Adherence to and invasion of mammalian cell lines by *Pasteurella multocida* B:2

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Dedication:

This thesis is dedicated to my father, mother, wife Rundk and my daughter Sidra, who are always sources of mental support, happiness, love and inspiration for my future.

It is also dedicated to my brothers, who have supported me throughout my academic career.

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Abstract

Haemorrhagic septicemia (HS), caused by the Gram-negative bacterium *Pasteurella multocida* serotype B:2, is an economically important disease responsible for morbidity and mortality of bovines, especially buffaloes, in countries of South or Southeast Asia and Africa. A feature of this disease is the rapid spread of infecting bacteria from the respiratory tract to the blood and lymph to cause a fatal septicemia. To pass into the blood stream, the bacteria must migrate through the epithelial layer into the pulmonary interstitium. Avian serogroup A strains of *P. multocida* have been reported to invade cultured mammalian cells, but the behaviour of other of serogroups has not been reported. The main object of the work was to confirm that HS strains of *P. multocida* B:2 have the capacity to invade and survive within cultured mammalian cells, such as J774.2 cells (mouse macrophage-like cell lines) and BL-3 cells (bovine lymphoma cell line). Invasion, defined as adhesion to, followed by uptake by, or entry into, J774.2 macrophage cells or BL-3 cells was determined by: (I) counting of viable intracellular bacteria after killing extracellular bacteria with polymyxin and gentamicin, (II) Transmission electronic microscopy. Comparison of the invasiveness of a B:2 HS strain and its *aro*A derivative JRMT12 with that of *P. multocida* A:3 and *E. coli* XL1-Blue, showed that both *P. multocida* B:2 strains invaded both types of mammalian cells more readily than *P. multocida* A:3 and that *E. coli* XL1-Blue was essentially non-invasive. Both strains of *P. multocida* B:2 could survive within J774.2 macrophage and BL-3 cells for at least 2 h. A longer-term survival experiment (up to 6 h incubation) indicated that the numbers of intracellular bacteria declined between 4 to 6 h post-infection. It was shown by TEM that a significant proportion of the *P. multocida* B:2 bacteria were found

within vacuoles in the cytoplasm of the mammalian cells with some free in the cytoplasm. A much reduced invasion capacity of *P. multocida* A:3 and *E. coli* XL1-Blue was detected. Different effects on the appearance and viability of J774.2 and BL-3 cells were observed by the trypan blue method and TEM when exposed to the *P. multocida* B:2 strains. Evaluation of cytotoxicity of *P. multocida* B:2 stains with J774.2 cells by the MTT assay produced unsatisfactory results.

Abbreviations

A:1	*Pasteurella multocida* biotype A capsular serotype 1
A:3	*Pasteurella multocida* biotype A capsular serotype 3
AR	Atrophic rhinitis
*aro*A	Aromatic amino acid metabolism gene
B:2	*Pasteurella multocida* biotype B capsular serotype 2
BA	Blood agar
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BL3	Bovine lymphoma cells
Bp	Base pair
°C	Degrees Celsius
CFU	Colony forming units
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
D. H_2O	Distilled water
E:2	*Pasteurella multocida* biotype E capsular serotype 2
FBS	Foetal bovine serum
FC	Fowl cholera
EDTA	Ethylenediaminetetraaceticacid
G	gram (s)
Gm	Gentamicin
h	Hour (s)
H_2O	Distilled water

HBSS	Hank's balanced salt solution
HS	Haemorrhagic septicaemia
KDa	Kilo Dalton
Kg	Kilogram
L	Litre
LB	Luria-Bertani broth
LD_{50}	half (50%) lethal dose
LPS	Lipopolysaccharide
µg	Microgram (s)
µl	Microlitre (s)
mg	Milligram (s)
min	Minute (s)
ml	Millilitre (s)
mm	Millimetre (s)
mM	Millimolar
ng	Nanogram (s)
nm	Nanometre (s)
OD	Optical density (absorbance)
OM	Outer membrane
OMP	Outer-membrane protein (s)
PBS	Phosphate- buffered saline
PCR	Polymerase chain reaction
pH	Hydrogen ion concentration
P.m	*Pasteurella multocida*
Pm	Polymyxin

p.s.i	Pounds per square inch
sec	Second (s)
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
TEM	Transmission Electron Microscopy
TNF-α	Tumour necrosis factor alpha
v/v	Volume/ volume ratio
w/v	Weight/ volume ratio

CHAPTER 1

Introduction

1.1 Family *Pasteurellaceae*

The family *Pasteurellaceae* was, until recently, comprised of 57 named bacterial species which have been isolated from human and various animal sources in three genera *Haemophilus*, *Pasteurella* and *Actinobacillus* (Korczak *et al*., 2004). At present, the taxonomic structure of the family *Pasteurellaceae* incorporates the organisms formerly known as [*Pasteurella*] *haemolytica* into the new genus *Mannheimia* (Angen *et al.*, 1999), and also includes the genera *Histophilus* (Angen *et al.*, 2003), *Gallibacterium* (Christensen *et al.*, 2003), *Volucribacter* (Christensen *et al.*, 2004), *Nicotella* (Kuhnert *et al.*, 2004), *Avibacterium* (Blackall *et al.*, 2005). Historically, members of the family *Pasteurellaceae* were classified on the basis of a limited number of phenotypic characteristics. In particular, organisms were assigned to the family on the basis of their requirements for the growth factors haemin and/or nicotinamide adenine dinucleotide and on the basis of their ability to cause disease in vertebrates (MacInnes and Borr, 1990). Major advances in understanding the phylogeny of the members of family *Pasteurellaceae* have come from DNA-DNA hybridization and from rRNA-DNA hybridization studies (De Ley *et al.*, 1990). These studies revealed that the members of the *Pasteurellaceae* were most closly related to member of the *Enterobacteriaceae*, the *Vibrionaceae*, the *Aeromonadaceae*, and the genus *Alteromonas* and also suggested that this family was very heterogeneous.

