A1	97	OVINE	1/32
A2	268	OVINE	NEAT
T10	112	OVINE	0
A1	439	RABBIT	1/128
A2	0010	RABBIT	NEAT
T10	440	RABBIT	1/16

Table 3.1. IHA titres of rabbit (used in chapter 5) and sheep antiserum (used in chapters 3, 6 and 7) raised against *P. haemolytica* and *P. trehalosi* serotypes and tested on homologous strains.
STD, standard antiserum.

3.3. Iron Restriction

The approach to iron restriction was to obtain maximum growth under the maximum stress of iron and/or divalent-cation restriction, as opposed to depletion. The first methods applied used chemically defined media (CDM, see Appendix I). Three different CDM which had been developed for *P. haemolytica* and closely related organisms were used. The serotypes were grown in the CDMs prepared with and

without the iron compound. Table 3.2 shows the effects of culture in the CDMs. The serotypes either grew in all media or in the case of CDM C did not grow at all.

SEROTYPE	CDM (A) + HAEMIN	CDM (A)	CDM (B) + Fe_2Cl_3	CDM (B)	CDM (C) + Fe_2SO_4	CDM (C)
A1	+++	+++	+++	+++	NG	NG
A2	+++	+++	+++	+++	NG	NG
T10	+++	+++	+++	+++	NG	NG

+++ - Growth
NG - no growth
Table 3.2. The growth of serotypes using three different chemically defined media (CDM), with and without an iron source. Results are the same for broth and agar plate culture.

Divalent cation chelators were employed to restrict iron availability. Two widely used chelators, namely αα dipyridyl and ethylenediamine Di (o-Hydroxyphenylacetic acid (EDDA), were screened using concentrations between 50 and 500μM. Table 3.3 shows the concentration of each chelator which arrested growth and also the concentration of ferric chloride needed to overcome the cessation of growth.

MEDIUM	SEROTYPE	CHELATOR MIC* FOR ARRESTING GROWTH	Fe_2Cl_3 CONCENTRATION ADDED WHICH REPLETES GROWTH
TSB/YE BROTH	A1	500μM ααdp	100μM
		400μM EDDA	200μM
	A2	450μM ααdp	100μM
		450μM EDDA	100μM
	T10	500μM ααdp	100μM
		400μM EDDA	150μM
TSB/YE AGAR	A1	250μM ααdp	100μM
		350μM EDDA	350μM
	A2	250μM ααdp	100μM
		450μM EDDA	450μM
	T10	350μM ααdp	100μM
		200μM EDDA	100μM

Table 3.3. Concentrations of chelator required for arresting growth of serotypes and concentrations of the amount of iron needed to reverse the effect.
*MIC - minimum inhibitory concentration, ie the lowest concentration of chelator which inhibited growth of the serotypes

MEDIA	SEROTYPE	CONCENTRATION OF CHELATOR	PASSAGE 1	PASSAGE 2	PASSAGE 3	PASSAGE 4	PASSAGE 5	PASSAGE 6	PASSAGE 7
TSB/YE AGAR	A1	150μM EDDA	+++	NG					
	A2		+++	NG					
	T10		+++	NG					
	A1	200μM αα dp	+++	+++	+++	+++	+++	NG	
	A2		+++	+++	+++	+++	+++	NG	
	T10		+++	+++	+++	+++	+++	+++	+++
TSB/YE BROTH	A1	200μM EDDA	+++	NG					
	A2		+++	NG					
	T10		+++	NG					
	A1	200μM αα dp	+++	+++	+++	+++	+++	+++	NG
	A2		+++	+++	+++	+++	+++	+++	NG
	T10		+++	+++	+++	+++	+++	+++	NG

Table 3.4. Passage of serotypes in broth and agar culture in the presence of iron chelators EDDA and αα dipyridyl (αα dp)
+++ - Growth, NG - no growth.

SEROTYPE	EDDA (150μM)	EDDA + Fe_2Cl_3	HEMIN (50μM)	HEMATIN (50μM)	OVINE HAEMOGLOBIN (0.125μM)	BOVINE HAEMOGLOBIN (0.125μM)	PORCINE HAEMOGLOBIN (0.125μM)
A1	NG	+++	+++	+++	+++	+++	+++
A2	NG	+++	+++	+++	+++	+++	+++
T10	NG	+++	+++	+++	+++	+++	+++

+++ - Growth, NG - no growth.
Table 3.5. Growth of serotypes after 1 passage in 150μM EDDA then transfer into broths containing no iron source or selected sources of iron.

The concentrations of chelators employed in Table 3.3 were deemed unusually high when compared to chelator concentrations used for other organisms and the variability of different concentrations of chelator for each serotype was not practical when making batches of media for screening iron compounds.

There was a possibility that iron was stored internally which has been shown for *Yersinia pestis* (Perry *et al,* 1993) and that the chelators were having a toxic effect rather than restricting growth, with subsequent repletion being neutralisation of the chelator. Lower concentrations of the chelators were used and the serotypes passaged from culture to culture which has been used for the oral pathogen *Bacteroides gingivalis* (Bramanti & Holt, 1990) thus utilising any possible internal storage of iron. Table 3.4 shows that passaging into iron-restricted broths did not affect growth of the serotypes with αα dipyridyl at the stated concentrations. EDDA, however, in both broth and agar would not allow growth as early as passage 2 and so was successful at preventing growth at the same (low) concentration of chelator. The system was tested by adding iron compounds to see if growth could be initiated. Table 3.5 shows that under these iron-depleted conditions, all serotypes were able to utilise hemin, hematin and ovine, bovine and porcine haemoglobin. This defined that 200μM EDDA produced iron restriction in all serotypes and if subcultured into 150μM EDDA the serotypes were thereby depleted of iron and if no iron source was available growth did not occur. This worked well with broth culture but agar culture combining chelators was not reproducible and with differing chelator concentrations between serotypes, the screening of iron compounds could be deemed impractical.

3.4. SDS PAGE

Bacterial envelopes were prepared from overnight cultures of serotypes A1, A2 and T10 in various media and in-vivo fluids. Fig 3.2 shows the Coomassie blue stained protein profiles of A1. Lane 1 which contains envelope preparations from NCS culture shows two extra protein bands at approximately 53 and 17 kDa, similar proteins are apparent in LS (Lane 2) growth but are at a slightly less molecular weight at 50 and 16 kDa. There were only two differences in iron-restricted culture with the loss of a 90 kDa protein and the appearance of a 70 kDa protein. No differences were observed in the envelope profiles of PBS and otbw cultures. Serotype A2 bacterial envelopes (Fig 3.3) showed an increase in a protein band at approximately 50 kDa in LS and a similar protein in NCS culture at 53 kDa. Proteins in A2 at 40 and 29 kDa are apparent in all samples and appear to be major OMPs in this serotype. Iron-restricted culture produced a protein profile very similar to that observed in LS with the exception of the appearance of a unique 70 kDa protein. Envelope profiles from PBS and otbw culture were also similar and shared profiles observed for NCS. T10 envelope profiles also showed many differences. Iron-restricted culture and PBS culture produced proteins of 80 and 100 respectivley both of which are present in LS, and no change was apparent in otbw. Similar increases in the proteins from serum cultures at 50 and 30 kDa are apparent (Fig 3 .4). Table 3.6 summarises the main envelope protein differences of the three serotypes.

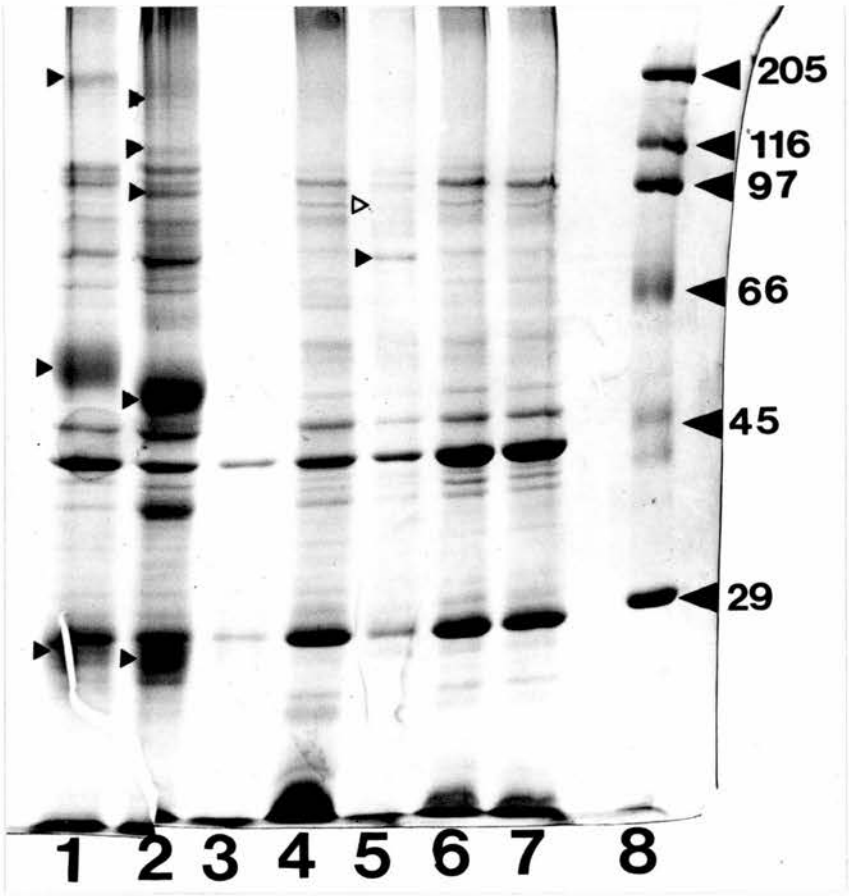


Fig 3.2. Coomassie blue stained SDS PAGE profiles of serotype A1 bacterial envelopes prepared from different culture fluids. Approximately 20μg loaded per well.

Growth in NCS (lane 1), LS (lane 2), otbw (lane 3), PBS stressed (Lane 4), TSB with 200μM EDDA (lane 5), TSB with 50μM iron (lane 6), TSB (lane 7) and molecular weight markers (lane 8). ▷ denotes missing band, ► denotes extra band when compared to TSB culture.

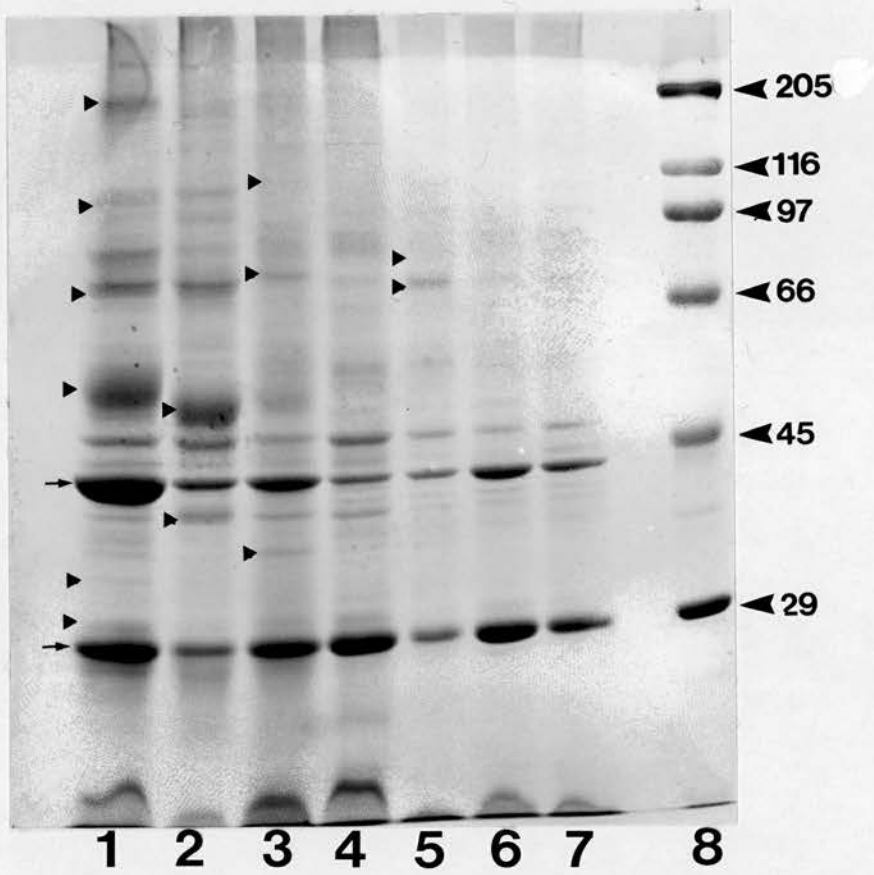


Fig 3.3. Coomassie blue stained SDS PAGE profiles of serotype A2 bacterial envelopes prepared from different culture fluids. Approximately 20μg loaded per well.

Growth from NCS (lane 1), LS (lane 2), otbw (lane 3), PBS stressed (Lane 4), TSB with 200μM EDDA (lane 5), TSB with 50μM iron (lane 6), TSB (lane 7) and molecular weight markers (lane 8). ▷ denotes missing band, ► denotes extra band when compared to TSB culture.

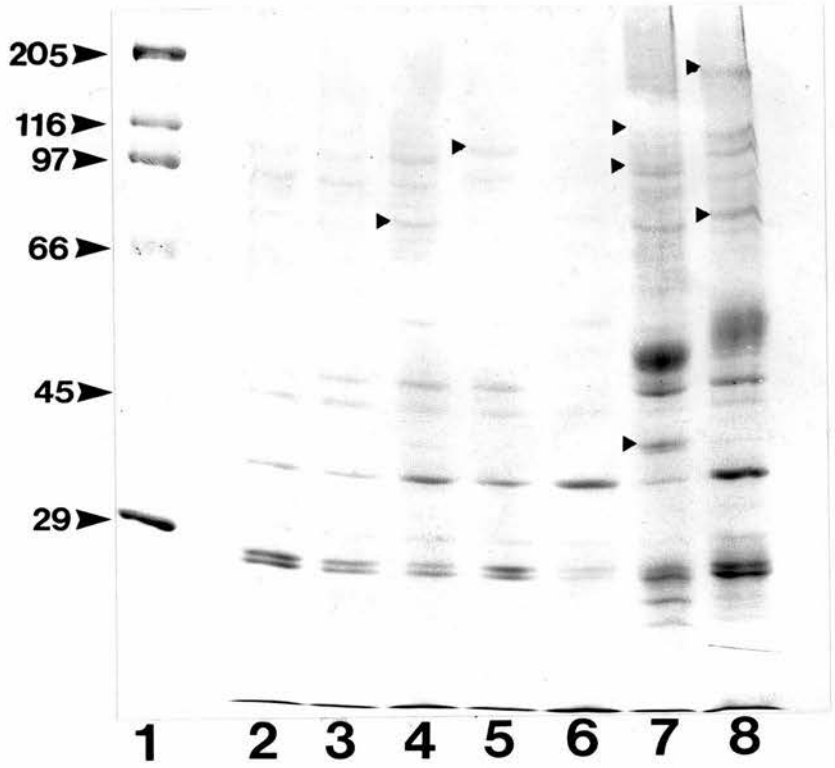


Fig 3.4. Coomassie blue stained SDS PAGE profiles of serotype T10 bacterial envelopes prepared from different culture fluids. Approximately 20μg loaded per well.

Molecular weight markers (lane 1), growth in TSB (lane 2), TSB with 50μM iron (lane 3), TSB with 200μM EDDA (lane 4), PBS stressed (lane 5), otbw (lane 6), LS (lane 7) and NCS (lane 8). ▷ denotes missing band, ► denotes extra band when compared to TSB culture.

Molecular weights (kDa) of missing and extra proteins when cultured in:

Serotype	TSB with EDDA	PBS	otbw	LS	NCS
A1	90 * 70			200 116 100	205 70
A2	100 77 70 65	116 77 30	116 30	100 77 65 32	205 200 116 100 77 65 30
T10	80	100		100 95 80 40	82

Table 3.6. Summary of missing (denoted by *) and extra proteins based on proteins present in TSB culture observed on Coomassie stained SDS PAGE of A1, A2 and T10 envelope preparations from different culture fluids.

Serotype A1, A2 and T10 cell contents (all other components left over after the envelope fraction has been removed) are shown in Fig 3.5, 3.6 and 3.7 respectively. Serotype A1 (Fig 3.5) showed a missing protein of 97 kDa in iron-restricted, otbw and LS cultures. Many extra proteins were observed from iron-restricted culture all in the 25-50 kDa range. All culture conditions showed an extra 30 kDa protein. A2 (Fig 3.6) shows proteins >205 kDa approximately and between 30 and 55 kDa in both serum cultures. Proteins of 90 and 55 kDa were missing from iron-restricted culture and no change in protein profiles were apparent in PBS and otbw cultures. T10 showed extra proteins from iron-restricted culture, PBS, LS and NCS at 40, 35 and 30 kDa.

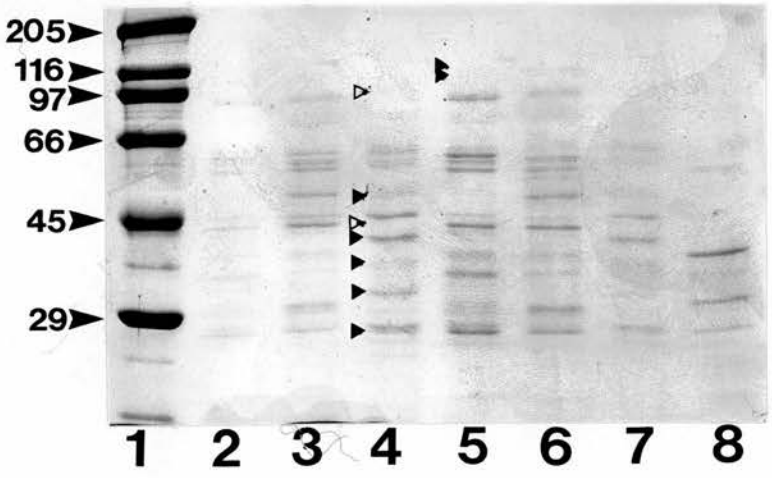


Fig 3.5. Coomassie blue stained SDS PAGE profiles of serotype A1 bacterial cell contents prepared from different culture fluids. Approximately 20μg loaded per well. Molecular weight markers (lane 1), TSB (lane 2), TSB with 50μM iron (lane 3), TSB with 200μM EDDA (lane 4), PBS stressed (lane 5), otbw (lane 6), LS (lane 7) and NCS (lane 8). ▷ denotes missing band, ► denotes extra band when compared to TSB culture

Fig 3.6. Coomassie blue stained SDS PAGE profiles of serotype A2 bacterial cell contents prepared from different culture fluids. Approximately 20μg loaded per well. Growth in NCS (lane 1), LS (lane 2), otbw (lane 3), PBS stressed (lane 4), TSB with 200μM EDDA (lane 5), TSB with 50μM iron (lane 6), TSB (lane 7) and molecular weight markers(lane 8). ▷denotes missing band, ► denotes extra band when compared to TSB culture.

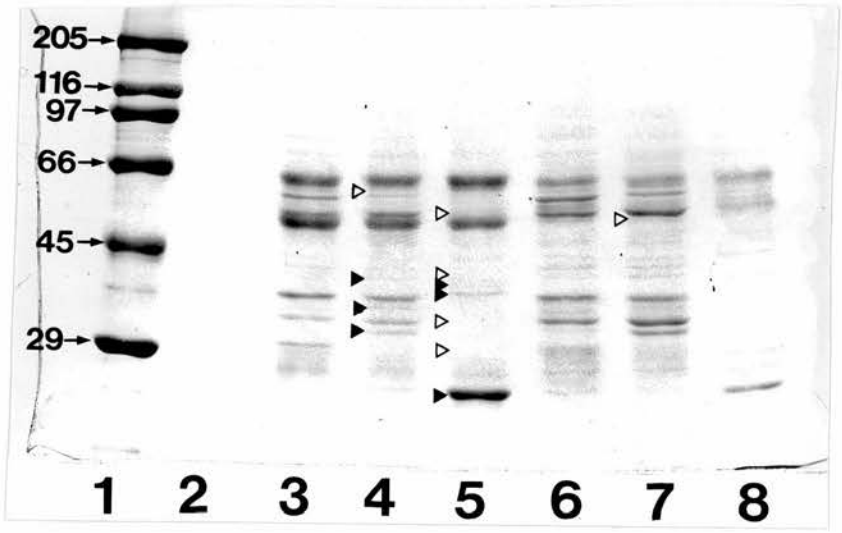


Fig 3.7. Coomassie blue stained SDS PAGE profiles of serotype T10 bacterial cell contents prepared from different culture fluids. Approximately 20μg loaded per well. Molecular weight markers (lane 1), TSB (lane 2), TSB with 50μM iron (lane 3), TSB with 200μM EDDA (lane 4), PBS stressed (lane 5), otbw (lane 6), LS (lane 7) and NCS (lane 8). ▷ denotes missing band, ► denotes extra band when compared to TSB culture.

Many proteins were missing, notably the 60 kDa from iron-restricted, PBS and LS culture. The protein profile of PBS and NCS cultured organisms was similar and no differences were observed for proteins from otbw cultures. Table 3.7 provides a summary of protein profiles observed of the three serotypes.

Molecular weights (kDa) of missing and extra proteins when cultured in:

Serotype	TSB/EDDA	PBS	otbw	LS	NCS
		116			
A1		100			
	97 *		97 *	97 *	
	50	50			
	40			40	
	35			35	
	30	30	30	30	30
	25				
				>205	>205
	90 *				
	55*			55	
A2				45	45
				35	35
				32	32
				30	30
	60 *	60 *		60 *	
		50 *			
				48 *	48 *
		42 *			42 *
	40	40			40
		39			39
T10		37			37
	35	35		35	35
		32 *			32 *
	30	30		30	30
		29 *			29 *
		17			17

Table 3.7. Summary of missing (denoted by *) and extra proteins (based on proteins present in TSB culture) observed on Coomassie stained SDS PAGE of A1, A2 and T10 cell contents from different culture fluids.

3.5. Western blotting

Ovine convalescent serum used as a probe on envelope fractions of the serotypes are seen in Figs 3.8, 3.9 and 3.10 shows A1, A2 and T10 respectively. Serotype A1 showed the lack of recognition of a 90 kDa band in PBS culture. A 55 kDa band was however recognised from all culture conditions. A2 showed 110 kDa band recognised in all ruminant fluids but not the envelopes of in-vivo isolated organisms (these organisms were from freeze dried stocks of pleural fluid collected cells, Donachie and Gilmour, 1988). The 77 kDa band was present in all culture conditions as was the 30 kDa band (except PBS). T10 showed no responses which were different from broth culture in iron-restricted and PBS. The serum cultures showed extra bands recognised at 200 and 100 kDa. A LMW (low molecular weight) protein was missing from recognition in otbw and a 70 kDa in LS. Table 3.8 summarises the recognition of antigens of the three serotypes by their respective homologous antiserum.

	Molecular weights (kDa) of missing and extra bands when cultured in:					
Serotype	TSB/EDDA	PBS	otbw	LS	NCS	In-vivo
A1		90 *				
	55	55	55	55	55	
A2			110	110	110	
	77	77	77	77	77	77
						60 *
				40		
	30		30	30	30	30
T10				200	200	
				100	100	
				70 *		
			LMW *			

Table 3.8. Summary of missing (denoted by *) and extra proteins (based on proteins present in TSB culture) observed on Western blots of A1, A2 and T10 envelope preparations from different culture fluids and probed with homologous convalescent lamb serum.

Figs 3.11-3.13 shows the recognition of cell content antigens by homologous antiserum. A1 showed an extra band recognised in cultures with extra iron at 140 kDa. With iron-restricted culture bands are not recognised at 85 and 60 kDa and extra bands are observed having the same molecular weights as NCS culture. There was no change in PBS or otbw culture. A2 showed an extra band recognised at 50, 40 and 70 kDa the latter was also present in both serum cultures. The 35 kDa band was missing from all cultures except PBS. T10 showed a 40 kDa extra band present in broth with extra iron culture and PBS. Whereas in iron-restricted and otbw culture an extra band was observed at 46 kDa. All ruminant fluids showed extra bands in the 10-20 kDa range and both serum cultures produced recognition of a 100 kDa band.

Fig 3.8. Western blot of serotype A1 envelopes prepared from different culture fluids and probed with homologous convalescent lamb serum (this chapter).

Molecular weight markers (lane 1), TSB (lane 2), TSB with 50μM iron (lane 3), TSB with 200μM EDDA (lane 4), PBS stressed (lane 5), otbw (lane 6), LS (lane 7) and NCS (lane 8). ▷ denotes missing band, ► denotes extra band when compared to TSB culture.

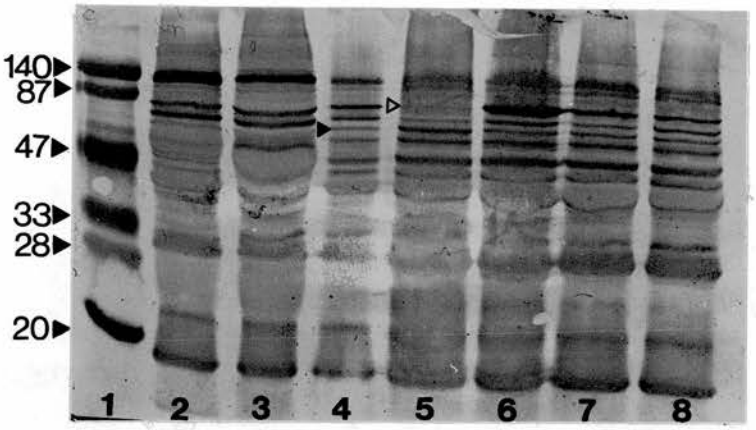
Fig 3.9. Western blot of serotype A2 envelopes prepared from different culture fluids and probed with homologous convalescent lamb serum (this chapter).

In-vivo isolated (lane 1), NCS (lane 2), LS (lane 3), otbw (lane 4), PBS stressed (lane 5), TSB with 200μM EDDA (lane 6), TSB with 50μM iron (lane 7), TSB (lane 8) and molecular weight markers (lane 9). ▷ denotes missing band, ► denotes extra band when compared to TSB culture.

Fig 3.10. Western blot of serotype T10 envelopes prepared from different culture fluids and probed with homologous convalescent lamb serum (this chapter).

NCS (lane 1), LS (lane 2), otbw (lane 3), PBS stressed (lane 4), TSB with 200μM EDDA (lane 5), TSB with 50μM iron (lane 6), TSB (lane 7) and molecular weight markers (lane 8).

▷ denotes missing band, ► denotes extra band when compared to TSB culture.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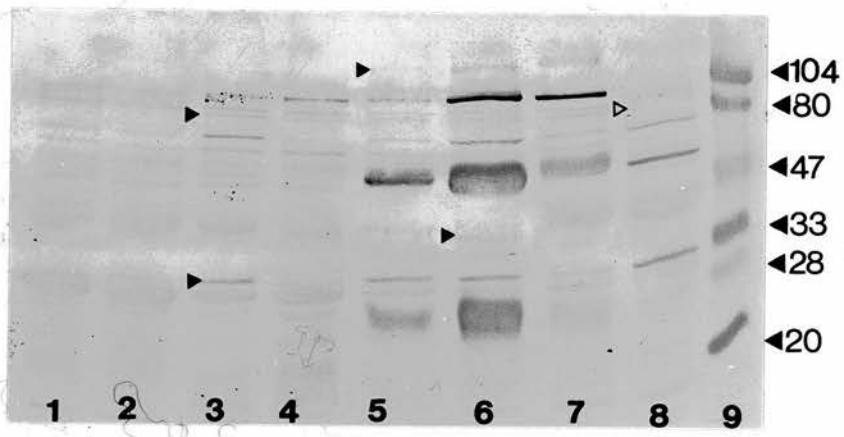
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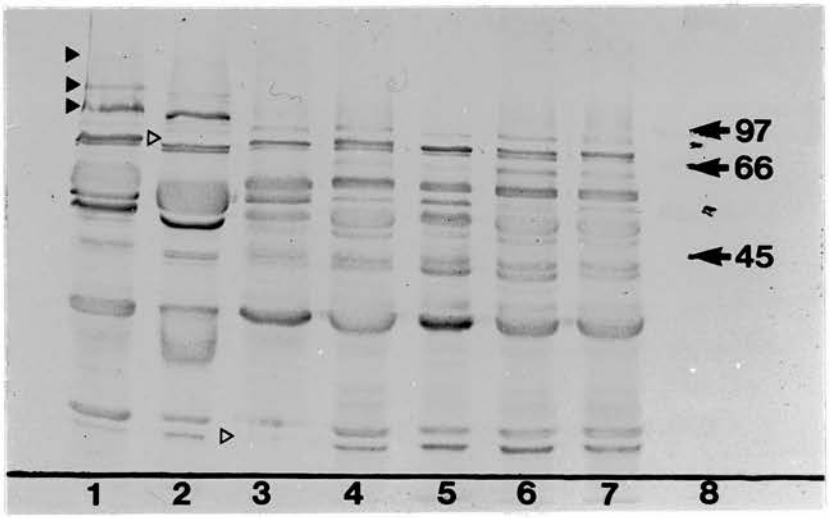
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Fig 3.11. Western blot of serotype A1 cell contents prepared from different culture fluids and probed with homologous convalescent lamb serum (this chapter).

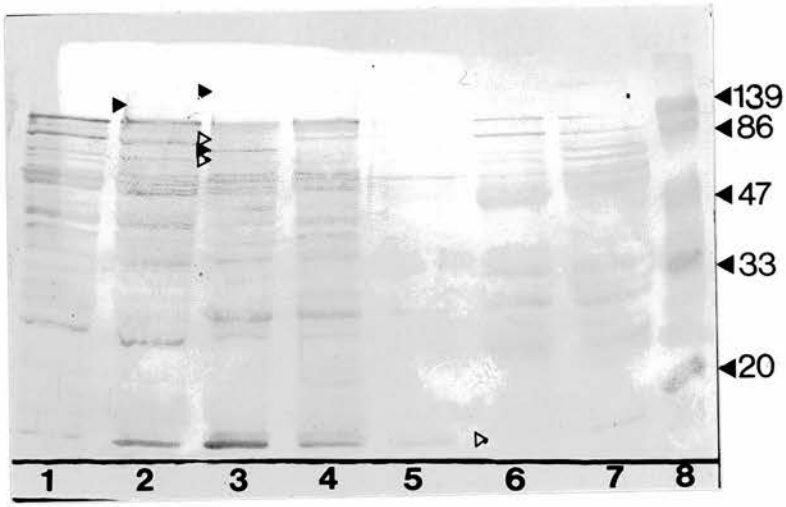
NCS (lane 1), LS (lane 2), otbw (lane 3), PBS stressed (lane 4), TSB with 200μM EDDA (lane 5), TSB with 50μM iron (lane 6), TSB (lane 7) and molecular weight markers (lane 8). ▷ denotes missing band, ► denotes extra band when compared to TSB culture.

Fig 3.12. Western blot of serotype A2 cell contents prepared from different culture fluids and probed with homologous convalescent lamb serum (this chapter).

In-vivo isolated (lane 1), NCS (lane 2), LS (lane 3), otbw (lane 4), PBS stressed (lane 5), TSB with 200μM EDDA (lane 6), TSB with 50μM iron (lane 7), TSB (lane 8) and molecular weight markers (lane 9). ▷ denotes missing band, ► denotes extra band when compared to TSB culture.

Fig 3.13. Western blot of serotype T10 cell contents prepared from different culture fluids and probed with homologous convalescent lamb serum (this chapter).

NCS (lane 1), LS (lane 2), otbw (lane 3), PBS stressed (lane 4), TSB with 200μM EDDA (lane 5), TSB with 50μM iron (lane 6), TSB (lane 7) and molecular weight markers (lane 8). ▷ denotes missing band, ► denotes extra band when compared to TSB culture.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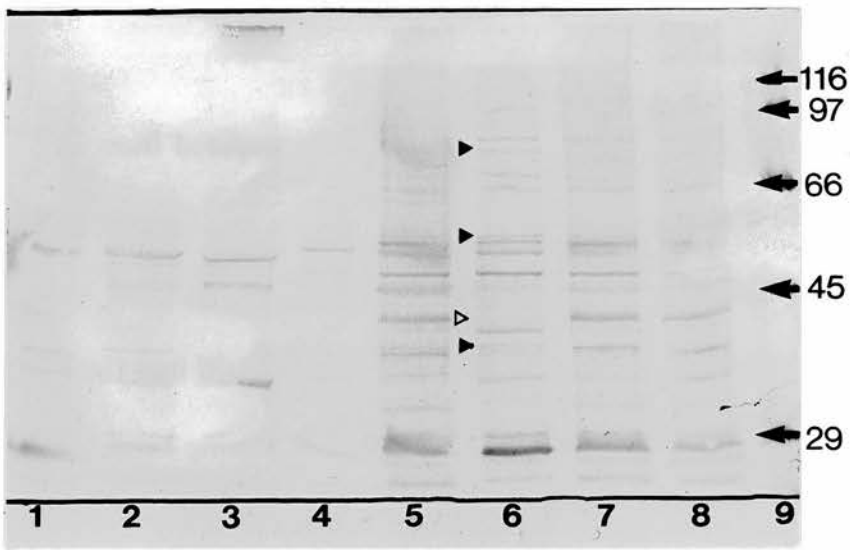
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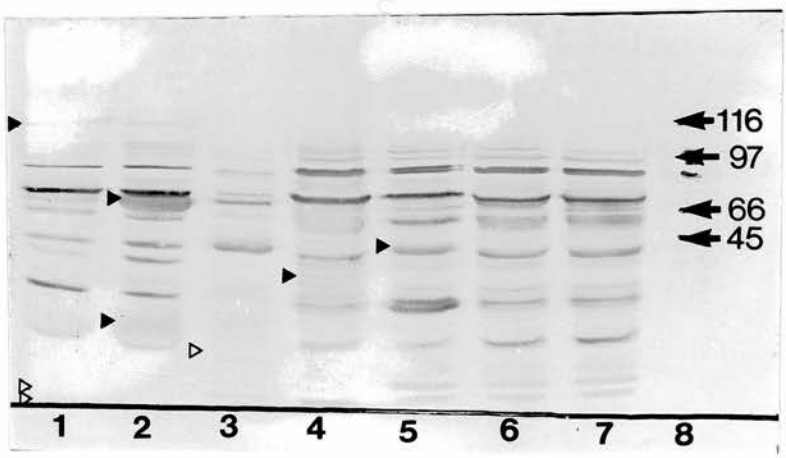
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The major differences of IgG recognition of each of the serotype grown in different culture conditions are summarised in Table 3.9.

Serotype	Molecular weights (kDa) of missing and extra bands when cultured in:						
	TSB with iron	TSB/ EDDA	PBS	otbw	LS	NCS	In-vivo
A1		HMW			HMW	HMW	
	140						
		85 *					
		70				70	
		60 *					
					LMW *		
A2		70			70	70	
		50					
		40					
		35 *		35 *	35 *	35 *	35 *
T10					100	100	
					70		
		46		46			
	40		40				
					35		
				30 *			
				10-20 *	10-20 *	10-20 *	

Table 3.9. Summary of missing (denoted by *) and extra proteins (based on proteins present in TSB culture) observed on Western blots of A1, A2 and T10 cell contents from different culture fluids and probed with homologous convalescent lamb serum.

Due to some of the culturing being carried out in "in-vivo" fluids, Western blots using anti-sheep conjugate as a probe to assess the presence of host proteins bound to

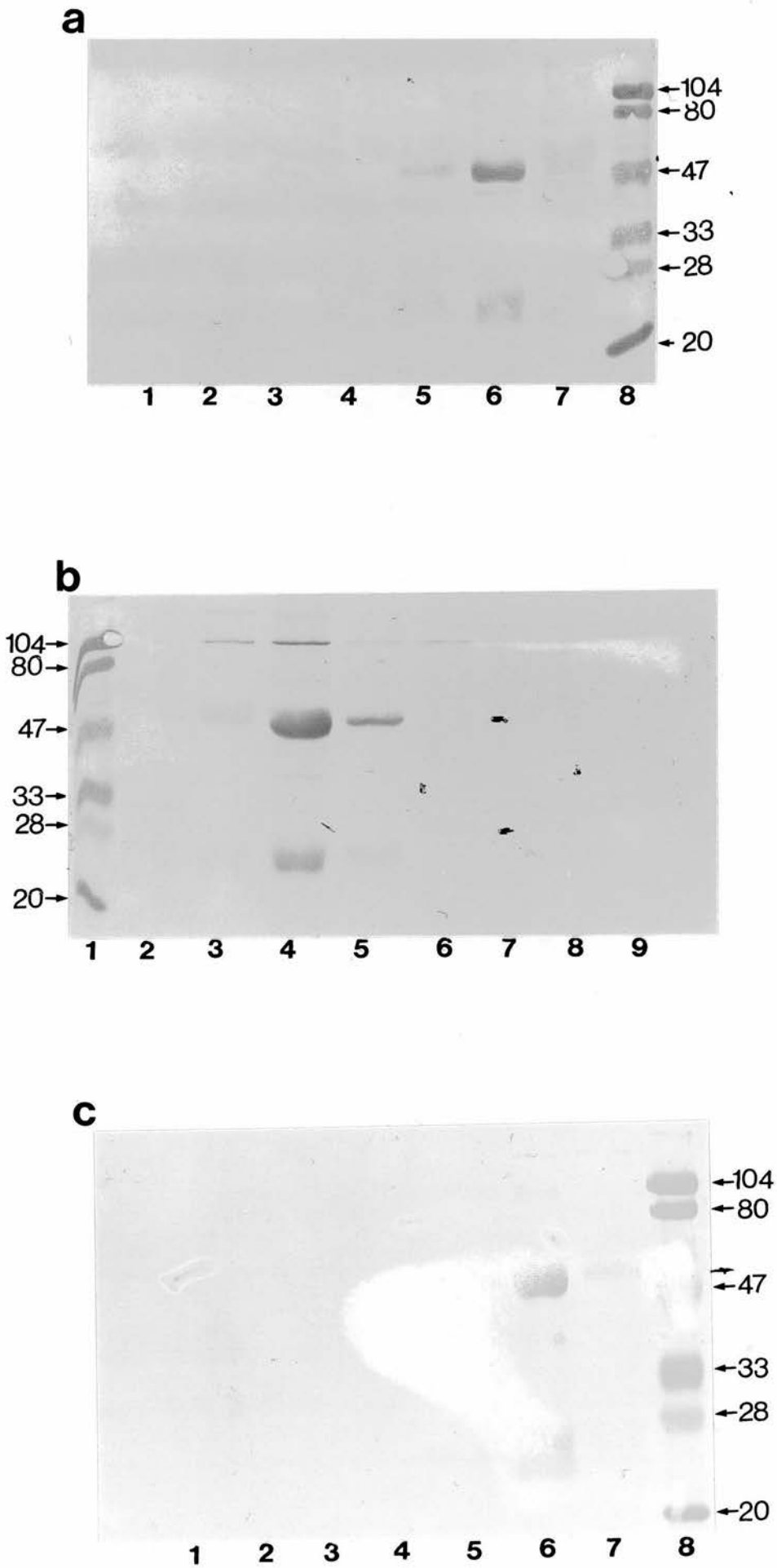
the bacterial proteins, were carried out. Fig 3.14 a-c shows the envelopes of the three serotypes. In A1 lanes containing samples from in-vivo fluid culture have recognised bands only at molecular weights of approximately 112, 50 52 and 29 kDa. In A2 envelopes bands at 53, 52 and 30 kDa are recognised. T10 also had bands of approximately the same molecular weights at 53, 52, 28 and 25 kDa. The cell contents of the three serotypes (Fig 3.15 a-c) show A1 had bands of 53, 52 and 28 kDa. A2 had approximate bands at 112, 52, 50 and 20 kDa whilst those of T10 were present also at 52, 50 and 28 kDa.

Fig 3.14. Western blots of envelopes of serotypes A1 (a), A2 (b) and T10 (c) grown in different culture fluids and probed with donkey anti-sheep conjugate to identify host bound protein.

(a) TSB (lane 1), TSB with 50μM iron (lane 2), TSB with 200μM EDDA (lane 3), PBS stressed (lane 4), otbw (lane 5), LS (lane 6), NCS (lane 7) and molecular weight markers (lane 8).

(b) Molecular weight markers (lane 1), In-vivo (lane 2), NCS (lane 3), LS (lane 4), otbw (lane 5), PBS stressed (lane 6), TSB with 200μM EDDA (lane 7), TSB with 50μM iron (lane 8) and TSB (lane 9).

(c) TSB (lane 1), TSB with 50μM iron (lane 2), TSB with 200μM EDDA (lane 3), PBS stressed (lane 4), otbw (lane 5), LS (lane 6), NCS (lane 7) and Molecular weight markers(lane 8).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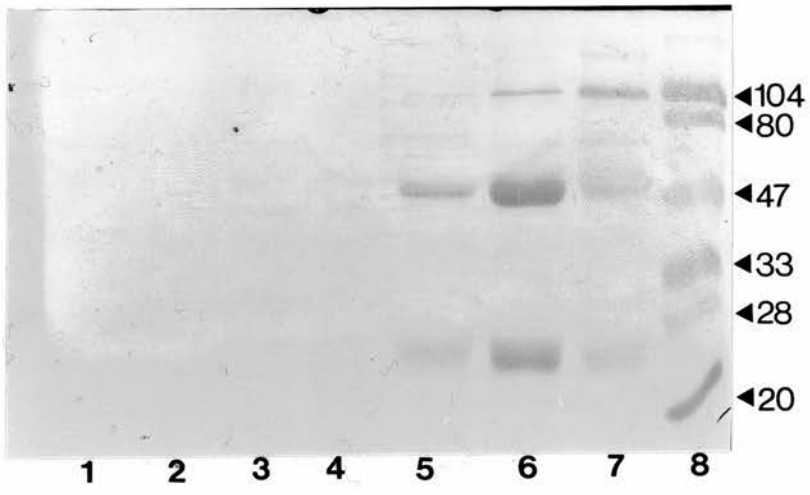
1 2 3 4 5 6 7 8

Fig 3.15. Western blots of cell contents of serotypes A1 (a), A2 (b) and T10 (c) grown in different culture fluids and probed with donkey anti-sheep conjugate to identify host bound protein.

(a) TSB (lane 1), TSB with 50 μM iron (lane 2), TSB with 200μM EDDA (lane 3), PBS stressed (lane 4), otbw (lane 5), LS (lane 6), NCS (lane 7) and molecular weight markers (lane 8).

(b) Molecular weight markers (lane 1), In-vivo (lane 2), NCS (lane 3), LS (lane 4), otbw (lane 5), PBS stressed (lane 6), TSB with 200μM EDDA (lane 7), TSB with 50μM iron (lane 8) and TSB (lane 9).

(c) Molecular weight markers (lane 1), NCS (lane 2), LS (lane 3), otbw (lane 4), PBS stressed (lane 5), TSB with 200μM EDDA (lane 6), TSB with 50μM iron (lane 7) and TSB (lane 8).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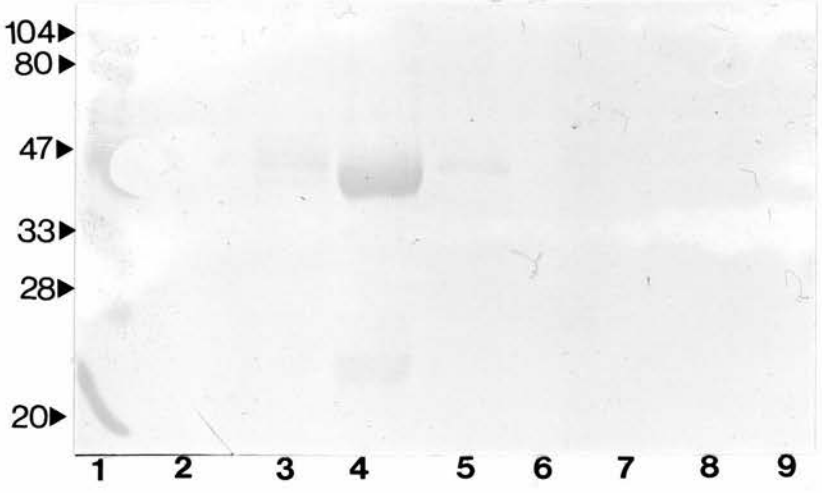
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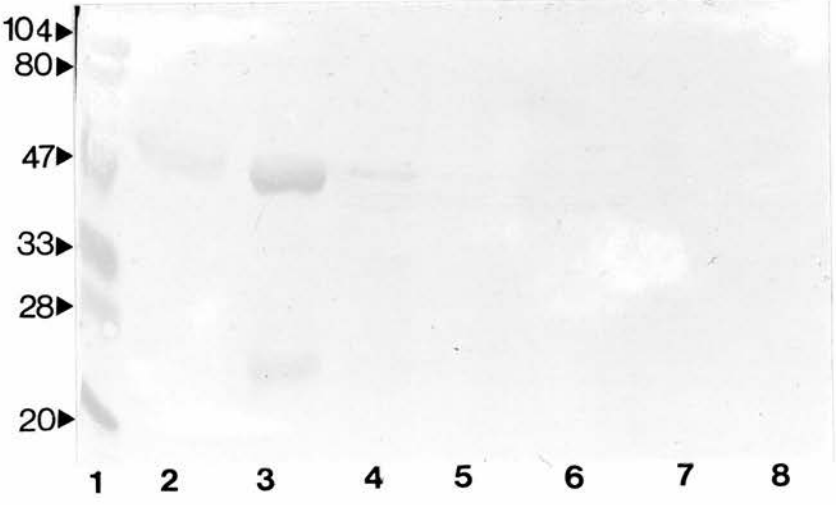
1 2 3 4 5 6 7 8 9


104
80
47
33
28
20
1 2 3 4 5 6 7 8

The cell contents were probed with a monoclonal antibody raised against an A2 serotype 35 kDa IRP (Lainson et al 1991). Fig 3.16 (a) shows that all growth conditions produced a protein in A2 with which the Mab reacted with the exception (Lane 8) of in-vivo isolated bacteria. All growth conditions with serotypes A1 and T10 were negative for recognition with the 35 kDa Mab. When the envelope fractions were probed (Fig 3.16 b) the results for A2 were the same as the cell contents where no recognition of the protein in in-vivo bacteria was observed. The envelopes of A1, however, showed recognition of a band at the correct molecular weight from TSB, TSB with iron and restricted, LS and NCS culture only (Fig 3.16c). T10 also possessed one band of the correct molecular weight in the sample from otbw culture (results not shown).

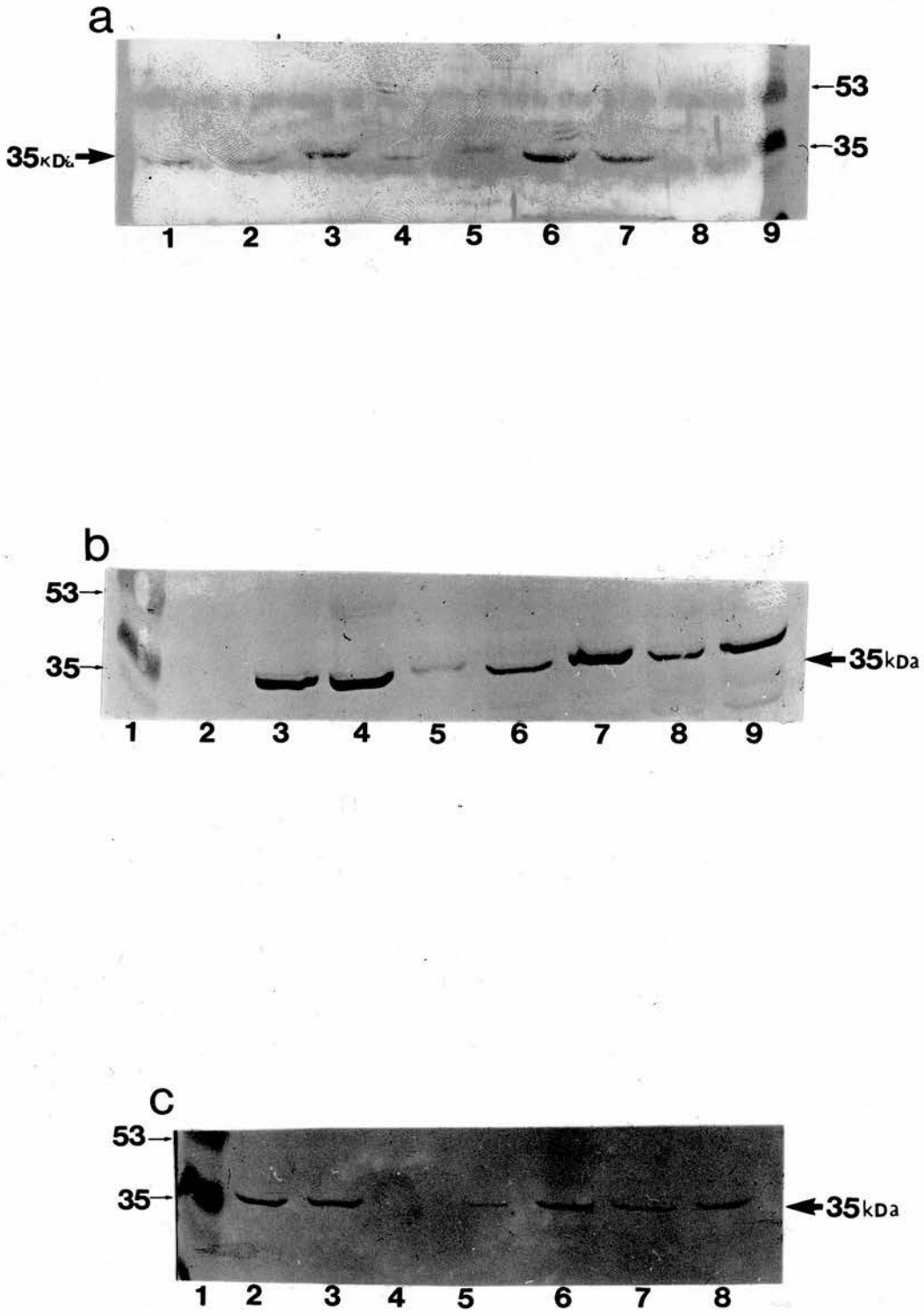
A rabbit polyclonal antiserum raised against a 100 kDa protein, proposed as a haemopexin receptor (Davies et al 1996), was used to probe A2 envelopes. A reaction (Fig 3.17) was observed in iron-restricted and LS growth conditions with a slight reaction in TSB and in-vivo growth conditions. No reaction was observed in A1 or T10 fractions.