Members of the *Pasteurellaceae* are small Gram-negative rods, which are generally isolated from mucosal membranes and tissue of birds and mammals including, occasionally, man. There is a strong host association that has probably co-evolved with

the corresponding host. While most of the species are commensals, there are a few that act as pathogens (Bisgaard, 1993; Mario and Mikael, 2002) (Table 1). These include the human pathogens *Haemophilus influenzae*, which causes neonatal meningitis and otitis media, and *Actinobacillus actinomycetemcomitans*, which causes juvenile periodontitis. Important animal diseases are caused, for example, by *Mannheimia haemolytica* (shipping fever of cattle), *Pasteurella multocida* (atrophic rhinitis in swine, fowl cholera, snuffles in rabbits and haemorrhagic septicaemia in cattle and buffaloes) and *Actinobacillus pleuropneumoniae* (pleuropneumonia in pigs) (Korczak *et al.*, 2004).

1.1.2 The genus *Pasteurella*

The genus *Pasteurella* has recently been investigated and the taxonomical position of *P. multocida* has been defined (Dziva *et al.*, 2008). *P. multocida* is the type species of the genus and currently includes *P. multocida* subsp. *multocida*, *P. multocida* subsp. *septica*, *P. multocida* subsp. *gallicida*. These are the most frequently isolated species in human infections (Escande and Lion, 1993). A new subspecies of *P. multocida*, subspecies *tigris*, has been designated and isolated from a tiger-bite wound in a child (Capitini *et al.*, 2002), but this finding has not been validated by further investigation. Two additional species designated *Pasteurella* species A and B were consider to be members of the genus but remain unnamed (Peter *et al.*, 2005; Dziva *et al.*, 2008). The principle characteristics of members of the genus are that they are non-motile, Gram-negative rods or coccobacilli, facultatively anaerobic and most species are oxidase-positive, catalase positive, alkaline phosphatase positive, reduce nitrate and they display a characteristic bipolar staining with Leishman or methylene blue stains (Peter *et al.*, 2005).

Table 1. Members of the *Pasteurellaceae* family and the diseases they cause in their respective hosts (Mario and Mikael, 2002).

Organism	**Host**	**Disease**
Haemophilus		
H. ducreyi	Human	Chancroid
H. influenzae capsular type B (Hib)	Human	Meningitis, septicaemia, and epiglottitis
H. influenzae non-typable *(NTHi)*	Human	Otitis media, sinusitis, conjunctivitis and acute lower respiratory tract infection
Actinobacillus		
A. actinomycetemcomitans	Human	Juvenile and adult periodontitis
A. pleuropneumoniae	Swine	Pleuropneumonia
Pasteurella		
Pasteurella (Mannheimia) haemolytica	Bovines and sheep	Pneumonia
P. multocida	Most animals, occasionally human	Meningitis, fowl cholera, atrophic rhinitis, snuffles, haemorrhagic septicaemia, and pneumonia

1.1.3 The species *Pasteurella multocida*

Louis Pasteur recognized and identified this organism over 125 years ago (Harper *et al.*, 2006). Members of this species are distributed throughout the world, among varieties of both terrestrial and aquatic species of mammals and birds (Rimler and Rhoades, 1989). The species *P. multocida* now includes a heterogeneous group of Gram-negative bacteria that are inhabitants of the upper respiratory tract of various animals (Dziva *et al.*, 2008), some of which are human pathogens causing infection of bite wounds, respiratory disease and infections of the central nervous system (Kristinsson, 2007). The organisms can occur as commensals in the naso-pharyngeal region of apparently healthy animals and they can be either primary or secondary pathogens in disease processes of a variety of domestic and wild mammals and birds (Harper *et al.*, 2006).

1.1.3.1 Cultural characteristics and colonial morphology

P. multocida can grow on rich laboratory media like brain heart infusion agar (BHIA), sheep blood agar (SBA), and chocolate agar. A suitable medium for growth of *P. multocida* is Casein Sucrose Yeast (CSY) agar containing 5% sheep blood. The organisms is non-haemolytic and does not grow on MacConkey's agar (Oie, 2004; Shivachandra *et al.*, 2006; Dziva *et al.*, 2008), or other types of selective or differential enteric media (Peter *et al.*, 2005). Bovine or ovine blood agar has consistently yielded reasonable success in isolation *Pasteurella* species (Dziva *et al.*, 2008). On blood agar, pure colonies of *P. multocida* are round, grey in colour, mucoid or non-mucoid with a typical sweetish odour like *E. coli*, perhaps due to the formation of large amounts of indole by the organism. Major variations in colony morphology have been observed for *P. multocida*, some of which are host related (Dziva *et al.*, 2008).

Cultures on agar medium may develop into either of two principal colony forms, smooth and rough. Smooth colonies are mucoid and their characteristics can range from cultures whose colonies are discrete, circular, and convex and have a slight mucus-like consistency, to cultures whose colonies are confluent, markedly moist and viscous. Rough colonies, which consist of filamentous non-capsulated cells have been described as being flat with slightly raised central papillae and flattened, irregularly serrated edges or discrete, raised, circular, rough and slightly dry (Rimler and Rhoades, 1989). Mucoid colonies are often obtained from pneumonic lesions in cattle, pigs and rabbits whilst non-mucoid colonies most often are recovered from poultry.

Colonies of serogroup A, D and F strains display a pearl-like iridescence in oblique transmitted light while colonies of *P. multocida* serotype B and E are usually grey and viscous, small, glistening mucoid dew-drop-like colonies on blood agar plates. All are catalase and oxidase positive and reduce nitrate and are non-motile and non-haemolytic on blood agar. Glucose, fructose, galactose, mannitol, sucrose are fermented (Shivachandra et al., 2006).

1.1.3.2 Serotype identification

In 1981, a standard system for the identification of *P. multocida* serotypes was recommended that utilized both the Carter capsular typing identified by the letters A, B, D, E and F (Carter and Chengappa, 1981), and the Heddleston somatic typing system which identified 16 different somatic types numbered 1 to 16 (Heddleston *et al.*, 1972). Haemorrhagic septicaemia strains were designated as serotypes B and E (Carter, 1955), with a combination of Carter types and somatic antigen typing defining Asian and African HS strains as 6:B or 6:E, respectively (Namioka and Murata, 1961), while in the

latter system they are designated B:2 and E:2, respectively (Oie, 2000). The type-specific antigen (Carter type B or E) refers to the bacterial capsule, whereas the O-group antigen (Heddleston type 2) refers to the lipopolysaccharide component (Shah *et al.*, 1996).

1.2 Molecular biology of *Pasteurella*

1.2.1 *Pasteurella multocida* genes

The recent application of molecular methods such as the polymerase chain reaction, restriction endonuclease analysis (REA), ribotyping, pulsed-field gel electrophoresis, gene cloning, recombinant protein expression, mutagenesis, plasmid and bacteriophage analysis and genomic mapping, have allowed progress towards a better understanding of the molecular biology of *P. multocida* (Gichohi, 2001). For example, REA has proved to be a valuable tool for investigating outbreaks of pasteurellosis. Several restriction enzymes have been used for DNA fingerprinting of *P. multocida* by REA, with *Hha*I and *Hpa*II yielding the most informative and distinguished profiles from a wide range of serotypes (Hunt *et al.*, 2000). Plasmids have been recovered from *P. multocida* strains isolated from various animal species (McGee and Bejcek, 2001). These plasmids conferred antibiotic resistance (R factors) as well as encoding virulence factors. For example, plasmids correlated to complement resistance are particularly conserved in avian isolates (McGee and Bejcek, 2001). Plasmid profile was considered as a virulence marker of *P. multocida* as well as an essential epidemiological tool for identifying different strains from the same phenotype. *P. multocida* can be transformed with plasmids by the electroporation process which subjects the bacterial cell membrane to a short electric shock leading to bacterial DNA uptake (Rubies *et al.*, 2002).

May *et al*. (2001) reported the genome sequence of *P. multocida* serotype A (Pm 70), which has a single circular chromosome of 2,257,487 base pairs and contains 2,014 predicated protein-coding genes. Two genes resemble the filamentous haemagglutinin (*fha*) genes found in the whooping cough bacterium, *Bordetella pertussis*. The FHA protein helps the pertussis bacterium to adhere to host cells, and the *P. multocida* versions of these proteins may play a similar role, making them promising vaccine targets (May *et al.,* 2001). The other striking feature of this genome is that 2.5% of the genes produce proteins that are predicted to be involved in transport of iron into the cell or its processing. Iron is an essential nutrient for nearly all organisms because of its presence in the electron transport chain producing ATP. In *P. multocida* (Pm 70), 174 genes showed at least a two-fold change in gene expression on iron depletion; 53% showed a decrease in activity and 47% showed an increase. About 16 genes showing higher activity at low iron levels are thought, based on comparison with other organisms, to code for iron transport proteins (May *et al.,* 2001).

1.3 Virulence-associated factors

Many members of *Pasteurellaceae* family express fimbriae at their surface, in addition to surface polysaccharides and outer membrane proteins, which are often involved in adherence to host cells (Mario and Mikael, 2002; Tomer *et al.*, 2004). However, the molecular mechanisms of *P. multocida* pathogenesis are still largely unknown, and only a few virulence factors have been identified. These factors include toxins, capsule, iron acquisition proteins (section 1.2.1), haemagglutinins (Boucher *et al*., 2005), lipopolysaccharide (endotoxin), fimbriae and outer-membrane proteins (Mullen *et al*., 2007). Some organisms of this family produce haemolytic and/or cytotoxic protein(s) of the RTX (repeats in toxin) family, including *Actinobacillus*

pleuropneumoniae and *A. suis* (Mario and Mikael, 2002) and *Pasteurella haemolytica* (Coote, 1992). It is thought that these factors allow *P. multocida* to multiply in the lungs of animals and contribute directly or indirectly to septicaemia.

1.3.1 Capsule

P. multocida capsule structure has a pivotal role in the determination of the serogroup type of the bacteria. Polysaccharides constitute the major part of the capsule and in some cases they may be associated with lipoproteins. Five capsular serotypes were identified namely, A, B, D, E and F (Carter, 1967). The capsule lies outside the outer membrane and is composed of highly hydrated polyionic polysaccharides to protect against dehydration of the bacteria (Roberts, 1996). Virulent strains of *P. multocida* are usually capsulated and this can be seen in organisms isolated from diseased tissue and after laboratory culture. Several techniques have been used to demonstrate capsules for light microscopy. The alcian blue, Congo red and crystal violet methods have been used for direct staining of the capsule (Boyce et al., 2000). Presence of the capsule is assumed to be important for transmission of *Pasteurella* from host to host or for enhanced survival in the environment and has also been shown to have antiphagocytic activity (Boyce *et al.*, 2000). The composition of the *P. multocida* capsule has been investigated only in serogroups A, B, D and F, and the chemical composition of serogroup E capsule remains unknown. The composition and structure of the capsular material found in *P. multocida* serotype A, D and F are very similar to mammalian glycosaminoglycans and consist mainly of hyaluronan, heparosan and unsulphated chondroitin, respectively (Pandit and Smith, 1993; Rimler and Rhoades, 1994; Boyce *et al.*, 2000). *P. multocida* serogroup B capsule was shown to contain fructose, mannose, glucose, glucosamine and polysaccharide and also to contain 0.5% protein and 1.7% nucleic acid (Boyce *et al.*,

2000). The complete nucleotide sequence of the capsule biosynthetic locus of *P. multocida* X-73 (A:1) and *P. multocida* M1404 (B:2), has been determined. The locus of *P. multocida* X-73 (A:1) consists of 11 ORFs and that of *P. multocida* B:2 consists of 15 ORFs in 3 regions. Functions for the encoded proteins have been proposed for seven of the B:2 ORFs. Regions one and three each contain a total of six genes, involved in transport of polysaccharide, while region two contains nine genes which are postulated to be involved in the biosynthesis of polysaccharide in *P. multocida* B:2 (Boyce *et al*., 2000).

In addition, Rimler and Rhoades (1989) believe that the capsule antigen of *P. multocida* is associated with lipopolysaccharide (LPS), as well as non-antigenic polysaccharide material. However, the capsule-specific antigen and LPS can be absorbed onto erythrocytes from crude cell extract. Passive haemagglutination tests with serum containing antibodies against both the capsule-specific antigen and LPS usually show a reaction only with the capsule-specific antigen. The high antibody levels do not correlate with protection against experimental challenge (Rimler and Rhoades 1989).