Fig 3.16. Western blot of serotype A2 cell contents and A2 and A1 envelopes from different culture fluids and probed with a monoclonal antibody raised against an A2 35 kDa IRP (Lainson *et al,* 1991).

(a) A2 cell contents of: TSB (lane 1), TSB with 50μM iron (lane 2), TSB with 200μM EDDA (lane 3), PBS stressed (lane 4), otbw (lane 5), LS (lane 6), NCS (lane 7), in-vivo isolated (lane 8) and molecular weight markers (lane 9).

(b) A2 envelopes: molecular weight markers (lane 1), in-vivo isolated (lane 2), NCS (lane 3), LS (lane 4), otbw (lane 5), PBS stressed (lane 6), TSB with 200μM EDDA (lane 7), TSB with 50μM iron (lane 8), and TSB (lane 9).

(c) A1 envelopes: molecular weight markers (lane 1), NCS (lane 2), LS (lane 3), otbw (lane 4), PBS stressed (lane 5), TSB with 200μM EDDA (lane 6), TSB with 50μM iron (lane 7), and TSB (lane 8).


a
35kDa
53
35
1 2 3 4 5 6 7 8 9
b
53
35
35kDa
1 2 3 4 5 6 7 8 9
c
53
35
35kDa
1 2 3 4 5 6 7 8

Another polyclonal rabbit antiserum was raised against the two transferrin binding proteins at 100 and 70 kDa (tbp1 and tbp2 respectively). Fig 3.18 a-c shows in serotype A1 that the 100 kDa was recognised in all lanes except from growth in LS. The 70 kDa protein was also in all lanes except from otbw growth. The A2 serotype had a reaction with the 100 kDa protein in lanes when the bacteria were grown in TSB, LS, NCS and in-vivo isolation. The 70 kDa was present with all growth conditions except PBS-stressed bacteria. Although T10 has been shown to possess different IRPs (Murray et al 1992) the 70 kDa protein was shown to be present in the serum grown bacteria.

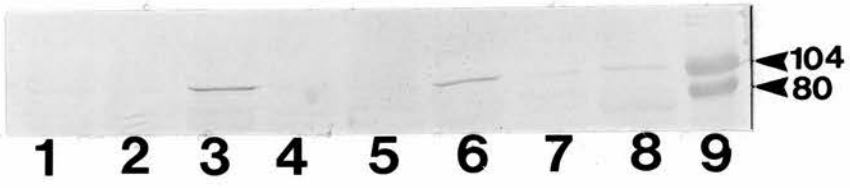


Fig 3.17. Western blot of serotype A2 envelope preparations from different culture fluids and probed with a rabbit antiserum raised against an A2 100 kDa haemopexin receptor (Davies *et al,* 1996).

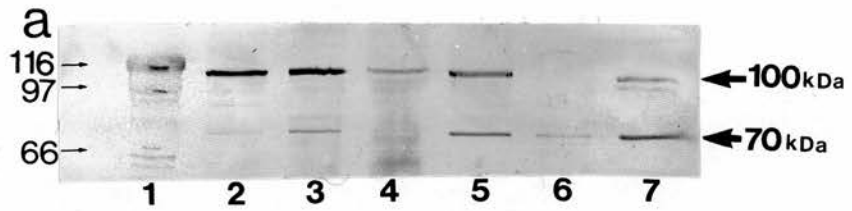
TSB (lane 1), TSB with iron (lane 2), TSB with EDDA (lane 3), PBS stressed (lane 4), otbw (lane 5), LS (lane 6), NCS (lane 7), in-vivo isolated (lane 8) and molecular weight markers (lane 9).

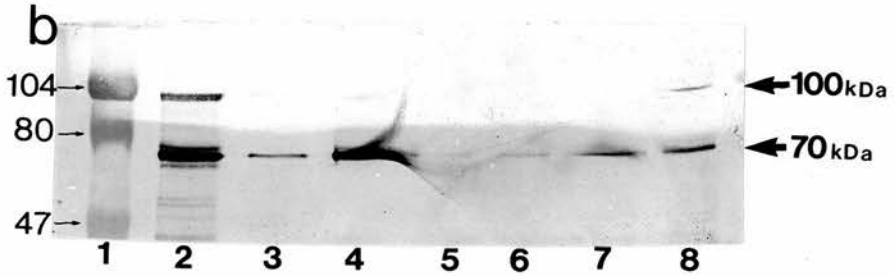
Fig 3.18. Western blot of serotype A1 (a), A2 (b) and T10 (c) envelope preparations from different culture fluids and probed with a rabbit antiserum raised against A1 100 kDa and 70 kDa transferrin binding proteins.

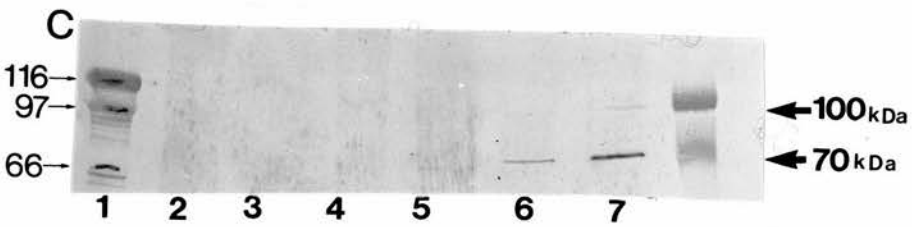
(a) Molecular weight markers (lane 1), TSB (lane 2), TSB with 200μM EDDA (lane 3), PBS stressed (lane 4), otbw (lane 5), LS (lane 6) and NCS (lane 7).

(b) Molecular weight markers (lane 1), TSB (lane 2), TSB with 200μM EDDA (lane 3), PBS stressed (lane 4), otbw (lane 5), LS (lane 6), NCS (lane 7) and in-vivo (lane 8).

(c) Molecular weight markers (lane 1), TSB (lane 2), TSB with 200μM EDDA (lane 3), PBS stressed (lane 4), otbw (lane 5), LS (lane 6) and NCS (lane 7).


a
116
97
66
100kDa
70kDa
1 2 3 4 5 6 7


b
104
80
47
100kDa
70kDa
1 2 3 4 5 6 7 8


c
116
97
66
100kDa
70kDa
1 2 3 4 5 6 7

3.6. Lipopolysaccharide analysis

Lipopolysaccharide (LPS) was extracted from overnight cultures of all three serotypes using a rapid hot-phenol method. Fig 3.19 shows the LPS from all serotypes grown under all the different culture conditions. All three serotypes show reduced presence of core polysaccharide when grown in otbw, whereas in btbw no LPS can be observed in any serotype using this extraction method even though growth was substantial. Serotypes A1 and A2 have similar LPS profiles with reduced core polysaccharide, from LS cultures. There is more core present in the NCS grown samples and TSB with or without iron restriction produced the largest amount of observable LPS. T10 LPS was similar in serum cultured samples and TSB cultures with core and O-antigen visible.

The same samples were Western blotted with antiserum raised against A1 (Fig 3.20) and recognition of A1 LPS from NCS, TSB and iron-restricted culture were recognised. These samples also recognised some O-antigen which was not previously visible on the stained gel and indicates that under certain growth conditions this A1 strain can possess O-antigen. The A1 antiserum cross-reacted with A2 core LPS from the same growth conditions as the A1 serotype and there was no recognition of T10 LPS.

A2 antiserum (Fig 3.21) recognised all the core LPS of A2 samples except the btbw grown samples. The antiserum cross-reacted with A1 grown in NCS, TSB and iron-restricted culture but recognised only core LPS. Again, no cross-reaction was observed with T10.

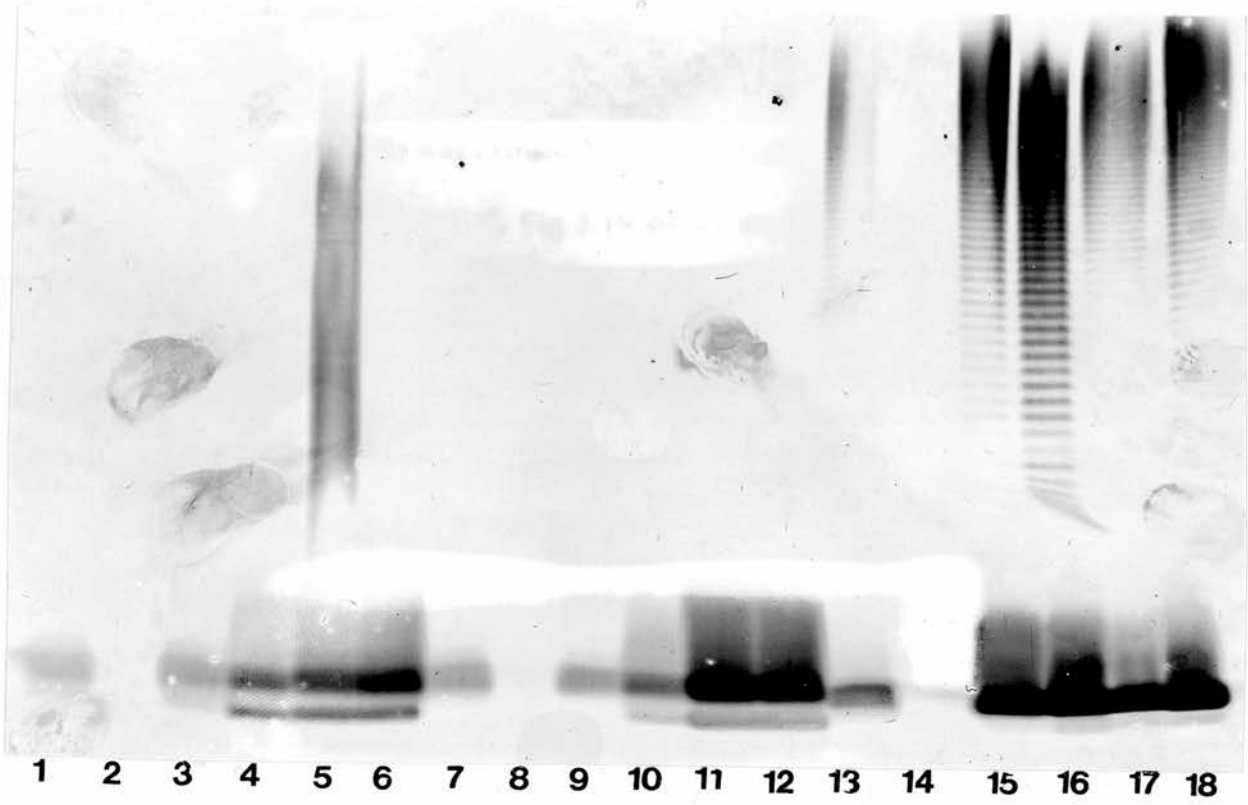


Fig 3.19. Silver stained PAGE gel of LPS samples of A1 (lanes 1-6), A2 (lanes 7-12) and T10 (lanes 13-18) from different culture fluids.

Various amounts were loaded onto the gel based on previous gels.

A1 from otbw (lane 1), btbw (lane 2), LS (lane 3), NCS (lane 4), TSB with 50μM iron (lane 5), TSB with 200μM EDDA (lane 6), A2 from otbw (lane 7), btbw (lane 8), LS (lane 9), NCS (lane 10), TSB with 50μM iron (lane 11), TSB with 200μM EDDA (lane 12), T10 from otbw (lane 13), btbw (lane 14), LS (lane 15), NCS (lane 16), TSB with 50μM iron (lane 17) and TSB with 200μM EDDA (lane 18).

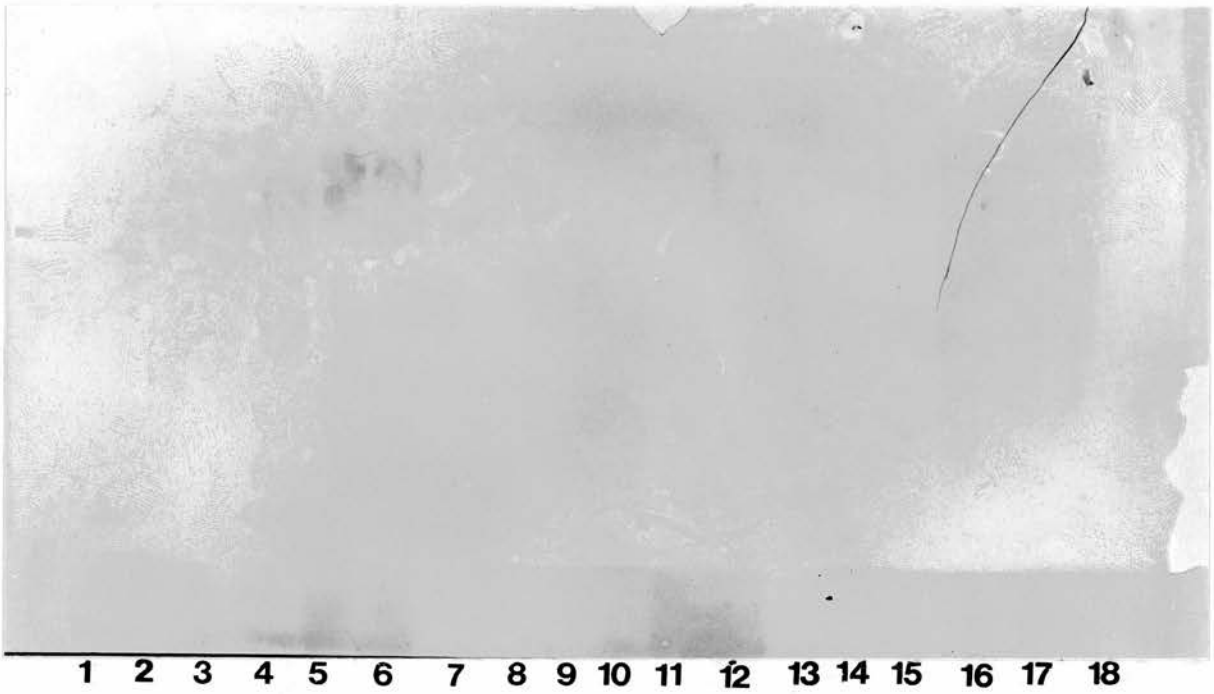


Fig 3.20. Western blot of LPS samples of A1 (lanes 1-6), A2 (lanes 7-12) and T10 (lanes 13-18) from different culture fluids and probed with convalescent lamb serum raised against A1 (this chapter).

A1 from otbw (lane 1), btbw (lane 2), LS (lane 3), NCS (lane 4), TSB with 50μM iron (lane 5), TSB with 200μM EDDA (lane 6), A2 from otbw (lane 7), btbw (lane 8), LS (lane 9), NCS (lane 10), TSB with 50μM iron (lane 11), TSB with 200μM EDDA (lane 12), T10 from otbw (lane 13), btbw (lane 14), LS (lane 15), NCS (lane 16), TSB with 50μM iron (lane 17) and TSB with 200μM EDDA (lane 18).

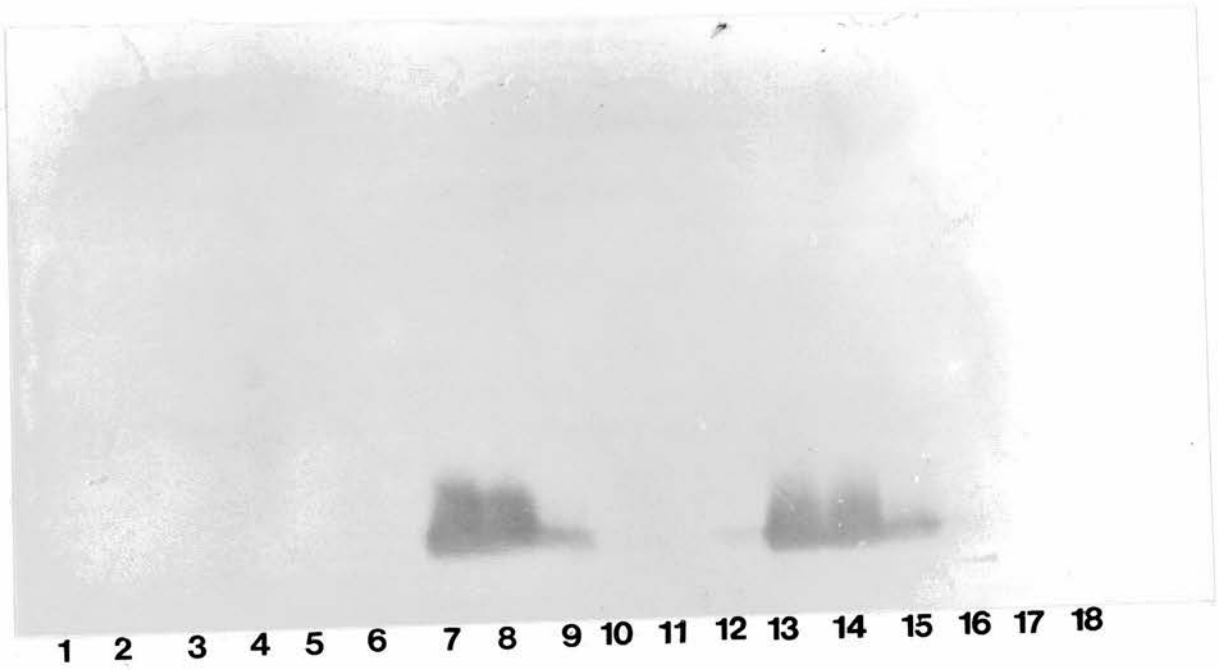


Fig 3.21. Western blot of LPS samples of A1 (lanes13-18), A2 (lanes 7-12) and T10 (lanes 1-6) from different culture fluids and probed with convalescent lamb serum raised against A2 (this chapter).

T10 from TSB with 200μM EDDA (lane 1), TSB with 50μM iron (lane 2), NCS (lane 3), LS (lane 4), btbw (lane 5), otbw (lane 6), A2 from TSB with 200μM EDDA (lane 7), TSB with 50μM iron (lane 8), NCS (lane 9), LS (lane 10), btbw (lane 11), otbw (lane 12), A1 from TSB with 200μM EDDA (lane 13), TSB with 50μM iron (lane 14), NCS (lane 15), LS (lane 16), btbw (lane 17) and otbw (lane 18).

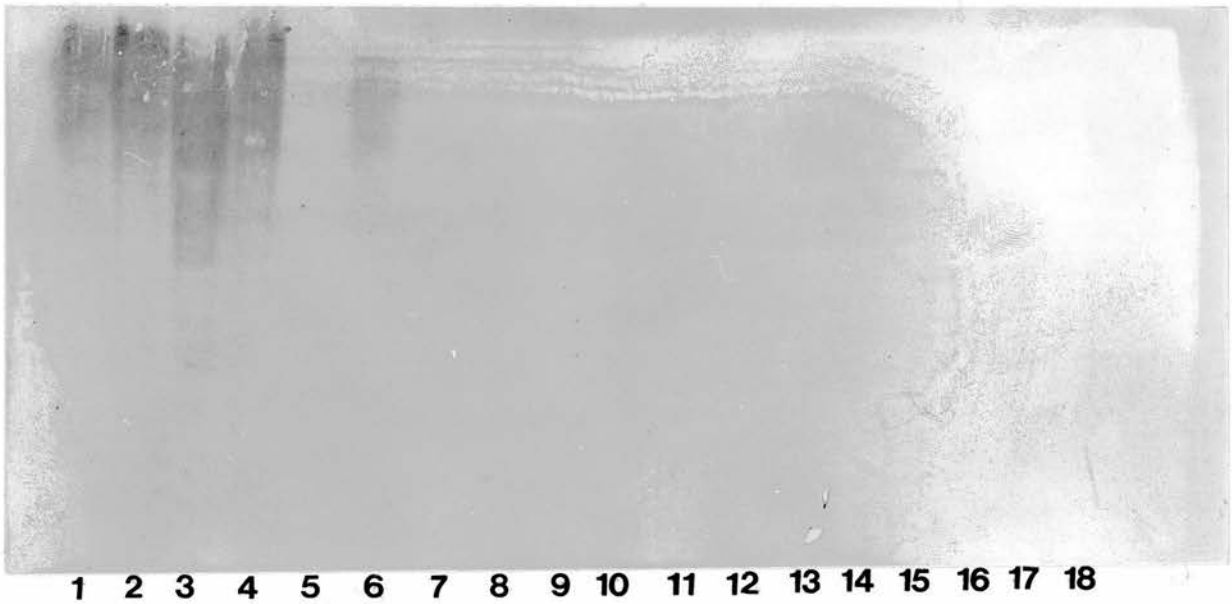


Fig 3.22. Western blot of LPS samples of A1 (lanes 13-18), A2 (lanes 7-12) and T10 (lanes1-6) from different culture fluids and probed with convalescent lamb serum raised against T10 (this chapter).

T10 from TSB with 200μM EDDA (lane 1), TSB with 50μM iron (lane 2), NCS (lane 3), LS (lane 4), btbw (lane 5), otbw (lane 6), A2 from TSB with 200μM EDDA (lane 7), TSB with 50μM iron (lane 8), NCS (lane 9), LS (lane 10), btbw (lane 11), otbw (lane 12), A1 from TSB with 200μM EDDA (lane 13), TSB with 50μM iron (lane 14), NCS (lane 15), LS (lane 16), btbw (lane 17) and otbw (lane 18).

Using T10 antiserum (Fig 3.22) there was recognition of LPS in all T10 samples except those from btbw culture. Only the O- antigen was recognised on the blots and no cross-reaction with A1 and A2 was observed.

Table 3.10 shows the endotoxin unit measurements of the extracted LPS. This is essentially a measurement of the toxicity of the Lipid A portion of LPS.

SEROTYPE	SAMPLE	DILUTION	ENDOTOXIN μg/ml
A1	OTBW	1/ 1000000	15
	BTBW	1/100	0.01
	LS	1/1000000	23
	NCS	1/1000000	90
	TSB + Fe	1/1000000	230
	EDDA	1/1000000	259
A2	OTBW	1/1000000	10
	BTBW	1/100	0.01
	LS	1/1000	1.8
	NCS	1/1000000	31
	TSB + Fe	1/1000000	67
	EDDA	1/1000000	44
T10	OTBW	1/1000000	51
	BTBW	1/1000	0.064
	LS	1/1000000	204
	NCS	1/1000000	576
	TSB + Fe	1/1000000	436
	EDDA	1/1000000	467

Table 3.10. The amounts of endotoxin present in serotypes grown in different media.

The endotoxin measurements correspond to the relative amounts of LPS observed on the stained gel. This indicates that the culture conditions can affect the amounts and therefore the subsequent toxicity of the bacterial LPS molecule.

3.7. Capsule polysaccharide analysis

Capsular polysaccharide was observed using the Maneval stain. Table 3.11 shows the variation seen in capsule size. Fig 3.23 a-e shows the observed capsules in relation to the tabulated grouping.

CULTURE CONDITIONS	SEROTYPE	CAPSULE SIZE
TSB/YE	A1	+
	A2	+/-
	T10	+
TSB/YE / EDDA	A1	+
	A2	+
	T10	++
TSB/YE / + Fe_2Cl_3	A1	+
	A2	+/-
	T10	+
OTBW	A1*	++
	A2	++
	T10	+/-
BTBW	A1	++
	A2	+
	T10	+/-
LS	A1	+/-
	A2	++
	T10	+/-
NCS	A1	+/-
	A2	+
	T10	++

+/- small, partial and no obvious capsule
+ medium capsule
++ large capsule
++ * largest capsule

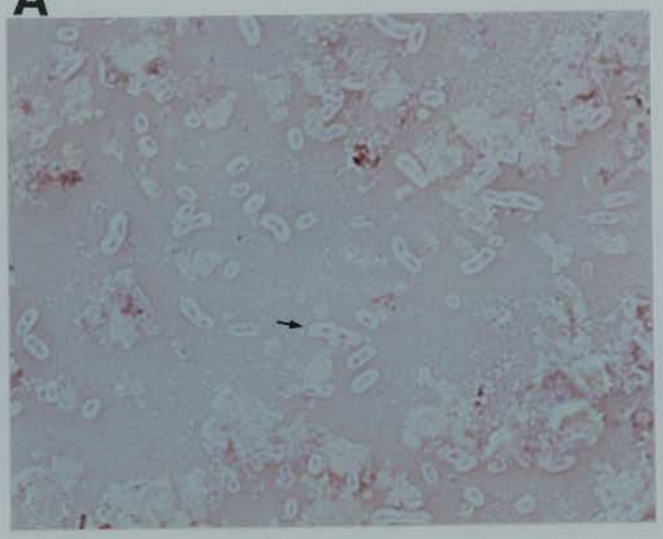
Table 3.11. Capsule presence in serotypes A1, A2 and T10 from different growth conditions.

The largest capsule was that belonging to serotype A1 grown in btbw. The other capsules were divided into three categories namely, large, medium and small, partial and no observable capsule.

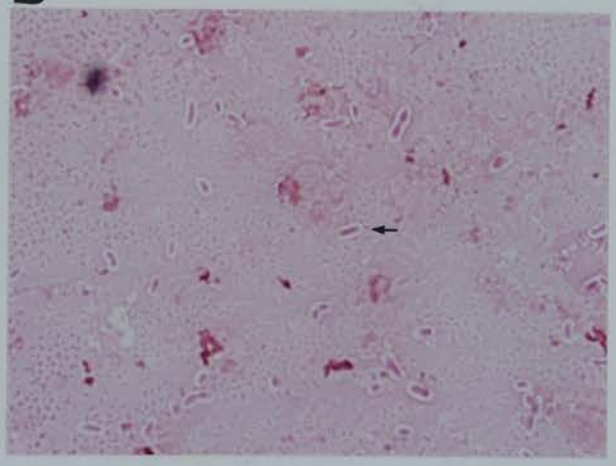
There was no pattern to the presence of capsular size and the culture medium. There was a trend towards less capsular material present on bacteria from in-vitro cultures. There was no relationship with a serotype and any particular capsule size.

Fig 3.23. Photographic examples of capsular polysaccharide sizes from different culture fluids and stained with Maneval's stain and observed at 100x magnification. (A) A1 from otbw the largest capsule observed, (B) A2 from btbw showing large capsule, (C) A2 from otbw showing medium capsule, (D) T10 from btbw showing small and partial capsule present and (E) T10 from LS shows what was regarded as areas with no obvious capsule present.

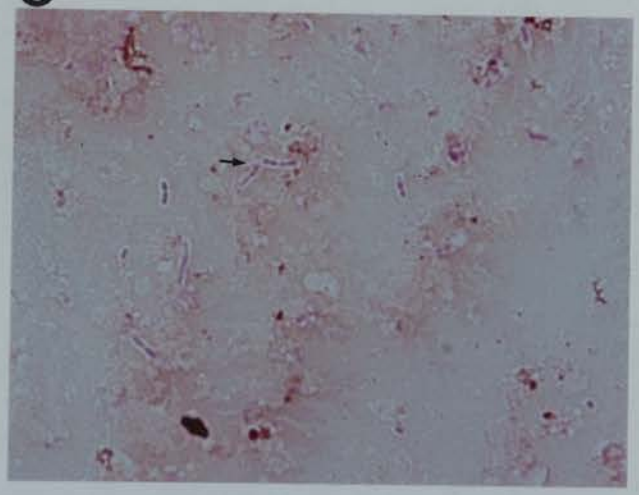
A



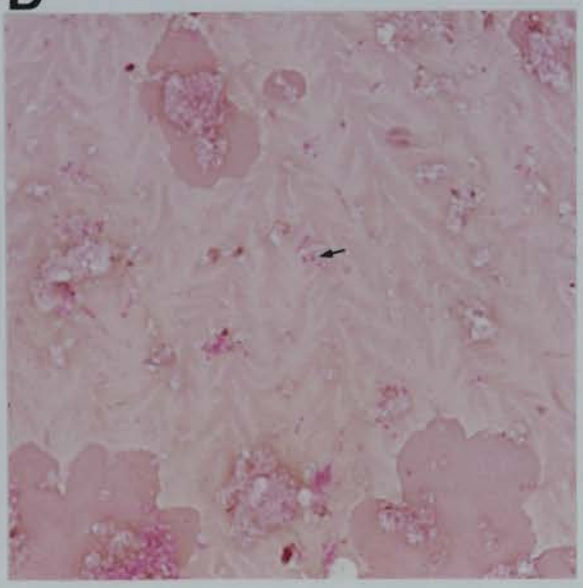
B



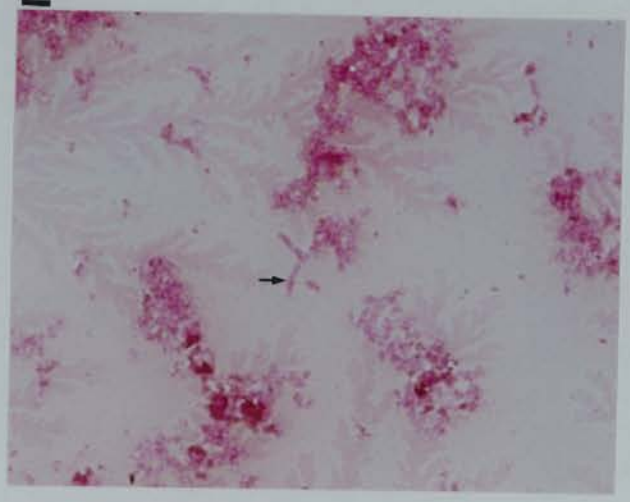
C



D



E



3.8. Leukotoxin analysis

Culture supernatants from four hour cultures (overnight for broth containing hemin) of the three serotypes grown in various fluids was analysed for the presence of leukotoxin using a cytotoxicity assay.

SEROTYPE	GROWTH MEDIUM	PERCENT VIABLE BL-3 CELLS
A1	TSB/YE	34
A2	TSB/YE	35
T10	TSB	55
A1	EDDA	19
A2	EDDA	19
T10	EDDA	20
A1	BTBW	100
A2	BTBW	100
T10	BTBW	100
A1	OTBW	98
A2	OTBW	94
T10	OTBW	100
A1	LS	100
A2	LS	100
T10	LS	100
A1	NCS	100
A2	NCS	98
T10	NCS	97
A1	HAEMIN	46
A2	HAEMIN	40
T10	HAEMIN	33

Table 3.12. The effects of culture supernatant on the viability of BL-3 cells. Percentage viability is based on cells with culture supernatant alone (100% viable) and Triton X100 killed cells (100% dead cells). EDDA at 200μM and Haemin at 50μM.

Table 3.12 shows that with all serotypes only culture supernatants from TSB, iron-restricted broth and broth containing hemin were toxic for BL-3 cells. This indicates that leukotoxin was actively toxic only in these cultures. The in-vivo fluid cultures showed no cytotoxicity for BL-3 cells although different growth rates may have meant that leukotoxin was produced at a different time other than that tested .

Leukotoxin was prepared from each of the three serotypes and compared. Leukotoxin preparations were run on SDS PAGE and Western blotted. A polyclonal rabbit antiserum raised against PAL2 leukotoxin (recombinant A1 leukotoxin) was used to probe the preparations as this is the only serum which recognised the leukotoxin protein from all three serotypes. Fig 3.24 shows the Western blot with recognition of all the serotypes' leukotoxin at relatively similar levels. The protein present in the preparations was 10.8 mg, 10.6 mg and 10.9 mg for A1, A2 and T10 respectively. This suggests that there was equal amounts of leukotoxin present in the preparations. Table 3.13 shows that the cytotoxicity for BL-3 cells of the leukotoxin preparations of A1 and A2 was similar with 93% and 88% killing respectively. T10 leukotoxin showed only 35% killing and so appeared significantly less toxic. This may indicate that the T10 leukotoxin is not as potent as the toxin from the other two serotypes. Using ovine convalescent serum, A1 leukotoxin was neutralised (74%) by homologous antiserum at 1/200 dilution. The ability to neutralise A1 leukotoxin with the heterologous antiserum of A2 and T10 was only 30% and 41% respectively when those antisera were at 1/50 dilution. The comparative neutralisation, at 1/200 dilution

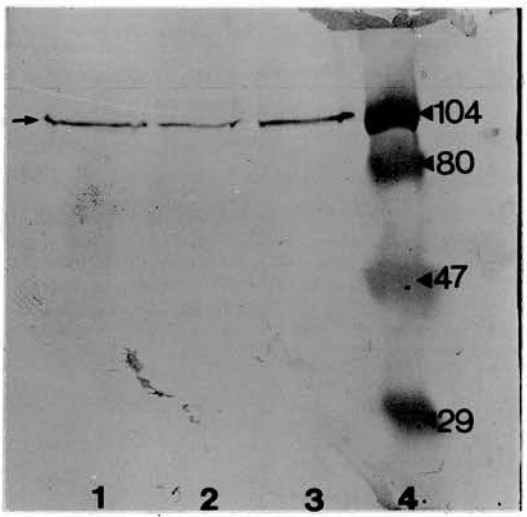


Fig 3.24. Western blot of serotypes A1, A2 and T10 leukotoxin preparations probed with rabbit antiserum raised against recombinant leukotoxin.

10µl were loaded into each well. T10 leukotoxin (lane 1), A2 leukotoxin (lane 2), A1 leukotoxin (lane 3) and molecular weight markers (lane 4).

of the antiserum, of A1 was 4% and 2% for A2 and T10 respectively. Serotype A2 leukotoxin was neutralised by homologous antiserum (1/200 dilution) at 55%, whereas the heterologous antiserum of A1 and T10 neutralised at 62% and 65% respectively, all at the same dilution.

SAMPLE	ANTISERUM (DILUTION)	% VIABLE CELLS	% NEUTRALISATION
A1	NA	7	NA
A1	A1 (1/50)	71	69
A1	A1 (1/100)	74	72
A1	A1 (1/200)	76	74
A1	A2 (1/50)	35	30
A1	A2 (1/100)	14	8
A1	A2 (1/200)	10	4
A1	T10 (1/50)	45	41
A1	T10 (1/100)	33	28
A1	T10 (1/200)	9	2
A2	NA	12	NA
A2	A1 (1/50)	71	67
A2	A1 (1/100)	41	34
A2	A1 (1/200)	66	62
A2	A2 (1/50)	77	74
A2	A2 (1/100)	74	71
A2	A2 (1/200)	61	55
A2	T10 (1/50)	67	63
A2	T10 (1/100)	69	64
A2	T10 (1/200)	73	65
T10	NA	65	NA
T10	A1 (1/50)	73	73
T10	A1 (1/100)	65	70
T10	A1 (1/200)	62	55
T10	A2 (1/50)	23	63
T10	A2 (1/100)	70	64
T10	A2 (1/200)	36	64
T10	T10 (1/50)	65	69
T10	T10 (1/100)	70	60
T10	T10 (1/200)	72	56

NA, not applicable

Table 3.13. Cytotoxicity of leukotoxin preparations from serotypes and the neutralising capacity of homologous and heterologous antiserum.

T10 leukotoxin was similar to A2 leukotoxin in that 1/200 dilution of the antiserum resulted in 56%, 55% and 64% neutralisation when A1, A2 and T10 antiserum were reacted against T10 leukotoxin.

Although cell lines (in this case BL-3 cells) are useful to compare effects of different treatments, they do not represent the in-vivo cells which are affected by interactions with Pasteurellae toxins or other cell products. Ovine and bovine macrophages were isolated from lungs to investigate in vitro the effect of leukotoxin on isolated in-vivo cells.

MACROPHAGE SOURCE	LEUKOTOXIN SAMPLE	ANTISERUM (AT 1/50)	NUMBER LIVE CELLS	NUMBER DEAD CELLS	% KILLING	% NEUTRALISATION
OVINE	A1	NA	37	63	55	NA
	A2	NA	34	66	46	NA
	T10	NA	26	74	68	NA
	A1	A1	73	27	NA	80
	A2	A2	80	20	NA	95
	T10	T10	64	36	NA	67
BOVINE	A1	NA	25	75	59	NA
	A2	NA	48	52	21	NA
	T10	NA	45	55	26	NA
	A1	A1	58	42	NA	91
	A2	A2	61	39	NA	100
	T10	T10	59	41	NA	87

NA - Not applicable.

Table 3.14. The effect of leukotoxin on ovine and bovine macrophages and the subsequent effects of homologous antiserum.

Table 3.14 shows that the percentage killing of ovine macrophages was similar at 55, 46 and 68% for A1, A2 and T10 respectively. Although the killing of ovine macrophages as compared to BL-3 cells was increased with T10 leukotoxin, killing by A1 and A2 leukotoxin was lower. Neutralisation was much higher than with BL-3 cells as: A1 gave 80%; A2 gave 95% and T10 gave 67% neutralisation with homologous antiserum.

The bovine macrophages showed a slight increase in killing by A1 leukotoxin when compared to ovine macrophages but the effects of A2 and T10 leukotoxin are greatly reduced at 21% and 26% respectively. Again, the neutralising capacity of homologous antiserum was higher than that for BL-3 cells and ovine macrophages with 91, 100 and 87% neutralisation for A1, A2 and T10 respectively.

3.9. Discussion

The production of antiserum against the three serotypes was successful. IgG responses increased over time and a number of antigens were recognised. The ovine antiserum also possessed leukotoxin neutralising antibody and bactericidal activity (latter work not in this thesis, M.Maley, pers comm). When the serum was tested in the IHA (a measure of anti-capsule antibodies) against the standard antiserum used for typing Pasteurellae serotypes differences were observed. The rabbit antisera was similar to the standard antisera except for the presence of a higher titre in the case of anti-A1 serum. Antibodies against the A2 serotype in all the sera tested were poor and in the standard antiserum even though when previously tested the titres were much higher. There is a possibility that prior to necropsy the high titre present was due to the presence of IgM antibodies which were susequently lost, with the result that the serum taken at post mortem does not possess a high titre. A2 has been shown to possess a capsule which does not induce a good immune response. However, antibodies against A2 capsular antigen correlate with protection against pasteurellosis (Adlam *et al.*, 1987; Mustafa, 1995). Recovery from, or resistance to, pasteurellosis challenge during the production of antibodies in this study, appears not to correlate with antibodies to the capsule antigen. This may be the reason why the ovine anti-capsular antibodies are low in comparison to the rabbit antibody titre as pasteurellosis is not a disease of the latter although the rabbits are usually hyperimmunised.

Although the serotypes were successfully grown in iron-restricted conditions the use of chemically defined medium was not successful due possibly to trace amounts of divalent cations present in some of the other constituents of the medium. These could

possibly have been removed by passing the medium down a Chelex 100 column (which removes iron) or even by passage of the strains in successful iron-restricted media, treatment that was eventually adopted for depletion of intracellular iron and which appears to have stimulated iron regulated protein expression.

For this study chemical chelators αα dipyridyl and EDDA were used. There was a general trend towards growth of the serotypes in broth at higher concentrations of αα dipyridyl than EDDA and the αα dipyridyl allowed sequential passage up to seven times. It appears from this study that there may be internal storage mechanisms available for iron in Pasteurellae as evidenced by the ability to deplete completely on passage of the cultures through successive chelated broths. The differences between the mechanisms of the chelators, αα dipyridyl chelates internally and EDDA chelates externally may explain some of the results. As αα dipyridyl is internalised in the bacteria, some of the earlier concentrations could have been toxic or may not be as efficient in chelating from an intracellular position. Conversely EDDA has the iron bound before it has a chance to be delivered to the bacteria and is a better chelator for this type of study as shown in the results. The passage experiment allowed a level of iron restriction to be achieved to the point where the bacteria could no longer live without an additional iron source present. This allowed the screening of some iron compounds not previously shown to be able to replete the growth of Pasteurellae under restriction. Although haemoglobin and haem containing compounds have been shown to enhance infection and toxicity with Pasteurellae (Chengappa *et a.,* 1983; Gentry *et al.,* 1986) they have not been tested in vitro as the sole sources of available iron. It is interesting that unlike the situation with the acquisition of iron from transferrin, which is host specific, it appears that all serotypes can utilise any species'

haemoglobin. The haem molecule alone which in-vivo probably comes from haemoglobin can also support the growth of all three serotypes. Leukotoxin which shows a broader target cell specificity than previously thought has been implicated in the haemolysing effect on red blood cells (Murphy *et al.,* 1995). However, leukotoxin activity was low, and haemolysis of red blood cells was observed in the uninoculated media controls. The results implicate leukotoxin involvement of the lysis of red blood cells was based upon examination of β-haemolysis. This link with haemolysis, however, needs more attention as other authors (Chidambaram *et al.,* 1995) have shown haemolytic activity by leukotoxin-negative mutants to be similar to the wild type strain. The acquisition of iron by utilising haem compounds needs more attention as different mechanisms from those previously described for transferrins are being employed.

The SDS PAGE protein profiles of all the serotypes differed depending on the fluid which sustained growth. Iron-restricted cells from the serotypes showed the presence of IRPs at the correct molecular weight described previously (Donachie & Gilmour, 1988; Deneer & Potter, 1989; Davies *et al.,* 1992; 1994a), T10 possesses an IRP at approximately 80kDa which could be the 78 IRP described by Murray *et al.* (1992). A1 possessed proteins of approximately 70, 50, 40, 35, 30 and 25 kDa while with A2 proteins 77, 70 and 65 kDa were identified T10 proteins of approximately 80, 40, 35 and 30 kDa were identified. Most of the IRP proteins were present in the serum cultures especially those grown in NCS. The similarity of protein profiles from NCS-grown bacteria and in-vivo isolated bacteria has been described by Davies *et al.* (1994a) with growth conditions in both cases also stimulating previously described IRPs. He also found the the expression of IRPs from in-vivo cells was not as strong as those from iron-restricted culture which may explain why most differences are

observed in the iron-restricted cultures. It may be the case that, due to the iron acquisition mechanisms which are present in Pasteurellae serotypes, in-vivo fluids or natural in-vivo growth does not constitute a growth restriction and the expression of IRPs is therefore lower. Another possibility is that the in-vivo environment is extremley complex and iron restriction is just one variable which initiates the expression of new proteins. Both A1 and A2 had obvious proteins missing from iron-restricted culture in comparison to iron replete media (TSB) at approximately 97 and 45 kDa in A1 and 90 and 55 kDa in A2. The precise nature of these proteins is unknown but they may not be required during the process of scavenging for iron but are expressed again once the cells are iron sufficient.

Most of the information on IRPs present came from Western blots using convalescent sera. Only the 70 kDa protein was recognised in serotype A1 whereas in A2 proteins at 77 kDa which were also identified in in-vivo isolated cells were apparent and those 70, 50 and 40 kDa proteins were also recognised. T10 possessed IRP bands which were recognised at 100, 70, 35 and 46 kDa some of which were present in serum and tbw cultured samples. Monoclonal antibodies and specific antiserum helped to determine which proteins are expressed in certain fluids. The 35 kDa IRP was present in all A2 samples in envelopes and cell contents except in in-vivo isolated cells. This suggests that the 35 kDa protein is not necessarily iron restricted (in contrsat to Lainson *et al,* 1991) but may well be an iron regugulated protein. The same result was also observed in Western blots with convalescent serum failing to recognise a protein of approximately 35kDa in iron-restricted, serum cultures and in-vivo isolated cells. It may be that in-vivo conditions modify slightly the protein so that it becomes unrecognisable to antibodies. It may also be that the protein is not expressed in vivo and that previous recognition by convalescent serum (Lainson *et al.,* 1991) may be

due to other 35 kDa proteins present at the same molecular weight. The protein was also observed in A1 envelopes samples and in T10 grown in otbw. The presence of the protein has not previously been shown in other serotypes. This may be due to the culture conditions and hence variation in the epitopes present on the proteins resulting in recognition by antibodies. The position as the protein is present in the envelopes, is also different to the proposed position by Lainson *et al.* (1991) for A2, so that the mechanisms of iron acquisition may differ slightly between serotypes.

The difference in iron acquisition mechanisms is also observed with the presence of the haemopexin receptor which has been shown only in ovine strains and so is not present in the A1 serotype as this is a bovine isolate. T10 expresses different IRPs and does not appear to possess this protein. The receptor was present in iron-restricted, LS and in-vivo A2 cells, highlighting the similarity of iron-restricted and serum cultures with in-vivo cells (Davies *et al.,* 1992). The reason why the protein is not expressed in NCS may be due to the species specificity reported for the protein. The receptors are found in strains isolated from ovine cases only, so it appears there is no requirement for a receptor of that kind for strains growing in a bovine environment and is therefore not expressed in bovine serum-grown cells. The transferrin binding proteins were also present in differing combinations in all serotypes. As the antibodies were raised against the A2 proteins slight serotype differences in epitopes may indicate that the protein is not present when it actually is.

All serotypes possessed high molecular weight proteins, especially in bacteria from serum cultures. Proteins at this molecular weight have been described in serotype A1 from in-vivo grown cells (Confer *et al.,* 1992). A2 envelope proteins of 40 and 29 kDa could be similar to the 41 and 29.5 kDa major OMPs described by Morton *et al.,* 1996. Another two A2 proteins at 100 and 65 kDa were described also by Sutherland

et al., (1990) in in-vivo cells. A 116 kDa protein present in preparations of A1 bacteria could be the A1 specific protein described previously (Gonzales-Rayos *et al.,* 1986).

The main proteins expressed during growth of all serotypes in serum appeared to differ by approximately 2kDa. This indicates that the environment of growth may change the proteins slightly possibly by glycosylation, which then affects the molecular weight of the protein which can then be identified wrongly as a new protein. Another problem of protein identification and comparisons with other studies is those proteins which reside at the same molecular weight. In A2 there are three proteins present at 70 kDa and two at 100 kDa (Donachie, 1994; Davies *et al.,* 1996). This may interfere with antibody recognition and shows the need to separate these proteins and produce specific antibodies to use as tools before screening for their presence in in-vivo cells.

This study is in agreement with Sutherland *et al.* (1990) and Davies *et al.* (1992) in the identification of host proteins bound to the surface of the bacteria. The bands between 50-52 and 20-28 kDa present in all serotypes are probably the IgG light and heavy chains. Other host proteins were also bound. This observation may be important as the host proteins may be bound to important antigens. In contrast, the bacterial proteins may not be important antigenically and divert antibody responses away from the impotant antigens. Only with direct comparison with in-vivo grown cells can an indication of an in-vivo fluid representing bacteria in-vivo be made. Serum-grown bacteria possessed proteins which appeared to resemble those proteins which have been described previously as belonging to in-vivo bacteria. Growth of serotypes in otbw did not produce unique protein profiles. PBS-stressed cells appeared to produce less of most proteins but there appeared no induction of stress proteins, although

further separation was not carried out beyond identifying envelope and cell contents. A2 bacteria from isolation in vivo also had little reaction with antibody on Western blots, which may indicate changes in vivo which helps to evade immune recognition. Morton *et al.* (1996) indicated that the variations in strains, extraction procedures, gel densities and molecular weight standards can make large differences in comparisons with previously described proteins.

Growth in btbw was shown to disrupt production of LPS dramatically in all serotypes. This may be due to the presence of antibodies interfering with the development of the LPS or to a nutritionally deficient medium, which would prevent synthesis of the molecule. A1 LPS has been identified in-vivo in the bovine respiratory environment (Whiteley *et al.*, 1990) so the absence of LPS could be a unique feature of growth in this particular tbw. It was thought that the LPS could not be observed on silver stained gels due to changes on the molecule to adapt to a different growth environment. The endotoxin amounts show that endotoxicity was present, although low, and so this was probably not the case. It is more likely there was an overall reduction in total LPS molecules on the bacteria and that the method employed may have added to the lack of observation. Western blotting showed previously unseen O-antigen on the A1 LPS molecule. In certain media A1 will produce an O- antigen side chain. O-antigen was also observed in the T10 samples which has been shown to be of smooth type LPS. O-antigens are important as they have been shown to reduce the accessibility of antibodies to the core region (where the endotoxic Lipid A is present) (Nelson *et al.*, 1991). This may account for the differences observed in reported bactericidal activities of antibodies. LPS of *P. haemolytica* has been shown to be the binding site for bactericidal antibodies (Sutherland *et al.*, 1988; Brogden *et al.*, 1992). The antibodies work probably by destabilising the LPS. This effect as described by

Sutherland *et al.* (1989) does not happen in *P. trehalosi* due probably to the diversion of antibodies away from the core towards the O- antigen. The lack of recognition by homologous and heterologous antiserum of the core of T10 LPS may give some indication of the differences between disease syndromes, although O-antigen specificity is well documented (Rietschel *et al.,* 1990). Systemic T disease has been shown to be a classic endotoxaemia (Hodgson *et al.*, 1993), therefore the raising of antibodies to the core may be important in protection. LPS has been shown to bind host proteins (Morrison, 1990) and although this was not apparent from the gel profiles it may explain the O-antigen presence only on the A1 serotype in certain growth fluids. Confirmation of the presence of different serum constituents attached to the molecule would need chemical analysis of the structures especially of isolated in-vivo bacteria.