Capsular variants of *P. multocida* serotype B have been shown to reach a lower titre in the blood of infected animals and have an approximately 10^5-fold higher LD_{50} (Snipes and Hirsh, 1986; Jacques *et al.*, 1993). This indicates as important role for the capsule in the pathogenesis of *P. multocida* serotype B as capsular variants are strongly attenuated in mice and have increased sensitivity to phagocytosis by murine macrophages. A virulence assay in mice indicated that a capsular variant of *P. multocida* B:2 was 10^6-fold less virulent than the encapsulated counterpart (Boyce and Adler, 2000). The capsule is also thought to be involved in bacterial avoidance of phagocytosis and

resistance to complement (Noel *et al.*, 1992; Jacques *et al.*, 1993; Shimoji *et al.*, 1994). Some studies have also indicted a relationship between capsule thickness and resistance to phagocytosis (Harmon *et al.*, 1991; Boyce *et al.*, 2000).

1.3.2 Lipopolysaccharide (LPS)

LPS, responsible for the 1-16 somatic serotypes, is a critical virulence determinant in *P. multocida* and a major antigen involved in host protective immunity. It consists of lipid A, a core oligosaccharide and a polysaccharide side-chain (O-antigen). In other mucosal pathogens, variation in LPS or lipooligosaccharide structure typically occurs in the outer core oligosaccharide regions due to phase variation (Harper *et al.*, 2007). There are conflicting reports as to the endotoxic properties of LPS isolated from avian *P. multocida;* chicken embryos and mice are highly susceptible, but turkey poults are relatively resistant (Harper *et al.*, 2006). In the HS-associated *P. multocida* serotype B, LPS is thought to be responsible for toxicity and to play an important role in the pathogenesis of the disease (Horadagoda *et al.*, 2002). *P. multocida* B:2 bacteria aspirated into lungs of buffaloes prompted the appearance of LPS in the blood and induced a response consistent with endotoxic shock. It was concluded that LPS is a major virulence factor and plays an essential role in causing HS in buffaloes (Horadagoda *et al.*, 2002). Antibody reacting with the LPS of type A strains has given protection against murine and rabbit infection, whereas antibodies to LPS appear to play a subordinate role in protection with type B (Harper *et al.*, 2006).

1.3.3 Fimbriae

Surface components such as capsule and outer membrane proteins have been the subject of a number of studies to further elucidate the pathogenic mechanisms of *P. multocida*, but adhesins of this bacterium have received little investigation. Bacterial adherence to host cells or surfaces is often an essential first stage in disease; it localizes pathogens to an appropriate target tissue. Adhesion to host cells may result in internalisation; either by phagocytes or by bacterial-induced endocytosis known as invasion. Thus, attachment by fimbriae to host surfaces is usually correlated with virulence (Heckels, 1989). *P. multocida* is considered to colonize the nasal mucosa of susceptible animals. Electron microscopy has shown fimbriae on *P. multocida* serotype A harvested from rabbit pharyngeal cells, pig tonsilar and nasal cells, and nasal and tracheal cells of calves (Glorioso *et al.*, 1982; Ruffolo *et al.*, 1997). In an in *vitro* trial using newborn pigs it was shown that serogroup D bacteria were adherent to the turbinate area and showed fimbriae when observed by scanning electron microscopy (Pijoan and Trigo, 1990). Although there are no data conclusively on fimbriae of serogroup B, fimbriae-like filamentous appendages were observed on the surface of HS-causing *P. multocida* B:2, although further characterization of this structure was not undertaken (Tomer *et al.*, 2004).

1.3.4 Outer membrane proteins (OMPs)

The outer membrane of Gram-negative bacteria contains lipopolysaccharide, several minor proteins and a limited number of major proteins of 34 to 42 kDa present in very high copy numbers (Lee et al., 2007), some of which are generally considered to play an important role in pathogenesis (Bosch *et al.*, 2004). Generally, seven such OMPs have

been found in *P. multocida*, namely: OmpH, OmpA, Omp87, Omp16, P1pB, Lpp and G1pQ (Coony and Lo, 1993; Kasten *et al*., 1995; Ruffolo and Adler, 1996; Luo *et al*., 1997; Gatto *et al.,* 2002; Lo *et al.,* 2004). OMP profiling also offers a relatively quick way to establish relationship between strains. OmpH and a heat-modifiable OMP of *P. multocida* (OmpA) have provided an OMP typing scheme. Based on the electrophoretic separation of these 2 major OMPs and other minor ones, Davies *et al.* (2003) demonstrated up to 19 OMP types among avian strains of *P. multocida* serotype A. High resolution OmpA and OmpH profiling of bovine isolates from England and Wales revealed no correlation with disease-status or geographic origin (Davies *et al.*, 2004). It has been suggested that OMP profiling could provide a non-serological technique for identifying HS strains of *P. multocida* (Dziva et al., 2008). Several studies have indicated that OMPs of *P. multocida* 6:B were involved in resistance to phagocytosis by murine peritoneal macrophages and possessed immunogenic properties (De Alwis, 1990a; Srivastava, 1998; Basagoudanavar *et al.*, 2006). A recent study investigated the generation of immune responses to OMPs in cattle and in buffaloes and revealed that OMPs of *P. multocida* may be important contributors to the pathogenic potential of disease-causing bacteria and possesses immunogenic properties that might be useful in vaccines against HS (Basagoudanavar *et al*., 2006).

The Pm 70 genome sequence identified genes for putative haemoglobin and haemin binding proteins, such as a heme acquisition system receptor homologue, suggesting that *P. multocida* might also use free haemin as an iron source (Bosch *et al*., 2004). Iron is essential for bacterial growth and replication as well as for establishment of infection. *P. multocida* serotype B:2,5 strains have been shown to produce transferrin-binding membrane proteins (Tbp1 and Tbp2) (Veken *et al.,* 1994). An 82-kDa iron-regulated OMP was found which specifically binds bovine transferrin. Iron-regulated OMPs of *P.*

multocida are reported to be expressed in *vivo* and may be involved in cross-protective immunity against pasteurellosis (Basagoudanavar *et al*., 2006). The composition of the *P. multocida* outer membrane varies only marginally upon infection of the host, suggesting that most surface components involved in pathogenesis are either expressed constitutively, up-regulated only during the early stage of infection or that their change in expression is below current detection limits (Ratledge and Dover, 2000).

1.3.5 *P. multocida* toxins

Most *P. multocida* strains are not known to express toxins, with the only well-characterized toxin found in serogroup D strains (Rimler and Brogden, 1986). The *Pasteurella multocida* toxin (PMT) is generally thought to be responsible for the clinical and pathological aspects of atrophic rhinitis (Kamp and Kimman, 1988; Chanter, 1990), and inhibits osteoblast differentiation and bone formation. Inhibition of the bone nodule formation by PMT requires the activation of the Rho GTPase (Harmey *et al*., 2004), but it is not yet known exactly how PMT influences Rho-dependent signaling, although Rho is not a direct target of PMT (Oswald *et al*., 2005). PMT independently stimulates at least two different signalling pathways through the heterotrimeric GTPase Gq and the small GTPase Rho.

P. multocida serogroup A (X-70) and serogroup B toxicity on host cells has been reported. The toxic activity of HS strains of *P. multocida* serotype B was examined in a mouse model. The mice were injected intraperitoneally with 10^2 cells of *P. multocida* B:2,5. After 6 h, the experiment revealed strong induction of cytoplasmic vacuolation in peritoneal macrophages. Additional *in vitro* experiments with a macrophage cell line,

incubated with different serogroups of *P. multocida,* A, B, E, and D, indicated that macrophage vacuolation was associated only with serogroup B and E HS strains (Shah et al., 1996). When *P. multocida* A X-70 was used to infect *Acanthamoeba polyphaga* or *Hartmanella vermiformis* trophozoites, the bacteria were found to be located intracellularly when examined via transmission electron microscopy. The *P. multocida* cells were visualized in membrane-bound vacuoles within the cytoplasm of the amoebal cells. Within the vacuoles, bacteria divided, then lysed and exited their amoeba hosts. There was a large decrease in the percentage of viable host cells (1.6% viable) at 18 h post-infection, compared to amoeba incubated in the absence of *P. multocida* (33% viable) (Hundt and Ruffolo, 2005).

1.4 Invasion of mammalian cells by bacteria

1.4.1 Interaction between eukaryotic cells and bacteria

The first specific interaction between a pathogenic micro-organism and its host entails attachment to the eukaryotic cell surface which may result in internalisation, either by phagocytic or by bacterially-induced endocytosis, a process known as invasion (Falkow *et al.*, 1992). Invasive bacteria seem to have evolved two major types of uptake. First, there is the Zipper mechanism that involves contact between bacterial ligands and cellular receptors, a system used by *Yersinia* and *Listeria* (Figure 1). *Y. enterocolitica* and *Y. pseudotuberculosis* have an outer membrane protein, invasin, that mediates entry into epithelial cells by binding tightly to a $ß_1$ integrin on the cell surface. Invasin mediates bacterial uptake by zippering the host cell membrane around the bacterium as it enters (Brett and Cossart, 1997). The intracytoplasmic domain of the $ß_1$ integrin interacts with the cytoskeleton by binding to actin-binding proteins such as talin and α- actinin.

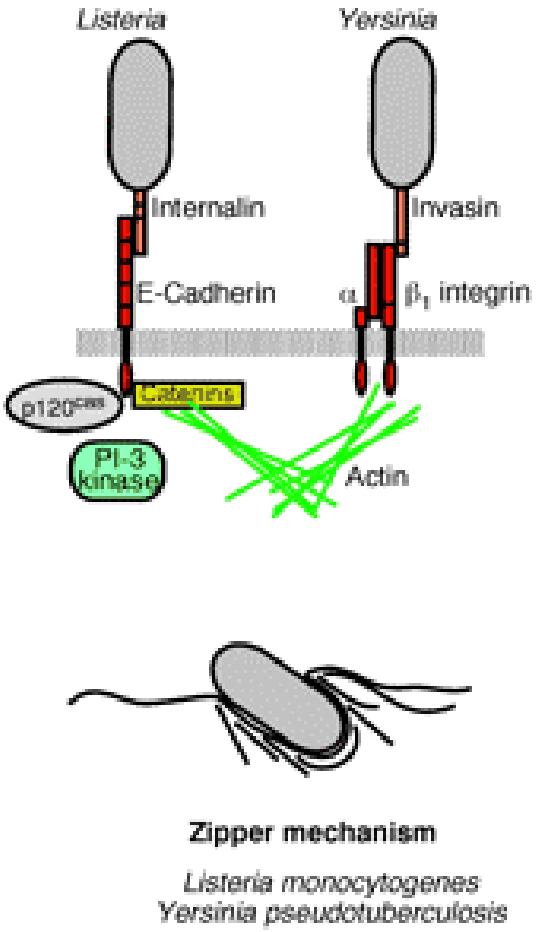


Figure 1: Zipper mechanism of bacterial cell entry into mammalian cells

Yersinia has an outer membrane protein, invasin, which mediates attachment and entry into epithelial cells by binding to a $ß_1$ integrin on the cell surface. This integrin interacts with the cytoskeleton by binding to α-actinin that contributes to internalisation. For Listeria, E-cadherin is a transmembrane cell adhesion protein, which through a complex with internalin and catenins involved in homophilic cell-cell interactions, activates phosphate: dlyinosit 3-kinase (PI 3-kinase) that mediates uptake by an unknown mechanism (Brett and Cossart, 1997).

The second type of uptake mechanism is the Trigger mechanism, used by *Salmonella* and *Shigella*, where bacteria send signals to the cells to induce membrane and cytoskeletal rearrangement, allowing virtually passive entry of bacteria (Brett and Cossart, 1997) (Figure 2). Both processes ultimately use the actin cytoskeleton to produce pseudopod extension, and thus the signalling mechanisms are likely to converge at some point (Swanson and Baer, 1995). The Zipper mechanism produces a small pseudopod relative to the particles, and the trigger mechanism elicits a large one. Secretion systems are known to play a central role in bacteria and host cell interaction (Oswald *et al.*, 2005). Invasion of *Salmonella* and *Shigella* into cultured cells are similar and both species use type III secretion systems to directly inject signaling proteins into target cells (Ogawa and Sasakawa, 2006). A type III secretion system is one in which the bacterium produces pore-forming proteins that create a pore spanning not only the bacterium's cytoplasmic membrane and outer membrane, but also the plasma membrane of the host cell. This allows the bacterium to deliver proteins directly from its cytoplasm into the cytoplasm of the host cell (Preston, 2007).