Confer *et al.* (1992) and Sutherland *et al.* (1990) both reported no differences between in-vitro and in-vivo LPS. The two authors reported also that there was no difference between capsule sizes. Gatewood *et al.* (1994) found that capsule size differed depending on the growth conditions with A1 and suggested the capsule was changing in response to growth conditions. It is possible that large capsules observed in this study were due to the presence of antibodies which may have initiated capsular swelling. Gilmour *et al.* (1985) reported that A1 could possess an irregular capsule. This study reported that there appeared no degradation of capsule in 18 hour cultures as reported by Corstvet *et al.* (1982). Gilmour *et al.* (1985) also reported that *P. haemolytica* capsules may appear in different forms, either as aggregations or as a discrete continuous envelope and this may reflect the differences observed here in different media. *P. trehalosi* were reported to possess thinner capsules which may be

why capsules in this study were relatively poor in most media in comparison to A1 and A2.

The relevance of these different size capsules would have to be evaluated in experiments where capsules are thought to be important such as phagocytosis experiments. Although capsules can enable bacteria to resist phagocytosis the process may be enhanced by deposition of C3b complement component. Some capsules such as meningococcal group b, with which A2 shares a capsule structure, fail to activate complement (Adlam *et al.,* 1987; Moxon & Kroll, 1990). In vivo, however, Sutherland *et al.* (1990) observed that A2 were aggregated together with glycocalyx type structures and these may have been important in resistance to phagocytosis. There is generally much more investigation required into the functions of bacterial capsules as the presence of capsules can be contradictory to generally held ideas of capsule function as discussed by Moxon & Kroll (1990). The evidence that capsules impair phagocytosis, interfere with complement and reduce the immunogenicity of an organism are known. However, the majority of infections which occur through contiguous spread of *Haemophilus influenzae* (otitis media, sinusitis and pneumonia) are caused by capsule deficient strains.

The identification of leukotoxin in the supernatants of different growth conditions was variable. Broth cultures at four hours incubation produced evident cytotoxicity for BL-3 cells, but cytotoxicity was also present in 18 hour cultures which contained hemin. Iron compounds have enhanced the cytotoxicity of *P. haemolytica* (Chengappa *et al.,* 1983; Al-sultan & Aitken, 1984; Gentry *et al.,* 1986). However, little data is available on the amounts of leukotoxin produced in iron-restricted cells. If leukotoxin is responsible for the haemolytic effect observed in Pasteurellae it would make sense that leukotoxin production is increased under iron-restriction in-vivo to

acquire an iron source in the form of haem from red blood cells. Gatewood *et al.* (1994) established that production of leukotoxin was relatively independent of growth conditions and more closely associated with growth rate. There was no cytotoxicity present in any of the in-vivo fluids in any of the serotypes. This may be due to the presence of neutralising antibodies although a neutralisation assay combining these fluids suggested that this may be true only for lamb serum. Other in-vivo components may bind and inactivate the leukotoxin or the conditions may not have stimulated leukotoxin production. Whiteley *et al.* (1990) showed the presence of leukotoxin in-vivo and studies at MRI have shown that leukotoxin is present and active in pleural fluid, so inactivation is the most probable explanation.

Protein content and Western blots indicated similar amounts of leukotoxin present in preparations from the three serotypes. Although A1 and A2 possessed similar cytotoxic activity against BL-3 cells the activity of T10 leukotoxin was comparatively low. Neutralisation of leukotoxin revealed that A1 possibly possesses a unique major neutralising epitope as evidenced by the lack of cross neutralisation by A2 and T10 antiserum. Heterologous neutralisation of A2 and T10 leukotoxin was similar to homologous neutralisation. This indicates that antibodies raised against A1 leukotoxin are probably important in infection as they will cross-react with other serotypes, but antibodies to other serotype leukotoxins will not protect against A1 leukotoxin. As A1 is the most prevalent serotype in bovine pneumonic pasteurellosis and as much of the pathology of the disease is due to leukotoxin and other excreted products it is clear why the vaccines which contain leukotoxin in a supernatant mixture appear to be so effective.

BL-3 cells are useful for toxicity assays but tell us little about the interactions of *P. haemolytica* and *P. trehalosi* with cells in-vivo. Macrophages of both ovine and

bovine origin were less affected by the leukotoxin of all three serotypes in comparison to BL-3 cells. Killing of ovine macrophages and neutralisation were similar in all three serotypes although heterologous antiserum neutralisation was not carried out. In bovine macrophages A1 leukotoxin killing was markedly higher than A2 and T10 which indicates some species specificity of serotype A1 for bovine macrophages. This may explain the high incidence of A1 in bovine cases of pasteurellosis. One of the important observations is that although the killing was lower in macrophages than BL-3 cells, the neutralising capacity of the antiserum was much higher, indicating a possible interaction between macrophage and antibody which enhances protection from the effects of leukotoxin.

The fluids used are, however, not applicable unless they are representative of the organism in-vivo. Many differences have been identified during the analysis of these serotypes grown in "in-vivo" fluids they do appear to resemble in vivo organisms especially when grown in serum. This would increase the knowledge of Pasteurellae and make analysis easier if the bacteria could be grown in in-vivo-like conditions in vitro.

CHAPTER 4

SURVIVAL OF *P. HAEMOLYTICA* AND *P. TREHALOSI* IN 'IN-VIVO' FLUIDS

4.1. Introduction

Iron-restricted proteins produced by bacteria in-vivo provide an understanding into the situation whereby nutrients are present but not readily utilisable. This may well be the normal situation for bacteria in-vivo with respect to their requirement for many types of nutrients. Bacteria which colonise tissue surfaces in a host will no doubt be under stress not only from defence mechanisms but also from low nutritient availiability. The nutrients may be restricted by host mechanisms or through competition from other bacterial species present. Pathogens such as *Pasteurellae* spp, which commonly colonise the tonsils and nasopharynx of ruminants, survive in this environment in low numbers. Bacteria are not always found and their appaerence during nasal swabbing fluctuates. Little is known about the bacteria in this state. Due to the differences observed in the protein profiles detailed in the last chapter, it may be that some proteins are important in survival in those particular fluids. This study will monitor whether the use of "in-vivo" fluids, which would naturally be present in vivo, would enable the serotypes to withstand nutritional stress and aid recovery on the addition of nutrients.

4.2. Growth and survival monitoring by viable counts

Serotypes A1, A2 and T10 were inoculated into various 'in-vivo' fluids, incubated at 37° C and sampled at selected time periods to determine viable counts on blood agar. The growth and survival graphs (Fig 4.1. a-d, for raw data see Appendix II) indicate the ability of the serotypes to grow in such fluids. In ovine tracheobronchial washings (otbw, Fig 4.1a) all serotypes showed initial growth equal to that observed in laboratory media.

Fig 4.1a-d. Graphs showing the growth and survival of serotypes A1, A2 and T10 in ruminant fluids at 37° C. Bacteria were inoculated into 5ml of the fluids and sampled at various times (from duplicate cultures), diluted and plated (in triplicate) onto 7% sheep blood agar for viable counts (counts are expressed as the mean). Fresh media was added on days 244 and 247 in otbw and days 153 and 154 in btbw.

Fig 4.1a GROWTH AND SURVIVAL OF SEROTYPES IN OVINE TBW

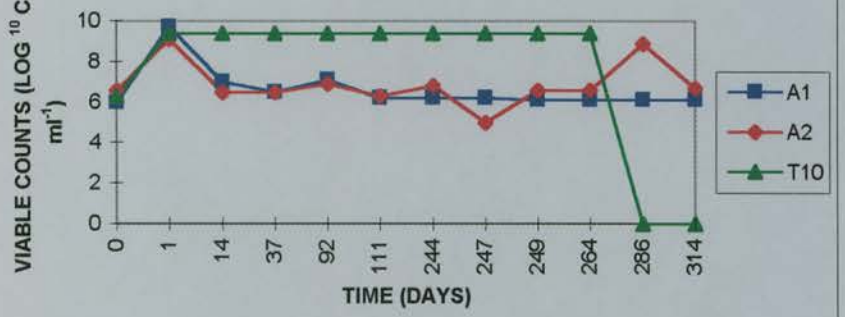


Fig 4.1b GROWTH AND SURVIVAL OF SEROTYPES IN BOVINE TBW

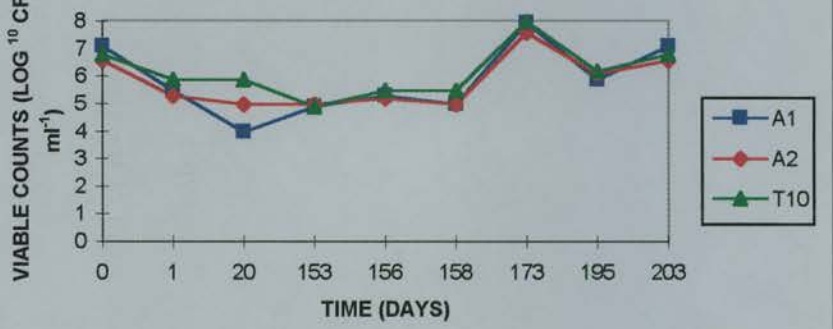


Fig 4.1c GROWTH AND SURVIVAL OF SEROTYPES IN LAMB SERUM

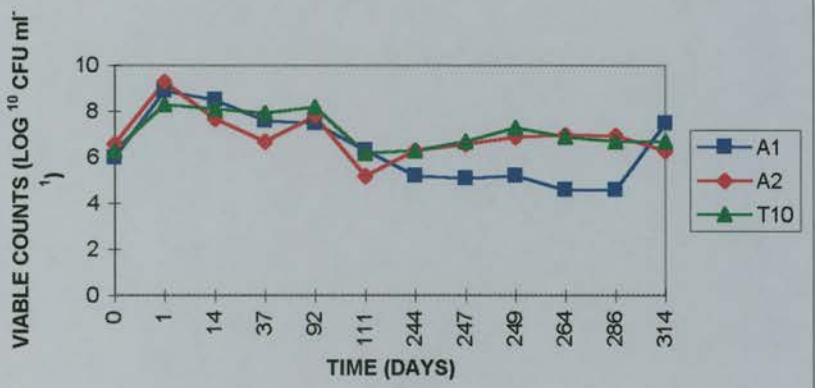
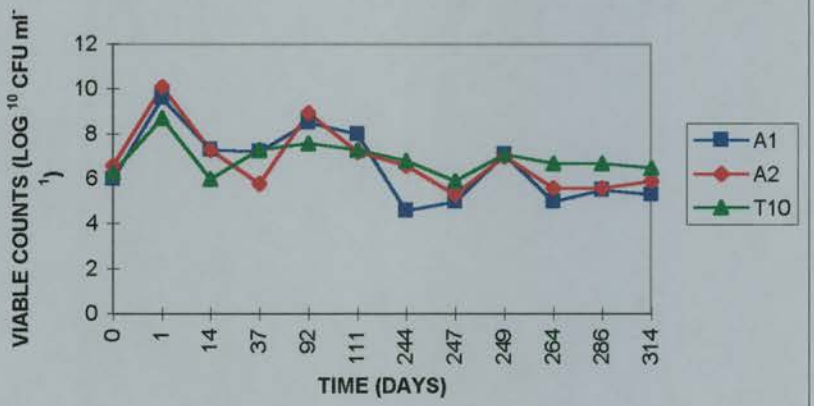


Fig 4.1d GROWTH AND SURVIVAL OF SEROTYPES IN NEWBORN CALF SERUM



The appearance of micro-colonies with serotype T10 was apparent at day 14 and lasted until day 264. As micro-colonies could not accurately be counted by eye, and there was no obvious loss of viability, the last viable count was deemed not to have altered. A1 and A2 showed a periodic rise and fall in viability (which is probably what T10 actually did) with A1 then showing extended micro-colony appearance after 244 days. There was a requirement to assess whether the addition of fresh fluids (the same fluid which was already present) would have any effect (increase viability, change in colony morphology and/or result in a decline in viability). The addition of fresh media (approximately 100µl/ ml of fluid present) on days 244 and 247 resulted in a lag phase before any reaction was observed. T10 declined rapidly and A1 did not change, apart from a brief return to 'normal' colony morphology. A2, however, increased in viability. The lack of viability of T10 is attributed to the loss of growth on sheep blood agar plates. It was still viable when inoculated into nutrient broth. In bovine tracheobronchial washings (btbw, Fig 4.1b) no initial growth was observed and all three serotypes had similar viability patterns with a rise and fall in viability between 10^6 and 10^4 cfu/ml. The addition of fresh serum on days 153 and 154 caused a rise in viable counts with all serotypes and this rise was higher than the original inoculum (which was not observed at 24 hours incubation). Growth in lamb serum (LS) or newborn calf serum (NCS) was relatively steady over 314 days with all serotypes. The lack of micro-colony formation and the consequent ease of counting colonies meant that fresh serum was not required to see if the bacteria were capable of resuscitation to normal colony morphology.

Fig 4.2a-d. Graphs showing the survival of serotypes A1, A2 and T10 in ruminant fluids at 37° C. Bacteria were inoculated into 5ml of the fluids at a higher inoculum than the growth and survival experiment and sampled at various times (from duplicate cultures), diluted and plated (in triplicate) onto 7% sheep blood agar for viable counts (counts are expressed as the mean). Fresh fluids were added on days 337 and 340 in otbw and on days 153 and 156 in btbw.

Fig 4.2a SURVIVAL OF SEROTYPES IN OVINE TBW

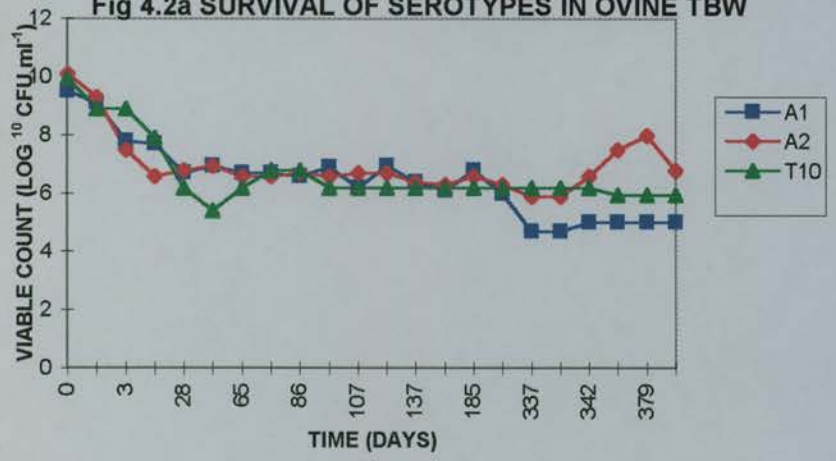


Fig 4.2b SURVIVAL OF SEROTYPES IN BOVINE TBW

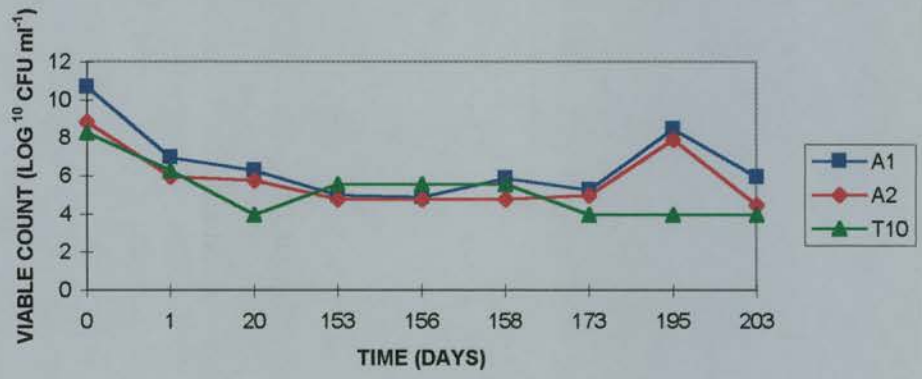


Fig 4.2c SURVIVAL OF SEROTYPES IN LAMB SERUM

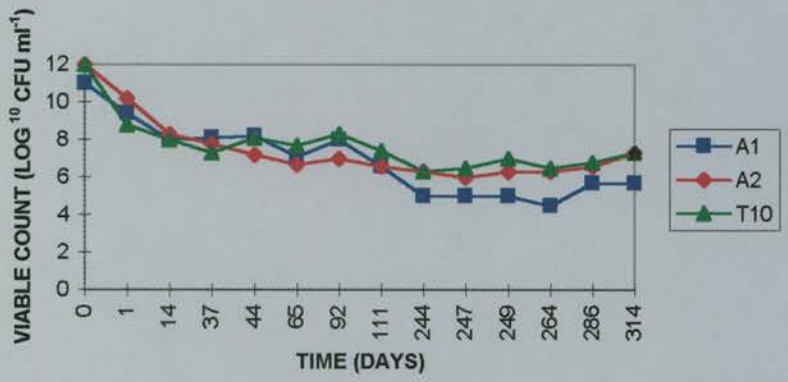
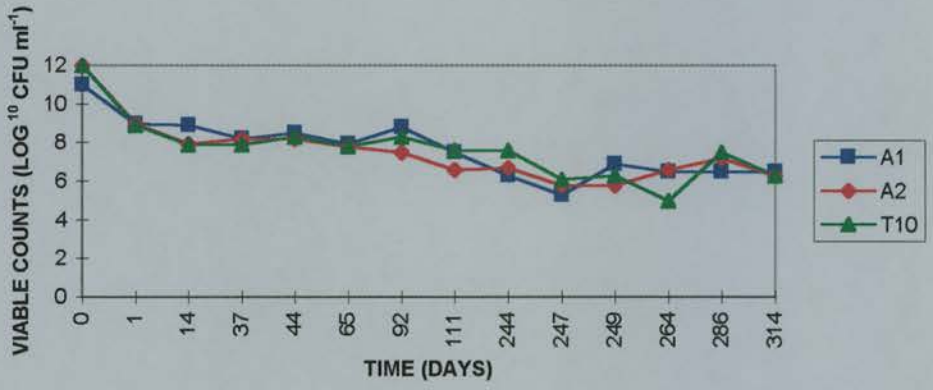


Fig 4.2d SURVIVAL OF SEROTYPES IN NEWBORN CALF SERUM



The survival experiments (Fig 4.2. a-d, for raw data see Appendix II) dealt with a higher initial inoculum to see if survival over a long period of time was inoculum dependent. In otbw (Fig 4.2a) an initial drop in viability was observed followed by a pattern of rise and fall in viability as seen with the growth experiments. T10 again had micro-colony morphology on days 107- 342, and 379-407, A1 had micro-colony morphology on days 340 and 357-407. The addition of fresh serum on days 337 and 340 caused a brief return to 'normal' colony morphology with A1 and T10 and an increase again in viability for A2. The survival pattern in btbw (Fig 4.2b) was again similar to that observed previously with a large slow decline in viability over time with T10 micro-colonies on days 156-173 and 195-203. This time however both A1 and A2 showed an increase in viability on the addition of fresh media on days 153 and 156 whereas T10 appeared to diminish with only micro-colonies observed and 'normal' colony type observed only on day 173 which was 17 days after the last addition of fresh media. The serum cultures were no different from those observed in the growth and survival graphs.

When representative nutrients were analysed (Table 4.1) it was found that the nutritional contents differed between the sera and tbws.

Nutrient	Bovine tbw	Ovine tbw	Newborn calf serum	Lamb serum
Protein g/L	0.07	0.84	71	81
Glucose mmole/L	0.02	0.07	4.03	2.2
Iron μmole/L	1.38	1.59	14.7	27.9

Table 4.1. Representative nutrient levels of 'in-vivo' fluids used in growth and survival of serotypes.

The survival behaviour of the serotypes is not easily comparable between the different fluids because of nutritional differences. The high levels present in serum may be the

reason why replenishment was not required. Although the levels of glucose and iron in the tbws are not dissimilar, protein levels were much higher in otbw than btbw and probably account for the inability to support growth and extended survival.

4.3. Survival in fluids from other species

To address the question of species specificity of survival in in-vivo fluids tbw and serum from a number of different species were used, along with control bacteria. The fluids were all adjusted to approximately the same levels based on protein measurements (Table 4.2).

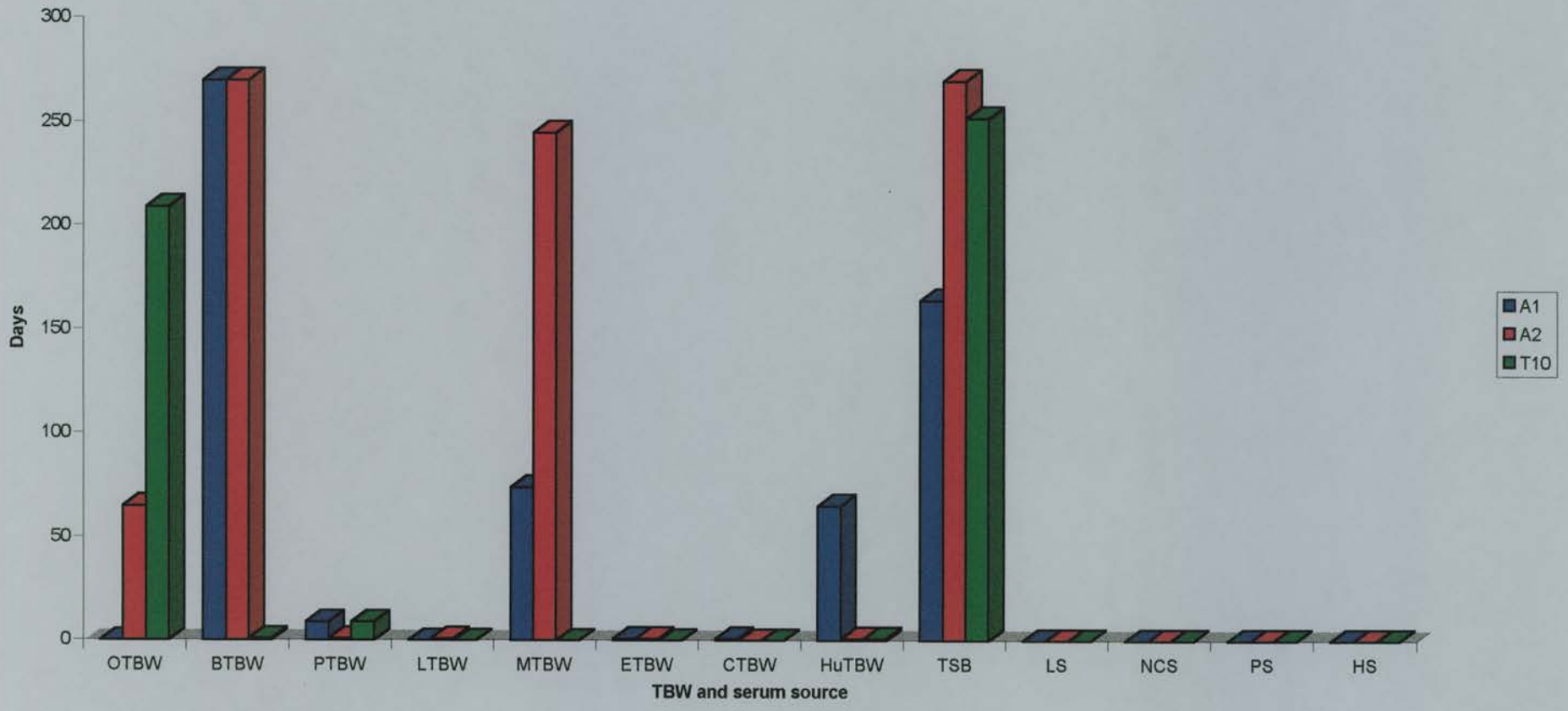
Fluid	Protein mg/ml	Glucose mmole/L	Iron µMole/L
LS (Lamb serum)	0.10	0.009	1.07
NCS (Newborn calf serum)	0.06	0.009	2.30
PS (Porcine serum)	0.13	0.023	0.67
HS (Horse serum)	0.08	0.010	7.33
otbw (Ovine)	0.13	0.006	5.51
btbw (Bovine)	0.11	0.011	0.75
ptbw (Porcine)	0.04	1.068	3.13
ltbw (Laprine)	0.06	0.029	3.19
mtbw (Murine)	0.12	0.079	2.44
etbw (Equine)	0.13	0.067	4.11
ctbw (Canine)	0.08	0.028	1.68
hutbw (Human)	0.04	0.009	0.66
TSB	<0.01	0.729	5.16

Table 4.2. Nutrient values of all tbws and sera used in an experiment to investigate species specificity role in survival in in-vivo fluids.

Fig 4.3 shows survival in otbw (A2 and T10), btbw and mtbw (A1 and A2), ptbw (A1 and T10), A1 in hutbw and all three serotypes in diluted TSB. Serum at this level would not support any survival. Control organisms, *Neisseria meningitidis* and

Haemophilus somnus did not survive for 24 hours in any fluid at any temperature. *E. coli* O157 survived in all fluids at 37^{0}C and 4^{0} C for at least 65 days.

Fig 4.3. Survival of serotypes A1, A2 and T10 in tbws and serum from different species at 37°C. Fluids were adjusted to approximately the same protein concentration then inoculated with the bacteria and sampled as previously described (Fig 4.1). Viability was identified by inoculating 10μl into 10ml of nutrient broth and incubating overnight at 37°C. This was carried out as colonies on agar were not visible.


300
250
200
150
100
50
0
Days
OTBW
BTBW
PTBW
LTBW
MTBW
ETBW
CTBW
HuTBW
TSB
LS
NCS
PS
HS
TBW and serum source
A1
A2
T10

FLUID	SEROTYPE	DAYS SURVIVAL ROOM TEMP	AT 4°C
otbw	A1	1	1
	A2	0	9
	T10	0	1
btbw	A1	1	1
	A2	1	9
	T10	0	9
ptbw	A1	1	1
	A2	1	9
	T10	0	1
ltbw	A1	0	1
	A2	0	9
	T10	0	1
mtbw	A1	0	0
	A2	0	1
	T10	0	9
etbw	A1	1	1
	A2	1	9
	T10	0	1
ctbw	A1	0	0
	A2	0	1
	T10	0	1
hutbw	A1	0	1
	A2	0	9
	T10	0	1
TSB	A1	0	0
	A2	0	1
	T10	0	1
LS	A1	0	0
	A2	0	1
	T10	0	1
NCS	A1	0	1
	A2	1	9
	T10	0	1
PS	A1	0	1
	A2	1	9
	T10	0	1
HS	A1	0	0
	A2	0	9
	T10	0	1

Table 4.3. Table showing days survival of serotypes inoculated into different tbw and sera and incubated at room temperature (approximatley 25°C) and 4° C. Viability was identified as in Fig 4.3.

When the incubation temperature of the fluids was lowered (Table 4.3) however, A1 survived at room temperature in otbw and A2 survived in NCS and PS which gave no indication of viability when incubated at 37^0 C.

The results for the 4^0C culture were even more dramatic and showed increased survival time for serotypes in fluids they previously did not survive in, especially serotype A2. This supports the evidence from Western blots that the serotypes were inhibited by something other than IgG bactericidal activity. The blots (Figs 4.4-4.6) show that there is no correlation between the ability to grow and/or survive and IgG recognition.

Fig 4.4. Western blots of envelope preparations of serotype A1 and probed with different tbws and serum to assess IgG recognition of proteins. (conjgates used at 1/200 dilution)

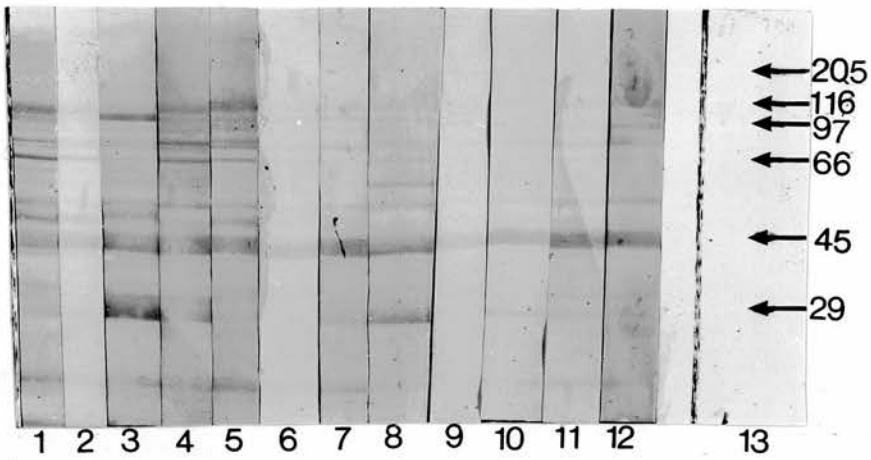
LS (lane 1), NCS (lane 2), PS (lane 3), HS (lane 4), otbw (lane 5), btbw (lane 6), ptbw (lane 7), etbw (lane 8), mtbw (lane 9), ltbw (lane 10), hutbw (lane 11), ctbw (lane 12) and molecular weight markers (lane 13).

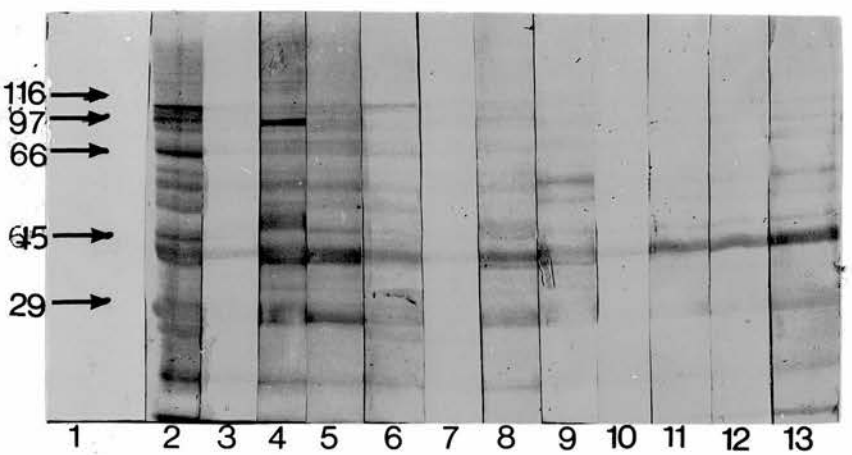
Fig 4.5. Western blots of envelope preparations of serotype A2 and probed with different tbws and serum to assess IgG recognition of proteins.

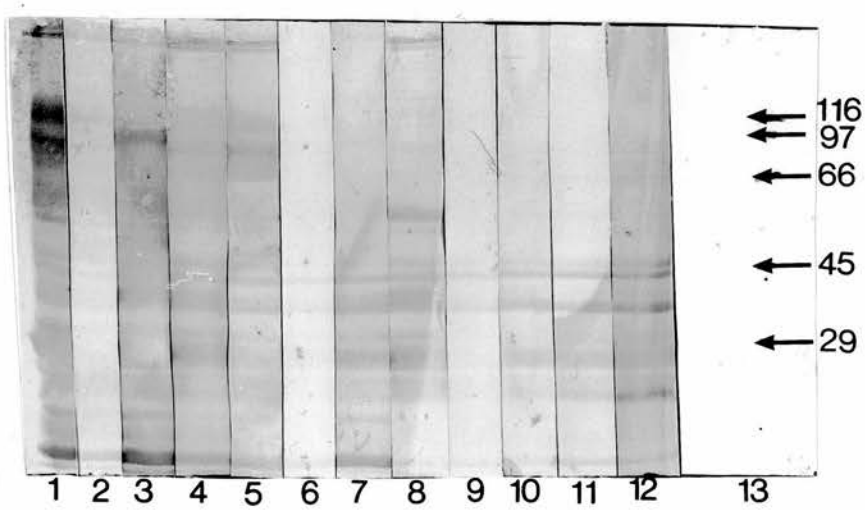
Molecular weight markers (lane 1), LS (lane 2), NCS (lane 3), PS (lane 4), HS (lane 5), otbw (lane 6), btbw (lane 7), ptbw (lane 8), etbw (lane 9), mtbw (lane 10), ltbw (lane 11), hutbw (lane 12) and ctbw (lane 13).

Fig 4.6. Western blots of envelope preparations of serotype T10 and probed with different tbws and serum to assess IgG recognition of proteins.

LS (lane 1), NCS (lane 2), PS (lane 3), HS (lane 4), otbw (lane 5), btbw (lane 6), ptbw (lane 7), etbw (lane 8), mtbw (lane 9), ltbw (lane 10), hutbw (lane 11), ctbw (lane 12) and molecular weight markers (lane 13).


205
116
97
66
45
29
1 2 3 4 5 6 7 8 9 10 11 12 13

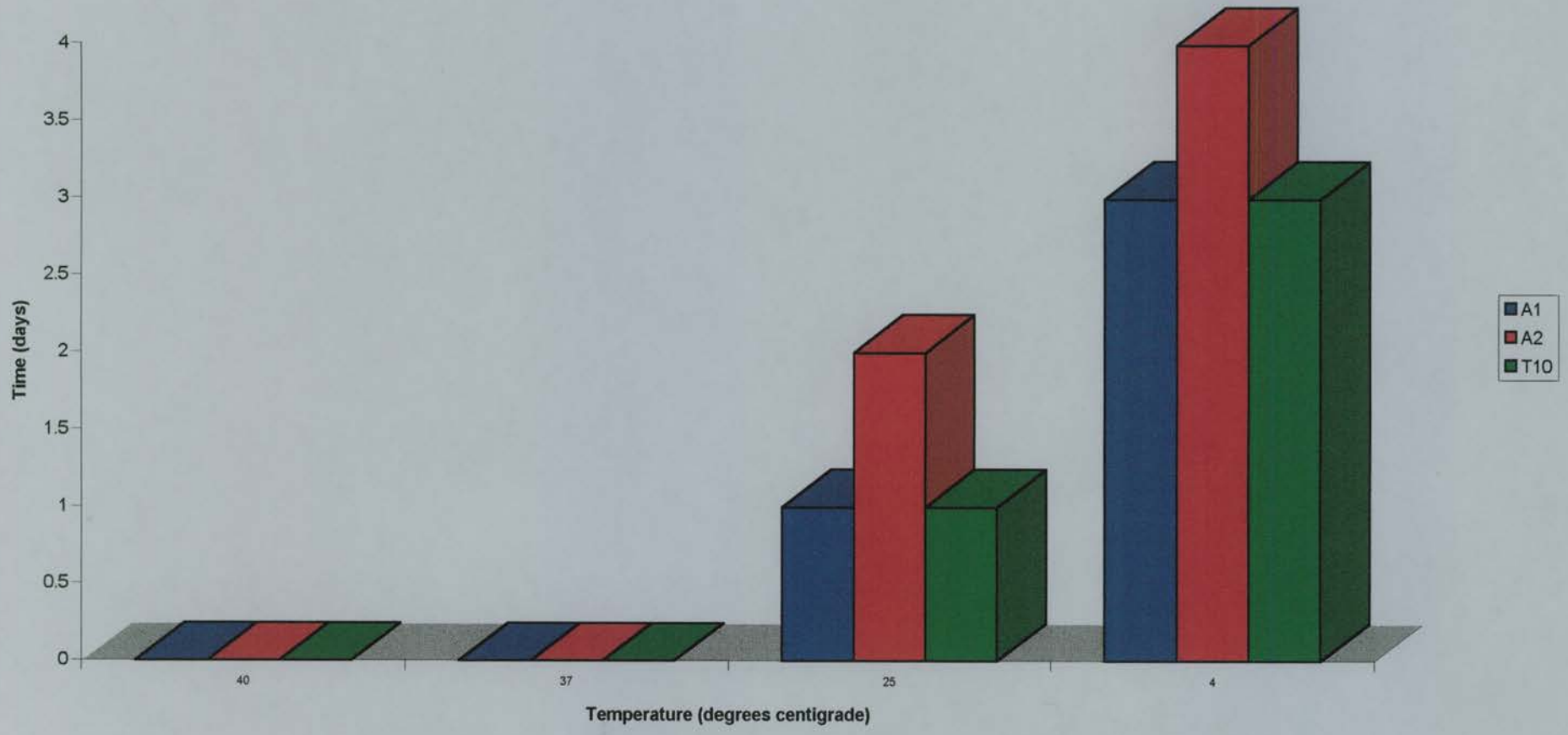

116
97
66
45
29
1 2 3 4 5 6 7 8 9 10 11 12 13


116
97
66
45
29
1 2 3 4 5 6 7 8 9 10 11 12 13

4.4. Survival in natural water

Survival has been shown in very low nutrient conditions and at low temperatures suggesting that pasteurellae may survive in natural water. Natural water was collected (Loch Etive, Scotland) and filtered through a 0.2μm filter. The serotypes were inoculated into the water as in previous survival experiments and after incubation at different temperatures were sampled for viability. As with the previous experiment the low nutrient levels resulted in loss of colonies on agar and broth inoculation was therefore used to identify viable organisms. Fig 4.7 shows that survival of up to 4 days for serotype A2 was possible in lake water which contained no detectable protein. The lower the temperature that the cultures were incubated at the longer the survival. Inoculation and growth in broth also shows the ability to resuscitate quickly in the presence of a nutrient influx and raised temperature.

Fig 4.7. Survival in fresh water of serotypes A1, A2 and T10 at different temperatures. Cultures were set up as previously described (Fig 4.1) and sampled daily for viable counts and for growth in broth at 37^0 C.


Time (days)
4
3.5
3
2.5
2
1.5
1
0.5
0
40
37
25
4
Temperature (degrees centigrade)
A1
A2
T10

4.5. Analysis of bacterial morphology

One of the most important observations during survival was the change in colony morphology from 'normal' to micro-colonies (Fig 4.8). The difference in size and numbers present is quite different and these colonies did not grow when diluted and were only observed on agar when plated straight from the culture. These colonies when subcultured onto blood agar regained their normal colony morphology. Gram's stain was used to compare micro-colonies to 'normal' colonies. Fig 4.9 shows that serotype A2 micro-colonies were only slightly different when compared to 'normal' colonies but they were less pleomorphic in size and shape. Figs 4.10 and 4.11 show A1 and T10 respectively forming long chains of elongated bacteria with small pleomorphic bacteria apparent in the background. When examined using electron microscopy with negative staining A1 and A2 (Fig 4.12 a and b) four hour cultures show typical pleomorphism of the bacteria. At higher magnification all three serotypes show the typical differences (Fig 4.13 a-f), which were also observed using light microscopy , in the size and shape of the bacteria when three hour cultures and micro-colony bacteria are compared.

When bacteria taken straight from starvation fluids were examined they would not take up Gram's stain and could not be observed. However, the use of Maneval's stain overcame this problem and Fig 4.14 shows very small bacteria closely associated and enclosed in what appears to be a glycocalyx of biofilm type structure. If this structure contained capsular polysaccharide this may explain how samples straight from culture and micro colonies still serotyped in the IHA.

Fig 4.8. Example of micro-colonies. This sample is serotype T10 from long term culture next to an overnight culture from TSB. Samples were dropped neat (10 µl) onto sheep blood agar and incubated overnight at 37^0 C.

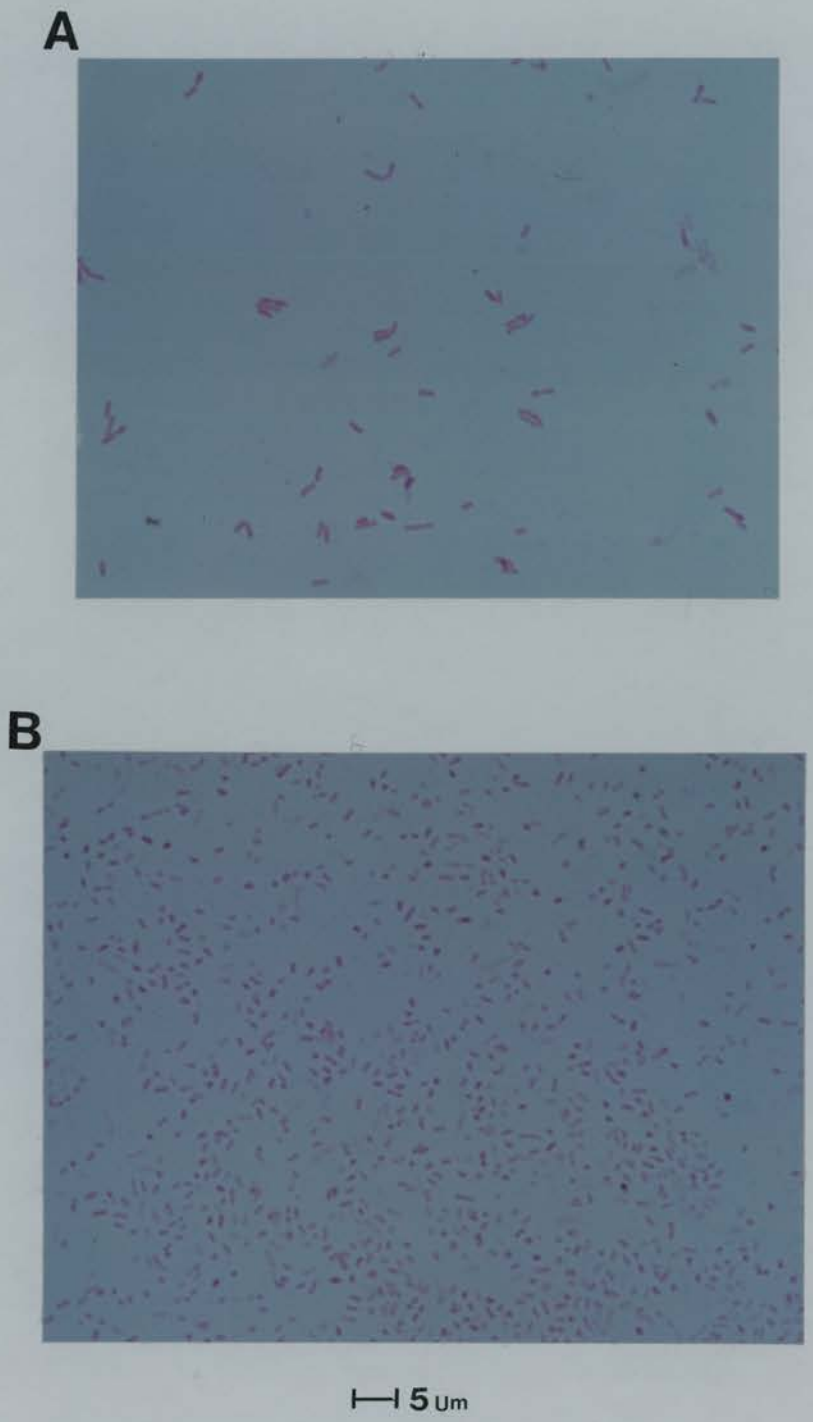


Fig 4.9. Serotype A2 from (A) 'normal' colonies and (B) from micro-colonies. The samples were Gram stained.

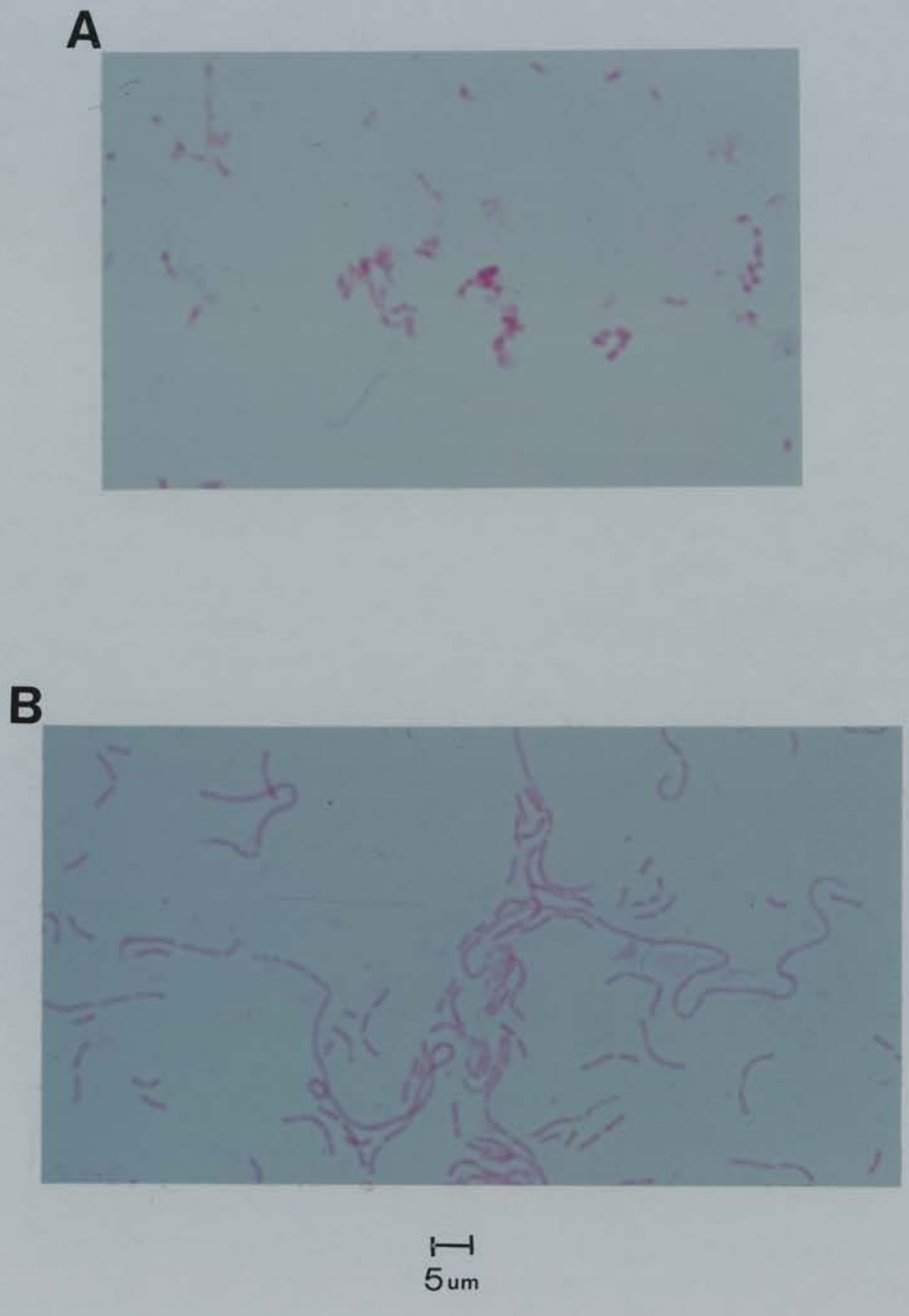


Fig 4.10. Serotype A1 from (A) 'normal' colonies and (B) from micro-colonies. The samples were Gram stained.

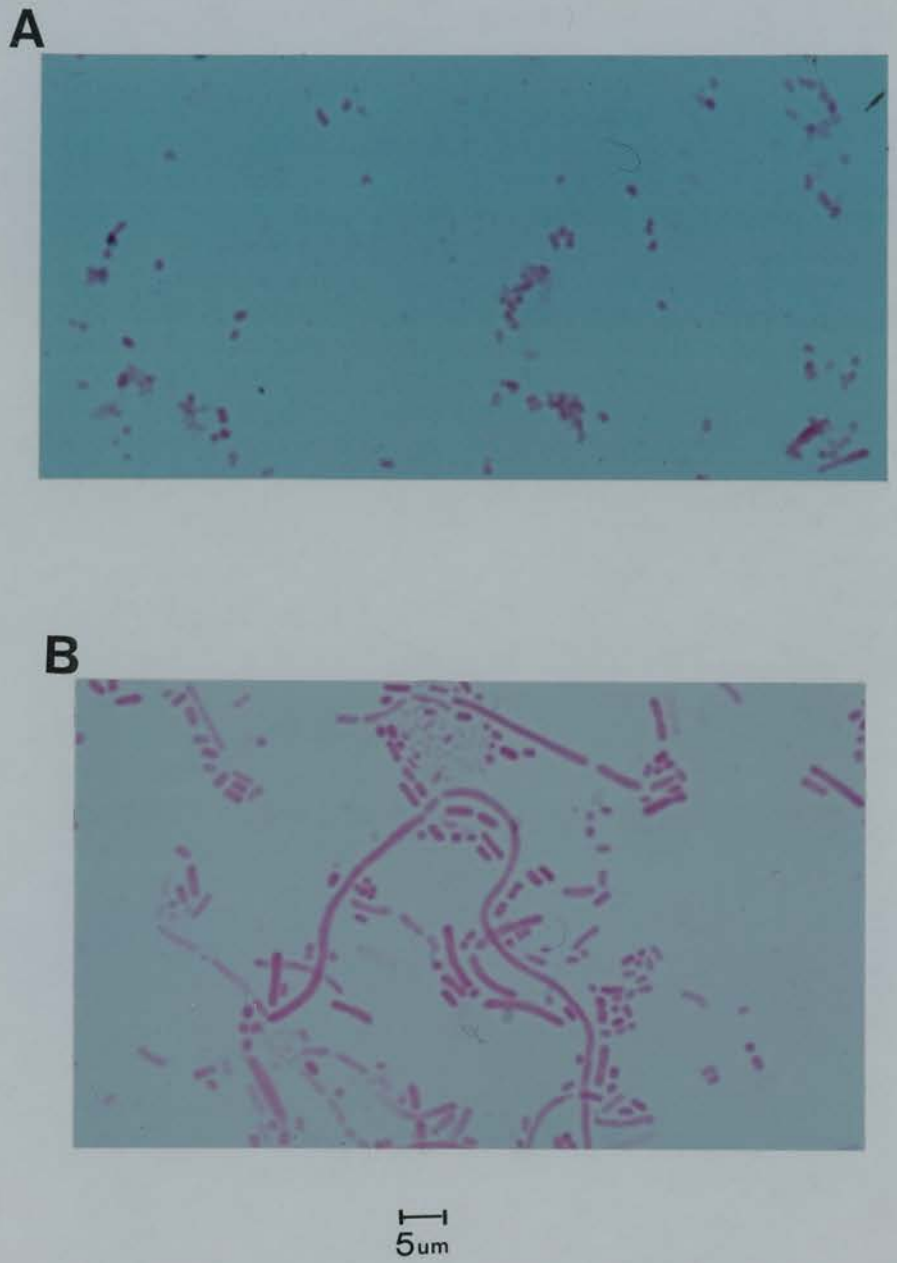


Fig 4.11. Serotype T10 from (A) 'normal' colonies and (B) from micro-colonies. The samples were Gram stained.

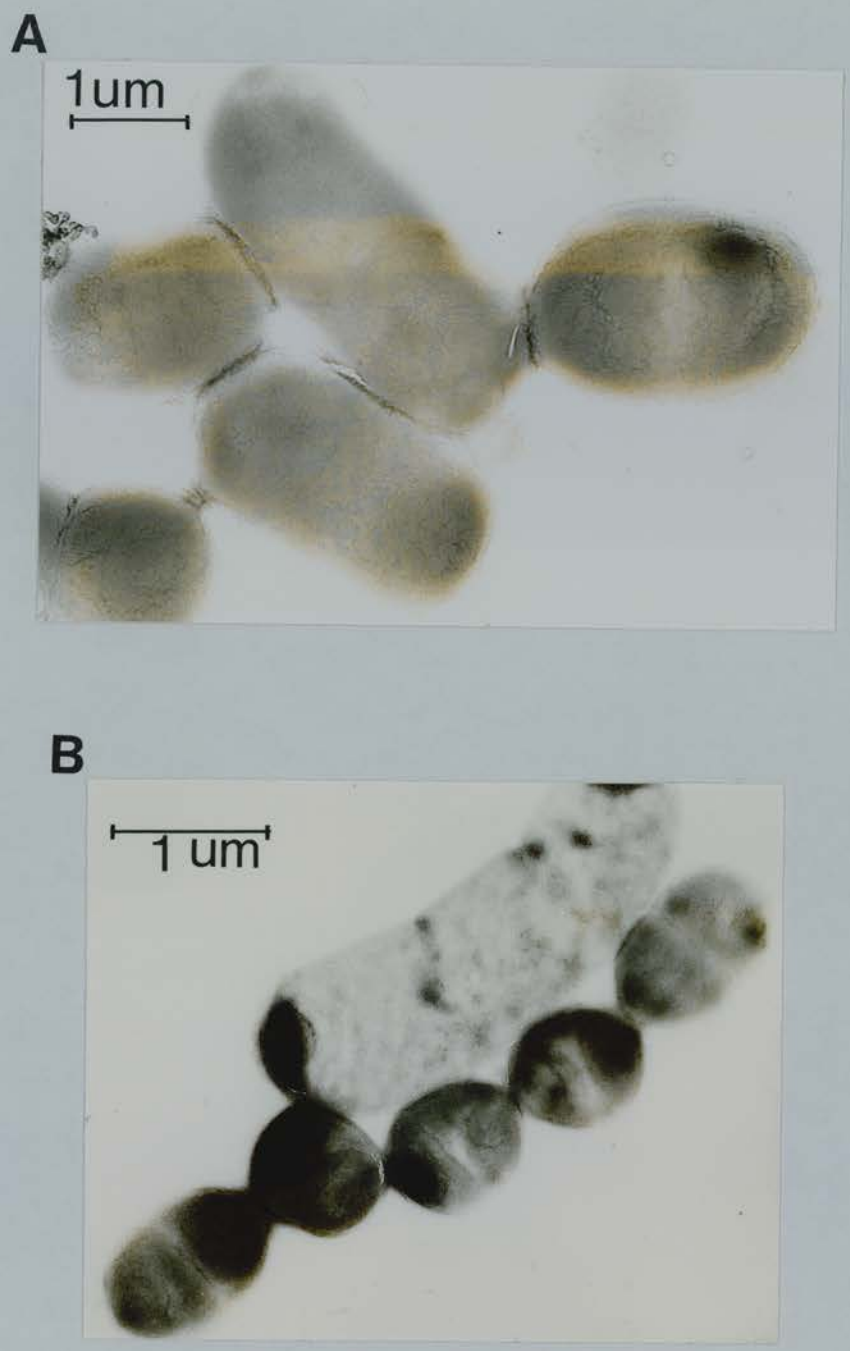
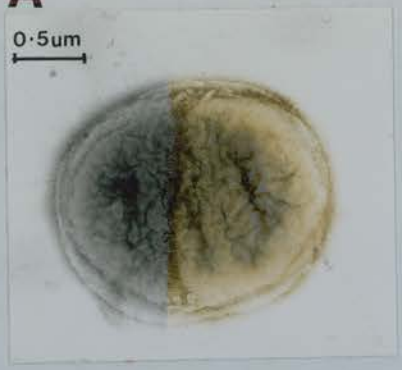


Fig 4.12. Negative stained electron microscopy of A1 (A) and A2 (B) four hour cultures in TSB showing typical polymorphism with long bipolar stained bacteria and small dividing cocci shaped bacteria.

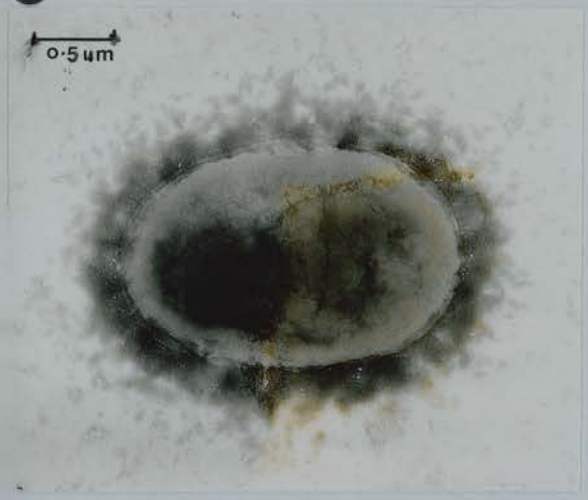
Fig 4.13. Negative stained electron micoscopy comparing bacteria from four hour cultures with micro-colony bacteria. A1 four hour (A), A1 micro colony (B), A2 four hour (C), A2 micro colony (D), T10 four hour (E), T10 micro colony (F). All bacteria were typical of those which were present on the grids. The micro-colony samples however did have a tendancy to be present in long chains with no obvious joins which has been observed in the light microscopy photographs. These chains were too long to photograph in any greater detail by EM, therefore, single bacterium were chosen.

A



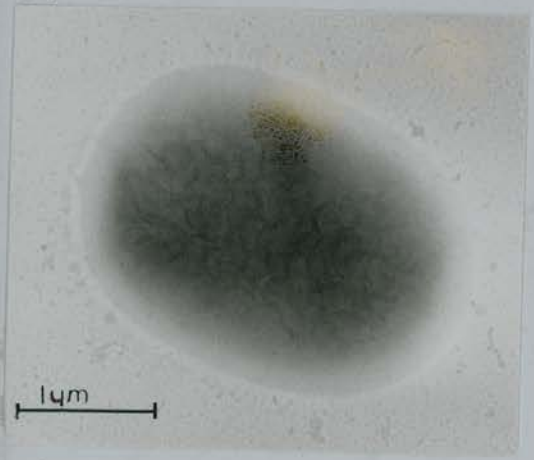
B

C

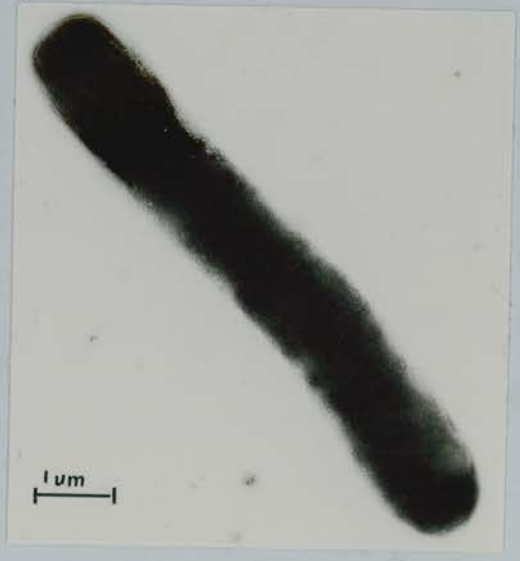


D

E



F



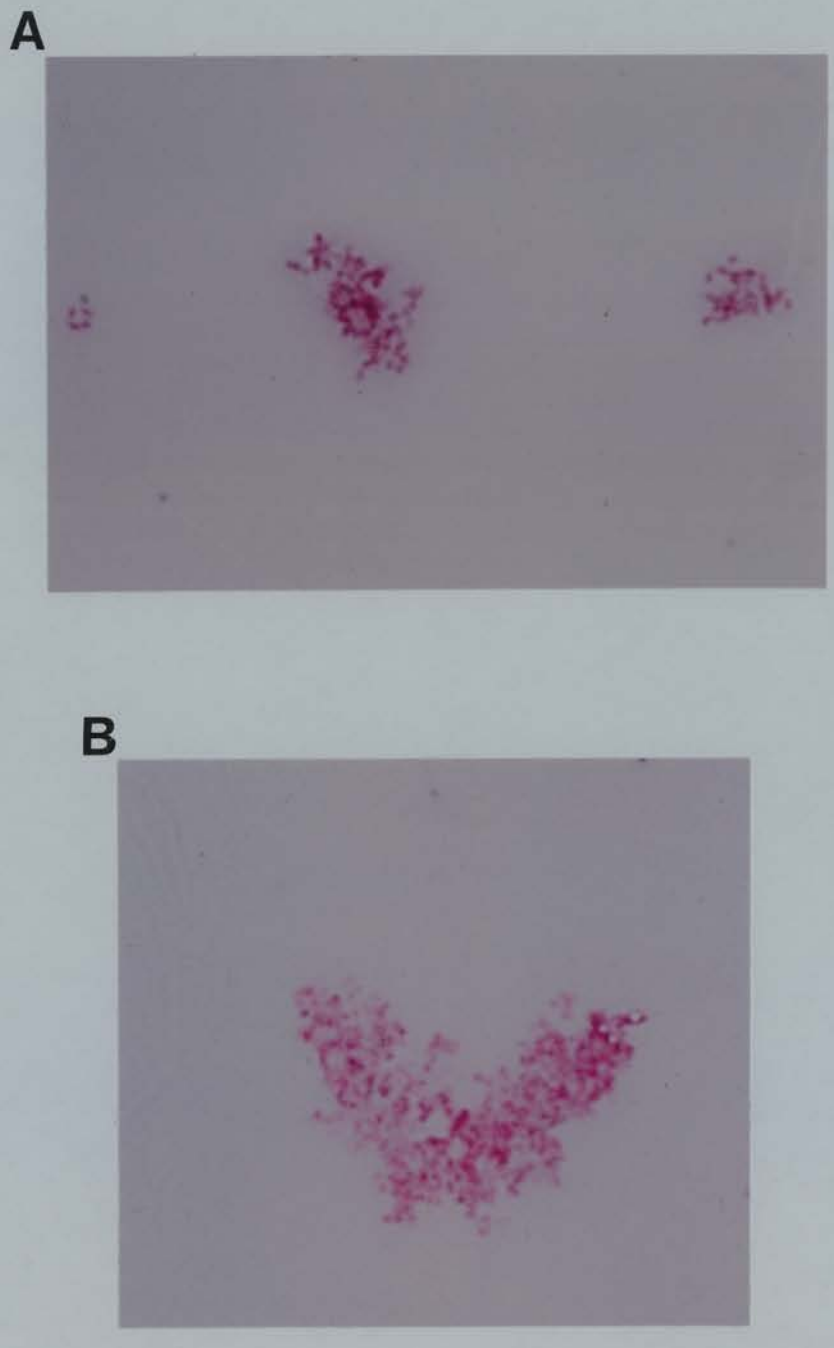


Fig 4.14. Maneval's stain of serotypes A1 (A) and T10 (B) directly from long term cultures without subculture. The pictures show the bacteria are much smaller and closely associated in what appears to be a glycocalyx type structure.

4.6. Discussion

The results of this study show that *P. haemolytica* and *P. trehalosi* have the ability to survive for long periods of time in relatively low nutrient 'in-vivo' fluids. Unlike most other bacteria, shown to have this capacity, the *Pasteurellae* spp are not regarded as being prevalent in the environment. Bacterial survival has been demonstrated in different environments such as soil, natural and marine waters (Morita, 1988). Stored soil samples analysed after 54 years showed bacteria oxidising sulphur and producing CO_2. *Bacillus* spp, Coryneforms and *Streptococcus* spp from 70 year old soil have been isolated. Ant-300 a marine vibrio survived 2.5 years in a starvation menstrum but would possibly have remained viable much longer. Also a marine pseudomonad remained viable at ~10^5 cfu ml^{-1} for over 1 year (Novitsky & Morita, 1976). Morita (1993) cited an experiment by Iacobellis and DeVey who showed that 14 isolates of *Pseudomonas syringae* subsp. *syringae* survived in distilled water for 24 years. The bacterial viable counts averaged 10^8 cfu ml^{-1} and dropped only two or three logs over the time period and also retained their antigenic properties.

Few studies have investigated the capacity of bacteria (especially those which are pathogenic for humans and animals) to survive in body fluids. However, Blasser *et al.,* (1980) studied the growth of *Campylobacter fetus* subsp. *jejuni* in HCl (to mimic gastric juice), human bile, urine, bovine milk and stream water. The organism survived for 30 minutes (pH dependent), 2 months, 5 weeks, 22 days and 4 weeks respectively. Porter & Wardlaw (1994) used tbws from different species and showed 7 days survival for *Bordetella bronchiseptica* and *B. avium* in all species' tbw tested. In contrast, the survival of *B. pertussis* was less than 2 days in human, mouse, sheep and chicken tbws whereas the bacteria were detectable in horse, dog and rabbit tbws after

2 days but not at 7 days. Growth did not occur with *B. pertussis* whereas prior to survival *B. bronchiseptica* grew in sheep, human, mouse, rabbit and chicken tbws. There may have been some effect of individual fluids as one human tbw tested did not allow growth or survival beyond day 2 for all species of bordetellae tested.

Morita (1985) produced a model graph showing four patterns of survival: a) increase in numbers followed by decline; b) rapid dying; c) increase of viable cells followed by a constant level; d) no dying or increase. These patterns however may depend on the nutrients available, state of the organism and the inoculum. In this study the growth and survival experiment would fit comfortably into Morita's classification 'a' except in the case of btbw, whose low nutrient content could not sustain growth of *P. haemolytica* and *P. trehalosi*. Roszak & Colwell (1987a) explained that laboratory media maintains organisms at 72g of carbon/litre compared to oligotrophic conditions found in the natural environment of 1-15mg carbon/litre. Morita (cited 1993) presented work carried out by Zobell and Grant in 1942 who studied growth using only 0.1mg glucose/litre for the marine bacteria *Staphylococcus citreus, Proteus vulgaris, E. coli* and *Lactobacillus lactis*. However, 10-25µg carbon/litre was needed for *Aeromonas hydrophilia* and *Pseudomonas aeruginosa* and this highlights the differences between bacteria and their specific minimal growth requirements. The survival experiment described here did produce a decline from the high inoculum, but this was not rapid and the viability settled out to between 10^5 - 10^7 cfu ml^{-1} for all serotypes in all fluids.

The survival patterns of the graphs were not constant with regard to viable counts. *P. haemolytica* and *P. trehalosi* isolates tested showed the characteristic rise and fall in viability. This has been termed cryptic growth where dead cells supply nutrients for the viable cells that remain and hereby induce a growth spurt (Roszak & Colwell,

1987a). Postgate & Hunter (1962) calculated that at least 50 bacteria which have died and leaked their nutrients are needed to support the growth of one bacterium.

Sporadic or long term loss of 'normal' colony morphology and the inability to culture on agar or in broth was a feature of starvation especially for serotypes A1 and T10. It is important to show that starvation cells can be resuscitated if they show different colony morphology or become non-culturable in/on their usual isolation media. Postgate & Hunter (1962) described *Aerobacter aerogenes* as being dead when they failed to multiply, but noted that some cells retained their osmotic barriers and could therefore be alive. These were described by Kaprlyants *et al.,* (1993) as dormant forms of non-sporulating bacteria which have formed due to low substrate conditions and were not able to produce colonies on agar surface without a prior resuscitation phase. Morita (1985) cited research on the study of marine organisms from the Gulf coast. Post filtration of the water took place then the samples had to be incubated in dilute nutrient broth for at least 21 days at 21 or 35^0 C which was then then used as an inoculum for dilute nutrient agar plates. Vibrios, *Aeromonas* spp, Pseudomonads and *Alcaligenes* spp were all isolated and developed into normal sized bacteria upon nutrient conditioning. Resuscitation has been shown to result in the usual lag, exponential phase with the lag phase directly proportional to the length of the prior starvation period (Morita., 1985). However, Novitsky & Morita (1976) grew starved Ant-300 without a significant lag phase and they regained normal size and shape within 48 hours and Roszak & Colwell (1987a) showed that seawater populations can respond kinetically to organic enrichment within a period of ~12 hours. Magarinos *et al.* (1994), studying dormant *Pasteurella piscicida,* found that it was difficult to ascertain whether the non-culturable cells were resuscitated, grew or did both when optimal conditions were restored.

This study shows that although the micro-colonies observed on agar, when the organisms seemed nutritionally stressed, were very small, the microscopic examination revealed larger than normal bacteria. Bretz (1962) using slide cultures reported bacteria that he described as moribund and which were observed to swell only during the period required for the other cells to divide. These cells eventually formed micro-colonies and were therefore considered viable. Kogure *et al.* (1979) developed a technique for counting viable non-culturable bacteria with the use of nalidixic acid and yeast extract. Nalidixic acid is an antibiotic which inhibits DNA gyrase, and thus DNA synthesis, but the presence of yeast extract allows nutrient uptake into the starving cell. This results in large elongated bacteria which can be clearly observed microscopically. The descriptions of these organisms are very similar to the micro-colonies observed in this study. The fact that subculture reveals a return to 'normal' colony morphology suggests that the bacteria are in a dormant starvation phase before the nutrients affect the bacteria and allow division to take place. As pointed out by Roszak & Colwell (1987b), the actual number of bacteria, using the above method and other specially developed detection methods, exceed the numbers indicated by viable counts.

The described behaviour of changes in morphology point to signalling mechanisms. These signals must allow sensing of low nutrient states to induce starvation mechanisms and *vice versa* once nutrients become available as described by NystrÖm (1993). These signals have been called chemical crosstalk (Pennisi, 1995), cell density sensing (Gomer, 1994) or quorum sensing (Claiborne-Fuqua, 1994). Kaprelyants & Kell (1996) uses the term microendocrinology, as bacterial growth has been shown to respond to hormones, cytokines and pheromones all of which have implications for pathogens residing in-vivo such as Pasteurellae.

With the addition of nutrients in this study a rapid increase in viability was not always observed. The response was either slow (from 2-10 days) or a decline in viability or loss of 'normal' colony morphology. The decline of viability after a resuscitation period may indicate behaviour described by Postgate & Hunter (1962) as substrate accelerated death. The induction of growth via added nutrients is so rapid (in the context of starvation behaviour) that the nutrients are exhausted quickly and cannot maintain cell division so enter into decline unless fresh nutrients are continually supplied. This suggests that *P. haemolytica* and *P. trehalosi* possess the ability to survive for long periods but not if the starvation regime is intermittent and only if the influx of nutrients is constant as is the case with bacteria inoculated into nutrient broth for resuscitation.

Signalling mechanisms which allow bacteria to multiply in a given environment may have some importance in the relative species specificity shown by the serotypes in different tbws and serum. The results showed that this was not due to an IgG bactericidal effect and so was probably a nutritional signal as the amounts of nutrients that would have allowed growth did not differ. Growth and survival may be superior for bacteria in the fluids from the preferred host due to the signalling mechanisms being unique to the host. In the case of Pasteurellosis the disease is limited to ruminants and the growth in murine tbw may be due to the reproducibility of Pasteurellosis in a mouse model. This does not explain the survival in human tbw by serotype A1. The fact that *B. bronchiseptica* grew in 7 different species of tbws was proposed to reflect its broad host range. However, *B. avium, B. pertussis* and *B. parapertussis* showed no species specificity (Porter & Wardlaw, 1993). The lack of survival in sera may be due to the dilution effect on any growth signals present.

Low temperatures in the environment usually coincide with low nutrient availability, so it is not surprising that these situations are comparable. Viable Pasteurellae were evident in fluids at low temperatures which previously at higher temperatures would not allow survival. Kjelleberg *et al.,* (1987) stated that the low temperatures may help to decrease endogenous metabolism to a low rate which matches the maintenance energy requirements and improves the chances of prolonged viability. Blasser *et al.,* (1980) in similar work also showed that at 4^0C *Campylobacter fetus* subsp. *jejuni* survived significantly longer in faeces, water (4 weeks), milk and urine.

The ability to survive in water is another feature of lowering temperatures which was seen with *P. haemolytica* and *P. trehalosi*. Porter *et al.,* (1991) and Porter & Wardlaw (1993) showed that *B. bronchiseptica* not only grew in PBS, reagent grade water, lake and pond waters but retained viability for 24 weeks. Although the serotypes did not survive as long as other organisms have, a few days is still a window of time in which an animal could pick up the organism from body fluids present in the field, accommodation or in drinking water. This work is in agreement with that of Porter & Wardlaw (1993) that previous obligate, non-invasive, respiratory tract parasites may survive in environments outside the host respiratory tract thereby creating potential reservoirs of disease. The reservoir state of bacteria which can become viable non-culturable (VBNC) is extremely important to public health and to that of animals. This appears not to be the case for pasteurellae and a more extensive investigation is required. The intermittent ability to culture from starvation cultures suggests that there may be such a state. Magwood *et al.* (1969) reported that in the nasal passages of calves the bacterial flora fluctuated in species and numbers, and although *P. haemolytica* could dominate the flora it could also not be detected for weeks at a time. Pass & Thompson (1971) also failed to consistently culture *P. haemolytica* in daily

swabs from known colonised animals. Although this situation can be interpreted in different ways it may indicate that if competition increases and nutrients are scarce a VBNC state could be induced in-vivo and this is worthy of further research.

The changes observed in morphology for *P. haemolytica* and *P.trehalosi* were encouraging in their adaptation to starvation. The cells straight from tbws and sera showed smaller bacteria than the normal pleomorphic cells of *P. haemolytica* and *P. trehalosi*. Size reduction is one of the more common responses to starvation and serves to reduce the surface area to volume ratio and so employ an efficient nutrient and metabolic state in the organism. The aggregation in a polysaccharide-like matrix (as observed with Maneval's stain) was reminiscent of biofilms and looks remarkably similar to the in-vivo *P. haemolytica* A2 collected by Sutherland *et al.* (1990). Brown & Williams (1985) state that the formation of biofilms in a polysaccharide matrix is the mode of growth for all but a small fraction of bacteria in the natural environment. Coglan (1996) states estimates that 99% of the planet's bacteria live in biofilms. Although not demonstrated in this study, an increase in hydrophobicity is also a well documented survival mechanism and is also associated with polysaccharide matrices (Kjelleberg & Hermansson, 1984). The lack of ability to observe the bacteria by Gram's stain indicates changes in the cell wall, especially the peptidoglycan layer which has been reported for other organisms (Morita 1988; 1993; Östling *et al.,* 1993). This state is different from the micro-colony appearance discussed earlier.

One of the reasons that these states of survival are important is that many pathogenic bacteria have the ability to maintain virulence for their hosts during survival or VBNC states. Examples include *Pasteurellae piscicida* and *Shigella dysenteriae* (Magarinos *et al.,* 1994; Rahman *et al.,* 1996). The ability of *P. haemolytica* and *P.trehalosi* to adapt with starvation/survival mechanisms requires further investigation. The areas of

most importance are whether starvation cells resemble those which colonise the upper respiratory tract and can cause disease in animals.

CHAPTER 5

THE USE OF IMMUNOMAGNETIC SEPARATION TECHNIQUES FOR IN-VIVO CAPTURE OF *P. HAEMOLYTICA* AND *P. TREHALOSI*

5.1. Introduction

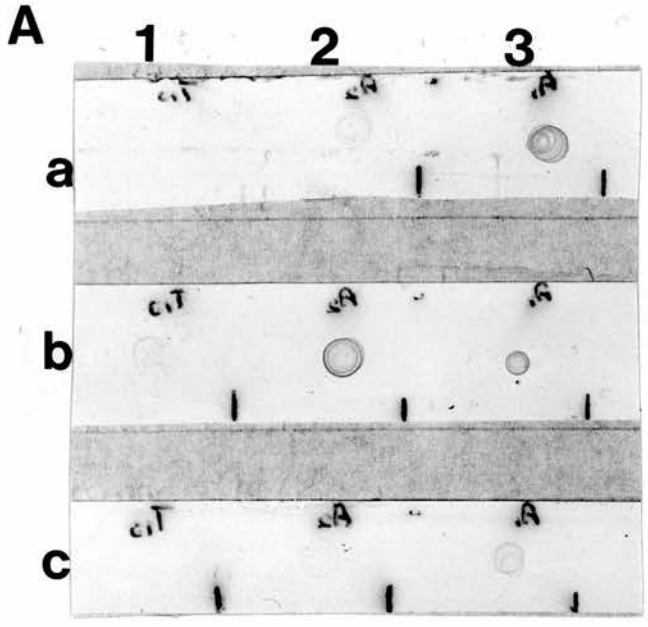
The previous two chapters discussed the employment of in-vivo fluids to mimic an in-vivo environment in order to culture bacteria. A technique was required to isolate *Pasteurella* spp from both diseased tissues in the lower respiratory tract and colonised tissues in the upper respiratory tract. This would enable a more accurate assessment of the changes in virulence factors observed in chapter 3 and if colonising bacteria resemble those observed during survival culturing. Immunomagnetic separation is a commonly used technique in the diagnosis of bacteria in food and fluids (Olsvik *et al.*, 1994) and is based on the specificity of the antibody employed to capture the target organism. The possibility that this could be used to isolate target bacteria from a mixture of host proteins and other bacteria for subsequent analysis would overcome many of the drawbacks of isolation of in-vivo bacteria.

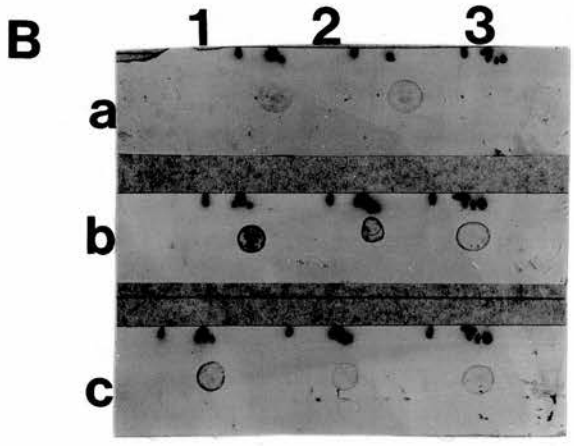
5.2. Adsorption of antiserum

The first step in immunomagnetic separation (IMS) is the production of antibodies with which specific capture takes place. Earlier studies (Chapter 3) describe the production of rabbit antiserum raised against each of the three serotypes to be investigated. Although each separate serum is raised against a specific serotype, cross-reacting antibodies will be present and must be removed to increase the specificity.

Fig 5.1 shows antiserum recognition using dot blots of all three serotypes and the subsequent decrease in cross reaction after absorption of the antisera against the serotypes which are not the target organism. The pre-bleed serum shows no antigenic recognition of the serotypes, whereas pre-absorbed sera show high degrees of cross reaction.

Fig 5.1. Dot blots of rabbit antiserum raised against A1 (A), A2 (B) and T10 (C) showing cross reactions with A1 (1), A2 (2) and T10 (3) of pre bleeds (a), pre adsorbed antiserum (b) and adsorbed antiserum (c).


A
1
2
3
a
b
c


B
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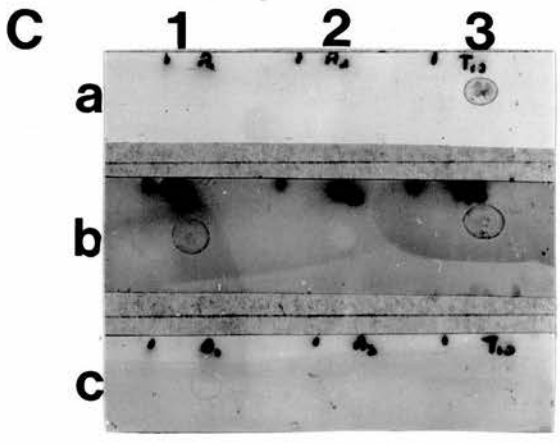
2
3
a
b
c


C
1
2
3
a
b
c

5.3. Standardisation of technique

The methods used in this chapter are summarised in Fig 5.2. Using the direct method bacterial recovery from beads was compared to that of ordinary direct plating techniques for viable counts to assess the sensitivity of the method. The results in Table 5.1 show the effectiveness of the bead capture in detecting bacteria beyond the levels obtained by traditional direct plating. This was apparent with all serotypes and the system was proven to be very sensitive. As the beads can bind approximately 4 bacteria/bead the potential numbers of colonies arising from the beads is underestimated because one bead will give rise only to one colony. Fig 5.3 shows fluorescently labelled bacteria attached to the beads

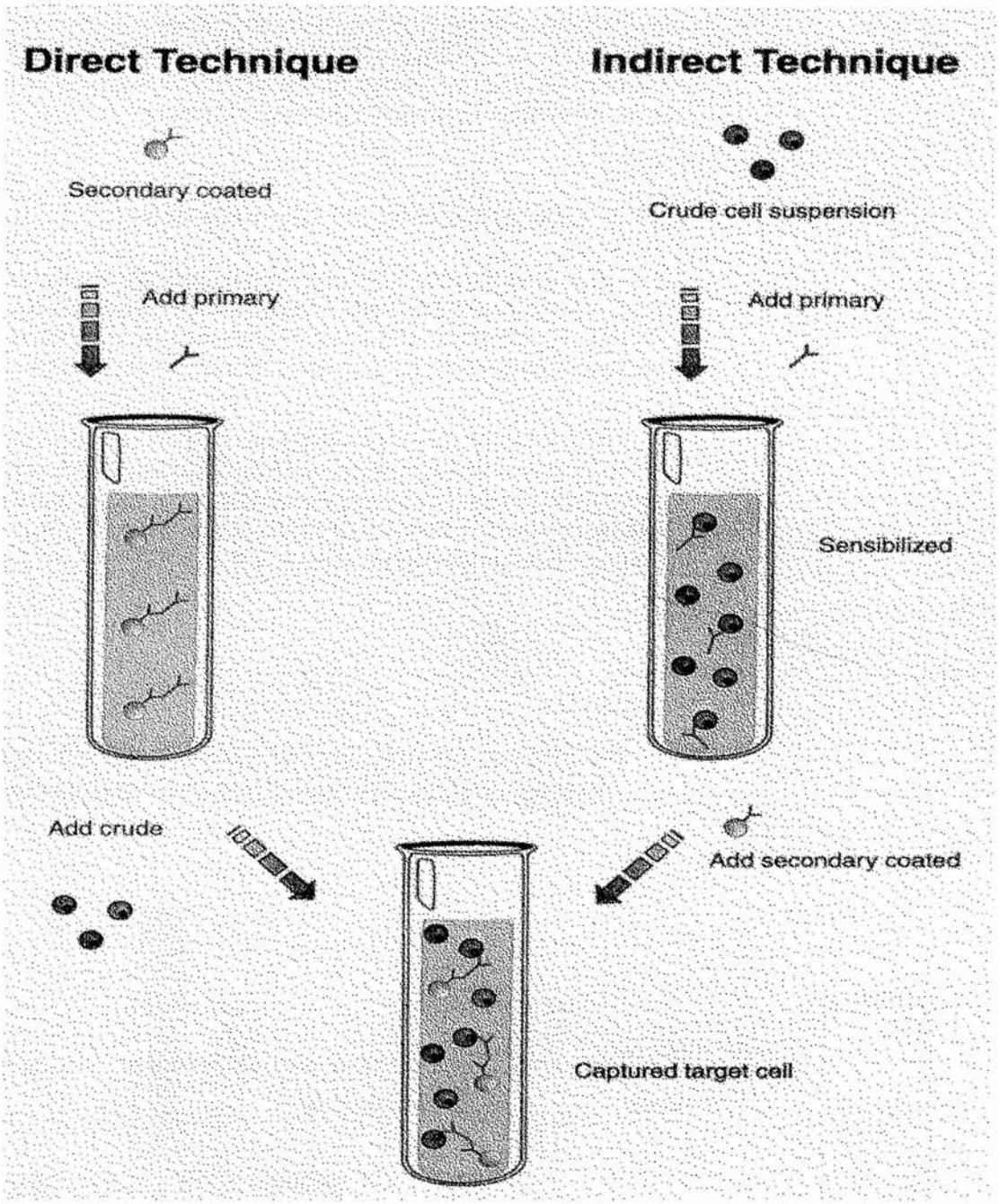


Fig 5.2. Diagramatic representation of the direct and indirect methods of IMS. (from Cell Separation and Protein Purification, Technical Handbook, second edition, Dynal)

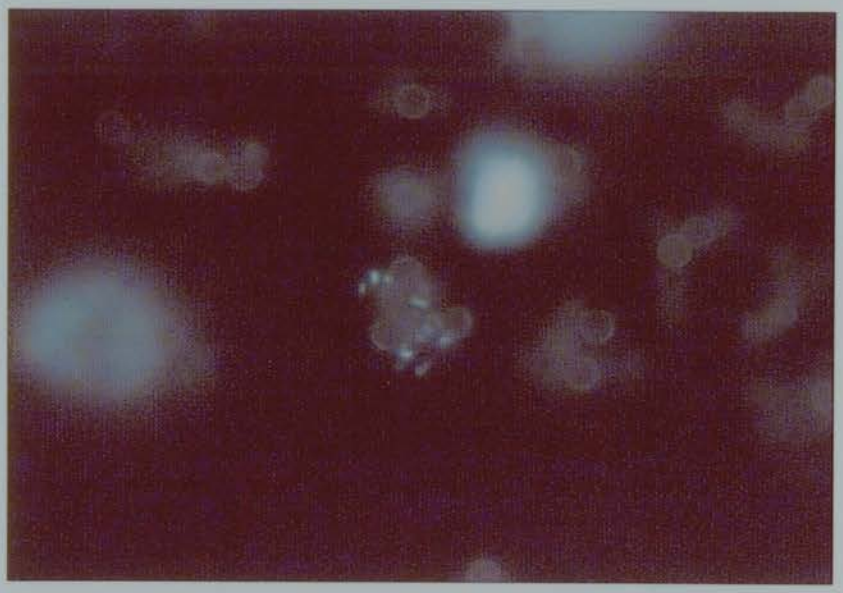


Fig 5.3. Photograph showing acridine orange stained A1 bound to immunomagnetic beads. Observed at x100 magnification and image captured with a single chip colour camera (Hitachi KPC 571) and image printed using a videoprinter (Sony CVP-ML).

SEROTYPE	DILUTION	DIRECT PLATING NUMBER OF COLONIES	IMS NUMBER OF COLONIES
A1	10^{-5}	CONFLUENT	CONFLUENT
	10^{-6}	CONFLUENT	CONFLUENT
	10^{-7}	12	4
	10^{-8}	3	10
	10^{-9}	0	4
	10^{-10}	0	35
A2	10^{-5}	CONFLUENT	CONFLUENT
	10^{-6}	CONFLUENT	CONFLUENT
	10^{-7}	17	31
	10^{-8}	3	12
	10^{-9}	0	1
	10^{-10}	0	1
T10	10^{-5}	CONFLUENT	CONFLUENT
	10^{-6}	CONFLUENT	CONFLUENT
	10^{-7}	23	CONFLUENT
	10^{-8}	6	80
	10^{-9}	1	11
	10^{-10}	0	12

Table 5.1. Comparison of direct plating method and recovery by IMS in different dilutions of *P. haemolytica* and *P.trehalosi* serotypes. Samples were diluted and half of the sample (0.5 ml) was exposed to the direct method of IMS isolation. When this was complete all samples were plated onto blood agar for colony counting.

The specificity of the anti-serotype antibody-coated beads was investigated in mixtures of all three serotypes with *E. coli* as a control in various combinations (Table 5.2). IMS with beads coated by A1or A2 antisera was successful in the specific isolation of the respective serotype only in the presence of each other. When present in the mixture serotype T10 or *E.coli* were always isolated with the target organism or were isolated in pure culture. The reverse was not true for T10 anti-beads which recovered T10 and no A types however when *E.coli* was present this was always isolated.

BACTERIA	ANTISERUM	BACTERIA RECOVERED
A1	A1	A1
A1, *E. Coli*		A1, *E. coli*
A1, A2		A1
A1, A2, T10		A1
A1, T10		A1, T10
A2	A2	A2
A2, *E.coli*		A2, *E.coli*
A1, A2		A2
A2, T10		T10
A1, A2, T10		A2, T10
T10	T10	T10
T10, *E. coli*		T10, *E. coli*
T10, A1		T10
T10, A2		T10
T10, A1, A2		T10

Table 5.2. Assessment of the specificity of each antiserum coated bead for the specific target in a mixture of bacteria. Using the direct IMS technique after mixing together various serotypes and *E. coli* the specificity of the antibody for the target organism could be made.

5.4. Reduction of non-specific binding

Treatments which had reduced or eliminated non-specific binding in other studies were assessed using A1 antiserum coated beads in an A1/*E. coli* mixture (Table 5.3).

ANTISERUM	TREATMENT	BACTERIA RECOVERED (number of colonies)
A1	PBS/Tw20 WASH	A1 (40), *E. coli* (8)
A1	PBS WASH	*E. coli* (10)
BEADS ALONE	PBS/Tw20 WASH	*E. coli* (2)
BEADS ALONE	PBS WASH	*E. coli* (1)
A1	SKIMMED MILK BLOCK	*E. coli* (30)
A1	RABBIT SERUM BLOCK	*E. coli* (50)
A1	NORMAL SHEEP SERUM BLOCK	*E. coli* (30+)
A1	HORSE SERUM BLOCK	*E. coli* (70+)
A1	NEWBORN CALF SERUM BLOCK	*E. coli* (150+)
A1	PROTAMINE	0
A1	GELATIN	*E. coli* (20)
A1	NONINDET P40	A1 (20), *E.coli* (15)
A1*	PBS/Tw 20	A1 (100), *E. coli* (1)

Table 5.3. The effects of various detergent washes, blocking steps and treatments on the recovery of A1 from a mixture of A1 and *E. coli* with A1 antiserum coated beads. A1*, A1 antiserum further absorbed against *E. coli, M. ovis* and *M. bovis*.

Addition of the detergent Tween 20 gave the best recovery by reducing the A1 to *E.coli* ratio to 40:8 whereas PBS alone produced only *E. coli* and the detergent Nonidet P40 was not as successful. The blocking steps had an increased recovery

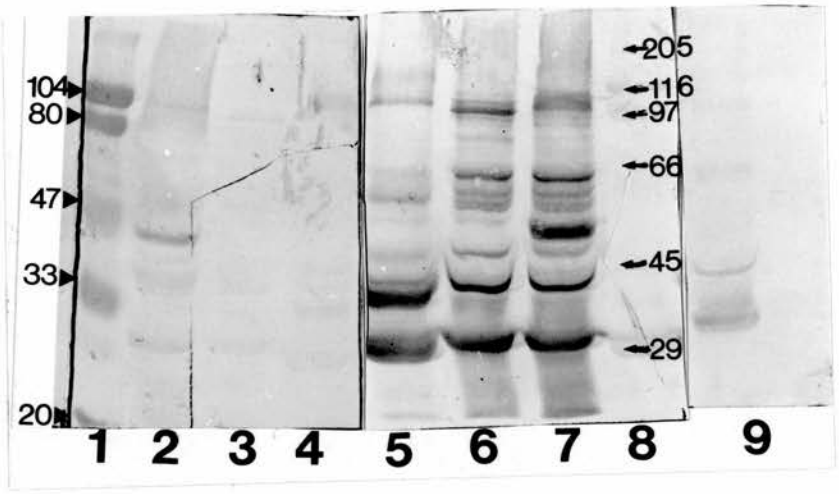
effect on *E. coli,* which was also shown to bind uncoated beads. Protamine also had a deleterious effect by reducing recovery to zero. Although the PBS/Tween recovery ratio of 40 A1 colonies to 8 *E. coli* colonies was a substantial reduction, further reductions in the *E. coli* count were considered essential.

Fig 5.4; 5.5 and 5.6 show the cross-reactions of the absorbed antiserum when reacted with envelope preparations of the three serotypes. It was evident that cross-reacting proteins were still recognised by the adsorbed antiserum and in turn these may be generally cross-reactive with other gram-negative bacteria. This could explain the isolation of *E. coli.* Another adsorption of the antiserum against *E. coli*, *M. ovis* and *M. ovis* cells was carried out and this resulted in a significant reduction of *E. coli* recovery (Table 5.3).

Fig 5.4. Western blots using pre bleed (lane 9), pre adsorbed (lanes 5-7) and adsorbed (lanes 2-4) rabbit antiserum raised against serotype A1 and used to probe envelope preparations of A1, A2 and T10 to monitor closely cross reating antibodies.
Molecular weight markers (lane 1), A1 (lane 2), A2 (lane 3), T10 (lane 4), T10 (lane 5), A2 (lane 6), A1 (lane 7), molecular weight markers (lane 8) and A1 (lane 9).

Fig 5.5. Western blots using pre bleed (lane 9), pre adsorbed (lanes 5-7) and adsorbed (lanes 2-4) rabbit antiserum raised against serotype A2 and used to probe envelope preparations of A1, A2 and T10 to monitor closely cross-reating antibodies.
Molecular weight markers (lane 1), A1 (lane 2), A2 (lane 3), T10 (lane 4), T10 (lane 5), A2 (lane 6), A1 (lane 7), molecular weight markers (lane 8) and A1 (lane 9).

Fig 5.6. Western blots using pre bleed (lane 1), pre adsorbed (lanes 2-4) and adsorbed (lanes 6-8) rabbit antiserum raised against serotype T10 and used to probe envelope preparations of A1, A2 and T10 to monitor closely cross-reating antibodies.
T10 (lane 1), T10 (lane 2), A2 (lane 3), A1 (lane 4), molecular weight markers (lane 5), T10 (lane 6), A2 (lane 7), A1 (lane 8) and molecular weight markers (lane 9).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205
116
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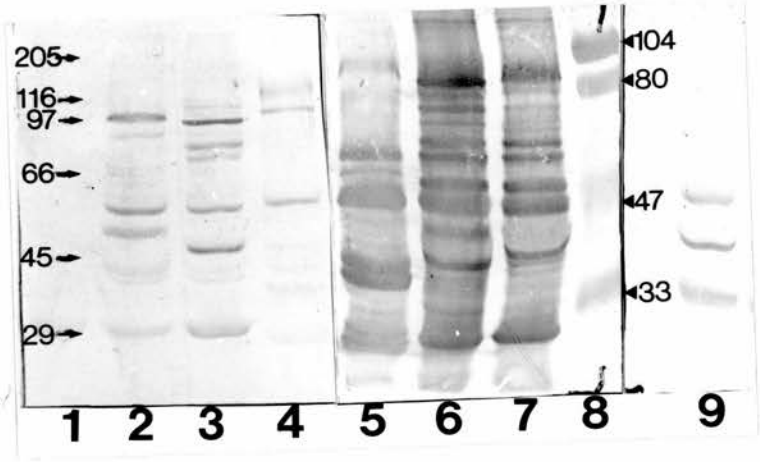
66
45
29
1 2 3 4 5 6 7 8 9


205
116
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66
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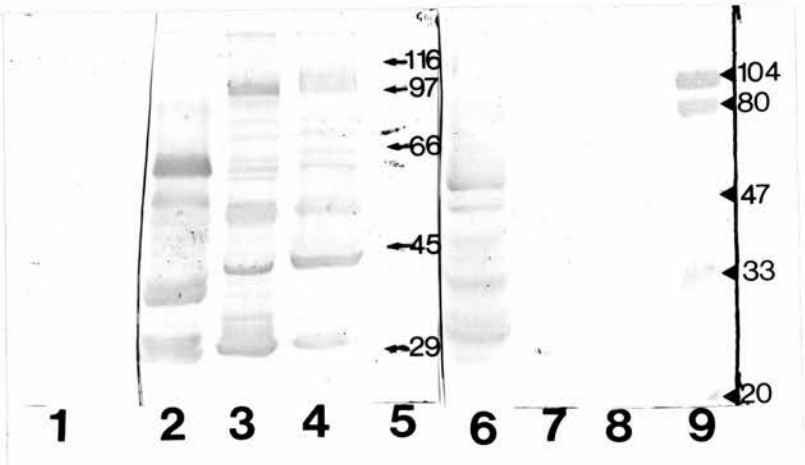
104
80
47
33
1 2 3 4 5 6 7 8 9


116
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1 2 3 4 5 6 7 8 9

Monoclonal antibodies were also investigated to see if the increased specificity of the antibody was an important factor in recovery. Table 5.4 shows that cross-reactions with T10 still occurred and the monoclonal antibodies against A2 were very poor. Both monoclonal antibodies against A1 capsule and A1 LPS were very effective. The simplicity of the indirect technique using Mab 7/45 meant that the antiserum was replaced with the Mab for further isolation of A1.

BACTERIA	MONOCLONAL ANTIBODY	RECOVERED BACTERIA
A2	32/4 ANTI A2 LPS	A2
A1, A2		A2
A2, T10		T10
A1, A2, T10		T10
A2	32/2 ANTI A2 30/40K LPS	A2
A1, A2		A1
A2, T10		T10
A1, A2, T10		A1, T10
A2	36/30 ANTI A2 40K LPS	A2
A1, A2		A1
A2, T10		T10
A1, A2, T10		A1
A2	36/34 ANTI A2 LPS	A2
A1, A2		A1
A1, T10		A1
A1, A2, T10		A1
A2	36/2 ANTI A2 40K	A2
A1, A2		A1
A2, T10		T10
A1, A2, T10		A1, T10
A2	37/2 ANTI A2 40K	A2
A1, A2		A1
A2, T10		T10
A1, A2, T10		A1
A2	7/13 ANTI A1 CAPSULE	A1
A1, A2, T10		A1
A1	7/45 ANTI A1 LPS	A1
A1, A2, T10		A1

Table 5.4. Table showing the specificity of recovery of target serotypes when beads are coated with monoclonal antibodies.

5.5 In-vivo isolation

The initial recovery of bacteria from animal tissue was very successful (Table 5.5). A1 was recovered easily from lung tissue (which appeared normal on gross inspection) of an experimental calf (C284) challenged with the same A1 strain in an earlier experiments.

ANIMAL NUMBER	TISSUE	ANTISERUM	BACTERIA RECOVERED
C284	CRANIAL LUNG	A1	A1
C284	CAUDAL LUNG	A1	A1
C282	CRANIAL LUNG	A1	0
C282	CAUDAL LUNG	A1	0
127	NASAL SWAB	A2	MIXED NASAL FLORA
268	NASAL SWAB	A2	MIXED NASAL FLORA, A2
98	NASAL SWAB	A1	MIXED NASAL FLORA
112	NASAL SWAB	T10	MIXED NASAL FLORA

Table 5.5. Table showing results of in-vivo isolation of target bacteria. All antiserum was absorbed polyclonal from rabbits as described in section 5.4 except for A1 which was the monoclonal antibody 7/13.

Calf 282 did not yield any organisms and was shown to be clear of bacterial infection by all methods employed. A1 recovery from a nasal swab of a lamb challenged with A1 bacteria did not produce any target bacteria. This was true also for the other nasal swabs except for animal 268 where A2 was identified along with other nasal bacterial flora. These results did not differ from those obtained by direct plating of nasal swabs. It was not definitely known if these animals were colonised and so the success of the experiment at this time was unknown.

5.6. Release of captured bacteria

It appeared that direct sampling of infected tissues was effective in isolating in-vivo organisms. Initial experiments on the analysis of these complexes was problematic as BSA and antibody in the mixture interfered with the analyses. This was rectified by omitting BSA from the post-capture steps and introducing procedures which detach the bacteria from the beads while leaving the antibody still attached through cross-linking (see Fig 5.7).