When *Shigella* contacts the epithelial cells of the colon, the type III secretion system delivers proteins such as IpaA (70 kDa), IpaB (62 kDa), IpaC (42 kDa) and IpD (37 kDa) (Sansonetti, 1998), into the epithelial cells enabling them to polymerise and depolymerise actin filaments (see legend to Figure 2). The cytoskeleton rearrangement is a key part of the pseudopod formation in phagocytic cells and enables phagocytes to engulf bacteria and place them in a vacuole (Coburn *et al.*, 2007). Ipa proteins have no signal peptide; upon contact of the bacterium with the cell surface, they are secreted, already folded, from the cytoplasm to the extracellular milieu without a periplasmic phase. In the extracellular medium, IpaB and IpaC form a complex (Sansonetti, 1998).

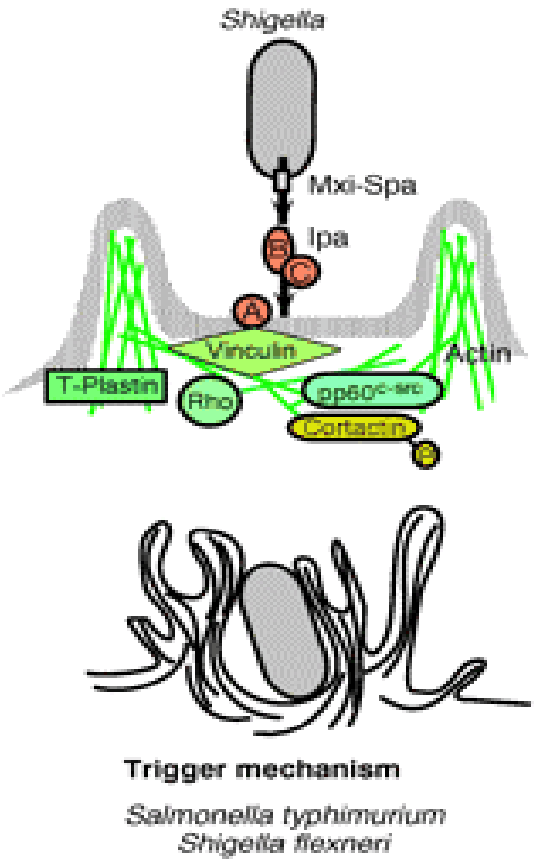


Figure 2: Trigger mechanism of bacterial invasion.

In *Shigella*, the mxi-spa secretion system and other secreted proteins (IpaA, -B, -C, -D), activate a host-signalling pathway resulting in bacterial uptake. Cortactin is an actin-associated protein and a substrate for the non-receptor tyrosine kinase Scr, which induces membrane ruffles. T-plastin appears to play a central role in mediating bacterial uptake, possibly by bundling newly formed actin filaments in the membrane extension. Another actin-binding protein, vinculin, colonizes to the site of entry and can be coimmunoprecipitated with IpaA.

Image was taken from (http://www.ncbi.nlm.nih.gov/pubmed/9115192).

This property of secretion upon contact is based on the expression and assembly of a type III secretory apparatus composed of about 20 different proteins called Mxi-Spa (membrane expression of antigens and secretion of protein antigens) which bridges the inner and outer membrane. Thus, in these examples, the bacterium is able to trick the epithelial cell into behaving like a phagocyte and engulfing the bacterium. The bacteria can then cause the vacuole membrane to rupture and escape into the cytoplasm were they begin to multiply.

1.4.2 Invasion and intracellular survival of *P. multocida*

P. multocida was, until recently, generally considered to be an extracellular pathogen associated predominantly with the respiratory tract of animal and human disease. However, a number of studies have shown that *P. multocida* may be found intracellularly within a variety of eukaryotic cells. For example, in an early study it was shown that *P. multocida* could multiply intracellularly in liver and spleens of experimentally infected turkeys at early stages of infection and then spread to the blood (Pabs-Garnon and Soltys, 1971). After intravenous inoculation of *P. multocida* serogroup A into turkeys, the number of *P. multocida* increased over 100 times in the liver and 75 times in the spleen 3 h after inoculation, but there was no increase in the blood. It was found that an increase in the number of organisms in the blood occurred rapidly between 22 and 28 hours after inoculation when the number of bacteria in the liver and spleen reached its peak. It appears that the bacteria from the liver and spleen invaded the blood before the death of the turkeys (Pabs-Garnon and Soltys, 1971).

Two serotype A:3,4 strains of *Pasteurella* that differed in virulence in turkeys were examined for their ability to adhere and invade different epithelial cell monolayers

grown in tissue culture (Lee *et al.*, 1994). Both organisms adhered to turkey kidney cells in comparable numbers but only the virulent strain was able to invade turkey cells, as well as porcine epithelial cells and feline epithelial cells in cell culture. Neither organism invaded rabbit epithelial cells (Lee *et al.*, 1994). This study was based on the gentamicin protection assay and consisted of enumerating intracellular bacteria by their resistance to the gentamicin-killing step of the assay, since it is expected that this antibiotic does not penetrate epithelial cells. In another study, the interaction of *P. multocida* serotype A:3 with turkey air sac macrophages (ASM) in culture was investigated and showed that the bacteria were capable of adhering in large numbers to the macrophages but were not internalized (Pruimboom, 1996). This author also showed that adherence of *P. multocida* A to these cells was mediated by capsular hyaluronic acid (Pruimboom *et al.,* 1996; Pruimboom *et al.*, 1999). *P. multocida* A:3 strain P1059 was shown to adhere to and invade polarized epithelial Madin-Darby canine kidney (MDCK) cells by interacting with host F-actin (Rabier *et al*., 1997). Bacteria were located inside the cells near the apical border by 30 min after exposure and were detected by confocal microscopy. This study demonstrated that bacteria appeared to enter cells by association with host actin. Treatment of MDCK cells with cytochalasin D for either 30 min or 24 h prior to infection disrupted the actin cytoskeleton and inhibited entry of *P. multocida* (Rabier *et al*., 1997).