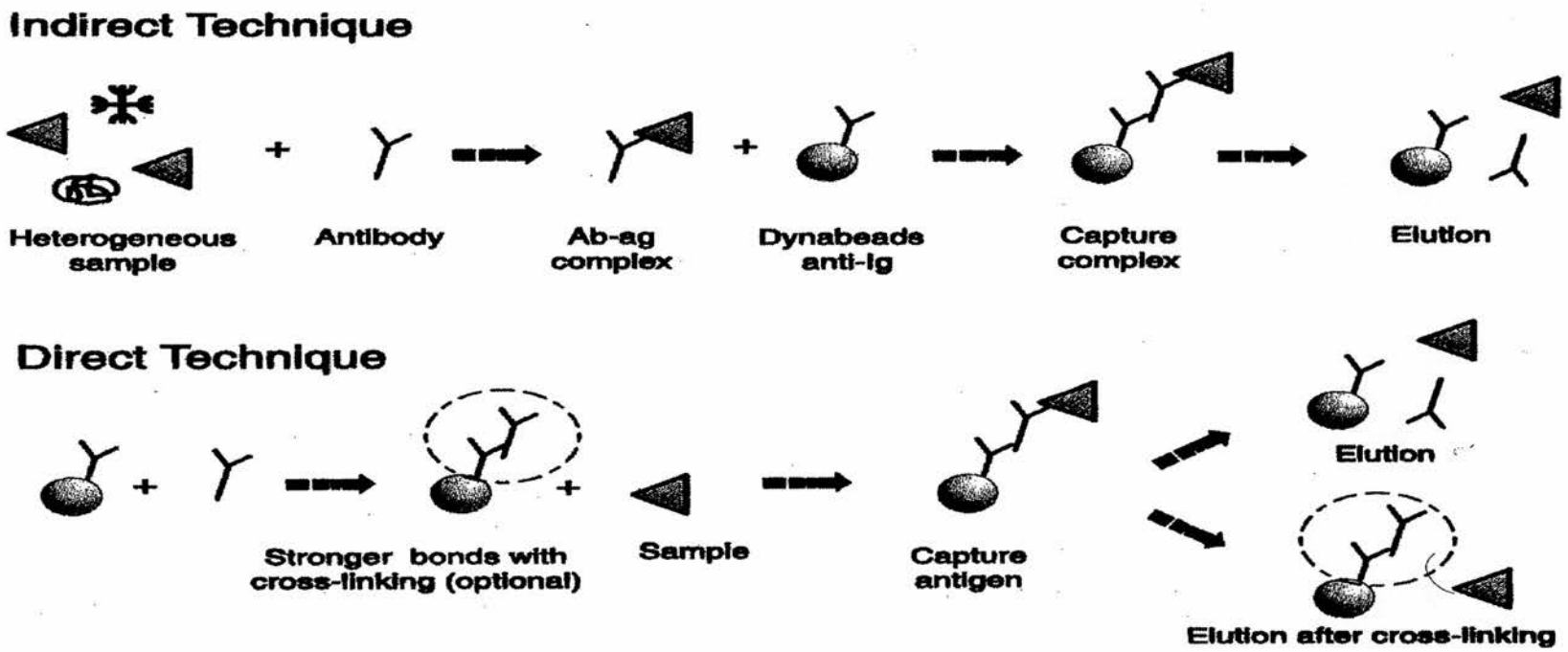


Fig 5.7: Graphic representation of the crosslinking and elution procedure (taken from Cell Separation and Protein Purification, Technical Handbook, second edition, Dynal).

In order to separate the bacteria from the antibody the principles of affinity column elution were applied. Standard elution buffers were added to the bead/target mixture. The beads were then separated using the magnet and these and the supernatant (containing bacteria but no antibody) fractions were compared for the presence of viable bacteria (Table 5.6).

TREATMENT	LOCATION OF BACTERIA	
	BEADS	SUPERNATANT
ACID GLYCINE (2.5M)	++	-
TRIETHYLAMINE (pH 11.5)	++	++
LITHIUM CHLORIDE (5M)	++	++
MAGNESIUM CHLORIDE (3.5M)	++	++
1% SDS	-	-
2M UREA	++	++
WATER	++	++
50% ETHYLENE GLYCOL (pH 11.5)	++	++

Table 5.6. The effects of elution reagents on the recovery of target bacteria. ++, substantial bacterial growth; -, indicates no growth.

Acid glycine did not allow separation of the bacteria from the antibody/bead complex. Using SDS both fractions resulted in loss of viability. All other treatments were effective but still appeared to retain as many bacteria as were eluted.

Using selected elution solutions this technique was applied to infected lung tissue for evaluation for in-vivo use. This time the tissue pellet was compared as well. Table 5.7 shows clearly that water, urea and magnesium chloride gave the greatest recovery of bacteria in the supernatant. Although there were more bacteria recovered in the supernatant fraction than the bead fraction with magnesium chloride and urea it was

decided to use water as this would have the least effect on the bacterial structures during elution.

TREATMENT	LOCATION OF BACTERIA		
	TISSUE PELLET	BEADS	SUPERNATANT
TRIETHYLAMINE	+++	+	++
MAGNESIUM CHLORIDE	+, CONTAMINANTS	++	+++
UREA	+, CONTAMINANTS	++	+++
WATER	+++	+++	+++
ETHYLENE GLYCOL	+++	+	-

Table 5.7. Comparison of the effect of elution reagents on the recovery of target bacteria directly from tissue and after crosslinking the specific antibody to the beads.

The crosslinking method using water as an elutant was applied to post mortem specimins used earlier in the study (except for the calf tissue).

ANIMAL NUMBER	TISSUE	CAPTURE ANTIBODY	DETECTABLE IN TISSUE	DETECTABLE BY BEADS
C35	CRANIAL LUNG	M Ab 7/13	+ (A1)	+ (A1)
C35	CAUDAL LUNG	M Ab 7/13	+ (A1)	+ (A1)
C35	TRACHEA	M Ab 7/13	+ (A1)	+ (A1)
C35	TRACHEA	M Ab 7/13	+ (A1)	+ (A1)
127	LUNG	SERUM	+ (A2)	+ (A2)
268	LUNG	SERUM	-	-
268	NASAL TURBINATE	SERUM	+ (A2)	+ (A2)
268	NASAL SEPTUM	SERUM	-	-
268	TONSILS	SERUM	+ (A2)	+ (A2)
112	LUNG	SERUM	-	-
112	NASAL TURBINATE	SERUM	-	-
112	NASAL SEPTUM	SERUM	-	-
112	TONSILS	SERUM	+ (T10)	+ (T10)

Table 5.8. Isolation of in-vivo bacteria from post mortem tissues prior to analysis using crosslinking of antibody and water as an elutant.

Table 5.8 shows the ability to detect pasteurellae straight from tissue homogenate which was plated onto blood agar and from beads which were mixed with tissue homogenate. A1 was isolated from different areas of the lung and the trachea of an experimentally infected animal. Lambs 127, 268 and 112 were the lambs used for convalescent antiserum production and at post mortem, lung, nasal septum, turbinates and tonsils were removed and prepared for isolation of target bacteria with IMS. Table 5.8 also shows that A2 was isolated from the lung of lamb 127 and from turbinates and tonsils of Lamb 268. T10 was also isolated from the tonsils of 112. The latter two animals were colonised.

5.7. Analysis of isolated in-vivo bacteria

Recovered bacteria were separated from the beads and analysed (after checking for purity by culture on blood agar) by SDS PAGE and Western blotting with in-vitro grown whole bacteria as a comparison.

When compared to in-vitro grown cells (Fig 5.8), serotype A1 from pneumonic lung tissue gave a protein profile which differed slightly. Three high molecular weight proteins were observed in the in-vivo bacteria (approx. 205, 200, >205 kDa) and these were not present in the in-vitro bacteria. Other proteins not present in-vitro were

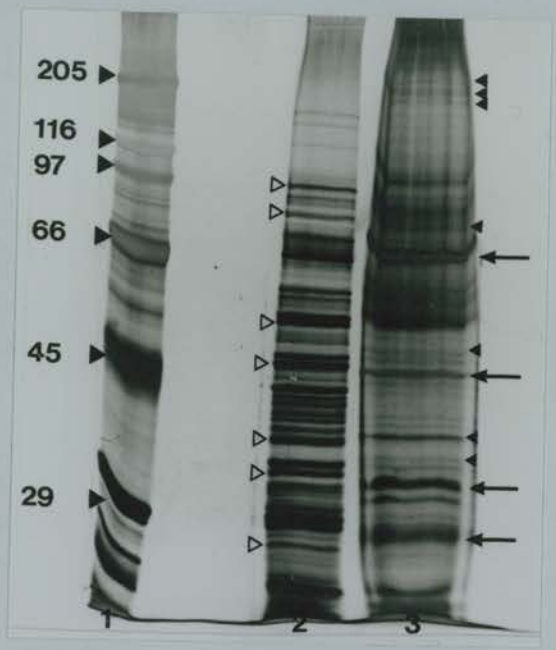


Fig 5.8. Silver stained SDS PAGE of serotype A1 whole cells from in-vivo and in-vitro growth.

Molecular weight markers (lane 1), A1 from broth culture (lane 2) and A1 from pneumonic lung tissue (lane 3). ▷ denoted bands present in broth cultured bacteria which are not present in in vivo bacteria, ► denoted bands present in in vivo bacteria which are not present in broth cultured bacteria, → indicates increases in proteins.

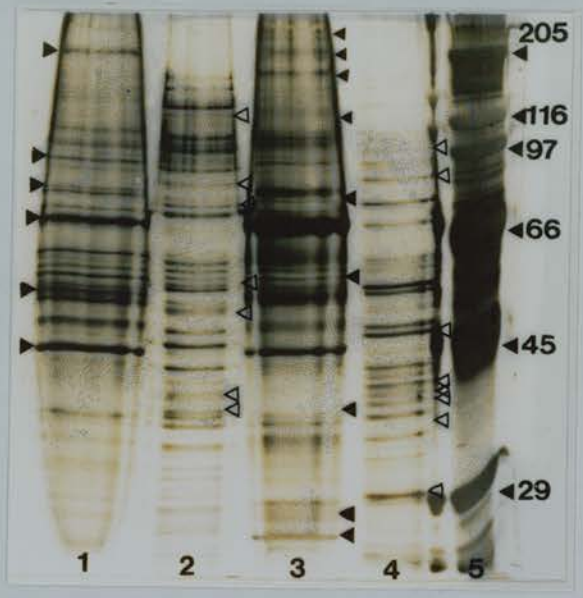


Fig 5.9. Silver stain of SDS PAGE of in-vivo and in-vitro isolated A2 and T10 whole cells.

T10 from tonsils (lane 1), T10 from TSB (lane 2), A2 from nasal turbinates (lane 3), A2 from TSB (lane 4) and molecular weight markers (lane5). ▷denoted bands present in broth cultured bacteria which are not present in in vivo bacteria, ► denoted bands present in in vivo bacteria which are not present in broth cultured bacteria. → indicates increases in proteins.

approximately 68, 48, 38 and 40 kDa. Many proteins, however, seemed to be missing from the in-vivo bacteria when compared with the in-vitro bacteria (approx. 100, 70, 50-55, 45, 37, 36 and 29). There were also some notable increases of protein at molecular weights of 66, 45, 35 and 30 kDa. Whether these were increases in existing proteins or new proteins is unknown. Serotype A2 isolated from nasal turbinates (Fig 5.9) also had high molecular weight proteins at 205 kDa and greater which did not appear in in-vitro samples. New proteins of approximately 120, 70, 50, 35, 28 and 17 kDa were apparent however, while proteins at 100, 80, 79, 45, 35-40 and 30 kDa were present in in-vitro but not in in-vivo samples. Serotype T10 from tonsils (Fig 5.9) had proteins of approximately 100, 68, 50 and a high molecular weight protein ~205 kDa present in in-vivo bacteria. In-vitro bacteria possessed proteins not present in in-vivo samples at 120, 80, 70, 52, 50, 40 and 35 kDa. Both A2 and T10 in-vivo bacteria showed increases in molecular weight regions of 66 and 45 kDa and these were the same as two of the increased proteins seen in A1.

Western blots of serotype A1 (Fig 5.10) in-vivo and in-vitro bacteria probed with anti-A1 sheep convalescent serum showed very little recognition of any in-vivo protein bands. Those which were recognised were also present in the in-vitro sample.

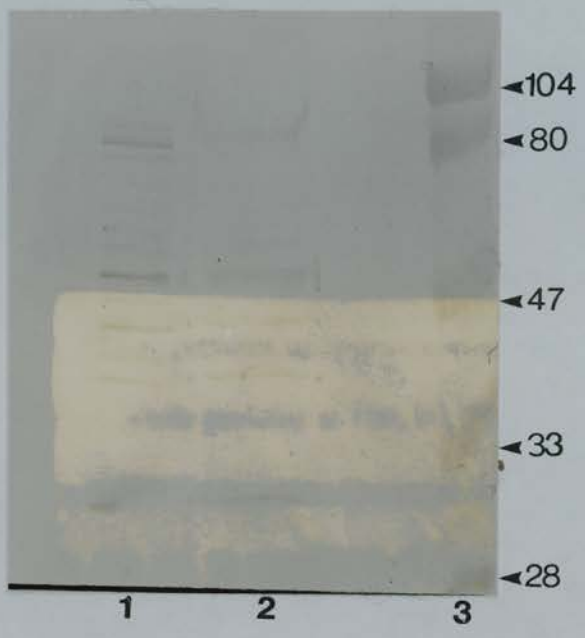
Fig 5.11 shows results for the same experiment with A2 probed with anti-A2 sheep convalescent serum. Again, recognition of proteins is very low in the in-vivo samples. One protein, however, was strongly recognised at approximately 40 kDa which is not equally recognised in the in-vitro sample.

Fig 5.10. Western blot of in-vivo and in-vitro A1 samples probed with convalescent lamb serum raised against serotype A1 (chapter 3).

A1 in-vitro (lane 1), A1 in-vivo (lane 2) and molecular weight markers (lane 3).

Fig 5.11. Western blot of in-vivo and in-vitro A2 samples probed with convalescent lamb serum raised against serotype A2 (chapter 3).

A2 in-vitro (lane 1), A2 in-vivo (lane 2) and molecular weight markers (lane 3).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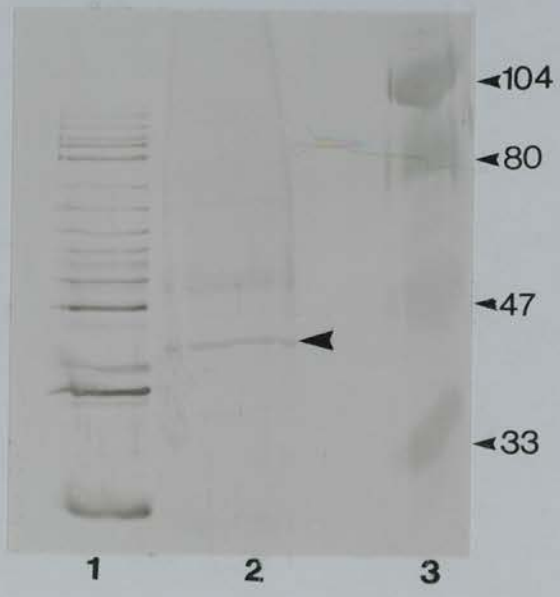
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5.8. Discussion

The application of the IMS technique to *P. haemolytica* and *P. trehalosi* was relatively successful. It was determined that the technique could detect the presence of serotypes at dilutions which conventional counting methods could not. Wright *et al.*, (1994) and others (Olsvik *et al.*, 1994; Safrik *et al.*, 1995) have shown that *E. coli* O157 isolation by IMS was more sensitive than direct culture. Cujoe *et al.* (1994) found that the subsequent culture was also important in detecting the target organism. Using IMS for the capture of salmonellae in foods he found that the technique was improved when the beads were plated onto XLD after capture, rather than direct culture on a selective agar. Authors such as Vermunt *et al.*(1992) found that IMS recovery of the target can be lower than in other systems available due to loss of bacteria during washing steps, with inhibited growth due to attachment to the bead. Prolonged incubation was found to increase numbers of salmonellae though recovery of non-target organisms showed an increase.

There was a problem with non-specific binding in this study with serotype T10 and *E. coli*. The nature of the binding is unknown and did not happen with A1 and A2 which would have been expected due to the apparent cross-reactivity between the two and their close relation to each other in comparison to T10 and *E. coli*. The situation with the non-specific binding was not improved with the introduction of monoclonal antibodies, although A1 anti-LPS and anti-capsule Mabs did appear to be superior in their affinity for the serotype and a lack of cross-reaction. *E. coli* had a binding affinity for the beads alone. Johne & Jarp (1988) found that although *S. aureus* did not bind straight onto the beads, if coated in FCS they would. This caused the contaminating presence of coagulase negative staphylococci, *Streptococcus* spp and *E.*

coli. Blocking steps had no effect on the non-specific binding even though authors such as Vermunt *et al.*(1992) and Mullins *et al.*(1995) found them successful. Some treatments and detergents such as the use of protamine (Okrend *et al.,* 1992) or NP-40 (Morgan *et al.,* 1991) have been attempted to increase recovery and reduce non-specific binding. In this study protamine resulted in no bacterial recovery and the NP-40 was no different from any of the other detergents. Wipat *et al.* (1994) stated that detergents can be used to reduce non-specific binding but that they can also affect the viability of the bacteria. As the use of Tween-20 has been shown to reduce contamination consistently (Wright *et al.,* 1994; Olsvik *et al.,* 1994; Safrik *et al.,* 1995) and was the most successful treatment in this study (even though not 100%) this technique was employed. The Western blots of envelopes from the three serotypes probed with the absorbed rabbit antiserum used for immunocapture do point to cross-reacting antigens remaining which may be why they were not particularly specific. Once further absorption was carried out, the non-specific binding was reduced to levels which were acceptable as far as contaminating organisms were concerned. It must be noted that a panel of non-related bacteria has not been screened and this could pose problems in the future. Concurrent direct plating of immunocaptured samples however, would always check this situation if it arises as contaminants would be observed.

Initial in-vivo isolation from lung samples of calves experimentally challenged with serotype A1 was extremely successful. All sites yielded pure cultures of *P. haemolytica* A1 whereas direct plating produced, as well as *P. haemolytica*, colonies which were regarded as contaminants. The ease by which contaminants were excluded using IMS could be due to the numbers present in this instance. In the case of pneumonic lung tissue the dominant organism present is that which is responsible for

the pneumonia, in this case *P. haemolytica*. The competition for binding sites on the beads is higher for non-target organisms. The same in-vivo isolation procedure was carried out on nasal swabs in an attempt to isolate colonising organisms. All animals produced mixed nasal flora from direct plating and from IMS isolation. Lamb 268 did have A2 colonies present but this was overwhelmed by other nasal bacteria. This is the opposite situation than that encountered with the lung tissue isolation.

P. haemolytica is probably in the minority in the total population of the nasal passages and although the specificity of the antibody on the beads may be higher for *P. haemolytica* the presence of many other organisms is greater and may well increase the likelihood of non-specific binding.

The later tissue analysis at necropsy sheds some light on why isolation from swabbing was not successful. Serotype A2 (127) was present only in the lung and T10 only in the tonsils which would not have been reached by swabbing. In lamb 268, A2 was present in the turbinate area and not the septum. A2 was previously isolated with mixed flora from nasal swabs and this result indicates that A2 was present alone in the turbinate while absent from the septum area. Quirie (1984) found that A2 was isolated commonly from ventral nasal meatus, floor of nasopharynx, lateral wall of nasopharynx and lateral wall of oropharynx but not from the dorsal nasal meatus, ventral turbinate (rostral and caudal) nasal septum or lateral wall of nasopharynx (opposing membranes pharyngeal septum). It is likely that the original swab was taken from an area of the nasal cavity in which A2 was present with other flora. Pass & Thompson (1971) pointed out that a single swabing was not representative of the nasal cavity as a whole. Magwood *et al.* (1969) showed that *P. haemolytica* was capable of dominating the nasal flora for several days at a time. Magwood's study was confirmed by Quirie (1984) who showed that the bacterial population could fluctuate

in species and numbers present. What could have happened with lamb 268 is that between swabbing and necropsy (assuming the swab was taken from the same site sampled at necropsy) the flora of the nasal cavity had changed dramatically with A2 present as the dominant bacteria. If it can be assumed that the sites of swabbing and necropsy tissue are not the same it calls into question the accuracy of swabbing as an indicator of the presence of certain bacteria in the nasal cavity and this is in absolute agreement with the findings of Pass & Thompson (1971).

The cross-linking procedure and subsequent elution of bacteria free of antibody and BSA, added to the clear visualisation of proteins on SDS PAGE. A1 organisms from pneumonic tissue differed markedly from in-vitro grown organisms, but convalescent antiserum did not recognise any proteins in the in-vivo sample that were not recognised in the in-vitro sample. This indicates none of the induced proteins were recognised by the antiserum although subtle changes in protein epitopes or glycosylation may mask them from antibodies. It may also be the case that the bacterium (a bovine isolate) from bovine tissue will not react as well with ovine antiserum as it would with a bovine convalescent serum. Previous data (Chapter 3) suggested that sufficient antibody recognition had occurred. Davies (1994b) developed an intraperitoneal implant chamber for the study of A1 in-vivo organisms. Significant antibodies against *P. haemolytica* were present pre-inoculation and these antibodies did not change in content either quantitatively or qualitatively over the course of the experiment. The Western blot profiles were not compared to in-vitro grown organisms and as only outer membrane proteins were analysed and not whole organisms, as in this study, little comparison can be made with the findings in this work. Davies (1994a) examined lung isolates of *P. haemolytica* A1 and showed that bacteria had reduced expression of 31 and 39.5 kDa proteins with enhanced

expression of proteins at 71, 77 and 100 kDa. The latter three proteins were probably IRPs and although present in one of the strains examined were not as evident in another strain. Reduction of an 18 kDa protein and the induction of one at 19 kDa is quite important as is the 52 kDa protein in the lung cells in comparison to the 50 kDa protein in the in-vitro cells. Davies suggested that the 19 kDa protein is novel and that the reduced expression of the 18 kDa protein is the same as the 17 kDa protein described by Donachie and Gilmour (1988) present in serotype A2. It may be that all these proteins are functionally the same and the in-vivo conditions, strain variation or SDS PAGE conditions differing slightly will make all the difference when an individual interprets results. In light of this, the A1 proteins in this study at 68 kDa in in-vivo only bacteria and 29 and 37 kDa in in-vitro bacteria may be similar proteins to the 70, 31 and 39.5 proteins of Davies. Confer *et al.* (1992) also examined *P. haemolytica* A1 in chamber implants in calves. As whole cells were analysed it is probably more appropriate to this study although it is questionable whether chamber grown bacteria and lung isolated cells are representative of each other (Davies 1994a suggests that they are). Confer found that using broth and solid media grown bacteria as a comparison to in-vivo, all growth conditions produced encapsulation with similar LPS profiles. In-vivo bacteria produced extra proteins > 150 kDa. Rabbit antiserum did not recognise any bands > 100 kDa but antiserum from calves did, suggesting these were probably precursor proteins not produced in-vitro.

The SDS PAGE profile of T10 isolated from the tonsils differs from that of the organism grown in-vitro. In reviewing the literature no studies have been carried out on the analysis of in-vivo growth or isolation of *P. trehalosi.* The pathology of the disease makes in-vivo isolation from systemically infected animals difficult. *P. haemolytica* serotypes causing bovine pneumonia have been isolated using lung

washes (plenty of contaminating host molecules present) and implant chambers (which may not represent the state of the organism in the lung) and ovine pneumonia serotype isolation has used chambers and pleural fluid of infected animals. *P. trehalosi* is not always found in large numbers in the lung and direct isolation from blood, spleen, liver and other tissues would have been problematic. IMS has enabled the isolation of T10 directly from tissue and is probably of use in other areas. Mazurek *et al.* (1996) enhanced and enriched the detection of *Mycobacterium tuberculosis* from CSF using IMS and Johne & Jarp (1989) isolated *S. aureus* from milk.

Serotype A2 envelopes from pleural fluid organisms have been studied by Donachie and Gilmour (1988). The identification of 100, 70 and 17 kDa proteins in-vivo may correspond to proteins of 70 and 17 kDa in this study. Sutherland *et al.* (1990) identified proteins of 105, 100, 95, 70, 66, 53 and 23 kDa (the latter two proposed to be heavy and light chain portions of IgG). The 70, 50 and 35 kDa in this study could correspond to the 70, 53 and 28 of the above in-vivo bacteria. The in-vivo bacteria also lacked 36 and 24 kDa proteins which may be functionally similar proteins to those in this work of 30, and 35-40 kDa proteins. Western blotting was not carried out on these samples and no comparisons can be drawn. Donachie and Gilmour (1988) showed a 55 kDa protein recognised on Western blots which was not observed by SDS PAGE. The induced 100 kDa protein on the other hand did not react with antiserum (same result as Davies 1994a). The analysis of A2 and T10 in this study deals with colonising bacteria. Many of the proteins from all serotypes, differ from the in-vitro proteins (or vice versa) by only a few kilodaltons in molecular weight and this implies that they are possibly the same protein with slight changes such as glycosylation or binding of host components. It is possible that just as bacteria grown

in vitro differ from those grown in vivo, then colonising bacteria may be different from their pathogenic state. Those bacterial mechanisms which protect the organism from destruction by the host, such as mimicry of host structures and capsules may become much more important in a colonising state. Sialic acid has been shown on the LPS of serotypes A1 and A5. The presence of sialic acid in prokaryotes is rare and when present is usually a constituent component of the capsule (Utley *et al.,* 1992). The colominic acid present in the capsule of A2 is non-immunogenic and has been implicated in the lack of immunity observed in bacterin vaccines (Adlam *et al.,*1987). Leukotoxin at sub-inhibitory concentrations has been shown to downregulate MHC Class II molecules in macrophages and so will interfere with antibody recognition (Hughes *et al.,*1994). Sutherland *et al.*(1990) showed results similar to Confer *et al.* (1992) in that LPS and capsules were unchanged in in-vivo bacteria. Sutherland *et al* also found that A2 aggregated together and may have prevented host defence mechanisms from being effective in removal of the organisms. The lack of available nutrients as evidenced by low numbers of bacteria present during colonisation and competition from other resident microbial flora suggests that colonising bacteria are probably better represented by those bacteria grown under starvation, stress and biofilm situations. In nature bacteria hardly ever grow in single species colonies and 99% of the planet's bacteria are thought to live in biofilms. 30 to 40 % of the proteins in *Ps. aeruginosa* have been shown to differ from those in biofilms and from free living bacteria (Coghlan, 1996).

This study has shown differences between serotypes tested with respect to comparison of in-vitro and in-vivo produced organisms. Western blots showed very little recognition of in-vivo antigens and only A2 possessed an antigen not recognised in vitro (T10 was not tested). The antiserum used to probe for A2 was from the same

animal that the bacteria were isolated from. This situation raises questions of convalescent states in pasteurellosis. The animal has been shown to be immune to challenge, shows antibody recognition of envelopes and other antigens, leukotoxin neutralising capacity and is bactericidal (Chapter 3; M. Maley pers comm). This however, does not prevent colonisation and suggests that serum antibody is not important for removal of colonising bacteria. Mucous membranes rely on IgA for effective removal of bacteria, rather than IgG which is derived from serum. It is not known whether IgA prevents colonisation. Vaccination of calves has been shown to inhibit colonisation, only serum IHA titres though (to monitor capsule recognition) were measured as a response. The role of IgA therefore remains unknown. M^{c} Vey *et al.* (1990) showed IgA to be serotype specific and with changing mucosal flora it may be difficult to maintain protection. If a convalescent animal is predisposed by other infections or environmental factors the possibility it will succumb to pasteurellosis (if it is colonised) is of concern in protection against the disease.

This study needs more attention in the areas of defining more clearly the differences between in-vitro, colonising and disease-state bacteria. The question of prevention of colonisation is also important and this appears to lie in finding more information about the role of IgA in the mucosal areas of ruminants, what will stimulate IgA and if IgA is essential in prevention of colonisation then the subsequent protection from pasteurellosis.

CHAPTER 6.

THE DETECTION AND CHARACTERISATION OF SUPEROXIDE DISMUTASE

6.1. Introduction

Investigations into superoxide dismutases in bacteria have shown some evidence to suggest that these enzymes may be important during disease especially in survival against the respiratory burst of phagocytic cells (Mandell, 1975; Beaman & Beaman, 1984). The identification of a copper/zinc SOD (still a particularly rare metal active site for a bacterial SOD) in *Haemophilus* spp (Kroll *et al* ,1991) and then further evidence that these enzymes were present in many mucosal colonising bacteria (Kroll *et al.*, 1995) suggested that this may also be the case for *Pasteurella* spp.

6.2. Demonstration of SOD activity in *P. haemolytica* and *P. trehalosi*

Bacterial lysates of *P. haemolytica* serotypes A1 and A2 and *P.trehalosi* serotypeT10 were assayed for superoxide dismutase (SOD) activity using a native PAGE gel and staining system. SOD activity was detected readily in crude lysates of all three serotypes (Fig 6.1). Only one zone of enzyme activity was apparent in A1 and A2 serotypes, the former migrating more rapidly than the latter. By contrast two zones of activity were detected in the T10 serotype, one of which migrated similarly to the that of A1 and the other migrating to a point between those observed for A1 and A2. The levels of activity were measured using an assay which is more sensitive than PAGE

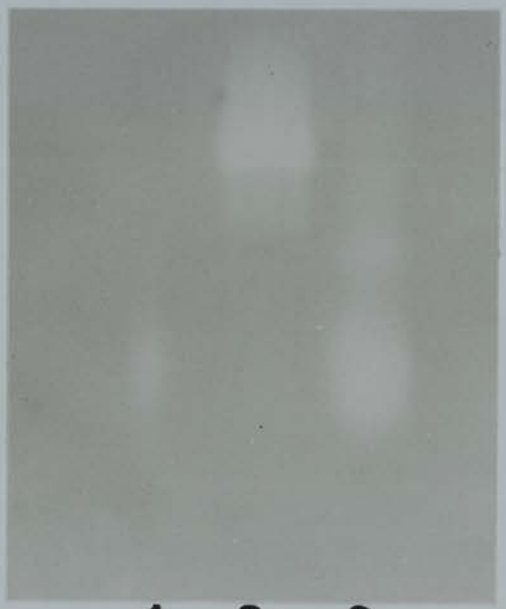


Fig 6.1. Native PAGE containing whole lysates of A1, A2 and T10 and stained for superoxide dismutase activity.

Serotype A1 (lane 1), Serotype A2 (lane 2) and Serotype T10 (lane 3).

but the differences in activity correlate with the strength of reaction observed on the gel. A2 activity (472.7 SOD units) was 27 times greater than A1 (17.35 SOD units) and 4 times greater than T10 (112.2 SOD units). This activity was not due to a greater protein concentration loaded onto the gel as A1, A2 and T10 had 0.15mg, 0.055mg and 0.045mg of protein loaded respectively.

6.3. Characterisation of SOD enzymes

Inhibition studies were used to identify the SOD isotype by specific inactivation (≥ 50%) of the metal active site. EDTA is used to commonly inhibit all SODs, H_2O_2 inhibits iron SODs and KCN inhibits copper/zinc SODs. Manganese SODs are resistant to the above allowing identification by a process of elimination.

Serotype	Control (H_2O)	KCN	H_2O_2	EDTA
A1	6.77 (0.7)	6.83 (0.6)	7.1 (0.5)	7.9 (4.2)
A2	274.82 (86.2)	51.23 (10.1)	296.32 (95.5)	221.32 (102)
T10	102.65 (28.2)	104.8 (27.9)	116.0 (32.9)	157.23 (71.9)

Table 6.1. Inhibition of SOD activity by various inhibitors.
Results are expressed as the means of four separate experiments (SOD units mg^{-1} protein) with the standard error of the mean in brackets. Inhibitors are at 5mM.

Table 6.1 (for raw data see Appendix II) shows that A2 was inhibited only by KCN (81.4%), thus defining it as a Cu/Zn SOD. This was also seen using the PAGE system (Fig 6.2) where other inhibitors had no effect. No inhibition of the SODs of any serotypes could be achieved with EDTA or H_2O_2 even at elevated molarities. Other metal chelators such as αα dipyridyl, EDDA and sodium azide (the latter an inhibitor of Fe and Mn SODs at 4 and 20 mM respectively) also failed to inhibit the enzymes.

Fig 6.2. Native PAGE showing A2 superoxide dismutase activity (lane 1) and loss of activity in the presence of potassium cyanide (lane 2).

6.4. Identification of *sod*C gene

The identity of the A2 SOD enzyme was confirmed by the development of a PCR based assay which recognises a fragment of the *sodC* gene (this gene codes for Cu/Zn SOD). Fig 6.3 shows the PCR product produced only in the lanes which contained A2 DNA with no product produced from samples of serotypes A1 and T10.

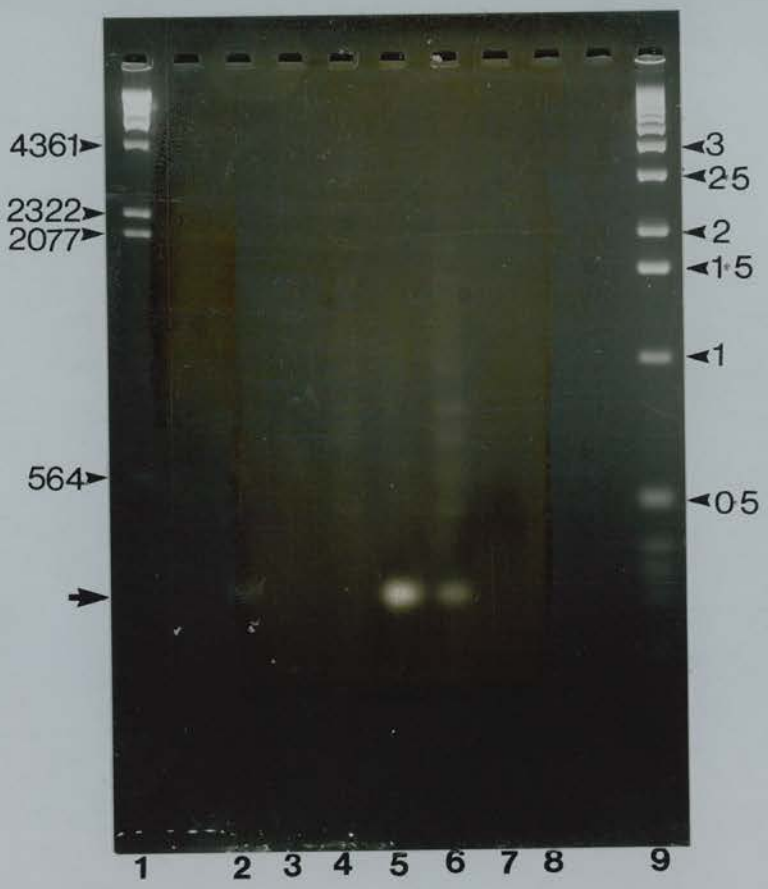


Fig 6.3. Agarose gel showing PCR product (arrow) of serotype A2 when amplified using nested primers specific for copper/zinc superoxide dismutase.

λ Hind III markers, bp (lane 1), positive control (a fragment of A2 which has been cloned following the production of a PCR product which has been confirmed by the use of nested PCR and Southern hybridisation to be part of the SOD gene) (lane 2), A1 (lanes 3 and 4), A2 (lanes 5 and 6), T10 (lanes 7 and 8) and DNA ladder markers, Kbp (lane 9).

6.5. Location of SOD

In order to identify the location of the enzyme in the bacterial cell, five methods were employed to obtain separate cytoplasmic and periplasmic fractions. The results can be seen in Table 6.2. With all three serotypes, those methods which employed the use of detergents produced unacceptable cytoplasmic contamination of the periplasmic contents. Chloroform proved slightly better but the high presence of MDH in the periplasm meant the correct location of the enzyme could not be determined with any confidence. The freeze-thaw technique, less harsh on the bacteria than detergents or solvents produced consistently low contamination of MDH in the periplasm of all serotypes. The SOD enzyme of serotypes A1and A2 were present in the periplasm 83.8% and 75.2% respectively, whereas the enzymes of T10 were predominantly present in the cytoplasm (59.1%). Although the amount of SOD in the periplasm of T10 was 40.9% and the activity of MDH was 33.3% the difference was not large enough to confidently consider that this serotype possessed a predominant periplasmic SOD.

SEROTYPE	METHOD	LOCATION	UNITS OF SOD ACTIVITY /mg PROTEIN	UNITS OF MDH ACTIVITY / mg PROTEIN	% SOD LOCATION	% MDH LOCATION
A1	CHAPS / LYSOZYME	PERIPLASM	8.8	0.07	57.3	92.1
A1		CYTOPLASM	7.6	0.006	46.3	7.9
A2		PERIPLASM	122.2	0.3	80.5	78.9
A2		CYTOPLASM	29.7	0.08	19.5	21.1
T10		PERIPLASM	97.1	0.2	85.3	66.7
T10		CYTOPLASM	16.8	0.1	14.7	33.3
A1	EDTA / LYSOZYME	PERIPLASM	124	0.2	86.5	99
A1		CYTOPLASM	19.3	0.002	13.5	1
A2		PERIPLASM	14.5	0.04	51.2	80
A2		CYTOPLASM	13.8	0.01	48.8	20
T10		PERIPLASM	93.1	0.3	77.9	97.1
T10		CYTOPLASM	26.4	0.009	22.1	2.9
A1	CHLORO-FORM	PERIPLASM	68.9	0.03	76.7	60
A1		CYTOPLASM	20.9	0.02	23.3	40
A2		PERIPLASM	475.3	0.07	87.7	58.3
A2		CYTOPLASM	66.6	0.05	12.3	41.7
T10		PERIPLASM	35.1	0.05	47.2	83.3
T10		CYTOPLASM	39.3	0.01	52.8	16.7
A1	OSMOTIC SHOCK	PERIPLASM	49.9	0.01	70.2	76.9
A1		CYTOPLASM	21.2	0.003	29.8	23.1
A2		PERIPLASM	3.8	0.009	26.9	31
A2		CYTOPLASM	10.3	0.02	73.1	69
T10		PERIPLASM	59	0.2	59.9	90.9
T10		CYTOPLASM	39.4	0.02	40.1	9.1
A1	FREEZE THAW	PERIPLASM	52.3	0.01	83.8	2.4
A1		CYTOPLASM	10.1	0.4	16.2	97.6
A2		PERIPLASM	475.3	0.009	75.2	23
A2		CYTOPLASM	156.5	0.03	24.8	77
T10		PERIPLASM	24.6	0.04	40.9	33.3
T10		CYTOPLASM	35.5	0.08	59.1	66.7

Table 6.2. Comparisons of different fractionation methods to determine the location of SOD using MDH as a marker of cytoplasmic contamination.

6.6. Effects of iron restriction on SOD

Movement of SOD enzymes into the periplasm under certain growth conditions has been shown (Barnes *et al* 1996 Schnell *et al* 1995). As the bacterial cell's response to iron restriction in vivo is an important aspect of pasteurellae infection lysates of iron-restricted (in 200μM EDDA) *P. haemolytica* and *P. trehalosi* cells were assayed for SOD. These were compared with bacteria cultured in normal media. The location of the enzyme was also investigated using the freeze thaw method which was shown previously to give favourable results.

Sample	Units of SOD activity/mg Protein	
	Iron replete	Iron deplete
A1	6.78	0.019
A2	153.39	2.653
T10	84.88	1.588

Table 6.3. Comparison of SOD activity from serotypes grown with and without iron restriction.

Sample	Units of SOD activity/mg protein	Units of MDH activity/mg protein	% location of SOD	% location of MDH
A1 periplasm	0.147	0.03	9.4	79
A1 Cytoplasm	1.411	0.008	90.6	21
A2 Periplasm	53.81	0.001	99.4	8
A2 Cytoplasm	0.341	0.012	0.6	92
T10 Periplasm	0.611	0.017	78.1	45
T10 Cytoplasm	0.171	0.021	21.9	55

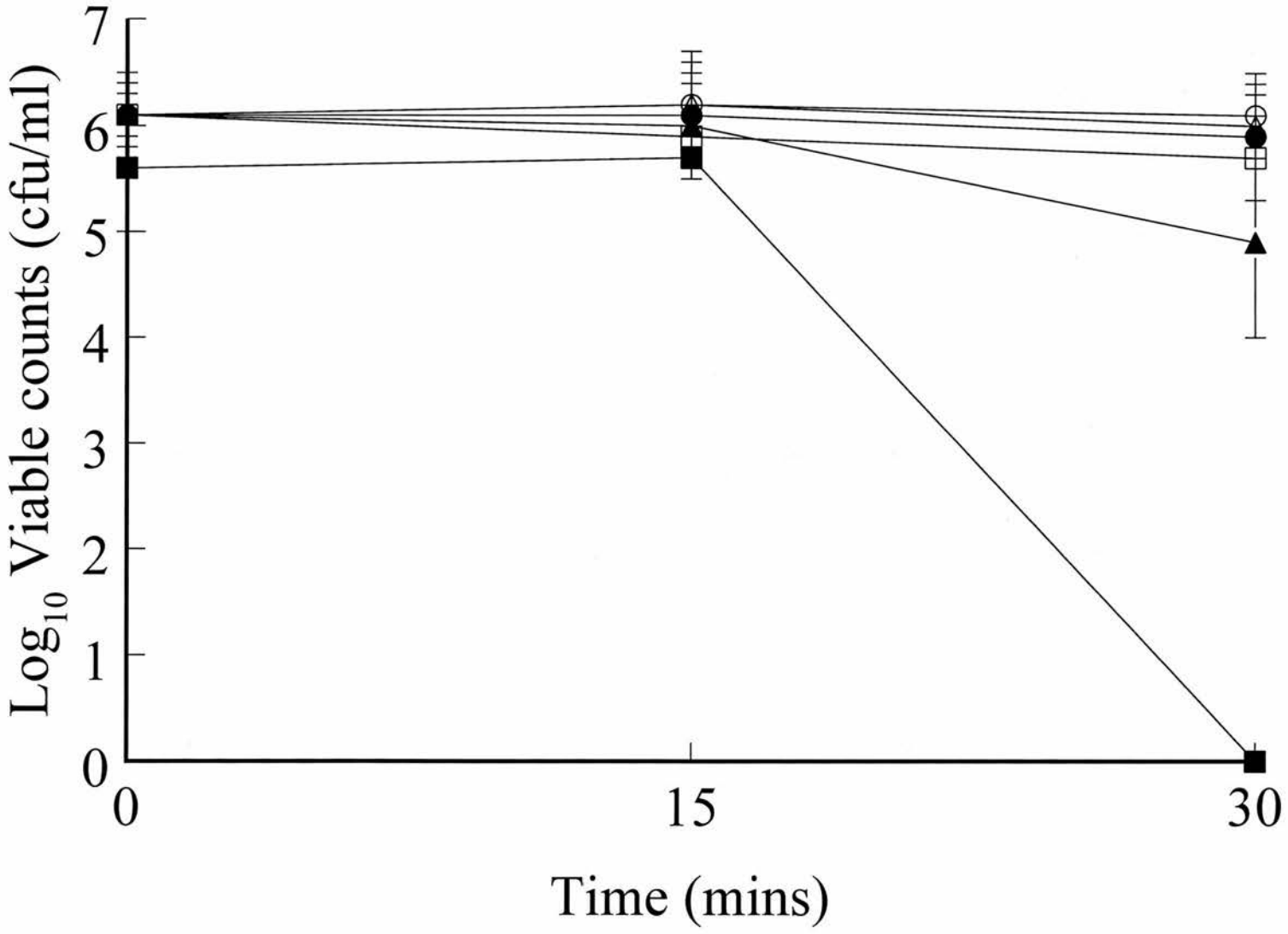
Table 6.4. Location of SOD activity from serotypes grown under iron restriction.

Table 6.3 shows the reduction in the units of SOD activity present in the bacteria. This was the situation for all of the serotypes when the bacteria are grown under iron restriction in comparison to those in ordinary culture. There is also a shift in the location of the enzyme and this can be seen in Table 6.4(for raw data see Appendix II). Most of the SOD activity in A1 is now restricted to the cytoplasm and in contrast, T10 now has predominantly periplasmic SOD. A2 SOD activity remains periplasmic.

6.7. Effects of exogenous superoxide on *P. haemolytica* and *P. trehalosi*

Changes in oxygen availability or environmental detection of superoxide may also trigger movement of the enzyme into the periplasm. A bactericidal assay was used to follow the survival of whole live bacteria in the presence of exogenous superoxide. This would indicate a possible mechanism of enzyme relocation into the periplasm under superoxide stress. Fig 6.4 shows that A2 and T10 had no significant difference in viability from that of the controls over a 30 minute period, a result which is consistent with the movement of the enzyme into the periplasm as seen under iron restriction. A1, however, did not survive 30 minutes in comparison to the control. When exogenous SOD was added into this milieu the effect on A1 was abrogated, showing that the loss of viability was due to damage caused by the presence of superoxide.

Fig 6.4. Survival of serotypes A1, A2 and T10 by viable count in the presence of exogenous superoxide. Controls (open symbols) omitted only xanthine oxidase and samples (bold symbols) contained xanthine oxidase to initiate superoxide generation. □, A1; Δ, A2; O, T10; ■, A1; ▲,A2; ●, T10.


7
6
5
4
3
2
1
0
Log10 Viable counts (cfu/ml)
0
15
30
Time (mins)

Lainson *et al* (1996) tested a large number of *P. haemolytica* and *P. trehalosi* serotypes for the prevalence of Cu/Zn SOD using correlation of PCR, southern hybridisation and inhibition with KCN. The results showed that all A2 strains tested were positive for the enzyme. A7 strains, however, gave both positive and negative results for Cu/Zn SOD suggesting another SOD type was present. It was thought that a comparison of these strains might give some information as to a specialised role for the Cu/Zn SOD. The bactericidal assay (Fig 6.5, for raw data see Appendix II) shows that strains M74 and L94f were still viable at 30 minutes and strains DO800 and L48c were not. The latter strains, however, were the strains which possessed the Cu/Zn SOD, so this does not correlate with the ability to survive the presence of exogenous superoxide. The importance placed on the presence and location of Cu/Zn SODs and the role of survival in macrophages was also investigated. Fig 6.6 (for raw data see Appendix II) shows survival in ovine alveolar macrophages. Although strain DO800 was still viable after 2 hours phagocytosis and is SOD positive, so was strain L94f and this strain is negative. The strains M74 (SOD negative) and L48c (SOD positive) also showed no survival following phagocytosis and do not correlate with the presence or absence of the enzyme. The pattern is similar in bovine alveolar macrophages (Fig 6.7, for raw data see Appendix II). Strains DO800 and L94f survived phagocytosis in bovine alveolar macrophages (the same was observed in OAM). M74 (Cu/Zn SOD positive) survived in the bovine cells and L48c (positive for Cu/Zn SOD) does not.

Fig 6.5. Survival of A7 strains in the presence of exogenous superoxide, two of which possess Cu/Zn SOD (DO800 and L48C) and two of which do not (M74 and L94F). Survival was monitored by viable counts. Controls are strains marked with an asterisk.