Association and invasion of bovine aortic endothelial cells (BAECs) by *P. multocida* B and *P. haemolytica* serotype A1 has also been studied utilizing the bacteria: cell ratio 100:1 and 50µg/ml of gentamicin to kill extracellular bacteria (Galdiero *et al.*, 2001). Electron microscopy demonstrated that both species were able to adhere to BAECs but it was reported that only *P. multocida* invaded BAECs while *P. haemolytica* was non-

invasive. Fewer internalized viable *P. multocida* B bacteria were detected at 6 h compared to the 2 and 4 h time points of invasion. The mammalian cell viability was checked by the lactate dehydrogenase (LDH) cytotoxicity assay, and it was reported that the cells were healthy after exposure to the bacteria (Galdiero *et al*., 2001). Some preliminary work examined uptake of *P. multocida* B:2 85020 wild-type and an *aro*A derivative JRMT12 into J774.2 macrophage-like cells at a bacteria/cells ratio 500:1 (Tabatabaei, 2000). This study used the antibiotics gentamicin and polymyxin B, each at a final concentration 350 μg/ml, to kill all extracellular bacteria. The results indicated that both strains were taken up into the cells and were able to survive for at least 2 h in the phagocytic cells.

1.5 Distribution and disease association

P. multocida (multocida literally meaning many killing) is a pathogen which causes disease of economic importance in a wide range of animals and can be the primary or secondary agent in the disease process. Often they are inhabitants of the upper respiratory tract of many vertebrate hosts including birds, cattle, swine, cats, dogs and rodents. Members of this species are responsible for a number of infections that normally are secondary to colonization of the upper respiratory tract, including avian cholera (in waterfowl, chickens and turkeys) caused by strains of serogroup A (Hirsh and Tim, 1990), respiratory disease and hemorrhagic septicaemias in ruminants (cattle, sheep, goats and buffaloes) caused by strains of serogroups A, B or E (De Alwis *et al.*, 1980), atrophic rhinitis in pigs and snuffles/septicaemias in rodents (mice and rabbits) caused by strains usually of serogroup D. These infections are primarily transmitted by the respiratory route and associated with crowding and other stressors. The pathogenicity of *P. multocida* serogroup F is associated with avian hosts (Haase *et al.*, 2006) and

rabbits (Jaglic *et al*., 2004). *P. multocida* is also a rare cause of infection in humans normally associated with dog or cat bites or scratches (Haase *et al.*, 2006). The human infections are characterized by rapid development of pain, erythema, swelling, cellulitis with or without abscess formation, and purulent or serosanguinous drainage at the wound site (Westling et al., 2000).

1.5.1 Bovine respiratory disease (BRD)

BRD is a highly infectious disease, primarily of cattle, affecting the lungs and occasionally the joints. Two bacteria, *Mannheimia* (*Pasteurella*) *haemolytica* type A1 and *P. multocida* serotype A, are generally associated with BRD or shipping fever in cattle and are often referred to as secondary bacterial invaders (Lillie, 1974; Irsik, 2007). These two bacteria are considered as part of the normal bacterial flora found in the upper respiratory tract of most cattle. Respiratory tract infections (pneumonic pasteurellosis) due to these two bacteria occur when the organism is inhaled. Under conditions of impaired pulmonary defences, a severe necrotizing pleuropneumonia develops. Spread of these organisms is by direct contact, or by ingestion of feed and water contaminated by nasal and oral discharges from infected cattle. Therefore *P. multocida* and *M. haemolytica* are easily spread between cattle, especially when calves are crowded (as in shipment) or closely confined (as in a dairy calf nursery) (Irsik, 2007). Pneumonia associated with either *M. haemolytica* or *P. multocida* often occurs when the animal's normal defences are compromised. It is possible that viruses, such as parainfluenza virus PI-3, play some role in shipping fever (Lillie, 1974; Mawhinney and Burrows, 2005; Irsik, 2007). The compromised defence mechanisms are thought to include damage to the cells lining the upper respiratory tract by viruses such as infectious bovine rhinotracheitis virus (IBR), PI-3, or bovine respiratory syncytial virus (BRSV). Damage

to the tracheal lining could also occur due to inhaled irritants such as exhaust fumes or dust. The respiratory defence mechanism could also be depressed due to immunosuppression associated with bovine viral diarrhoea (BVD) virus (Potgieter, 1997). The production of mucosal antibodies against the bacteria could be suppressed due to environmental or nutritional stress. When this defence is compromised, the bacteria become attached to and colonise the lining of the respiratory tract, reproduce rapidly and spread throughout the lungs. The clinical signs include lung damage associated with increased coughing and rapid breathing (Dowling *et al.*, 2002).

1.5.2 Fowl cholera (FC)

FC is a serious disease of poultry and can present in either acute or chronic forms. It is mainly caused by *P. multocida* serotypes A and F but, although *P. multocida* subspecies *multocida* is the most common cause of fowl cholera, *P. multocida* subspecies *septica* and *gallicida* may also cause the disease to some extent (Christensen and Bisgaard, 2000). In birds, it is widely believed that *P. multocida* enters the host via the respiratory tract. Rhoades and Rimler (1990) have demonstrated adhesion of *P. multocida* to turkey air sac macrophages. Although FC is considered a septicaemic infection, bacteria can only be isolated in large numbers from the blood of birds very late in infection, and it has been proposed that this late emergence of blood-borne bacteria is due to the rupture of liver and spleen phagocytes (Pabs-Garnon and Soltys, 1971; Harper *et al.*, 2006). Virulent *P. multocida* inoculated into the upper respiratory tract or trachea of turkeys can be subsequently detected in internal organs between 6 and 12 h post infection (Rhoades and Rimler, 1990). Obvious clinical signs of acute FC include facial oedema, swollen and oedematous wattles and combs, severe respiratory disorders, ruffled feathers, fever,

anorexia, diarrhoea, increased respiratory rate and mucous discharge from the mouth (Harper *et al.*, 2006; Woo and Kim, 2006).

1.5.3 Atrophic rhinitis (AR)

AR is a disease that profoundly affects the bone structure of the porcine snout. The aetiological agent currently considered to be the cause of growth retarding progressive AR is a toxigenic strain of capsular type D which colonizes the nasal cavity of pigs (Eamens *et al.*, 1988; Foged *et al.*, 1988). The pathological effects of *P. multocida* infection may be ascribed to the PMT toxin (see section 1.2.5) produced by this bacterium which is also known as dermonecrotic toxin (Foged, 1992). The toxin induces resorption of the nasal turbinates and other bone structures in the nasal cavity and impairs osteoblast bone formation (Foged, 1992; Magyar *et al.*, 2003). There are a variety of factors that pre-dispose animals to developing AR, including co-infection with *Bordetella bronchiseptica.* In pigs, the severe respiratory disease can be induced experimentally with *P. multocida* infection alone, and it is now accepted that in the naturally-occurring disease, the pivotal role for infection with *B. bronchiseptica* is to facilitate colonisation by *P. multocida* (Jordan and Roe, 2004). The disease is characterized by other symptoms including sneezing, nasal discharge, pneumonia, and reduced growth rates (Rutter, 1985).