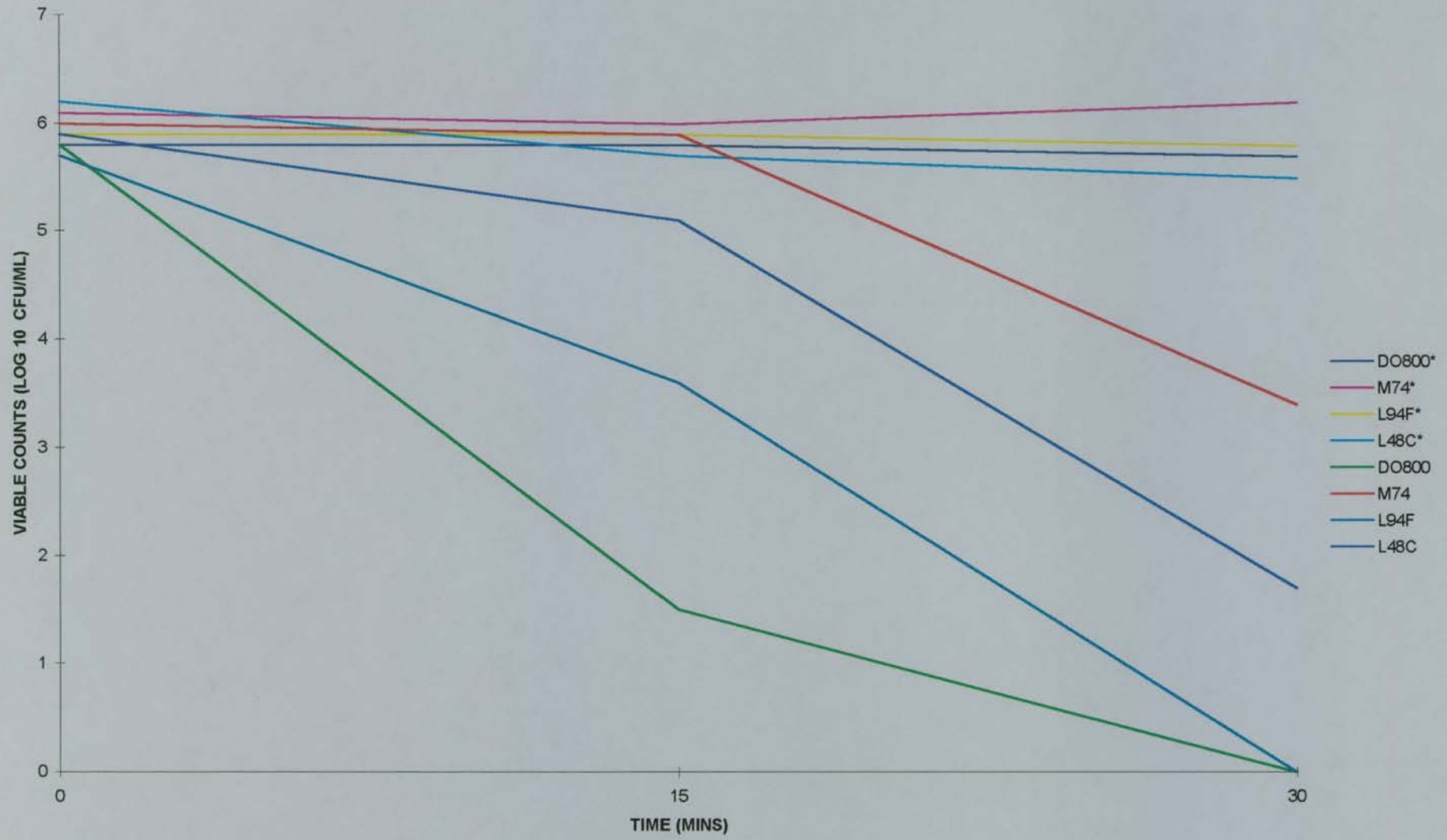

7
6
5
4
3
2
1
0
VIABLE COUNTS (LOG 10 CFU/ML)
0
15
30
TIME (MINS)
DO800*
M74*
L94F*
L48C*
DO800
M74
L94F
L48C

Fig 6.6. and Fig 6.7 show A7 Cu/Zn SOD positive and negative strains survival in ovine (Fig 6.6) and bovine (Fig 6.7) alveolar macrophages. Bacteria were incubated with macrophages at a ratio of 10:1 bacteria to macrophage for 2 hours. Gentamicin was added to kill any extracellular bacteria and then the macrophages were lysed and the bacteria counted using viable counts. Results are the mean of three separate experiments.

Fig 6.6. Phagocytosis survival at 2 hours by A7 strains in ovine alveolar macrophages.

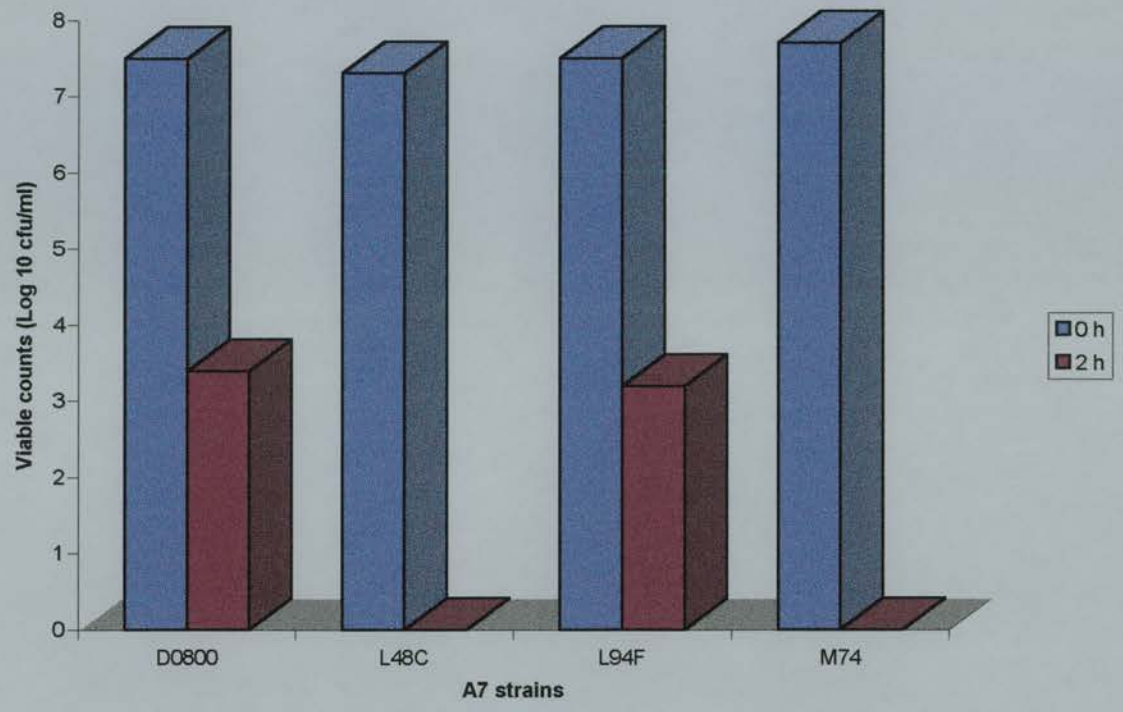
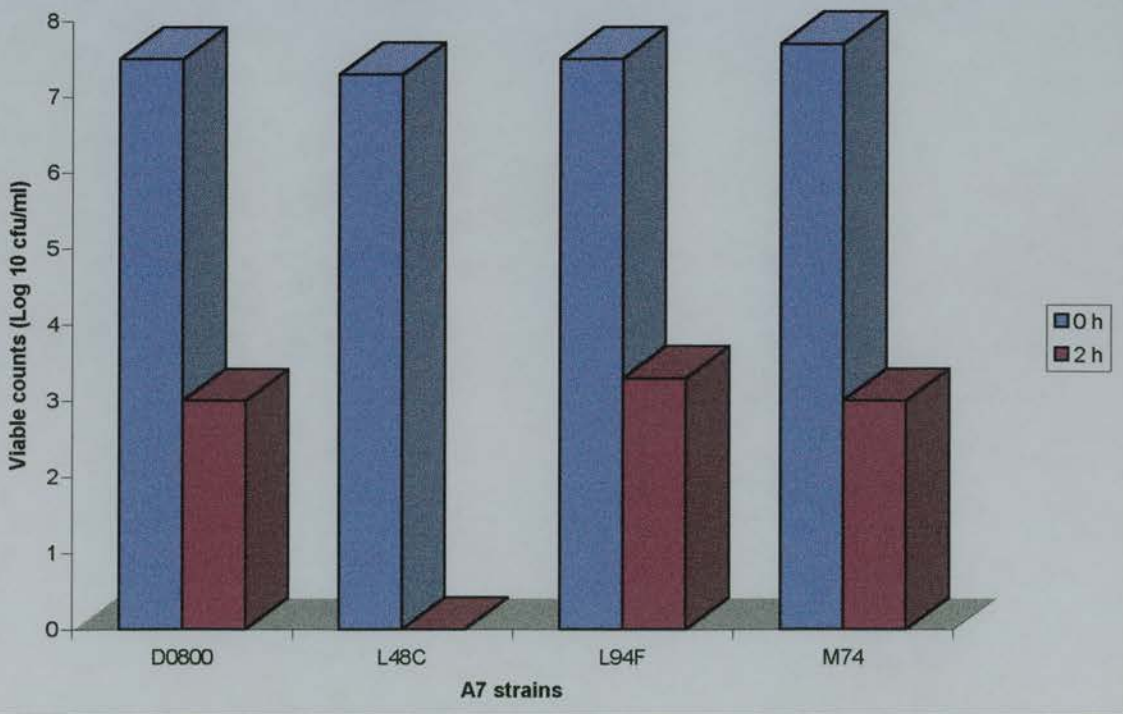


Fig 6.7. Phagocytosis survival at 2 hours by A7 strains in bovine alveolar macrophages



6.8. Serum antibody to SOD

The possibility that this enzyme may elicit an immune response during infection was also investigated. Convalescent ovine antiserum was pre-incubated with lysates and then run on native PAGE and stained for SOD activity. Fig 6.8 shows activities of each serotypes mixed with homologous convalescent antiserum. The active component appeared to be bound by a serum component and carried down the gel but was not inactivated. Serum alone did not show this effect. In order to see if this effect was due to immunoglobulin G (IgG), IgG was purified from the serum (Fig 6.9 shows the degree of purity of the IgG). The purified IgG was mixed and incubated with the lysates and treated in the same way as whole serum. Fig 6.10 shows the results obtained with serotype A2 as an example. The other serotypes showed the same results. There is no difference in the electrophoretic mobility of the enzyme indicating no binding of IgG.

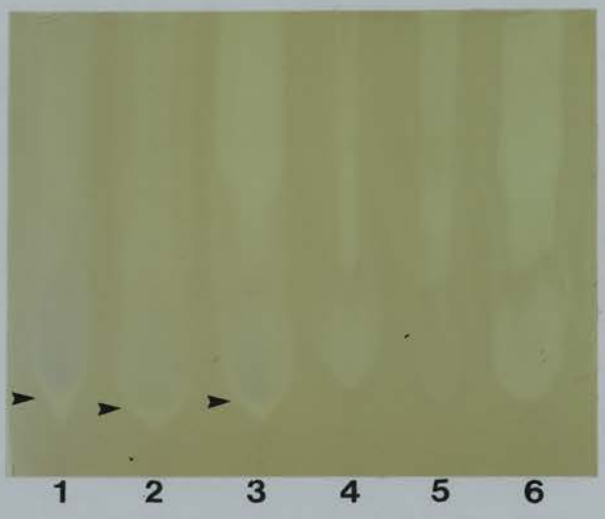


Fig 6.8. Native PAGE showing the effect of superoxide dismutase activity when pre incubated with homologous convalescent lamb serum (chapter 3).

A1 with A1 antiserum (lane 1), A2 with A2 antiserum (lane 2), T10 with T10 antiserum (lane 3), A1 antiserum (lane 4), A2 antiserum (lane 5) and T10 antiserum (lane 6). The arrows show superoxide dismutase activity carried with serum down the gel.

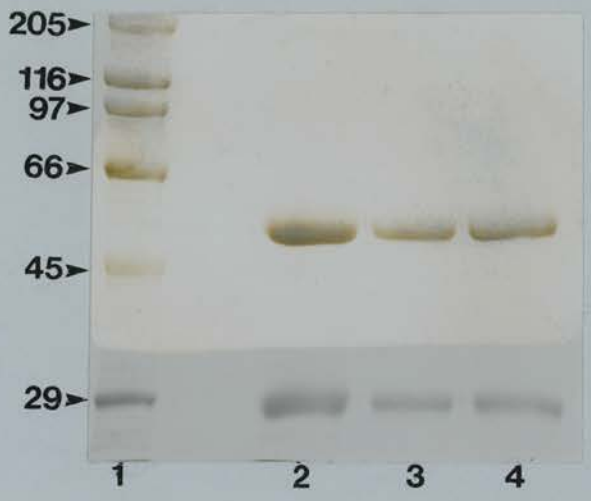


Fig 6.9. Coomassie stained SDS PAGE showing purified IgG (chapter 3). Molecular weight markers (lane 1), IgG from A1 convalescent lamb serum (lane 2), IgG from A2 convalescent lamb serum (lane 3) and IgG from T10 convalescent lamb serum (lane 4).

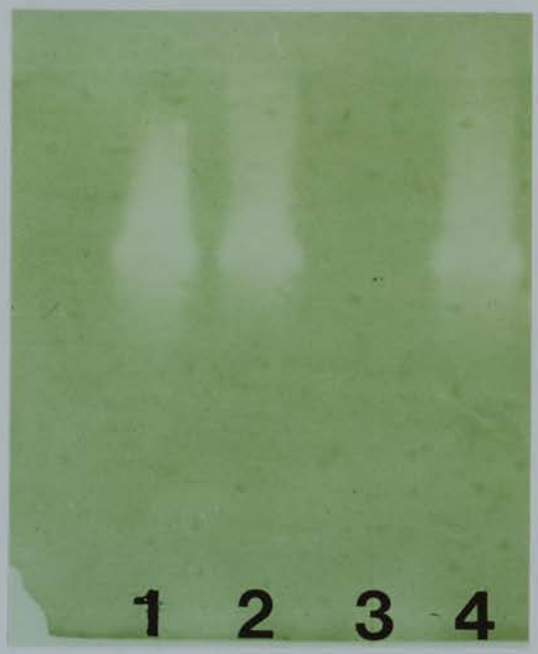


Fig 6.10. Native PAGE showing the effects of purified IgG on superoxide dismutase activity.

A2 whole lysate (lane 1), A2 lysate pre mixed with IgG purified from convalescent lamb serum raised against A2 (lane 2), IgG alone (lane 3) and A2 lysate pre mixed with IgG purified from lamb serum raised against orf virus (lane 4). A2 is shown here but all other serotypes tested showed the same reactions.

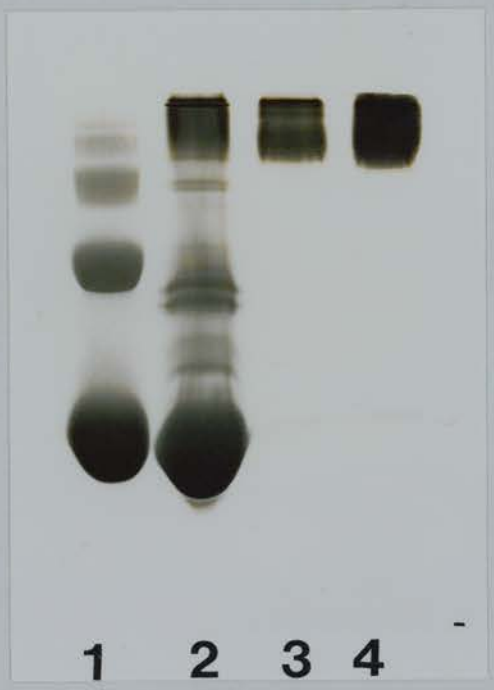


Fig 6.11. Coomassie stained Native PAGE to show where BSA (lane 1), whole serum (lane 2), convalescent lamb purified IgG (lane 3) and purified IgG from lamb serum raised against orf virus (lane 4) are positioned on the gel.

As the gel system may not be quite sensitive enough to detect minor changes in SOD activity the assay was used. The basis of the method relies on the affinity of Protein G for the IgG molecule. If IgG recognises and binds SOD, Protein G if present will bind IgG. Centrifugation then removes the Protein G complex and the supernatant is assayed for SOD activity.

Sample	Purified IgG source	Protein G *	Mean SOD units mg^{-1} protein (SE)
A1	A1 antiserum	-	4.47 (0.6)
	A1 antiserum	+	3.02 (0.4)
	control serum	-	4.84 (1.1)
	control serum	+	4.1 (0.7)
A2	A2 antiserum	-	21.21 (3)
	A2 antiserum	+	16.56 (4.8)
	control serum	-	23.64 (0.4)
	control serum	+	24.61 (3)
T10	T10 antiserum	-	9.99 (3.3)
	T10 antiserum	+	6.01 (1.97)
	control serum	-	8.2 (1.8)
	control serum	+	5.4 (1.3)

Table 6.5. The effect of specific IgG on SOD activity.
* -, no protein G; +, 100μg protein G. Results are mean values of 4 separate experiments. Control serum IgG was incorporated to ensure that any reduction in activity was due to specific IgG. Antibody binding of SOD without loss of activity was monitored by the presence or absence of Protein G.

Table 6.5 (for raw data see Appendix II) shows a reduction in enzyme activity when specific IgG and Protein G were present with serotypes A1 and A2 but not T10. This result was not statistically significant. It was therefore concluded that there was no specific IgG response. The binding component was further investigated. As serum albumin constitutes a large component of serum, bovine serum albumin was mixed with lysates and treated as previously on native PAGE. Fig 6.11 shows a native gel stained with Coomassie blue to show the electrophoretic mobility of albumin

Fig 6.12. Native PAGE showing BSA as the responsible serum component for the binding of superoxide dismutase.

A2 with BSA (lane 1), A2 with serum (lane 2), BSA alone (lane 3) and serum alone (lane 4).A2 was used as an example but all serotypes tested behaved in the same way.

Fig 6.13. Western blot on a Native PAGE gel probed with rabbit antiserum raised against the copper /zinc superoxide dismutase of *Haemonchus contortus*.

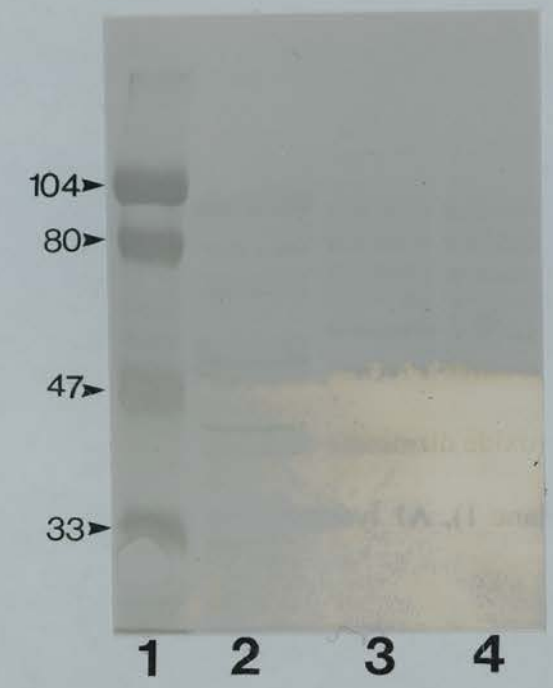
Molecular weight markers (lane 1), A1 lysate (lane 2), A2 lysate (lane 3) and T10 lysate (lane 4).

Note there is no recognition of a band in A2 which is not present in the other two serotypes.

Fig 6.14. Western blot on an SDS PAGE gel probed with rabbit antiserum raised against the copper /zinc superoxide dismutase of *Haemonchus contortus*.

Molecular weight markers (lane 1), A1 lysate (lane 2), A2 lysate (lane 3) and T10 lysate (lane 4).

Note there is no recognition of a band in A2 which is not present in the other two serotypes.


104
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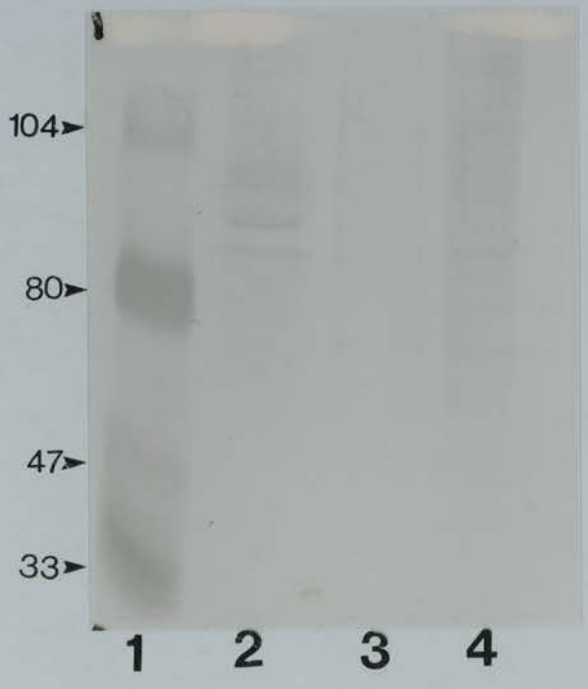
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compared to IgG and whole serum. Fig 6.12 then shows the SOD activity associated with the albumin and was identified as the component binding SOD (in the same way observed for whole serum). The activity seen on the gel correlates with the position observed on the Coomassie stained gel of the proteins and the expected position of the SOD if it had bound IgG.

Western blots of whole cell lysates of the three serotypes were probed with a rabbit antiserum raised against the recombinant Cu/Zn SOD of the nematode *Haemonchus contortus*. Fig 6.13 and 6.14 show that the antiserum did not recognise any Cu/Zn SOD epitopes on *P. haemolytica* A2 or any of the other serotypes. This was the case in both native PAGE (Fig 6.13) and SDS PAGE (Fig 6.14) gels showing that no cross reaction exists of the enzyme under the conditions tested.

6.9. Discussion

Detection of SOD in lysates using PAGE was a relatively simple procedure, although activity in A1 was so low that visual detection of this serotypes was not always reliable. To overcome this an assay was used alongside PAGE to confirm the results and to detect subtle changes not obvious using PAGE.

The relative mobilities of the enzymes from the three serotypes differed markedly as did the strength of the activity. A2 possesses one enzyme which has SOD activity greater than that of the other serotypes combined. This enzyme was also not induced under certain growth conditions as is the case with other organisms from the *Haemophilus, Actinobacillus* and *Pasteurella* (HAP) group (Kroll *et al.,* 1995) suggesting the enzyme is under the control of a strong promoter. The relative electrophoretic mobilities of the enzymes suggest that A1 and T10 share one of the SODs. The level of SOD activity in comparison to A1 (6 times greater) may be explained by the presence of two enzymes in T10. These differences suggest a diversity not only within the Pasteurellaceae but also within *P. haemolytica* itself.

Identification of the enzyme was a straightforward procedure with serotype A2. Both the gel and assay inhibition studies with KCN identified for the first time a Cu/Zn SOD in *P. haemolytica*. This was confirmed by PCR and southern hybridisation techniques developed by this group (Lainson *et al.,* 1996). Serotypes A1 and T10, however, were not inhibited by any of the standard inhibitors applied. Because T10 possesses two SODs, neither of which is a Cu/Zn SOD, it may be assumed to have one Fe and one Mn SOD, one of which is shared with serotype A1. This distinction between the inhibition of the Cu/Zn SOD and the lack of inhibition of the A1 and T10 enzymes may be due to structural differences. Cu/Zn and Fe/Mn are two separate

families of SODs, and there is a complete lack of DNA, protein or structural homology between the two. It may also be the case that the use of a whole lysate means that other enzymes may be present and these may have Cu, Zn, Mn or Fe at the active site which may interfere with inhibition. In the case of inhibition with H_2O_2, a characteristic catalase reaction (a violent evolution of bubbles) was observed with A1 and A2. This may explain the lack of inhibition of the A1 enzyme. In contrast, T10 is catalase-negative and so does not explain the lack of inhibition with this serotype. The levels of inhibitors investigated should have overcome any of these effects. A purified enzyme or active recombinant enzyme from A1 and T10 may help in their identification. A PCR based technique similar to that used for the Cu/Zn enzyme could be employed, as was the case for *Haemophilus* spp with unreproducible inhibition using KCN whereby the authors switched to a molecular identification system (Kroll *et al.,* 1995). The inhibitors EDTA and H_2O_2 have been used in numerous studies at the same and lower concentrations than those in this study, yet no inhibition was observed. The A2 SOD was markedly, but not completely inhibited by KCN indicating the analytical system was appropriate for the definition attempted. The insensitivity of the SODs to inhibition may indicate that the conformations of the bacterial enzymes, in particular at the active site differ from existing defined SODs. In contrast the active site of Cu/Zn SODs is highly conserved even though its other domains show little cross-reactivity between species (Hassan, 1989; Kroll *et al.,* 1995). This high degree of conservation may explain the clear inhibition with KCN observed with A2. A new type of SOD was described in 1983 by Beaman *et al.* for *Norcardia asteroides* and contained Mn, Fe and Zn. The enzyme was closely related to the Mn SOD of *Mycobacterium smegmatis.* It was inhibited by H_2O_2 and was therefore a Fe SOD but only 40% of the activity was associated with this. The SODs

of A1 and T10 remain uncharacterised and in the light of current knowledge are relatively novel.

Bacterial Cu/Zn SODs have been shown to be periplasmic. *Caulobacter cresentus* and *Brucella abortus* have been shown to possess two types of SOD with one (Fe or Mn) in the cytosol and a Cu/Zn SOD in the periplasm (Steinman & Ely., 1990; Stabel *et al.,* 1994). *Haemophilus* spp have been shown to secrete Cu/Zn SOD in *E. coli* minicells carrying the cloned *sod c* gene and the presence of an N-terminal leader peptide motif in other species has led to the authors' surmise that this is evidence for periplasmic location of the enzyme (Kroll *et al.,* 1991; 1995). Superoxide generated in the cytosol as a byproduct of normal respiratory metabolism does not pass through the membrane into the periplasm. Thus periplasmic SOD is thought to deal with externally generated superoxide (Hassan, 1979). Some organisms secrete SOD into the medium and these include *Mycobacterium tuberculosis* (Kusnose *et al.*, 1976) and *Norcardia asteroides* (Beaman *et al.,* 1990), whose secretion has been shown to protect it from the killing effects of neutrophils. Investigation of the location of *P. haemolytica* and *P.trehalosi* SODs was carried out in the light of the identification of a Cu/Zn SOD. Methods based on those described by Stabel *et al.* (1994) and Lainson *et al.* (1991), using various detergents and solvents were used to separate periplasmic and cytoplasmic fractions. The freeze thaw technique was reproducibly less harsh on *Pasteurella* spp and gave the least observable cytoplasmic contamination. This was controlled by the parallel measurement of malate dehydrogenase, an enzyme which takes part in Kreb's cycle and is unique to the cytoplasm. A1 and A2 had 83.8 % and 75.2% of SOD activity respectively therefore the SODs were periplasmic, whereas the T10 enzyme remained cytoplasmic (59.1% of activity). This result for A2 brings the serotype into line with other Cu/Zn SODs identified so far. A1 SOD location was

unexpected although the results correlated with these obtained by the chloroform method. The cytoplasmic location of T10, was surprising, especially as two enzymes were clearly present.

It was thought that the location may depend on the growth environment. The SOD of *C. cresentus* locates preferentially in the periplasm in stationary phase growth (Schnell & Steinman, 1995), whereas an environmental signal such as changes in oxygen or iron availability may stimulate secretion into the periplasm and this has been shown for the manganese SODs of the fish pathogen *Aeromonas salmonicida* (Barnes *et al*, 1996) and mucoid *Pseudomonas aeruginosa* (Polak *et al.,* 1996). Although the overall SOD activity from iron restricted cells was reduced for all serotypes, T10 did contain measurable activity located in the periplasm when cultured under these conditions. The location of A2 SOD had not changed but A1 SOD was confined to the cytoplasm. Another indication of induced transport across the inner membrane was when whole bacteria were assayed for their resistance to externally generated superoxide. T10 and A2 were unaffected by the presence of external superoxide, whereas A1 did not survive for 30 minutes. This indicates the possible movement of the enzymes due to environmental signals, which locate the enzymes where they are most required. In the case of A1, although it has been shown to be periplasmic in resting cells it is comparatively not of a high activity and so resistance is low in in-vitro assays. Serum bactericidal activity results in a loss of viability after only 15 minutes at dilutions of 1 in 100 (M. Maley, pers comm). It may be that, in-vivo, 30 minutes during infection may be long enough to ensure bacterial survival when structural features and virulence factors are taken into account. It now appears that it is possibly the location of the enzyme that is important and not so much the metal found at the active site.

The lack of techniques to produce isogenic mutants for pasteurellae led to the use of some A7 strains which were Cu/Zn SOD positive and negative. This could give some information on the possible importance of Cu/Zn SODs when directly compared to other strains which do not differ in any obvious way (M. Maley, pers comm). The results were surprising in that those which appeared resistant to the effects of superoxide were the strains negative for Cu/Zn SOD. Those strains which were positive showed a survival similar to that observed previously for A1. This adds weight to the assumption that other factors may be involved in resistance to superoxide. These strains were not assayed for their location of the enzyme which may play a more important role. Steinman (1993) investigating *C. cresentus* found that Cu/Zn SOD deficient mutants were also sensitive to citrate, which arose from a reduced affinity for magnesium and calcium. Those results suggested to the author that Cu/Zn SOD protects the cell envelope functions which bind Mg^{2+} and /or Ca^{2+} by the stabilisation of LPS, though this was deemed to be unlikely. However, if the enzyme was compartmentalised in the periplasm it could have some selective advantage. Simpson *et al.* (1989) showed that *Pseudomonas aeruginosa* alginate scavenges free radicals from macrophages. It is possible then, that, other polysaccharide molecules may have the same properties.

In-vitro assays with macrophage survival also gave no evidence as to the role of SOD in protection of possible oxygen-dependent killing mechanisms. Only two strains survived for 2 hours in both ovine and bovine macrophages and one was positive and one negative for Cu/Zn SOD. This situation could arise if some of the organisms entered via complement receptors which have been shown not to trigger the release of oxygen intermediates (Tsolis *et al.*, 1995). These strains were not opsonised and so this would be unlikely. Leukotoxin production will interference with macrophage

function and was possibly produced during the course of the assay. These results do indicate the presence of other factors in protection of the bacteria and question the importance placed upon Cu/Zn SODs. Latimer *et al.* (1992) produced a Cu/Zn SOD mutant in *Brucella abortus* which showed no difference in a mouse virulence model with the wild type, this was in agreement with Tatum *et al.* (1992) who showed that survival and growth of mutant strains were comparable to wild type strains in both HeLa cells and J774 macrophages. The study did reveal that in contradiction to Latimer's work, mutant strains in BALB/C mice had 10 fold lower splenic recovery of organisms of the vaccine strain used but the virulent strain splenic levels were again lower and spleen weights in mutants were consistently lower. Mandel (1975) investigating *Staphylococcus aureus* found no correlation between SOD content and virulence in mice. Exogenous SOD did not enhance mouse lethality whereas catalase did and although phagocytosis could not be impaired, on catalase addition the ability of the phagocytes to kill in-vitro was impaired. These results indicate the importance of catalase which can follow superoxide dismutation in catalysing the conversion of hydrogen peroxide to oxygen and water. Franzon *et al.* (1990) found that with *Shigella flexneri* the Fe SOD was important in pathogenesis and catalase appeared to make a limited contribution.

Analysis of immune reactions against SOD indicated that no specific response was raised against any of the enzymes tested. A study by Beck *et al.* (1990) identified an antigenic protein of *Brucella abortus* recognised by bovine convalescent serum and this turned out to be Cu/Zn SOD. *Brucella abortus* however, showed lack of immune response in-vivo and failed to demonstrate a cell-mediated response when synthetic peptides were used in cattle (Stevens *et al.,* 1994). When observing the effects of Cu/Zn SODs, the high degree of conservation of the active site (85%) must be taken

into consideration. The raising of antibodies against an enzyme which is also shared with the host could have catastrophic repercussions to the host. This may be why the Cu/Zn SOD rabbit antiserum did not cross react with the enzyme in A2 as those regions of surface antigenic domains to which a response would be raised are less conserved.

It is not known whether the binding of albumin is specific and /or significant. Albumin is recognised as a carrier protein but its role in binding SOD may be purely co-incidental. During acute phase response albumin scavenges divalent cations and may bind SOD because of the metal active site, but as the enzyme is not inactivated it is not particularly advantageous to the host.

The presence of periplasmic SODs has been proposed as important, not only during the protection of phagocytic respiratory burst, but also from oxygen radicals generated at the tissue surface and the mucus during colonisation (Pruitt *et al.,* 1994; Salyers & Whitt, 1994). It has also been suggested that SOD may not be as important in defence and it is the subsequent production of hydrogen peroxide (Kroll *et al.,* 1995) where the advantage lies. This has been shown to disrupt ciliary function (Burman & Martin, 1986) and would be advantageous for a respiratory pathogen like pasteurellae. The presence of a periplasmic Cu/Zn SOD in A2 and not in A1 shows a phenotypic difference which may confer an advantage for the organism in vivo. A2 is by far the most prevalent serotype isolated from nasopharyngeal carriers and ovine pneumonic and septicaemic cases. In *P.trehalosi*, T10 is also prevalent in tonsillar carriage and systemic disease cases. This may indicate that the periplasmic induction or presence of SOD may play a role in its ability to colonise and cause disease in areas of the host which are not under oxygen stress, or in the case of pneumonia those areas which are under oxygen stress.

Characterisation of the enzyme alone does not give a specific role for the enzyme in pathogenesis. The evidence for SOD mutants losing virulence is minimal, and it is hoped that this may be rectified when more organisms are studied. The differences between serotypes in this study do point specific roles for the enzyme and warrant further investigation.

CHAPTER 7

INTERACTION OF *P. HAEMOLYTICA* AND *P. TREHALOSI* WITH OVINE AND BOVINE ALVEOLAR MACROPHAGES

7.1. Introduction

The alveolar macrophage is the first line of host defence against bacterial invasion of the lower respiratory tract. Serotype A1 is an important pathogen in ruminants particularly in cattle. The interaction with bovine bronchoalveolar macrophages has been extensivley studied but few reports deal with the interaction of ovine macrophages with serotypes A2 and T10 both of which are significant serotypes as causative agents of pasteurellosis. This study sets out to compare the interaction of all three important serotypes with both ovine and bovine macrophages, especially in light of the data concerning superoxide dismutase production. In observing the effects of an interaction on both bacterial survival and effects on the macrophage it is hoped that these will produce greater knowledge of the interactions.

7.2. Survival of bacteria in macrophages

A phagocytosis assay was developed to determine the survival of *P. haemolytica* and *P. trehalosi* in ruminant macrophages. Live Pasteurellae cells were incubated with macrophages (isolated from healthy adult sheep) for a set period of time (either 15, 30, 45, 60, 90 or 120 mins), followed by addition of gentamicin to kill extracellular organisms. The only viable bacteria are those located internally which are then released from the macrophages (after cell lysis) and counted. Fig 7.1 (for raw data see Appendix II) shows the survival over time for serotypes A1, A2 and T10 in ovine

alveolar macrophages (OAM). After 2 hours incubation A2 showed the highest number of surviving bacteria followed by A1 then T10. Using bovine alveolar macrophages (BAM), A2 was again the best survivor followed by T10 with A1 showing no survival after 45 minutes (Fig 7.2, for raw data see Appendix II). Although A1 did not survive well in BAM, a 1 hour incubation period was chosen for further studies (this is a common incubation period for many studies which have been undertaken with macrophages and pasteurellae or components of pasteurellae) to monitor more closely the interactions taking place.

Listeria monocytogenes and *Listeria ivanovii* were used as controls. *Listeria monocytogenes* is a known intracellular pathogen of macrophages and other cell types, *Listeria ivanovii* in contrast is similar but is regarded as less virulent. Both species were only sampled at the end of the experiment (2 hours incubation) and showed little reduction in viability in comparison to the Pasteurellae tested.

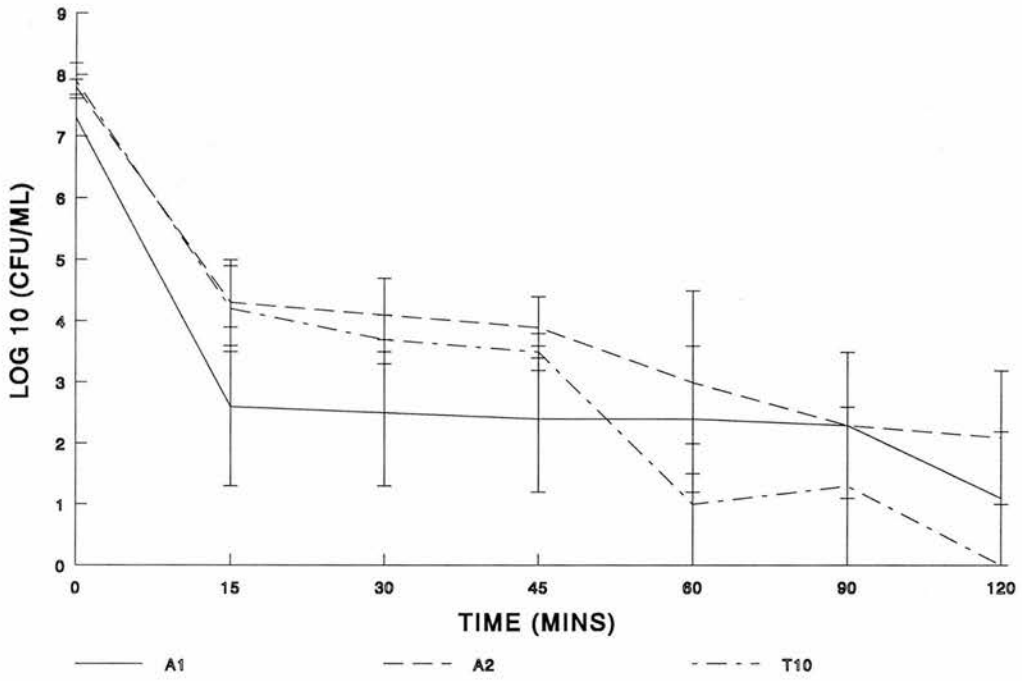

Fig 7.1. Phagocytic survival of serotypes A1, A2 and T10 by ovine alveolar macrophages. As controls the intracellular pathogen *Listeria monocytogenes* and another Listeria spp *L. ivanovii* were used but sampled only at the end of the experiment. The average inoculum (Log 10 cfu/ml) was *L. monocytogenes* - 8.3 and *L. ivanovii* - 8.4. After 2 hours incubation these were *L. monocytogenes* - 6 and *L. ivanovii* - 6.1.

Macrophages were incubated for various times with the serotypes then any extracellular bacteria were killed by the addition of gentamicin. The macrophages were washed then lysed and viability determined by plating onto blood agar to determine the number of live bacteria which were intracellular. Results were expressed as the mean of three separate experiments (see Appendix II).

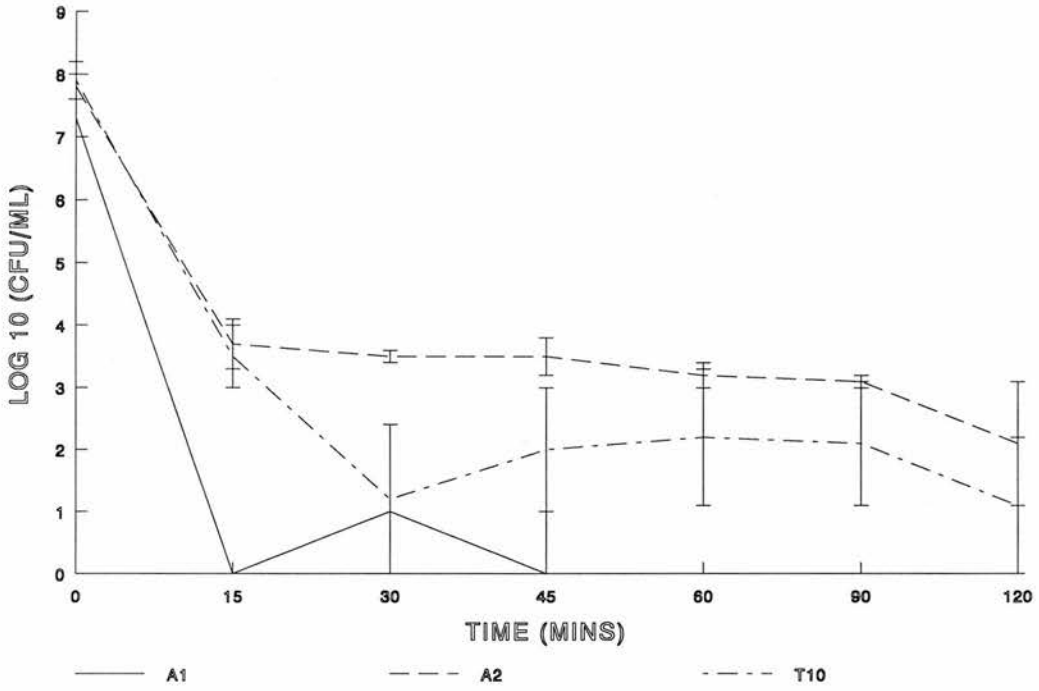

Fig 7.2. Phagocytic survival of serotypes A1, A2 and T10 by bovine alveolar macrophages. As controls the intracellular pathogen *Listeria monocytogenes* and another Listeria spp *L. ivanovii* were used but sampled only at the end of the experiment. The average inoculum (Log 10 cfu/ml) was *L. monocytogenes* - 8.3 and *L. ivanovii* - 8.4. After 2 hours i ncubation these were *L. monocytogenes* - 5.7 and *L. ivanovii* -6.1.

Macrophages were incubated for various times with the serotypes then any extracellular bacteria were killed by the addition of gentamicin. The macrophages were washed then lysed and viability was determined by plating onto blood agar to determine the number of live bacteria which were intracellular. Results were expressed as the mean of three separate experiments (see Appendix II).

7.3. Effects of opsonisation on phagocytosis

This section looked at the effects of various pre-treatments of bacteria upon subsequent survival of phagocytosis. Opsonisation by antiserum, heat-treated antiserum (to destroy complement) and tbws (to look at local lung responses) were carried out to ascertain which, if any, would affect phagocytosis and survival by the serotypes. In Fig 7.3, 7.4 and 7.5 (for raw data see Appendix II) when serotypes were incubated with ovine convalescent antiserum and various other pre-treatments, then added to OAM the number of bacteria which survived appeared to increase in comparison to unopsonised bacteria. Although the results differed in the amount of surviving bacteria when serotypes were compared (A2 and T10 surviving better than A1), pre-treatments had no significant effect on survival of each individual serotype.
In BAM (Figs 7.6, 7.7 and 7.8, see Appendix II) the serotypes, with respect to survival, were similar to the results found with OAM. Certain pre-treatments appeared to have deleterious or beneficial effects on bacterial survival but were not statistically significant. Results for serotype comparisons did again show that their survival differed from each other. A1 again showed little or no survival of phagocytosis whereas A2 survived better. T10 survived noticeably less than in OAM.

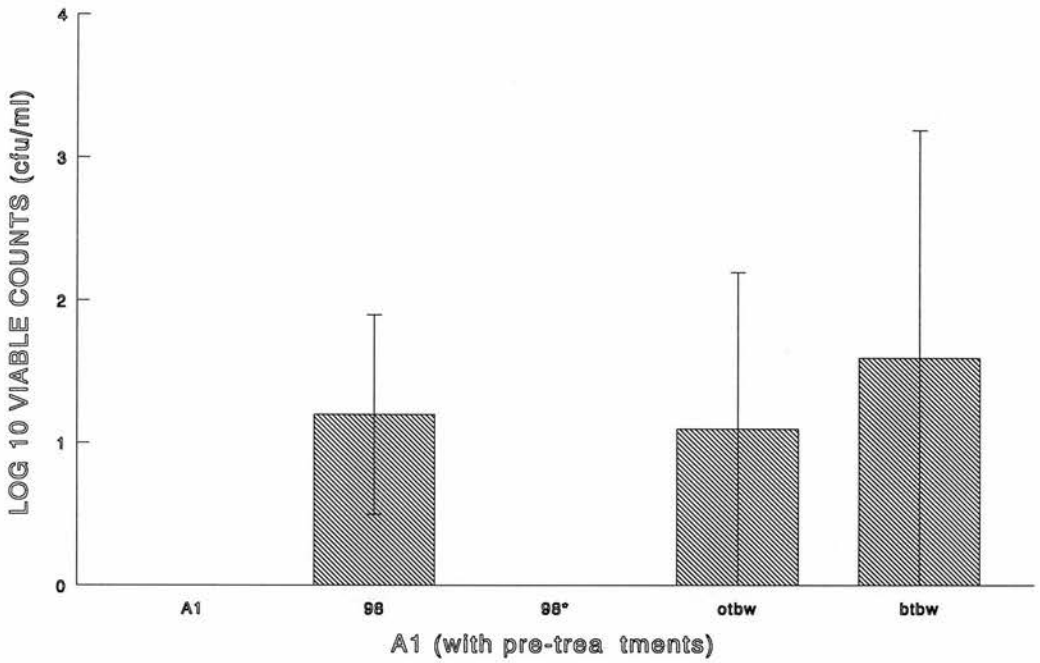

Fig 7.3. Survival of serotype A1 after 1 hour in ovine alveolar macrophages after being subjected to opsonins for 15 mins.

Serotype A1 was incubated alone, with convalescent antiserum (98), heat treated convalescent lamb serum (98*) and ovine and bovine tbws and incubated with ovine macrophages for 1 hour. Treatment post incubation is as described previously (Fig 7.1) and results are the mean of between 3 and 5 separate experiments (see Appendix II).

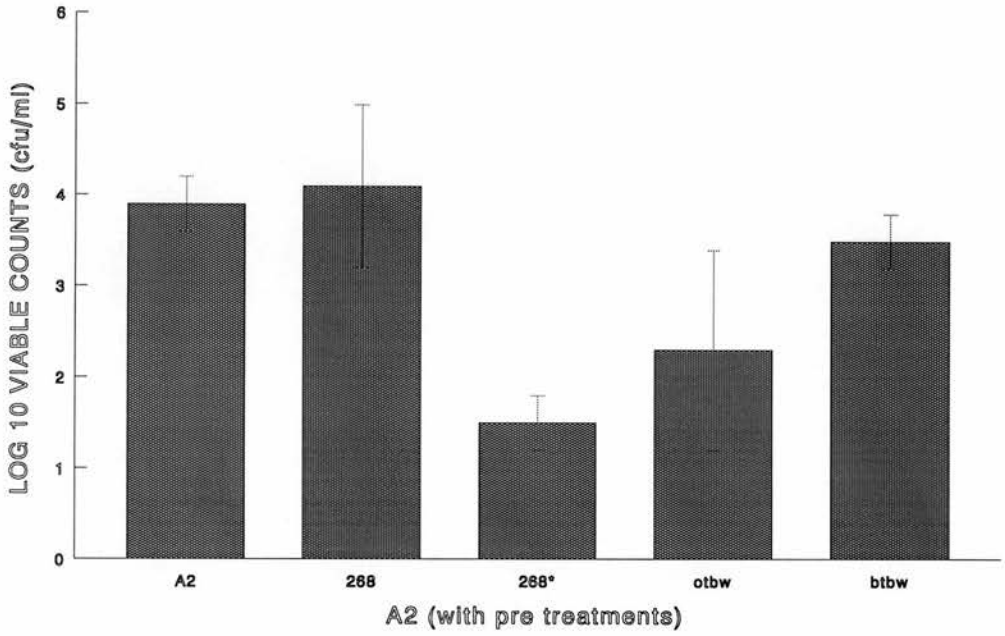

Fig 7.4. Survival of serotype A2 after 1 hour in ovine alveolar macrophages after being subjected to opsonins for 15 mins.

Serotype A2 was incubated alone, with convalescent antiserum (268), heat treated convalescent lamb serum (268*) and ovine and bovine tbws and incubated with ovine macrophages for 1 hour. Treatment post incubation is as described previously (Fig 7.1) and results are the mean of between 3 and 5 separate experiments (see Appendix II).

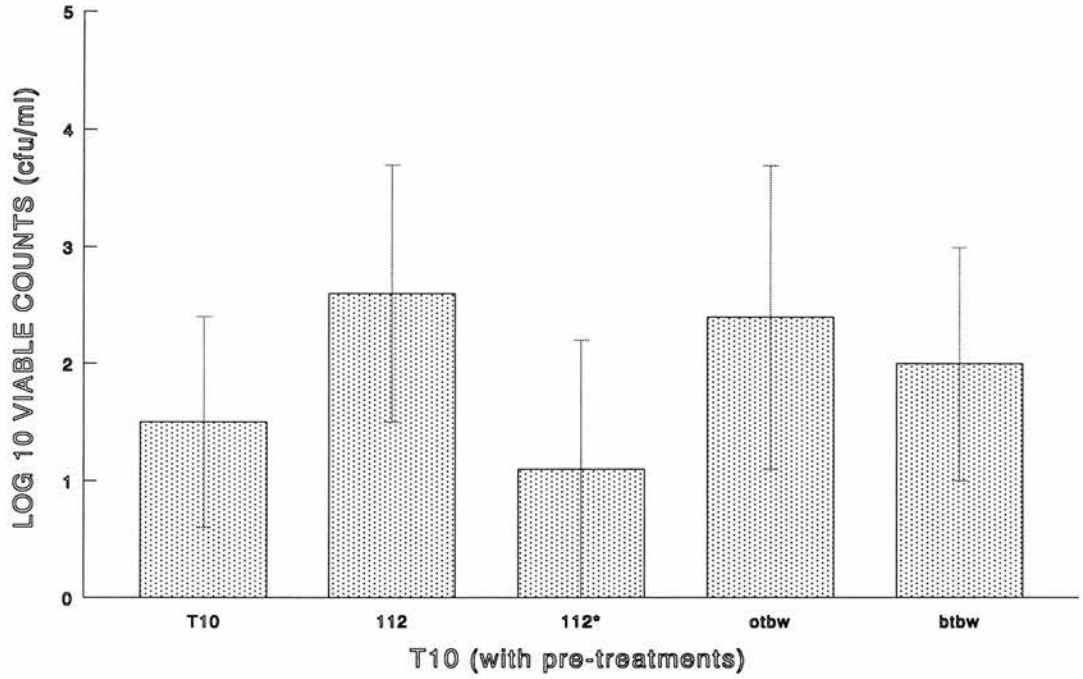

Fig 7.5. Survival of serotype T10 for 1 hour in ovine alveolar macrophages after being subjected to opsonins for 15 mins.

Serotype T10 was incubated alone, with convalescent antiserum (112), heat treated convalescent lamb serum (112*) and ovine and bovine tbws and incubated with ovine macrophages for 1 hour. Treatment post incubation is as described previously (Fig 7.1) and results are the mean of between 3 and 5 separate experiments (see Appendix II).

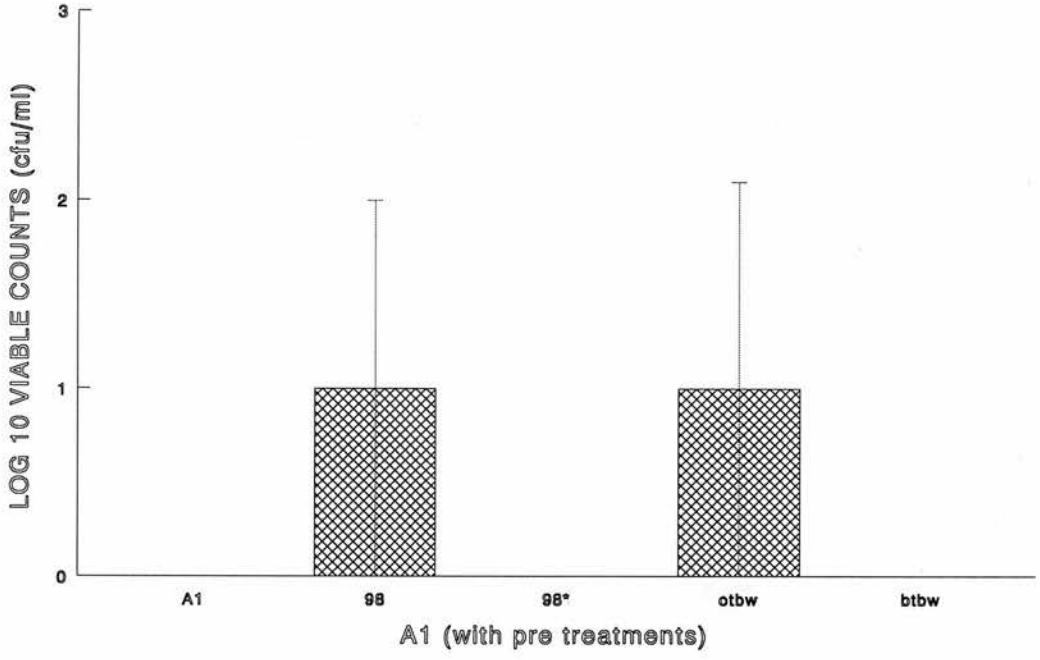

Fig 7.6. Survival of serotype A1 for 1 hour in bovine alveolar macrophages after being subjected to opsonins for 15 mins .

Serotype A1 was incubated alone, with convalescent antiserum (98), heat treated convalescent lamb serum (98*) and ovine and bovine tbws and incubated with bovine macrophages for 1 hour. Treatment post incubation is as described previously (Fig 7.1) and results are the mean of between 3 and 5 separate experiments (see Appendix II).

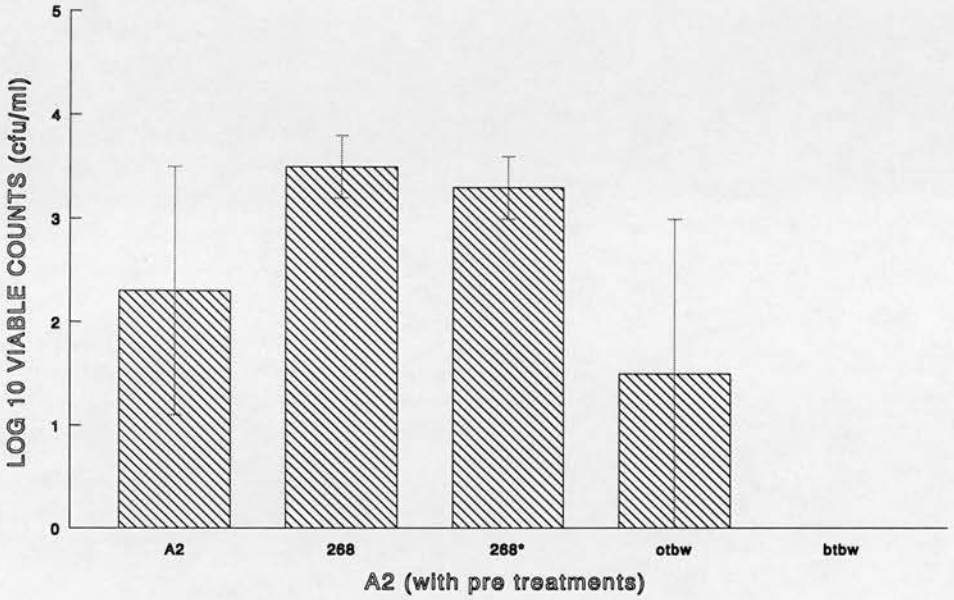

Fig 7.7. Survival of serotype A2 for 1 hour in bovine alveolar macrophages after being subjected to opsonins for 15 mins.

Serotype A2 was incubated alone, with convalescent antiserum (268), heat treated convalescent lamb serum (268*) and ovine and bovine tbws and incubated with bovine macrophages for 1 hour. Treatment post incubation is as described previously (Fig 7.1) and results are the mean of between 3 and 5 separate experiments (see Appendix II).

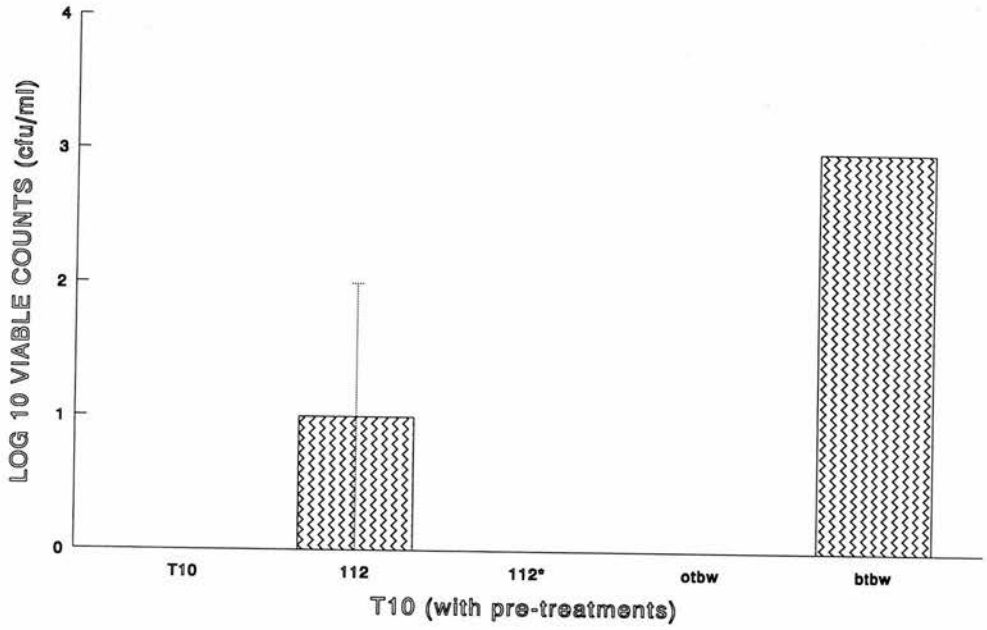

Fig 7.8. Survival of serotype T10 for 1 hour in bovine alveolar macrophages after being subjected to opsonins for 15 mins .

Serotype T10 was incubated alone, with convalescent antiserum (112), heat treated convalescent lamb serum (112*) and ovine and bovine tbws and incubated with bovine macrophages for 1 hour. Treatment post incubation is as described previously (Fig 7.1) and results are the mean of between 3 and 5 separate experiments (see Appendix II).

7.4. Cytotoxicity of the serotypes for macrophages

The possibility that macrophage death caused by cytotoxicity of bacteria for the macrophages may have been responsible for the low viable counts emerging from the previous study was investigated. If the internal bacteria lysed the macrophages they would have been released into the medium and killed by the gentamicin present. A commercial cytotoxicity kit which measures the release of lactate dehydrogenase from lysed cells was used to test this hypothesis. In OAM (Fig 7.9, for raw data see Appendix II) A1 showed the lowest killing of macrophages (10%) followed by A2 (20%) and T10 (50%). Opsonised antiserum did not alter the killing. With BAM (Fig 7.10, for raw data see Appendix II) the killing of macrophages was slightly lower than that observed in OAM but antiserum slightly increased killing in comparison to unopsonised bacteria. This slight increase was, however, similar to that of unopsonised bacterial effects in OAM. An experiment which allowed the overnight incubation of bacteria with macrophages in the presence and absence of gentamicin in the medium (data not shown) was carried out to record the final outcome of phagocytosis. Microscopic analysis showed the absence of macrophages, except in control wells (macrophages alone) indicating total lysis of all macrophages. The wells which contained no gentamicin showed observable bacterial turbidity, which on subculture produced pure cultures of each representative serotype. In contrast the wells which contained gentamicin in the medium showed no bacteria or macrophages.

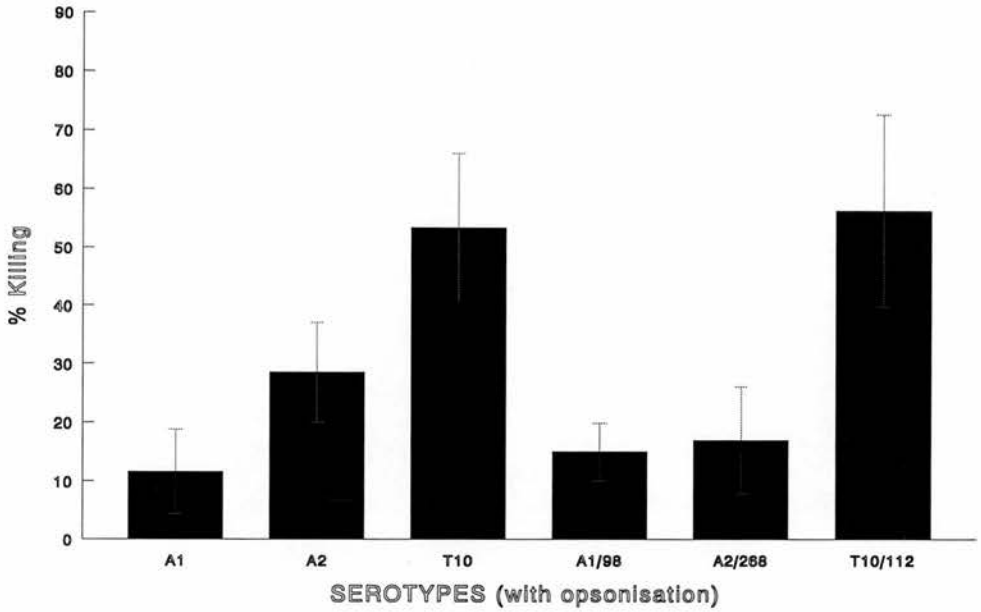

Fig 7.9. Killing of ovine alveolar macrophages by serotypes incubated alone or post opsonisation (indicated by the number of the homologous convalescent serum). Bacteria were incubated with the macrophages for 1 hour and the macrophages were then subjected to the cytotoxicity assay (Promega kit) as described in Chapter 2.

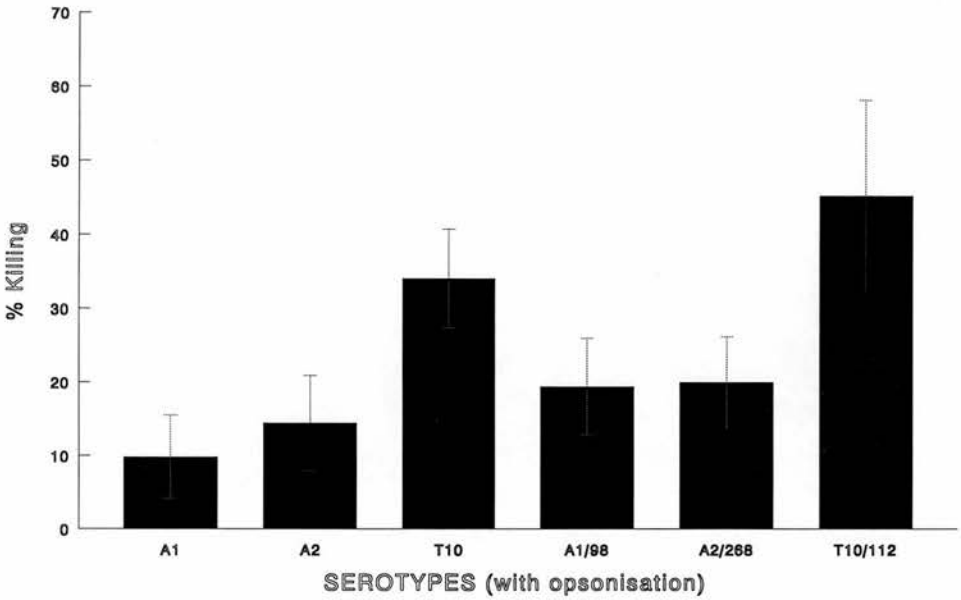

Fig 7.10. Killing of bovine alveolar macrophages by serotypes incubated alone or post opsonisation (indicated by the number of the homologous convalescent serum). Bacteria were incubated with the macrophages for 1 hour and the macrophages were then subjected to the cytotoxicity assay (Promega kit) as described in Chapter 2

This shows that all macrophages were eventually killed and on lysing, the cells were then released into the medium to re-infect other cells with the result that bacteria with no gentamicin present were allowed to continue replicating whereas those in the presence of gentamicin were killed.

7.5. Bacterial entry mechanisms into macrophages

Bacteria can enter cells via different mechanisms; direct invasion of cells (not applicable in this study), phagocytosis which uses actin microfilament formation within the cell and receptor-mediated endocytosis which employs microtubule formation via tubulin formation. The latter two mechanisms have been used extensively in the study of bacterial and virus uptake into cells via the use of specific inhibitors (Roof *et al.,* 1992; Schlegel *et al.,* 1982; Bass *et al.,* 1995). Cytochalasin D (CD) inhibits phagocytosis and monodansylcadaverine (MDC) inhibits receptor-mediated endocytosis.

INHIBITOR	SEROTYPE	VIABLE COUNTS (log 10 cfu/ml)	MEAN	SE
CYTOCHALASIN D	A1	8.7, 0, 3.3	4	2.5
CYTOCHALASIN D	A2	0, 0, 0	0	0
CYTOCHALASIN D	T10	0, 0, 0	0	0
MONODANSYL CADAVERINE	A1	0, 0, 0	0	0
MONODANSYL CADAVERINE	A2	3.6, 3, 3	3.2	0.2
MONODANSYL CADAVERINE	T10	0, 0, 0	0	0

Table 7.1. The effect of inhibitors on the uptake of *P. haemolytica* and *P.trehalosi* by ovine alveolar macrophages. Macrophages were pre-incubated with inhibitors for at least an hour prior to bacterial incubation.

In OAM (Table 7.1) T10 uptake appears to be prevented by both CD and MDC which indicates the organism employs both uptake mechanisms. A1 was inhibited only by MDC and A2 by CD indicating differing mechanisms of receptor-mediated uptake and phagocytosis respectively. The effects differed in BAM (Table 7.2) and were not as clear as in OAM therefore, the ability to draw conclusions is inappropriate.

INHIBITOR	SEROTYPE	VIABLE COUNTS (LOG 10 cfu/ml)	MEAN	SE
CYTOCHALASIN D	A1	0, 0, 0	0	0
CYTOCHALASIN D	A2	0, 0, 3	1	1
CYTOCHALASIN D	T10	0, 0, 3	1	1
MONODANSYL CADAVERINE	A1	0, 0, 3	1	1
MONODANSYL CADAVERINE	A2	0, 0, 0	0	0
MONODANSYL CADAVERINE	T10	0, 0, 0	0	0

Table 7.2. The effect of inhibitors on the uptake of *P. haemolytica* and *P.trehalosi* by bovine alveolar macrophages. Macrophages were pre-incubated with inhibitors for at least an hour prior to bacterial incubation.

7.6. Bacterial location during macrophage interaction

The use of a differential fixation technique and subsequent staining allowed the location of the bacteria to be monitored. The use of methanol which permeabilises the macrophage cell membrane allows the visualisation of both intracellular and extracellular bacteria. Fixation with formaldehyde allows extracellular bacteria only to

be observed. The difference, therefore, between the two allows a distinction between intra- and extracellular bacteria to be calculated. The differences in staining observation can be seen in Fig 7.11 a and b using T10 as an example.

SEROTYPE	% INFECTED MACROPHAGES	% INTRACELLULAR BACTERIA	% EXTRACELLULAR BACTERIA
A1	47.7	11.8	88.2
A2	69	70.2	29.8
T10	66.7	18.1	81.9

Table 7.3. Table showing the number of ovine macrophages infected with *P. haemolytica* and *P.trehalosi* and the subsequent ratio of intracellular and extracellular bacteria.

Table 7.3 shows that in OAM just under 50% of the total number of macrophages present were infected when incubated with A1. This infection rate was 69% with A2 and 66.7% with T10. There was a dramatic difference between the numbers of intracellular and extracellular bacteria present in the infected macrophages. A2 contained 70.2% intracellular organisms, whereas this was only 11.8% for A1 and 18.1% for T10. Consequently A1 and T10 had the majority of bacteria located extracellularly.

SEROTYPE	% INFECTED MACROPHAGES	% INTRACELLULAR BACTERIA	% EXTRACELLULAR BACTERIA
A1	45.7	16.2	83.8
A2	78.7	42.3	57.7
T10	70.3	22.6	77.4

Table 7.4. Table showing the number of bovine macrophages infected with *P. haemolytica* and *P.trehalosi* and the subsequent ratio of intracellular and extracellular bacteria.

The pattern was very similar in BAM (Table 7.4) with infected macrophages at 45.7%, 78.7% and 70.3% for A1, A2 and T10 respectively. A2 extracellular bacteria (57.7%) were higher than intracellular (42.3%). These numbers were still higher than those observed for A1 and T10 intracellular bacteria (A1-16.2%, T10-22.6%). A1 and T10 remained similar to OAM in the numbers of extracellular bacteria.

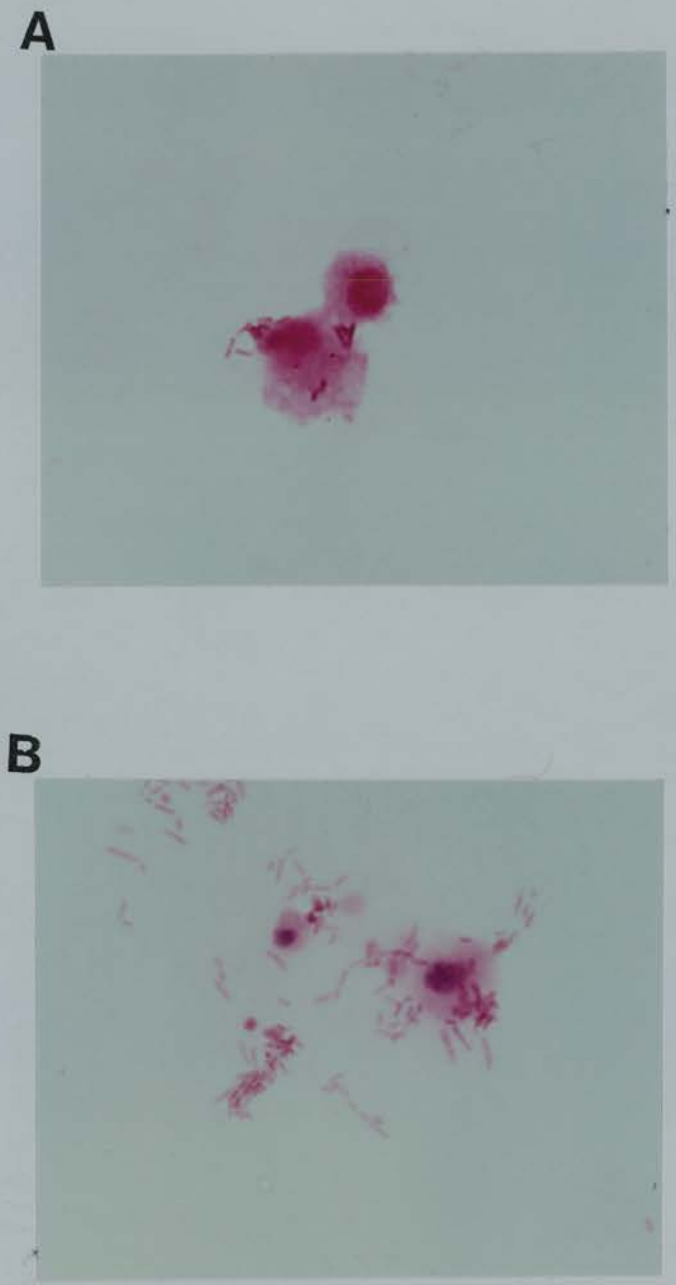

Fig 7.11. Photographic representation of T10 interaction with ovine alveolar macrophages showing differential fixation with formalin (A) and methanol (B). x 100 magnification.

7.7. Discussion

The intracellular survival of phagocytosed pasteurellae was monitored over a two hour period. Phagocytosis was a rapid event with intracellular bacteria detected at 15 minutes. The numbers of viable intracellular bacteria detected declined over the period of time monitored. A2 were detected in larger numbers than A1 and T10 in both OAM and BAM. Serotype A1 fared better than T10 in OAM whereas in BAM the situation reversed indicating the two serotypes had a reduced ability for survival in both macrophage types. A decline in Log 10 viable counts of 3-4 logs at 15 minutes indicated that a substantial number of the bacteria were extracellular at this point and were killed by the gentamicin present in the media. Contradictory results for *P. haemolytica* A1 phagocytosis in macrophages have been reported. Benson *et al.* (1978) showed a poor rate of phagocytosis, wheras in contrast Maheswaran *et al.* (1980) showed that 90% of the bacteria were phagocytosed. Wilkie *et al.* (1990) also showed rare ingestion of pasteurellae by BAM and the inability of macrophages to phagocytose in-vitro after in-vivo infection with *P. haemolytica* has also been reported (Walker *et al.,* 1980). Walker *et al.*(1984) compared log phase grown cells with those in decline phase and found that log phase bacteria were not phagocytosed whereas those in decline phase were. When comparing results, the source of macrophages, the bacterial growth phase, incubation times all have different effects on the outcome. Any prior exposure to pasteurellae will have an effect on the subsequent ability of macrophages to deal with the organism. In this study a pooled sample of bovine macrophages were used from frozen stocks. In contrast ovine macrophages showed poor viability and adherent capabilities from frozen stocks and

so fresh macrophages from different animals were used. This did show some degree of animal to animal variation, hence the use of repeated experiments and the inclusion of standard errors in results. The subsequent decline in bacterial numbers observed over time could be due to killing of bacteria by macrophages. However, killing of macrophages by bacteria could also cause a decline in bacterial numbers, as bacteria released from lysed macrophages encounter gentamicin-containing medium. Gentamicin leakage into macrophages has been identified as a cause of loss of viability of the intracellular bacterium *Listeria monocytogenes* when investigated in murine macrophages (Drevets *et al.* 1994). The *L. monocytogenes* and *L. ivanovii* controls in this experiment were unaffected by the presence of gentamicin in this study and may indicate a difference between the uptake of gentamicin by ovine, bovine and murine macrophages. It would also have been expected that if this was the method of killing, then the overnight incubation in the presence of gentamicin would result in some intact viable macrophages remaining. It must be taken into consideration, however, that if the toxicity of the bacteria was due to leukotoxin this would be unaffected by the presence of gentamicin once it is released by the organisms. Leukotoxin, also at low concentrations, could have many detrimental effects on macrophage function such as the downregulation of MHC class II molecules (Hughes *et al.,* 1994). As respiratory burst or other phagocytic killing mechanisms were not measured it is not known whether the macrophages were activated and therefore macrophage killing of the bacteria is also unknown. As the numbers of *L. monocytogenes* remained high at the end of the experiment it seems likely that destruction of the macrophages is the most likely cause of the decline in bacterial viability. This result indicates that pasteurellae has no capacity for extended survival in macrophages such as those reported for the intracellular pathogens *L.*

monocytogenes and *Rhodococcus equi* or the recently reported *Bordetella pertussis* which is a respiratory pathogen (Friedman *et al.,* 1992; DeChastellier & Berche, 1994; Hondalus & Mosser, 1994) due to its toxicity for macrophages.

A 1 hour incubation period was chosen to monitor the interactions of the bacteria and macrophages to gain a clearer insight into the mechanisms involved. As it was likely that a number of macrophages were being killed during the incubation period a cytotoxicity assay was used to measure this effect. Surprisingly few ovine macrophages were killed by serotypes A1 and A2 whereas at least half were killed by T10. The killing was increased slightly with A1 and T10 in the presence of antiserum but not with A2. The pattern was repeated with bovine macrophages with A2 antiserum this time increasing killing slightly. Sutherland & Donachie (1986), using bovine macrophages at a ratio of 1:1 bacteria to macrophage (lower than this study), found higher killing of macrophages with A1 and A2 with T10 giving similar results. The differences between percentage killing may be due to the detection method for dead cells. Sutherland & Donachie (1986) used trypan blue exclusion, a method which identifies dead cells by their permability to the dye and they appear blue under the microscope, whereas this study used LDH release, apparent only during cell lysis. A drawback of this assay in comparing the status of bacteria and macrophage at this time period is that damaged cells will lose their ability to adhere (Markham & Wilkie 1980a) and in assays measuring phagocytosis they will be removed during washing steps and account for the lack of observable macrophages. The cytotoxicity assay does not employ washing steps so the number of killed macrophages is a measure of dead cells but does not take into account for those which are damaged and have subsequently lost their ability to adhere.

Monitoring the effects of various pre-treatments on phagocytosis showed there was a trend for certain pre-treatments to have certain effects on the serotypes indicating the possible involvement of antibody and/or complement to varying degrees. However, when phagocytosis of opsonised A1 bacteria in OAM and BAM was investigated it was found that there was no significant difference in the treatments. Survival of A1 was repeatedly low, therefore, either A1 was not entering the macrophages (resistant to phagocytosis) and/or was engulfed and killed efficiently by the macrophages. The high level of survival with A2, regardless of pre-treatment, is in contrast to that of A1. A2 survived well and it appears this serotype must have been intracellular. The uptake was not affected by pre-treatments and was still phagocytosed in the absence of antiserum. This suggests other possible mechanisms such as a lectin, lipopolysaccharide binding or mannose/fucose- N-acetylglucosamine terminated glycoprotein-containing receptors on macrophages involved in uptake. T10 showed similar patterns of phagocytic uptake in OAM to that already observed for A2. In BAM T10 were similar to A1.

Sutherland (1989) demonstrated a significant increase in phagocytosis of A2 in OAM with opsonisation. Active serum complement was seen to induce significant opsonophagocytosis, although complement-mediated opsonisation could not be shown to be synergistic with antibody-mediated opsonisation. Results using purified IgG indicated this to be the major opsonin present in serum. The process of uptake via complement confers an advantage for some bacteria as entry via C3b and C3bi receptors inhibit the release of toxic oxygen species (Wright & Silverstein, 1983) in the phagocytes (ie. no respiratory burst). This may indicate that the uptake of A2 and T10 (in OAM) via a variety of mechanisms could still induce an oxidative burst and would therefore need to withstand this attack as the results of viable organisms have

suggested. Earlier results presented in this thesis may suggest a possible explanation in the presence of superoxide dismutase. The ability of A2 and T10 to withstand an attack by superoxide has been demonstrated, whereas A1 is not as capable at surviving in the presence of superoxide. If A1 has the capacity to enter macrophages via complement receptors which do not induce the release of superoxide, superoxide dismutase will not be required by the bacteria to protect them from the effects of respiratory burst, which may be the case.

The use of tbws on the effect of macrophage uptake was also investigated. These had no effect on survival of phagocytosis in comparison to the untreated bacteria. Markham & Wilkie (1980b) had reported the inhibition of phagocytosis in macrophages in the presence of broncho-alveolar washings. Although the predominant antibody present in BAW is IgG which, if specific for pasteurellae, would enhance uptake, a substantial amount of IgA is present and would block uptake as there are no IgA receptors on macrophages. There may be, therefore, inhibitory factors present in BAW (Markham & Wilkie, 1980b). They showed that serum antibody could compete with the inhibitory effect, indicating that during disease the increased presence of serum antibody could increase uptake. In the absence of serum antibody killing of pasteurellae would rely on bactericidal effects as reported by McDonald *et al.* (1983).

Different entry mechanisms into macrophages in the absence of antiserum was demonstrated for the serotypes in OAM but not as evident in BAM. Bacterial entry into cells has been investigated by a number of authors by using cytochalasin D (CD) and monodansyl cadaverine (MDC). *Brucella abortus* entry into vero cells (Detilleux *et al.* 1991) was inhibited by both CD and MDC, the latter in a dose dependent manner. CD was shown to inhibit uptake of *B. pertussis* in human macrophages with

MDC having no effect (Friedman *et al.* 1992). This was also the mechanism of entry for the organism in HeLa 229 cells and human respiratory epithelial cells (Ewanowich *et al.* 1989). A1 enters ovine macrophages by what appears to be receptor-mediated endocytosis (RME), whereas in bovine macrophages the mechanism was inconclusive. This is the opposite with A2 as phagocytosis occurs in ovine macrophages. T10 was inhibited by both treatments in ovine macrophages. There may be a lack of effect of the inhibitors on BAM and this needs more attention. As A1 and T10 were later found to be predominantly extracellular, entry is an ambiguous measurement with these serotypes. Other inhibitors could be employed and more experiments may pinpoint the exact mechanisms involved. The end result of internalisation is the same regardless of the mechanism and it is unknown whether the difference in uptake results in one type of killing mechanism being induced against which the bacteria has defences. A larger study would increase the understanding of the mechanisms and outcome, as would the extension of the study to other phagocytic cells.

The investigation of extracellular versus intracellular bacteria suggests that especially in serotype A2 there is no evasion of phagocytosis. Mustafa (1995) reported increased uptake of opsonised killed A2 with the serum containing high anticapsular titres. In bovine macrophages the prior incubation with purified capsular polysaccharide reduced the phagocytosis of A1 (Czuprynski *et al.,* 1991b). Both of these reports infer that capsule can hinder phagocytosis and that the presence of anticapsular antibody would abrogate that effect. The results presented here suggest that A2 is phagocytosed and can survive intracellularly in comparison to the other two serotypes. It appears that A1 and T10 do possess evasive factors and that they can exert an equally toxic effect on the macrophages by adhering to the surface of the

cell. The observation of A1 and T10 bacteria on the surface of the macrophages of both species may account for the lower recovery of viable bacteria from phagocytosis assays as they would be susceptible to the gentamicin. The possession of O-antigen LPS on A1 and T10 observed in an earlier chapter may indicate the possibility of binding of LPS to lipopolysaccharide binding protein which in turn binds to CD14 on the macrophage.

There are many differences between the interactions of the three serotypes investigated on the two macrophage species. These could be investigated further preferably with the use of isogenic mutants deficient in selected structures and virulence factors which would point to where these differences lie. The end result of the interaction is death of the macrophage regardless of these differences. The obvious difference in the A2 serotype of large numbers of intracellular bacteria which enter via a variety of mechanisms and which appear not to be affected by macrophage killing mechanisms deserves more attention. A1 and T10 possess mechanisms which enable them to effectively evade phagocytosis and this needs to be investigated further. The results indicate that the presence of antibody or complement does not enhance phagocytic clearance and specific antibodies may enhance this. Initial clearance in the lung may require high levels of bactericidal activity. It may be that cytokines play an important role in the induction of specific killing mechanisms and are not being stimulated or are being blocked by bacterial factors. Specific IgA at the upper respiratory tract (URT) mucosal surface to prevent colonisation would possibly reduce the cases of pasteurellosis. Such research would also lead into areas of predisposition to pasteurellosis by viral and bacterial agents.

CHAPTER 8

GENERAL DISCUSSION

The initial studies in this thesis focused on the use of serum and tracheobronchial washings as culture medium in order to imitate in-vivo growth conditions. Many of the induced proteins have been previously described but few have undergone extensive characterisation. Western blotting utilising convalescent lamb serum also revealed antibody recognition of antigens some of which have been described previously. These particular antigens are of interest in vaccine improvement and require further characterisation. Each serotype responded differently in each of the culture fluids represented by the differences observed in the bacterial and immunogenic profiles. Only A2 in-vivo bacterial cells were available for comparison with the culture fluid grown bacteria. The profiles of proteins from otbw and serum cultures were similar to the in-vivo grown samples particularly in terms of immunogenicity. The main focus were the IRPs as many of these proteins are already well characterised. In most cases serum-grown bacteria produced IRPs at molecular weights already reported. There was however, a lack of recognition of the A2 35 kDa IRP by the convalescent lamb serum. Whether the effects observed are due to induced or reduced expression or maybe just changes in the epitopes which the antibodies recognise remains unknown and deserves more attention. The capsular polysaccharide showed various size capsules depending on the culture medium, and the lack of observable LPS from btbw culture highlights adaptation to various environments by *Pasteurella* spp. Antigen recognition of LPS and leukotoxin showed little cross

reaction when samples were probed with heterologous antiserum and this may have important implications for protection.

The in-vivo fluids sustained viability of the three serotypes beyond that expected. There was obvious species specificity for survival only in ruminant and murine fluids which maintained viability and favoured survival in tbws rather than serum as survival fluids. This has implicated respiratory tract secretions in the ability to maintain bacteria in long-term survival at low nutrient concentrations. Survival in water was also observed and though this was only for a few days it has important implications for the spread of the disease by susceptible animals in the vicinity of an infected animal. The morphology of the bacteria changed dramatically and this is similar to that observed for other organisms undergoing starvation/survival. Serotype A2 was substantially more robust than the other serotypes. Whether this was linked to the fact that A2 is the most common nasopharyngeal isolate of both sheep and cattle is yet to be investigated.

Chapters 3 and 4 highlighted the requirement for a method where the isolation of in-vivo bacteria would have been useful for further analyses of the bacteria. The IMS technique was relatively successful with even non-specific binding reduced where there would not be any interference with analysis. This study highlighted the possible differences not only between in-vitro grown and in-vivo isolated bacteria but also between those which colonise and those which are present in diseased tissue. There was also evidence that colonising *P. haemolytica* and *P. trehalosi* possessed mechanisms which evade immune defences and the future of protection against pasteurellosis may be improved by stimulation of mucosal immunity and the prevention of colonisation.

Superoxide dismutase was readily detected in all 3 serotypes. A2 was different in that it possessed a periplasmic copper/zinc SOD. This enzyme was, however, no more effective against the effects of superoxide when compared to the enzyme in serotype T10. As whole bacterial lysates were used in this study, a purified enzyme or isogenic mutant deficient in the SOD gene would help in defining a role for the enzyme in pathogenesis.

The three serotypes again differed in their interaction with alveolar macrophages. These differences were observed mainly with serotype A2 which survived phagocytosis the longest. All serotypes caused the eventual death of all macrophages present. Phagocytic uptake and survival was high for A2 in the absence of convalescent serum components and this suggests differences in receptor uptake mechanisms. This was apparent by the substantially higher ratio of intracellular-located A2 in comparison to the other serotypes which were predominantly extracellular which for A1 and T10 suggest phagocytic evasion mechanisms. All serotypes eventually overcome the macrophage and extended investigations into the mechanisms involved and to stimulate factors which may participate in the effective removal of the bacteria.

This thesis has shown serotype A2 to be very different in all ways investigated from the other two serotypes. This is not entirely an isolated view. Davies & Donachie (1996) have recently shown that serotype A2 rRNA analysis places the serotype into a separate group. This ability of A2 to adapt and survive in the host results in an organism extremely pathogenic for sheep and it appears that protection against this serotype will prevent the majority of deaths from pasteurellosis. Serotype T10 was reclassified recently into a separate species and has not been extensively researched. It may in the future receive the attention it deserves due to the similarity of the disease

produced to septic shock in other species, and there are good animal models available for study in sheep. T10 is a successful organism as demonstrated by its ability to withstand superoxide attack and to cause substantially more damage to macrophages than the other two serotypes, even with the presence of a comparatively less potent leukotoxin. The importance of serotype A1 in bovine pasteurellosis cannot be ignored even though it appears to be deficient in effective mechanisms when compared to A2 and T10.

All three serotypes possess aspects which require more attention in order to understand the pathogenesis of the disease and allow progress towards better protection. It must be stressed that the in-vivo situation should always be prominent in this kind of research as the answers may lie with the preferred host species as well as with the bacteria.

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APPENDIX I

Chemically defined media

1) Method of Jackson & Burrows (1956)

4.12g KH_2PO_4 in 300ml of distilled water (pH 6.8 - 8.0 with NaOH)
2.24g Glucose (3.7ml of 60% solution) *
0.53g NH_4 CL
0.53g $(NH_4)_2$ SO_4 in 100ml of water
0.12g DL-phenylalanine
0.06g DL-methionine
0.048g L-cystine (10ml 0.48% solution) *
0.132g DL-glutamic acid
0.05g Glycine
0.048g DL-valine
0.027g L-leucine
0.055g DL-isoleucine
0.020g DL-serine
0.048g DL-threonine
0.25ml 0.1% pantothenate
1ml Biotin (1.25 mg/L)
0.1g Mg SO_4. $7H_2O$
1ml Zn SO_4. $7H_2O$ (0.01%)
1ml Mn SO_4. $7H_2O$ (0.01%)
1ml Lactic acid (4% v/v)
20g Agar

Medium was made up to 1L adjusted to pH 7 with NaOH.
Broth was made by omitting agar.
Iron replete contained 0.025g haemin in 0.01M NaOH, in iron restricted medium this was omitted.
* made separately and filter sterilised.

2) Method of Hu *et al.* (1986)

32.3g Na_2 HPO_4. $12H_2O$
1.36g KH_2PO_4
1.19g NaCL
0.25g Mg SO_4. $7H_2O$
6.0g Glucose
0.2g L-arginine
1.6g L-aspartic acid
0.12g L-cysteine hydrochloride
0.2g L-serine
0.15g L-glutamic acid
0.065g L-isoleucine
0.065g L-leucine
0.095g L-phenylalanine
0.09g L-tyrosine
0.002g Calcium pantothenate
0.005g Nicotinamide

0.0001g Thiamine hydrochloride
0.001g Orotic acid

15g of agar per litre was added to produce solid media.
This medium should contain <0.02μg/ml of iron so for replete broths and agar 50μM $FeCl_3$ was added.

3) Method of Wessman *et al.* (1966)

0.58g L-alanine
0.74g L-arginine HCl
1.28g L-aspartic acid
4.06g L-glutamic acid
0.36g Glycine
0.56g L-histidine HCl
1.10g L-isoleucine
1.66g L-leucine
1.48g L-lysine HCl
0.9g L-phenylalanine
1.92g L-proline
1.30g L-valine
0.88g L-threonine
0.29g L-tyrosine
0.314g L-cysteine HCl
2.10g Citric acid *
4.36g $K_2 HPO_4$ *
0.278g Fe SO_4 *
0.016g Mn SO_4 *
2.465g Mg SO_4
0.0025g Calcium pantothenate
0.005g Nicotinamide
0.02g Thiamine hydrochloride
10g D-galactose
1g D-glucose

pH to 7.4 and add 15g of agar for solid medium.
* in 100ml of distilled water pH to 6.5 with NaOH.
Fe SO_4 omitted in iron restricted media.

SDS PAGE for protein detection.

2 x 0.75 mm 10% gels

Resolving gel -	15.9ml	Distilled water
	10 ml	Buffer (1.91g tris, 0.4g SDS, water to 100ml, pH 8.8)
	12.7ml	Acrylamide (30% BDH)
	2.22g	Sucrose
	15µl	TEMED
	150µ	Ammonium persulphate

Stacking gel -	4ml	Distilled water
	5ml	Buffer (3.03g tris, 1g SDS, water to 100ml, pH 6.8)
	1ml	Acrylamide
	10µl	TEMED
	75µl	Ammonium persulphate

Electrode buffer -	14.4g	Glycine
	3.03g	Tris
	1g	SDS

Make up to 1L with distilled water.

Coomassie blue -	1g	Coomassie brilliant blue R250 (Sigma)
	100ml	Glacial acetic acid
	500ml	Methanol
	400ml	Distilled water

Dissolve overnight and filter through a Whatman's No 1 filter paper. Pour directly onto the gel and stain for a minimum of 2 hours.

Destain -	70ml	Glacial acetic acid
	230ml	Methanol
	700ml	Distilled water

Pour off stain, add destain and change until required staining is achieved.

Silver Stain - step 1-	50%	Methanol
	10%	Glacial acetic acid
step 2 -	5%	Methanol
	7%	Glacial acetic acid
step 3 -	10%	Glutaraldehyde

step 4 - Prolonged washing overnight.

step 5 -	500µl	1mg/ml dithiothreitol
step 6 -	0.1%	Ag NO_3

step 7 - Rinse in distilled water

step 8 - Rinse in developer (3% Na_2CO_3, 50µl of 37% formaldehyde in 100ml of water), develop to staining requirement.

step 9 - Terminate development with 2.3M citric acid.

Western Blotting

Electrode buffer	3g	Tris
	14.42g	Glycine
	250ml	Methanol
	To 1L with distilled water	

Blot wash buffer	5ml	Tween 20
	0.37g	EDTA
	20.45g	NaCl
	100ml	10x PBS (400g NaCl, 10g KCl, 57.5g Na_2HPO_4, 10g KH_2PO_4, distilled water to 5 litres)
	To 1L with distilled water	

PAGE for detection of LPS

2 x > 0.75 mm 14% gels

Resolving gel -	3.45ml	Distilled water
	17.5ml	Double strength buffer (90.855g Tris, 1L distilled water, pH8.8)
	12.25ml	40% acrylamide
	50µl	TEMED
	1.75ml	Ammonium persulphate (15mg/ml)

Stacking gel -	3.5ml	Distilled water
	5ml	Double strength buffer (3.03g Tris, 100ml distilled water, pH6.8)
	1ml	40% acrylamide
	20µl	TEMED
	0.5ml	Ammonium persulphate

Electrode buffer -	6.057g	Tris
	28.827g	Glycine
	2g	SDS

Make up to 2L with distilled water.

PAGE for SOD detection

2 x 0.75 mm 7% gels

Resolving gel -	4.85ml	Distilled water
	2.5ml	1.5M tris/HCl pH8.8
	2.5ml	30% acrylamide
	50µl	10% ammonium persulphate
	5µl	TEMED

Stacking gel -	6.1ml	distilled water
	2.5ml	0.5M tris/HCl pH 6.8
	1.3ml	acrylamide
	50µl	Ammonium persulphate
	10µl	TEMED

Electrode buffer -	9g	Tris
	43.2g	Glycine

Make up to 600ml with distilled water. Dilute 60ml with 240ml of distilled water to use.

RPMI 1640

RPMI 1640	164ml
FBS	20ml
Glutamine	4ml (14.6g/L)
Pen/Strep	2ml (10,000u/ml, 10,000mg/ml)
Nystatin	3ml (10,000u/ml)
Mercaptoethanol	1ml (10µl in 10ml PBS)
8% sodium bicarbonate	5ml
HEPES	2.4ml (2.5M)

APPENDIX II

Chapter 4.

All viable counts are the mean of triplicate counts from duplicate samples

* Denotes mixture of normal and micro-colonies
^{0} Denotes addition of fresh fluid
\ Denotes no growth on agar
mc denotes micro-colonies only

	Fig 4.1a	RAW DATA				
DATE	DAY	A1		A2		T10	
30/11/93	0	$1.1X10^{5}$		$4x10^{5}$		$2.2x10^{5}$	
1/12/93	1	$5X10^{8}$		$1.2x10^{8}$		$2.3x10^{8}$	
14/12/93	14	$1X10^{6}$		$3x10^{5}$		\	
6/1/94	37	$3X10^{5}$		$3x10^{5}$		mc	
2/3/94	92	$1.3X10^{6}$		$7x10^{5}$		\	
21/3/94	111	$1.6X10^{5}$		$2x10^{5}$		\	
22/7/94	244	mc	0	$6x10^{5}$	0	mc	0
25/7/94	247	mc	0	$1x10^{4}$	0	mc	0
27/7/94	249	$1.2x10^{5}$		$4x10^{5}$		mc	
9/8/94	264	mc	0	$4x10^{5}$		mc	0
31/8/94	286	\		$8x10^{7}$		\	
28/9/94	314	mc		$5x10^{5}$		\	

	Fig 4.2a	RAW DATA		
DATE	DAY	A1	A2	T10
31/8/93	0	$3X10^8$	$1.2x10^9$	$9x10^8$
1/9/93	1	$1.3X10^8$	$1.8x10^8$	$8x10^7$
3/9/93	3	$6X10^6$	$3x10^6$	$7x10^7$
6/9/93	6	$5X10^6$	$4x10^5$	$7x10^6$
28/9/93	28	$5X10^5$	$6x10^5$	$1.5x10^5$
27/10/93	59	$9X10^5$	$9x10^5$	$2.4x10^4$ *
2/11/93	65	$5X10^5$	$4x10^5$	$1.5x10^5$
10/11/93	73	$5X10^5$	$4x10^5$	$6x10^5$
23/11/93	86	$4X10^5$	$5x10^5$	\
1/12/93	94	$7X10^5$	$4x10^5$	$1.5x10^5$
14/12/93	107	$1.5X10^5$	$4.8x10^5$ *	mc
6/1/94	130	$9X10^5$	$5x10^5$	mc
13/1/94	137	$2.4X10^5$	$2.7x10^5$	mc
3/2/94	158	$1.3X10^5$ *	$1.8x10^5$ *	mc
2/3/94	185	$6X10^5$	$4x10^5$	mc
21/3/94	204	$1X10^5$	$2x10^5$	mc
22/7/94	337	$5X10^3$ 0	$7x10^4$ 0	mc 0
25/7/94	340	mc 0	mc 0	mc 0
27/7/94	342	$1x10^4$	$4x10^5$	\
9/8/94	357	mc 0	$3x10^6$	$9x10^4$
31/8/94	379	mc	$1x10^7$	\
28/9/94	407	mc	$6x10^5$	mc

	Fig 4.1b	RAW DATA		
DATE	DAY	A1	A2	T10
1/3/94	0	$1.2X10^6$	$4X10^5$	$6x10^5$
2/3/94	1	$3X10^4$	$3X10^4$	$8x10^4$ *
21/3/94	20	$1X10^3$ *	$1X10^4$	mc
22/3/94	153	$7X10^3$ 0	mc 0	$8x10^3$ 0
25/3/94	156	$2.2X10^4$ 0	$1.4x10^4$ 0	$3x10^4$ 0
27/3/94	158	$1.1X10^4$	$1x10^4$	$3x10^4$
9/8/94	173	$9X10^6$	$4x10^6$	$1x10^7$
31/8/94	195	$8X10^4$	\	$1.4x10^5$
28/9/94	203	$2X10^6$	$5x10^5$	$6x10^5$

	Fig 4.2b	RAW DATA					
DATE	DAY	A1		A2		T10	
1/3/94	0	$5X10^9$		$7X10^7$		$2x10^7$	
2/3/94	1	$1X10^6$		$1X10^5$		$2x10^5$	
21/3/94	20	$2X10^5$		$6X10^4$		$1x10^3$	*
22/7/94	153	$1X10^4$	0	$6X10^3$	0	$4x10^4$	0
25/3/94	156	$7X10^3$	0	mc	0	mc	0
27/3/94	158	$7X10^4$		$6x10^3$		mc	
9/8/94	173	$2X10^4$		$1x10^4$		$1x10^3$	
31/8/94	195	$3X10^7$		$9x10^6$		\	
28/9/94	203	$9X10^4$		$3x10^3$		mc	

	Fig 4.1c	RAW DATA			
DATE	DAY	A1	A2		T10
30/11/93	0	$1.1X10^5$	$4X10^5$		$2.2X10^5$
1/12/93	1	$8X10^7$	$2X10^8$		$2X10^7$
14/12/93	14	$3X10^7$	$5X10^6$		$1.2X10^7$
6/1/94	37	$4X10^6$	$5X10^5$	*	$9X10^6$
2/3/94	92	$3X10^6$	$6X10^6$		$1.6X10^7$
21/3/94	111	$2X10^5$	$3.1X10^5$		$1.5X10^5$
22/7/94	244	$1.5X10^4$	$1X10^6$		$2X10^5$
25/7/94	247	$1.2X10^4$	$4X10^5$		$5X10^5$
27/7/94	249	$1.5X10^5$	$7X10^5$		$1.8X10^6$
9/8/94	264	$4X10^3$	$1X10^6$		$8X10^5$
31/8/94	286	\	$9X10^5$		$5X10^5$
28/9/94	314	$3X10^6$	$2X10^5$		$5X10^5$

	Fig 4.2c	RAW DATA		
DATE	DAY	A1	A2	T10
30/11/93	0	$11X10^{9}$	$10x10^{10}$	$10x10^{10}$
1/12/93	1	$2.3X10^{8}$	$1.5x10^{9}$	$6x10^{7}$
14/12/93	14	$1.1X10^{7}$	$2x10^{7}$	$1x10^{7}$
6/1/94	37	$1.2X10^{7}$	$6x10^{6}$	$2x10^{6}$
13/1/94	44	$1.4X10^{7}$	$1.5x10^{6}$	$1.3x10^{7}$
3/2/94	65	$1.3X10^{6}$	$5x10^{5}$	$5x10^{6}$
2/3/94	92	$1X10^{7}$	$1.1x10^{6}$	$2x10^{7}$
21/3/94	111	$4X10^{5}$	$4x10^{5}$ *	$2.5x10^{6}$
22/7/94	244	$1X10^{4}$	$2x10^{5}$	$2.2x10^{5}$
25/7/94	247	mc	$1x10^{5}$	$3x10^{5}$
27/7/94	249	mc	$2x10^{5}$	$1x10^{6}$
9/8/94	264	$3x10^{3}$	$1.9x10^{5}$	$3x10^{5}$
31/8/94	286	$5x10^{4}$	$4x10^{5}$	$6x10^{5}$
28/9/94	314	mc	$2x10^{6}$	$2x10^{6}$

	Fig 4.1d	RAW DATA		
DATE	DAY	A1	A2	T10
30/11/93	0	$1.1X10^{5}$	$4X10^{5}$	$2.2x10^{5}$
1/12/93	1	$4X10^{8}$	$1.2X10^{9}$	$5x10^{7}$
14/12/93	14	$2X10^{6}$	$7X10^{5}$	$1x10^{5}$
6/1/94	37	$1.7X10^{6}$ *	$6X10^{4}$ *	$2x10^{6}$
2/3/94	92	$3X10^{7}$	$9X10^{7}$	$4x10^{6}$
21/3/94	111	$1.1X10^{7}$	$1.5X10^{6}$	$2x10^{6}$
22/7/94	244	$4X10^{3}$	$4X10^{5}$	$6x10^{5}$
25/7/94	247	$1X10^{4}$	$2X10^{4}$	$7x10^{4}$
27/7/94	249	$1.3X10^{6}$	$1.1X10^{6}$	$1.3x10^{6}$
9/8/94	264	$1X10^{4}$	$4X10^{4}$	$5x10^{5}$
31/8/94	286	$3X10^{4}$	mc	mc
28/9/94	314	$2X10^{4}$	$7x10^{4}$	$3x10^{5}$

	Fig 4.2d	RAW DATA		
DATE	DAY	A1	A2	T10
30/11/93	0	$11X10^{9}$	$10x10^{10}$	$10x10^{10}$
1/12/93	1	$1X10^{8}$	$1x10^{8}$	$7x10^{7}$
14/12/93	14	$8X10^{7}$	$9x10^{6}$	$8x10^{6}$
6/1/94	37	$1.4X10^{7}$	$1.4x10^{7}$	$7x10^{6}$
13/1/94	44	$2.8X10^{7}$	$1.5x10^{7}$	$2.2x10^{7}$
3/2/94	65	$9X10^{6}$ *	$6x10^{6}$	$6x10^{6}$ *
2/3/94	92	$6X10^{7}$	$3x10^{6}$	$2x10^{7}$
21/3/94	111	$3X10^{6}$	$4x10^{5}$	$4x10^{6}$ *
22/7/94	244	$2X10^{5}$	$5x10^{5}$	$4x10^{6}$
25/7/94	247	$2X10^{4}$	$6x10^{4}$	$1.3x10^{5}$
27/7/94	249	$7X10^{5}$	mc	$2x10^{5}$
9/8/94	264	$3X10^{5}$	$4x10^{5}$	$1x10^{4}$
31/8/94	286	mc	$1.5x10^{6}$	$3x10^{6}$
28/9/94	314	mc	$2x10^{5}$	$2x10^{5}$

Chapter 6. Raw data to Table 6.1 (Data are expressed as SOD units/mg^{-1} protein)

	H_2O			KCN			H_2O_2			EDTA		
EXPT	A1	A2	T10	A1	A2	T10	A1	A2	T10	A1	A2	T10
1	7.37	150.51	83.09	7.08	36.95	90.95	5.91	135.42	198.33	7.22	217.59	98.21
2	5.16	128.64	36.89	5.23	31.7	37.02	7.15	395.9	127.25	3.11	61.4	28.4
3	6.28	321.35	121.02	7.04	62.75	120.74	7.04	137.62	40.05	1.4	97.29	140.78
4	8.25	498.8	169.58	7.96	73.5	170.34	8.21	516.32	98.34	20.07	508.98	361.52

Chapter 6. Chapter 6 Raw data to Table 6.5. (Data are expressed as SOD units/mg^{-1} protein)

	ANTISERUM			ANTISERUM + PROTEIN G			SPF SERUM + PROTEIN G		
EXPT	A1	A2	T10	A1	A2	T10	A1	A2	T10
1	4.58	19.63	5.05	3.27	20.76	10.6	5.55	18.65	8.07
2	2.88	13.97	5.24	3.48	25.61	5.67	4.73	32.2	6.33
3	5.68	28.12	10.36	3.33	3.27	1.01	3.74	26.5	5.47
4	4.75	23.13	19.3	1.99	16.6	6.75	2.22	21.09	1.8

Chapter 6. Raw data for Fig 6.4 (Data are log^{10} cfu ml^{-1})

	CONTROL 0 MIN			CONTROL 15 MIN			CONTROL 30 MIN		
EXPT	A1	A2	T10	A1	A2	T10	A1	A2	T10
1	5.9	6	5.8	5.6	6	6	5.3	5.9	5.9
2	5.9	6.4	6.5	6	6	6	5.9	5.8	6
3	6.5	5.9	5.9	6	6.6	6.5	5.9	6.4	6.5

	ASSAY 0 MIN			ASSAY 15 MIN			ASSAY 30 MIN		
EXPT	A1	A2	T10	A1	A2	T10	A1	A2	T10
1	5.9	6.1	5.9	0	5.6	5.5	0	5	6.1
2	6	5.9	6	0	5	6.1	0	4	5.2
3	5	6.3	6.4	0	5.8	6.3	0	5.8	6.3

Chapter 6. Raw data for Fig 6.5 (Data are log^{10} cfu ml^{-1})

	CONTROL 0 MIN				CONTROL 15 MIN				CONTROL 30 MIN			
EXPT	DO800	M74	L94F	L48C	DO800	M74	L94F	L48C	DO800	M74	L94F	L48C
1	5.4	5.9	5.6	6.6	5.6	5.6	5.9	5.3	5.5	6	5.9	5.3
2	5.5	6	6	5.7	5.5	6.1	5.8	5.5	5.5	6	5.5	5.5
3	6.4	6.4	6	6.3	6.4	6.4	6	6.3	5.7	6.7	6	5.6

	ASSAY 0 MIN				ASSAY 15 MIN				ASSAY 30 MIN			
EXPT	DO800	M74	L94F	L48C	DO800	M74	L94F	L48C	DO800	M74	L94F	L48C
1	5.5	5.9	5.6	5.6	4.6	5.9	5.4	5	0	0	0	0
2	5.6	6	5.7	5.8	0	6.7	5.3	5.3	0	5	0	0
3	6.3	6.3	6	6.4	0	5.3	0	5	0	5.3	0	5

Chapter 6. Raw data for Fig 6.6 & 6.7. (Data are log^{10} cfu ml^{-1})

	0 h				2 h in OAM				2 h in BAM			
EXPT	DO800	M74	L94F	L48C	DO800	M74	L94F	L48C	DO800	M74	L94F	L48C
1	8	8	8	8	3.3	3.3	3	3.3	3.6	3	3	0
2	7	7.5	7.3	7.9	0	0	3.5	0	0	0	3	0
3	7.8	7.1	7.7	7	3	3	0	0	3	0	3	3

Chapter 7. Raw data for Fig 7.1 (Data are expressed as $\log^{10}$ cfu ml^{-1})

	0 MIN			15 MIN			30 MIN			45 MIN		
EXPT	A1	A2	T10	A1	A2	T10	A1	A2	T10	A1	A2	T10
1	7.3	8	8.5	4	5.5	5.3	3.8	4.6	4.3	3.8	4.1	3.9
2	7.3	7.5	7.6	0	3	3	0	3	3	0	3	3
3	7.3	7.8	7.7	3.7	4.5	4.2	3.6	4.7	3.8	3.5	4.5	3.5

	60 MIN			90 MIN			120 MIN		
EXPT	A1	A2	T10	A1	A2	T10	A1	A2	T10
1	3.5	4.3	3	3.9	3.9	0	3.3	3.3	0
2	0	0	0	0	0	0	0	3	0
3	3.6	4.8	0	3	3	3.9	0	0	0

Chapter 7 for Fig 7.2 (Data are expressed as $\log^{10}$ cfu ml^{-1})

	0 MIN			15 MIN			30 MIN			45 MIN		
EXPT	A1	A2	T10	A1	A2	T10	A1	A2	T10	A1	A2	T10
1	7.3	8	8.5	0	4.3	4.6	0	3.3	3.7	0	3	3
2	7.3	7.5	7.6	0	3.8	3	0	3.6	0	0	3.4	3
3	7.3	7.8	7.7	0	3	3	0	3.6	0	3	4	0

	60 MIN			90 MIN			120 MIN		
EXPT	A1	A2	T10	A1	A2	T10	A1	A2	T10
1	0	3	3.6	0	3.3	3.3	0	0	0
2	0	3	3	0	3	0	0	3.3	3.3
3	0	3.6	0	0	3	3	0	3	0

Chapter 7. Raw data for Figs 7.3-7.8. (Raw data are expressed as $\log^{10}$ cfu ml^{-1})

	OAM					BAM				
EXPT	A1	A1+ #	A1+ # *	A1+otbw	A1+ btbw	A1	A1+ #	A1+ # *	A1+ otbw	A1+ btbw
1	0	0	0	3.3	0	0	4	0	0	3
2	0	3	0	0	0	0	0	0	0	0
3	0	3	0	0	4.7	0	0	0	na	na
4	0	0	0	na	na	0	0	0	na	na
5	0	0	0	na	na	0	na	0	na	na

	OAM					BAM				
EXPT	A2	A2+ #	A2+ # *	A2+ otbw	A2+ btbw	A2	A2+ #	A2+ # *	A2+ otbw	A2+ btbw
1	3	3.5	0	3.5	3.5	0	3.3	3.5	3	0
2	3.9	4	4	3.3	3	3	4.3	3	0	0
3	4.2	3.9	4.5	0	3.9	4	3	na	na	na
4	4.5	4.5	na	na	na	na	3.3	na	na	na
5	na	4.5	na	na	na	na	na	na	na	na

	OAM					BAM				
EXPT	T10	T10+ #	T10+ # *	T10+ otbw	T10+ btbw	T10	T10+ #	T10+ # *	T10+ otbw	T10+ btbw
1	3	4.7	3.3	3	0	0	3	3	0	3
2	0	0	0	0	0	0	0	0	0	3
3	3	0	0	4.3	0	0	0	na	na	na
4	0	5	na	na	na	na	3	na	na	na
5	na	3.3	na	na	na	na	na	na	na	na

na - not applicable; # - homologous antiserum; # * - homologous antiserum heat treated.

Chapter 7. Raw data for Figs 7.9 and 7.10.

	OAM						BAM					
	Bacteria only			bacteria opsonised			bacteria only			Bacteria opsonised		
EXPT	A1	A2	T10	A1	A2	T10	A1	A2	T10	A1	A2	T10
1	39	41	77	24	49	92	12.2	0	46.7	18.7	0	68.9
2	8	1	67	9	14	78	0	2	9	11	12	36
3	11	20	11	27	26	29	5	12	43	38	32	72
4	0	31	38	0	0	6	0.9	34	38	1	24	0.4
5	0	50	74	15	0	77	31	24	34	30	32	49
6	na	na	na	na	na	na	8.2	12	29	17	17	38

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Divergent activity and function of superoxide dismutases in *Pasteurella haemolytica* serotypes A1 and A2 and *Pasteurella trehalosi* serotype T10

H.A. Rowe [a], D.P. Knox [a], I.R. Poxton [b], W. Donachie [a,*]

[a] *Moredun Research Institute, 408 Gilmerton Road, Edinburgh, Scotland EH17 7JH, UK*
[b] *Medical School, University of Edinburgh, Teviot Place, Edinburgh, Scotland EH8 9AG, UK*

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Abstract

Representative strains of *Pasteurella haemolytica* serotypes A1 and A2 and *Pasteurella trehalosi* serotype T10 were examined for the presence of superoxide dismutase. Visualisation of superoxide dismutase enzyme activity on polyacrylamide gels, and specific inhibition with potassium cyanide verified a copper/zinc (Cu/Zn) superoxide dismutase only in serotype A2 whereas serotypes A1 and T10 showed other superoxide dismutase activity. Using a simple freeze-thaw method the cellular location of superoxide dismutase enzyme activity was determined in all three serotypes. In serotypes A1 and A2 but not T10 superoxide dismutases were located in the periplasm. The viability of serotypes A2 and T10 cells in the presence of exogenous superoxide was unchanged over a 30 min period, whereas serotype A1 cells declined in viability between 15 and 30 min. Purified immunoglobulin from sheep convalescent serum did not reduce superoxide dismutase activity in the serotypes in an in vitro assay. The presence of this enzyme within the pasteurellae suggests a supportive role in the virulence of this major pathogen of ruminants.

Keywords: *Pasteurella*; Superoxide dismutase; Periplasm

1. Introduction

Pasteurella haemolytica, a Gram-negative bacterium responsible for pneumonia in all ages of sheep, comprises 13 identifiable serotypes of which A2 is the most prevalent cause of pasteurellosis in the U.K. *Pasteurella trehalosi*, which was until recently classified as the T biotype of *P. haemolytica* [1] causes systemic disease in young lambs and older sheep [2]. Both species colonise the upper respiratory tract of apparently healthy sheep, with *P. haemolytica* residing mainly in the nasopharynx and *P. trehalosi* in the tonsils.

Superoxide dismutases (SOD) are metalloenzymes that catalyse the conversion of highly toxic superoxide radicals to hydrogen peroxide and oxygen. Three distinct forms of SOD have been identified; iron (Fe), manganese (Mn) and copper/zinc (Cu/Zn). The first two are common in the cytoplasm of pro-

* Corresponding author. Tel.: +44 (131) 664 3262; fax: +44 (131) 664 8001.

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karyotes and Mn SOD is also present in the mitochondria of eukaryotes. Cu/Zn SODs are found in the cytoplasm of eukaryotes and were thought to be unique to these organisms until their discovery in the fish bacterium *Photobacterium leionathi* [3]. Since then, Cu/Zn SODs have been identified in an increasing number of bacteria, especially in Gram-negative bacteria and including *P. haemolytica* [4]. It is thought that possession of this enzyme contributes to the bacterium's ability to cause disease and/or survive in its host [5]. Although the potential role of SOD in pathogenesis has been investigated using molecular biology little is known about its role in vivo [3,6,7].

2. Materials and methods

2.1. Bacterial strains and culture

P. haemolytica serotype A1 (strain M4/1/2, isolated from calf lung), A2 (strain 124/92, isolated from sheep lung) and *P. trehalosi* T10 (strain 152/94, systemic disease isolate) were maintained at Moredun Research Institute as reference strains stored at −70°C. All bacteria were grown on 7% sheep blood agar (Oxoid) or in nutrient broth No. 2 (Oxoid) at 37°C overnight.

2.2. Bacterial lysates and native PAGE

These were prepared according to the method outlined by Lainson et al. [4].

2.3. SOD assay

This was carried out according to the manufacturer's instructions (RANSOD kit, Randox, Co., Antrim, U.K.). Samples were mixed with substrate, and xanthine oxidase was added to start the reaction. Absorbance of samples and standards were read at a wavelength of 500 nm after 30 min incubation at room temperature.

2.4. Malate dehydrogenase assay

The assay was carried out following the method described in the Worthington Enzyme Manual [8].

2.5. Inhibition studies of SOD

These experiments were carried out to identify the type of SOD in the bacteria. EDTA inhibits all SOD types, hydrogen peroxide inhibits Cu/Zn and Fe SODs and potassium cyanide inhibits only Cu/Zn SOD [3]. Prior to electrophoresis or assay, samples were incubated for 30 min with final concentrations, in aqueous solutions, of 5 mM potassium cyanide, 5 mM hydrogen peroxide or 5 mM EDTA. During gel staining the inhibitors were also present in the nitroblue tetrazolium (NBT) and riboflavin solutions.

2.6. Preparation of periplasmic and cytoplasmic fractions

Bacterial cultures (20 ml) were washed twice in phosphate buffered saline, pH 7.4 (PBS) and resuspended in 500 μl of PBS. The cell suspensions were then frozen at −20°C and thawed at 37°C at least 10 times. After centrifugation at 11 000 × *g* the supernatants (periplasmic fraction) were removed for assay. The remaining bacterial pellets were lysed using the bead-beating method described previously [4] to release cytoplasmic contents. All periplasmic and cytoplasmic contents were assayed for SOD activity and malate dehydrogenase (the latter acts as a cytoplasmic marker) [9].

2.7. Bactericidal assay

Bacterial cultures were grown in nutrient broth at 37°C with shaking for 4.5 h. The bacteria were washed twice in PBS and resuspended in 0.1 M phosphate buffer pH 7.5. Using a microtitre plate, the contents of control wells containing xanthine (0.05 mM), 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT, 0.025 mM) in 0.1 M phosphate buffer pH 7.5 and 0.002 units of xanthine oxidase were mixed and the production of visual formazan dye monitored to show that production of superoxide had occurred. As INT is toxic to *Pasteurella* this was omitted from the test wells. Control samples contained xanthine, buffer and bacteria at 10^5 cfu/ml^{-1}. Experimental wells had xanthine, xanthine oxidase and the same bacterial inoculum. The plate was incubated at 37°C and 10 μl samples were removed at times 0, 15 and 30 min. Samples were

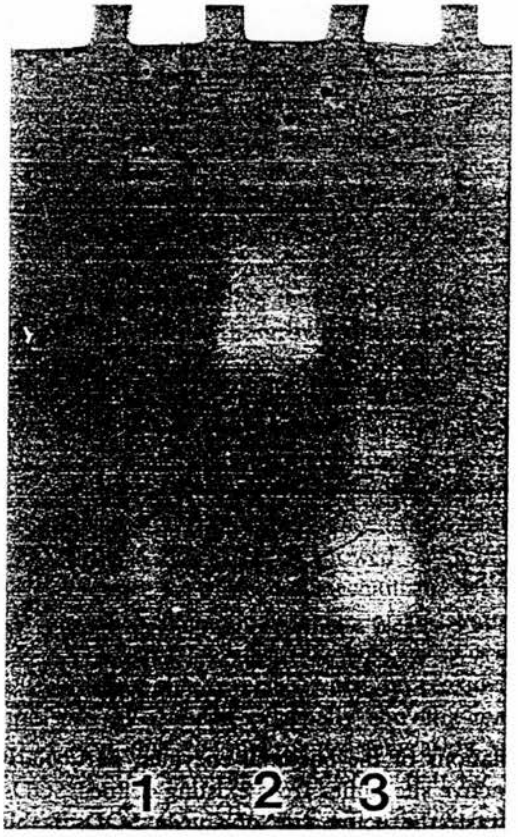


Fig. 1. Native PAGE gel of bacterial lysates (5 μl loaded) stained for SOD activity. Lane 1, serotype A1 (17.35 units SOD activity in 0.15 mg protein); lane 2, serotype A2 (472.7 units SOD activity in 0.055 mg protein); lane 3, serotype T10 (112.2 units SOD activity in 0.045 mg protein).

diluted serially, plated onto 7% sheep blood agar in duplicate and incubated at 37°C overnight.

2.8. *Reactivity with antiserum*

Antiserum was raised in specific pathogen-free lambs inoculated with live *P. haemolytica* or *P. trehalosi* cells. Briefly the lambs [10] were inoculated intratracheally with 2 ml and subcutaneously with 1 ml of a $10^6/ml^{-1}$ bacterial suspension at 0 and 3 weeks. A final intranasal inoculation with 2 ml of a $10^9/ml^{-1}$ bacterial suspension was administered 2 weeks later. Seroconversion of the lambs was monitored using western blot analysis of bacterial envelope preparations and an indirect haemagglutination assay. Negative control serum was obtained from specific pathogen-free lambs infected with orf virus.

To determine whether specific IgG bound to and inhibited SOD activity, 10 μl of sample was mixed in a total volume of 100 mg ml^{-1} of purified IgG [11] in 0.1 M Tris/HCl pH 8. This was incubated with mild agitation at room temperature for 1 h. Protein G (5 ml Protein G insolubilised on Sepharose 4B, Fast Flow, Sigma) was added and incubation continued for a further 30 min. Samples were centrifuged and the supernatant removed and assayed using the Ransod kit.

3. Results and discussion

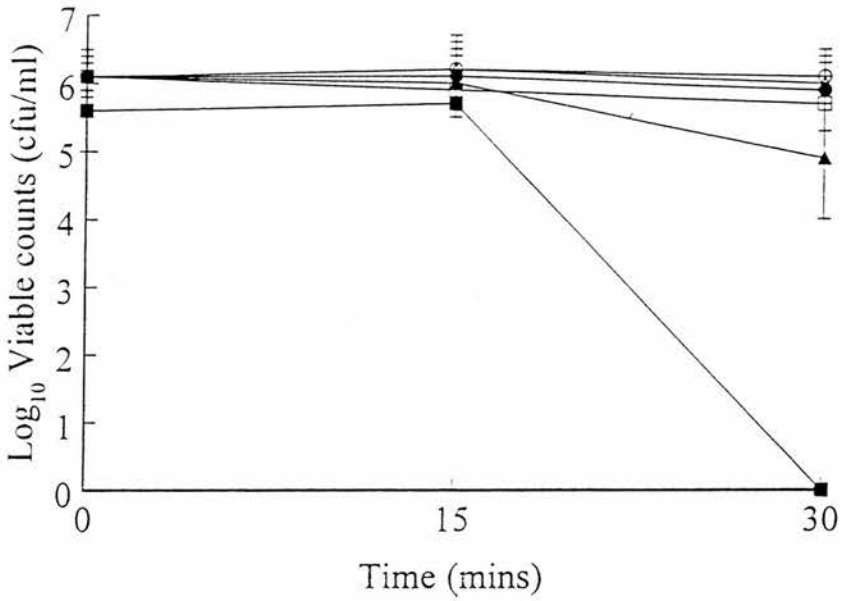
Superoxide dismutase activity was detected readily in crude lysates of all three serotypes (Fig. 1). Differences were observed in the electrophoretic mobilities of the enzymes from the three serotypes and T10 showed two zones of activity. The levels of activity measured in the assay correlated with the strength of reaction observed on the gel. The three serotypes therefore contain different isoforms and levels of activity suggesting a diversity not only within the Pasteurellaceae but also within *P. haemolytica* itself.

Inhibitors of SOD activity were used to identify SOD types. Table 1 shows clearly that potassium

Table 1
Superoxide dismutase activity in the presence of the inhibitors KCN, H_2O_2 and EDTA

Serotype	Control (H_2O)	KCN	H_2O_2	EDT
A1	6.7 (0.7)	6.8 (0.6)	7.1 (0.5)	7.9 (4.2)
A2	274.8 (86.2)	51.2 (10.1)	296.3 (95.5)	221.3 (102)
T10	102.6 (28.2)	104.8 (27.9)	116.0 (32.9)	157.2 (71.9)

Results are expressed as mean (4 separate experiments) SOD units mg^{-1} protein showing the standard error of the mean in brackets. Controls contained H_2O and the concentration of inhibitors was 5 mM in each case. Inhibition of enzyme activity is defined as a decrease of ≥50% of the control activity.



ig. 2. Survival of serotypes A1, A2 and T10 by viable count in the presence of exogenous superoxide. Controls (open symbols) omitted nly xanthine oxidase and samples (bold symbols) contained xanthine oxidase to initiate superoxide generation. □, A1; △, A2; ○, T10; , A1; ▲, A2; ●, T10.

yanide inhibited only A2 SOD activity (81.4% inibition) thus confirming it as a Cu/Zn SOD. No ıhibition of the SOD of any serotypes could be chieved with EDTA or H_2O_2 even at elevated moıities (data not shown). The inhibitors EDTA and $[_2O_2$ have been effective in numerous studies at the ıme or lower concentration than those in this study ɛt no inhibition was observed here. The A2 SOD as markedly, but not completely, inhibited by KCN dicating that the analytical system was appropriate ır the definitions attempted. The insensitivity of the her SODs to inhibition may indicate that the conformations of the bacterial enzymes, particularly at the active site, differ from existing defined SODs. In contrast, the active site of Cu/Zn SODs is highly conserved, even though its other domains show little antibody cross reactivity between species [3,5], and this may account for the clear inhibition by cyanide. The SODs of A1 and T10 remain uncharacterised and in light of current knowledge are relatively novel.

Bacteria have been shown to possess SOD enzymes in the periplasm and the periplasmic location of SOD has also been indicated by the presence of an

ble 2
easurement of SOD and malate dehydrogenase activity from preparations of periplasmic and cytoplasmic contents using a freeze-thaw thod

mple	Units of SOD activity mg^{-1} protein	Units of malate dehydrogenase activity mg^{-1} protein	% Location of SOD	% Location of malate dehydrogenase
periplasm	52.3	0.01	83.8	2.4
cytoplasm	10.1	0.4	16.2	97.6
periplasm	475.3	0.009	75.2	23
cytoplasm	156.5	0.03	24.8	77
) periplasm	24.6	0.04	40.9	33.3
) cytoplasm	35.5	0.08	59.1	66.7

ɛation is expressed as a percentage of the combined activity of both cytoplasmic and periplasmic contents.

N-terminal leader peptide sequence [5,12]. Superoxide generated in the cytosol does not pass through the cytoplasmic membrane into the periplasm. Thus periplasmic SOD is thought to deal with external superoxide [13,14]. This prompted investigation into the location of the enzyme in *P. haemolytica* and *P. trehalosi*. Table 2 shows that SOD activity was located in the periplasm of A1 (83.8%) and A2 (75.2%, Cu/Zn), but was in the cytoplasm of serotype T10 (59.1%). It is possible that the location of T10 SOD may depend on the growth phase of the bacteria as the SOD of *Caulobacter cresentus* locates preferentially in the periplasm in stationary-phase growth [13]. An environmental signal such as a change in oxygen availability may stimulate secretion into the periplasm although this may happen only in vivo. Survival of T10 in the presence of exogenously generated superoxide (Fig. 2) showed some indication of the presence of such a mechanism. Serotype A2 also possessed a good degree of resistance to superoxide whereas A1 did not survive beyond 30 min. This effect was reversed when superoxide dismutase was also added externally showing loss of viability was due to superoxide generation (data not shown). This experiment indicates that the possession of a Cu/Zn SOD or a periplasmic located enzyme is not indicative of protection from superoxide. In vivo, however, 30 min may be long enough to ensure the bacteria survive the encounter especially when structural features and bacterial virulence factors are taken into account.

To determine whether the enzyme elicited an immune response during infection, neutralisation of enzyme activity was attempted using purified IgG from homologous convalescent antiserum and compared with control serum from orf virus infected lambs. The results in Table 3 show a trend towards a reduction in enzyme activity when specific antiserum and protein G are present with serotypes A1 and A2 but not T10. This result was, however, not statistically significant. Specific antibodies neither neutralised enzyme activity nor bound the enzyme as similar activity was present when Protein G was incorporated into the mixture. The absence of a significant antibody reaction is not unusual. The lack of antibody response to SOD in vivo has been shown with *Brucella abortus*, and the same study also failed to demonstrate a cell-mediated immune response [6].

This study has shown that the Cu/Zn SOD of *P. haemolytica* serotype A2 is located in the periplasm and that A2 does not appear to contain a second SOD enzyme. The presence of the SOD enzyme can be important in protecting the bacteria not only during the respiratory burst of phagocytes but also from oxygen radicals generated at the tissue surface and mucus during colonisation [15]. It has been suggested that it may not be the production of SOD which is important, but the subsequent production

Table 3
The effect of specific IgG on SOD activity

Sample	Purified IgG source	Protein G*	Mean SOD units mg^{-1} protein (S.E.)
A1	A1 antiserum	−	4.5 (0.6)
	A1 antiserum	+	3.0 (0.4)
	Control serum	−	4.8 (1.1)
	Control serum	+	4.1 (0.7)
A2	A2 antiserum	−	21.2 (3)
	A2 antiserum	+	16.6 (4.8)
	Control serum	−	23.6 (0.4)
	Control serum	+	24.6 (3)
T10	T10 antiserum	−	10.0 (3.3)
	T10 antiserum	+	6.0 (1.97)
	Control serum	−	8.2 (1.8)
	Control serum	+	5.4 (1.3)

*−, no Protein G; +, 100 μg Protein G. Results are mean values of 4 separate experiments. Control serum IgG was incorporated to ensure that any reduction in activity was due to specific IgG. Antibody binding of SOD without loss of activity was controlled for by the presence or absence of Protein G.

of hydrogen peroxide [12]. This has been shown to interfere with ciliary function [16] and this disruption would be advantageous for a respiratory pathogen like *P. haemolytica*. Serotype A2 is by far the most prevalent serotype isolated from nasopharyngeal carriers and cases of pneumonic pasteurellosis [2] and the presence of the Cu/Zn enzyme in A2 and not in A1 may confer an advantage for the organism in vivo. The SODs from all serotypes tested do not appear to elicit an antibody response in vivo even though their periplasmic location would place them in contact with the hosts immune system. While location is important, the type of SOD present may confer an even greater advantage. The response of whole bacteria to the effects of extraneous superoxide indicates that resistance to superoxide cannot be explained by characterisation of the enzyme alone and other factors must be taken into consideration. Further investigation will clarify the putative role of SOD enzymes in pathogenesis.

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Occurrence of [copper, zinc]-cofactored superoxide dismutase in *Pasteurella haemolytica* and its serotype distribution

F.A. Lainson [a,*], N. Thomson [a], H.A. Rowe [a], P.R. Langford [b], K.D. Aitchison [a], W. Donachie [a], J.S. Kroll [b]

[a] *Moredun Research Institute, 408 Gilmerton Road, EH17 7JH Edinburgh, UK*
[b] *Imperial College School of Medicine of St. Mary's, Norfolk Place, London W2 1PG, UK*

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Abstract

Fifty-two ovine strains of *Pasteurella haemolytica* and *P. trehalosi* representing serotypes 1–16 were examined for the presence of [copper, zinc]superoxide dismutase DNA sequences. This was done using a combination of polymerase chain reaction with degenerate primers based on the sequence of the [Cu,Zn]superoxide dismutase gene (*sodC*) in related species and Southern hybridization using a fragment of *sodC* from *P. haemolytica* A2 serotype as a probe. Both detection methods identified a fragment of the *sodC* gene in 9/9 strains of *P. haemolytica* serotype 2 examined and in 5/8 strains of serotype 7. No evidence of this gene was found in any other serotype of *P. haemolytica* or in any *P. trehalosi* serotype. Comparison of DNA sequence showed near identity between *sodC* from the A2 and A7 serotypes of *P. haemolytica* and substantial similarity (70%) to *sodC* previously sequenced in *P. multocida, Haemophilus parainfluenzae and H. influenzae*. Analysis by gel electrophoresis of the superoxide dismutase activity present in cell lysates showed that one or more superoxide dismutase is present in all serotypes. However, cyanide-inhibitable activity, corresponding to [Cu,Zn]superoxide dismutase, was detected only in those strains of serotypes A2 and A7 which showed evidence of the *sodC* gene fragment.

Keywords: *Pasteurella haemolytica*; [copper, zinc]Superoxide dismutase; *sodC*; Ovine; Superoxide dismutase

1. Introduction

Pasteurella haemolytica and *P. trehalosi* are important bacterial pathogens of sheep. Both species are carried in a commensal state in the nasopharynx of apparently healthy animals [1], but each causes a different disease syndrome. *P. haemolytica*, of which there are 13 recognized serotypes, produces pneumonic disease whereas the four serotypes of *P. trehalosi* produce a septicaemic infection. The factors which cause the progression from commensal to pathogenic behaviour are uncertain, but some bacterial factors are recognised as putative virulence determinants. These include leukotoxin, iron-uptake proteins and lipopolysaccharide. Leukotoxin production has been shown to lead to activation of lung neutrophils [2], resulting in generation of an oxidative burst, production of oxygen-free radicals [3], and consequent host tissue damage. While the damage caused to host cells may facilitate bacterial survival, the products of the oxidative burst are potentially highly detrimental to

* Corresponding author.

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Fig. 1. Alignment of [Cu,Zn]SOD peptide sequence from *P. haemolytica* A2 with *P. multocida* [7] and *H. parainfluenzae* [5]. Sequence shown corresponds to residues 88–174 of the 187-amino-acid *H. parainfluenzae protein* [5]. Dots signify identity between adjacent sequences: black arrowhead indicates an invariant Zn^{2+}-ligand His; × indicates additional amino acids conserved across all known bacterial [Cu,Zn]SOD sequences, excluding the highly divergent *Caulobacter crescentus* peptide sequence; open arrowhead indicates a residue previously found to be invariant in bacterial SODs.

the infecting bacteria, and factors allowing organisms to withstand their action may potentiate virulence.

Superoxide dismutase (SOD) is a key metallo-enzyme involved in the detoxification of oxygen-free radicals. Single electron reduction of O_2, achieved through the action of cytochrome oxidase in the course of aerobic metabolism or by NADPH oxidase at the phagocyte membrane, generates superoxide radical anion, which in a further series of reactions leads to production of the highly toxic hydroxyl radical. Mechanisms to remove free superoxide are correspondingly widely found, three varieties of SOD being recognised. In the bacterial cytosol, iron- or manganese-cofactored SOD protects cellular proteins and DNA from free-radical attack. However, as the cytoplasmic membrane is impermeable to superoxide, these SODs cannot dismute superoxide produced outside the cell, for example by neutrophils, leaving the cell surface and periplasmic contents vulnerable. A novel SOD, cofactored by copper and zinc ([Cu,Zn]SOD) and encoded by the gene *sodC*, has been described in the periplasm of a wide range of Gram-negative commensal and pathogenic bacterial organisms, including members of the *Haemophilus–Actinobacillus–Pasteurella* family [4–9]. The part that this form of the enzyme plays in bacterial biology is unknown, but it is appropriately located to be involved in dismutation of superoxide produced in the course of host defence against bacterial infection and, as such, is a potential further determinant of bacterial virulence. A polymerase chain reaction (PCR) approach to the identification of *sodC* genes has been developed based on sequences conserved across a range of organisms [7]. Using this approach, coupled with Southern hybridization and activity assays, we demonstrate here the presence of [Cu,Zn]SOD in *P. haemolytica*, but not in *P. trehalosi*, and show that it has a restricted serotype distribution.

2. Materials and methods

2.1. Bacterial strains

Table 1 lists the ovine strains of *P. haemolytica* and *P. trehalosi* (serotypes 1–16) examined in this study. These were from a collection of reference strains held at Moredun Research Institute.

2.2. [Cu,Zn]SOD activity detection on PAGE

P. haemolytica was plated on blood agar and grown for 16 h at 37°C to form a bacterial lawn. Bacteria scraped from the surface of the agar were resuspended in 5 mM Tris-HCl buffer, pH 6.8, containing 1 mM EDTA, 0.5 mM NADP and DNAase at 0.5 mg/ml and disrupted with a reciprocating bead beater operating at 4000 cycles/min using washed 0.1 mm diameter 'zirconia' silica beads (Biospec Products Inc., cat. no. 11079101Z) suspended in 0.5 vol. of distilled water. Samples were centrifuged to remove cellular debris and the protein concentration of the supernatant determined using the BCA Protein Assay Reagents (Pierce). Supernates were then stored at −70°C. Total cellular proteins (10 μg) were separated on a non-denaturing PAGE gel with a 4.5% (w/v, acrylamide) stacking gel, pH 6.8, and a 10% resolving gel, pH 8.8. SOD activity was detected in the gel using the colorimetric method of Beauchamp and Fridovich [10]. For specific inhibition of [Cu,Zn]SOD activity potassium cyanide was added at a final concentration of 5 mM.

2.3. DNA manipulations

P. haemolytica strains were grown at 37°C overnight in shaken broth culture and genomic DNA, prepared using the Genome DNA Kit (Bio101), was used as template in PCR amplification and in Southern blot analysis. PCR was carried out using Taq polymerase and buffer (Boehringer) with degenerate oligonucleotide primers, 3′-univsod and 5′-univsod [7]. Thermal cycling conditions were 30 cycles of denaturation at 94°C for 1 min; annealing at 45°C for 1 min; extension at 72°C for 1 min, followed by a single extension at 72°C for 10 min. Confirmation of recovery of the *sodC* sequence from different strains was obtained by PCR using nested primers P6748 (AAGGTGGCAAGCTCACAGCAG) and P6749 (TTCAAGCGAGGGGCTAATACT), constructed on the basis of the DNA sequence determined from the cloned fragment of the *P. haemolytica* serotype A2 *sodC* gene. Thermal cycling conditions were as before except that the annealing temperature was raised to 51°C. PCR fragments were cloned into the vector pCRII (Invitrogen) and the resulting plasmids propagated in *E. coli* JM109. DNA sequence analysis was carried out using either M13 universal forward and reverse primers or primers constructed on the basis of determined sequence and the fmol DNA sequence system (Promega). For Southern blot analysis, DNA was transferred to Hybond N membrane (Amersham) as described by Smith and Summers [11]. DNA probes were cloned PCR fragments of *sodC* from *P. multocida* [7] or *P. haemolytica* serotype A2 strain 124/92 (pNTI) (this work). Probes were labelled with digoxygenin (DIG) using the DIG DNA random primed labelling system (Boehringer). Blots were processed using the DIG DNA luminescence detection system (Boehringer). For detection of *sodC* in genomic DNA autoradiographs were typically exposed for 1–16 h depending on the sensitivity required.

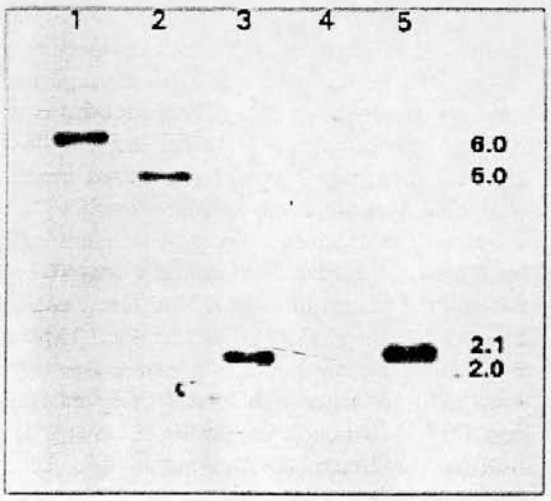

Fig. 2. Southern hybridization of *Hin*dIII-digested genomic DNA from representative *P. haemolytica* serotype A2 and A7 strains probed with the cloned *sodC* PCR product from *P. haemolytica* serotype A2 strain 124/92 (pNTI). Lane 1, serotype A2 strain 124/92; lane 2, A2 strain M65; lane 3, A2 strain M64B; lane 4, A7 strain L40I; lane 5, A7 strain D0800.

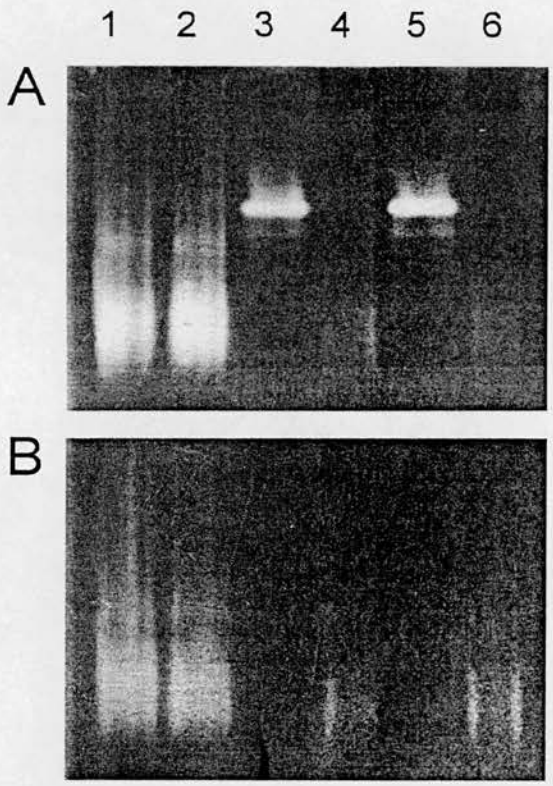

Fig. 3. Determination of SOD activity by gel electrophoresis. Lysates of each of the strains of *P. haemolytica* and *P. trehalosi* were separated by acrylamide gel electrophoresis and stained for superoxide dismutase activity without cyanide (A) or in the presence of 5 mM potassium cyanide (B). Lanes contain extracts from strains as follows: lane 1, serotype T10 strain L52E; lane 2, T3 strain M75; lane 3, A7 strain L45B; lane 4, A7 strain L40I; lane 5, A2 strain 124/92; lane 6, A1 strain L48D.

Table 1
Fifty-two strains of ovine *P. haemolytica* and *P. trehalosi*, grouped according to serotype. + or − indicates the presence or absence of *sodC* DNA sequences as determined by PCR or Southern hybridization, and [Cu,Zn]SOD activity as determined after non-denaturing PAGE

Species/serotype[a]	Strain	PCR	Hybridization[b]	[Cu.Zn]SOD activity
A1	D0652	−	−	−
	L48D	−	−	−
	L47	−	−	−
A2	L48A	+	+ (6.0)	+
	M53	+	+ (6.0)	+
	M64B	+	+ (2.0)	+
	M65	+	+ (5.0)	+
	M103A	+	+ (2.0)	+
	M105C	+	+ (6.0)	+
	L111N	+	+ (6.0)	+
	124/92	+	+ (6.0)	+
	D0136	+	+ (2.0)	+
T3	T635	−	−	−
	M75	−	−	−
	L94CC	−	−	−
T4	D0693	−	−	−
	L55	−	−	−
	M110A	−	−	−
A5	R341B	−	−	−
A6	D0135	−	−	−
	L48E	−	−	−
	M61A	−	−	−
A7	L48C	+	+ (2.1)	+
	L40I	−	−	−
	L45B	+	+ (2.1)	+
	M74	−	−	−
	L111H	+	+ (2.1)	+
	L131D	+	+ (2.1)	+
	L94F	−	−	−
	D0800	+	+ (2.1)	+
A8	D0200	−	−	−
	C53	−	−	−
A9	D0775	−	−	−
	L52I	−	−	−
	M76A	−	−	−
T10	D0770	−	−	NT
	L52E	−	−	−
	M114	−	−	−
A11	B561	−	−	−
	L52G	−	−	−
	L98B	−	−	−
A12	D0132	−	−	−
	L59E	−	−	−
	M58	−	−	−
A13	D0804	−	−	−
	L59D	−	−	−
	L62B	−	−	−

Table 1
(*continued*)

Species/serotype[a]	Strain	PCR	Hybridization[b]	[Cu,Zn]SOD activity
A14	N513	–	–	–
T15	D0885	–	–	–
	L52N	–	–	–
	M113A	–	–	–
A16	N-236	–	–	–

[a] A denotes *P. haemolytica* and T, *P. trehalosi*.
[b] The figures in parentheses indicate the approximate size (kbp) of the restriction fragment identified by hybridization.
NT = not tested.

3. Results

The *sodC* gene was identified provisionally in *P. haemolytica* serotype A2, strain 124/92, by Southern hybridization using a gene probe prepared by PCR from *P. multocida* [7](data not shown). The same degenerate oligonucleotide primers, 3′-univsod and 5′-univsod, that had been used to recover *sodC* sequence from *P. multocida* were used in PCR with templates of chromosomal DNA from strains representing each of the serotypes of *P. haemolytica* and *P. trehalosi* (Table 1). By analogy with *P. multocida*, recovery of a band of 304 bp was taken as preliminary evidence of the presence of *sodC*. Such a band was seen only with A2 and A7 serotypes of *P. haemolytica*.

The PCR product from strain 124/92 was excised from an agarose gel, cloned in the plasmid vector pCRII to produce pNT1, and its identity as a fragment of *sodC* confirmed by nucleotide sequencing, the deduced amino acid sequence being 71% identical to the *P. multocida* and *H. parainfluenzae* sequences (Fig. 1). Nested *sodC* oligonucleotide primers P6748 and P6749 were synthesized based on the 124/92 sequence and used in PCR to confirm, by production of a band of 169 bp, that the PCR products obtained with 3′-univsod and 5′-univsod were in each case derived from *sodC*. The PCR product from the serotype A7 strain D0800 was also cloned (pNT2) and sequenced, and the sequence compared to that from the serotype A2 strain 124/92. The two were almost identical (98% identity at nucleotide level, 100% identity at deduced amino acid sequence level). DNA sequences have been submitted to the EMBL database. Accession numbers are U59225 for the A2 serotype and U59226 for the A7 serotype.

To ensure that local sequence variation was not responsible for failure to detect *sodC* sequences by PCR, the complementary Southern hybridization approach was used. Genomic DNA samples from representative strains of *P. haemolytica* and *P. trehalosi* were digested with *Hin*dIII and examined by Southern hybridization using pNT1 as a probe (Fig. 2, Table 1). The distribution across strains of the *sodC* gene identified by hybridization was identical to that determined by PCR. Positive signals were obtained from 9/9 of the A2 serotype strains and from 5/8 A7 strains tested. In each case the probe reacted with a single fragment. In the A2 strains the fragment size showed variation: in five strains the *sodC* gene was located on a 6 kb fragment, in one strain on a 5.0 kb and in three strains on a 2.0 kb fragment. In each of the A7 hybridization-positive strains the fragment was 2.0 kb (Fig. 2). None of the other serotypes showed a hybridization-positive fragment even after extended autoradiographic exposure.

SOD activity corresponding to *sodC* expression was sought in all strains by detection of enzymatic activity in polyacrylamide gel after non-denaturing PAGE. Fig. 3 shows the pattern of expression of SOD activity in various strains of *P. haemolytica* and *P. trehalosi*. Only serotypes A2 and A7 exhibited a band of SOD activity which could be inhibited by KCN, indicating that this was [Cu,Zn]SOD (results summarised in Table 1).

4. Discussion

[Cu,Zn]SOD activity and the *sodC* gene were detected in all *P. haemolytica* A2 serotype strains (9/9)

and in some A7 serotype strains (5/8). None of the strains in other serotype groups of *P. haemolytica* or *P. trehalosi* showed evidence of [Cu,Zn]SOD activity or of possession of the *sodC* gene. Two methods were used to screen strains for a fragment of the *sodC* gene. PCR detection provided a rapid screening method but has the disadvantage that it may not identify the *sodC* gene where there is localized sequence heterogeneity at a PCR primer site. This technique was therefore complemented by Southern blot hybridization using as a probe the cloned *sodC* PCR fragment recovered from *P. haemolytica* serotype A2 strain 124/92. However, in all the *P. haemolytica* and *P. trehalosi* strains examined, PCR and Southern hybridization identified the same set of strains. Additionally, Southern blot analysis showed that there is variation in the size of the *Hin*dIII restriction fragment that the *sodC* gene is located on in the A2 serotype. DNA sequence analysis demonstrated a very high degree of similarity (98%) between A2 and A7 *sodC* fragments. These sequences also show a substantial degree of similarity with the *sodC* gene in the related bacterial species, *H. parainfluenzae* and *H. influenzae* (71%) [5]. In each of the strains containing the *sodC* gene fragment, corresponding [Cu,Zn]SOD activity was also identified. This situation differs from *H. influenzae* phylogenetic division II of serotypes a, b, e and f where, despite the *sodC* gene sequences being present, [Cu,Zn]SOD activity cannot be detected [5]. In the case of type b this has been ascribed to a point mutation affecting a crucial Cu^{2+} ligand. Resolution of SOD activity by PAGE indicated that more than one form of SOD is present in *P. haemolytica*. While only A2 and A7 serotype strains have [Cu,Zn]SOD activity, all serotypes express an additional form of SOD which is not inhibitable by cyanide. Similarly *P. trehalosi* strains contain non-[Cu,Zn]SOD activity. This has not been characterized here but presumably represents iron- or manganese-SOD.

In diseases caused by *P. haemolytica* and *P. trehalosi*, bacterial survival in the presence of activated neutrophils is a central feature. The leukotoxin of *P. haemolytica* A1, implicated in the pathogenesis of bovine pasteurellosis, is a potent neutrophil activator [2,3], and similarly neutrophil activation is a feature of ovine pneumonic pasteurellosis caused by a range of *P. haemolytica* serotypes. This activation of neutrophils is attended by a burst of production of superoxide and more toxic free-radical products of its further reaction with hydrogen peroxide, but factors in pasteurella strains conferring resistance to their bactericidal action remain to be characterized. We suggest that [Cu,Zn]SOD may play a part in this. [Cu,Zn]SOD has been shown to be periplasmic, or such a position is deduced by the presence of a leader peptide directing export from the cytoplasm in the translated protein sequence, in all bacterial examples studied [5,9,12–15], and it would seem reasonable to infer that the pasteurella [Cu,Zn]SOD is similarly located. A protective role has been demonstrated for surface-exposed bacterial SOD in *Nocardia asteroides*, which shows reduced survival within macrophages when the enzyme is inactivated by bound monoclonal antibody [16]. It would be going too far to suggest that [Cu,Zn]SOD is a crucial determinant of pasteurella virulence, as its distribution appears to be restricted to A2 and certain A7 serotype strains of *P. haemolytica*, while all serotypes can be found as animal colonists or pathogens. Rather, it might be that the enzyme modulates virulence, enhancing the survival of serotypes in which it is found and so increasing the risk of disease. It is striking that A2 and A7 serotypes are those most commonly isolated from ovine infection in Britain, with A2 accounting for 54% and A7 for 5% of clinical referrals of ovine isolates to Moredun Research Institute in 1982-1993 [1]. It should be informative to characterize further strains from diverse origins to establish whether there is a true correlation between expression of [Cu,Zn]SOD and productive infection.

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