1.5.4 Haemorrhagic septicaemia (HS)

1.5.4.1 The disease

HS is an acute fatal disease, mainly of cattle and buffaloes, that is of great economic importance particularly in south-east Asia, the Middle East and some regions of Africa (de Alwis *et al.*, 1990). It is a primary pasteurellosis characterised by a terminal

septicaemia and high mortality caused by *P. multocida* serotype B:2 which is more prevalent in Asia, or by serotype E:2, which has been reported only in Africa (Wijewardana, 1992). The disease is spread by direct and indirect contact; the source of infection is infected animals or carriers (Oie, 2000). Studies have shown that the carrier status is usually involved in disease outbreaks and the offending bacterium can be transmitted to other animals (Hiramune and De Alwis 1982). The carriers are identified by either the presence of the organism in the nasopharynx or the organism is detectable in the lymphoid organs of the upper respiratory tract (Hiramune and De Alwis 1982). The tonsils are the most consistent site for the persistence of the organism in a carrier state in cattle (De Alwis and Wijewardana 1990; Wijewardana, 1992). Under stressful conditions, such as inclement weather, overcrowding and transportation, carriers are believed to transmit the organism to susceptible animals, which leads to outbreaks of HS (De Alwis *et al.,* 1990b).

1.5.4.2 Pathogenesis

It is not known where the organism lodges and multiplies during the early clinical phase of HS, but a feature of the disease is the rapid spread of infecting bacteria from the respiratory tract to the blood and lymph that causes a fatal septicaemia in less than 48 h. The bacteria must migrate through the epithelial layer into the pulmonary interstitium (Shah *et al.*, 1996; Tabatabaei *et al.*, 2002). It has been observed that virulence of the organism is correlated with the production of hyaluronidase although it is not known whether or how this is connected with pathogenesis (Wijewardana, 1992). Endotoxin (LPS) on the other hand is believed to play a role in the production of the pathological lesions and death (Wijewardana, 1992). HS-causing bacteria aspirated into the lung of buffaloes can lead to the appearance of LPS in the blood and can induce a systemic

response similar to endotoxic shock, resulting in the rapid death of challenged animals. This endotoxaemia would seem to be a major factor in the pathogenesis of HS in buffaloes (Horadagoda *et al*., 2002).

1.5.4.4 Clinical presentation

Some characteristics and clinical features aid in the recognition of HS. The clinical signs of the typical disease caused by *P. multocida* B:2 or E2 strains include a rise in temperature, respiratory distress with nasal discharge, frothing from the mouth, which leads to recumbency and death (Oie, 2004). The majority of cases in cattle and buffaloes are acute or peracute, with death occurring from 6-24 h after the first recognized signs (Benkirane and De Alwis 2002). In most cases, there is an oedematous swelling of the head, neck, and brisket region. Incision of the swelling reveals a clear or straw-coloured serous fluid (Oie, 2004).

1.5.4.3 Epidemiology

HS is endemic in most tropical areas, including Asia, Africa, China, India, Indonesia, Mongolia, Myanmar, Philippines, Sri Lanka and Malaysia and causes high mortality in the major susceptible animal species, cattle and water buffaloes. Pigs, sheep, goats, deer and camel are also susceptible to infection and disease (Blackall *et al.,* 2000) and deer with septicaemic disease have been identified in the United Kingdom (Pedersen *et al.,* 2003). There is some evidence that buffalo are more susceptible than cattle and that, in both species, young adult animals are more susceptible than older animals (De Alwis, 1990b). Higher incidence of HS is associated with moist, humid conditions (Benkirane and De Alwis, 2002). In the United States, the disease has been confirmed only in American bison. The disease is thought to be endemic in one large herd of North

American range bison; however, epidemics appear to be rare, for example in 1912, 1922, and 1965. A *P. multocida* isolate from the 1922 outbreak, a serotype B:2, is maintained in the USDA culture collection as a reference strain. Although outbreaks of HS have been reported in sheep and swine, it is not a frequent or significant disease. Cases have been reported in deer, elephants and yaks. There is as yet no evidence of a reservoir of infection outside the principal hosts: cattle, water buffaloes, and bison (Oie, 2004).

Outbreaks can occur at all times of the year but those occurring during the wet season tend to spread, presumably due to the longer survival of the organisms under moist conditions (Oie, 2004). Outbreaks are usually correlated with the onset of rain, which is normally preceeded by a long dry period with limited food. These adverse climatic factors and accompanying nutritional deficiencies may well precipitate the activation of the carrier to become a source of infection for susceptible animals.

1.5.4.5 Diagnosis

To identify HS-causing organisms, a wide range of laboratory diagnostic tests have been developed which mainly include culture on specific media such as Blood Agar (BA) or on Brain Heart Infusion Agar (BHIA), and biological tests after isolation of the causative agent. Freshly isolated colonies on Casein Sucrose Yeast (CSY) agar enriched with blood are approximately 2 mm in diameter after 24 h at 37ºC (Wijewardana, 1992). Serological tests have been also employed, e.g. rapid slide agglutination tests or agar gel precipitation tests. An ELISA test using a live or formalin-inactivated suspension of *P. multocida* as coating antigen was developed. Regardless of the capsular serogroup associated with HS, the assay was reported to have a specificity of 99% and a sensitivity of at least 86% (Dziva *et al*., 2008). Molecular methods such as PCR, ribotyping or

restriction endonuclease analysis are epidemiologically useful because they are more rapid and enable strain differentiation within serotypes and hence some epidemiological inferences, for investigations extending beyond routine diagnosis (Benkirane and De Alwis, 2002).

1.5.4.6 Treatment

HS is a primary bacterial disease and, theoretically, could be effectively treated by the wide range of antibiotics currently available. However, treatment is constrained by a host of practical considerations. Animals can be cured only if they have been treated in the very early stages of the disease (Benkirane and De Alwis 2002). Early detection and effective treatment are achieved through regular checking of rectal temperatures of in contact animals. Usually, chemotherapy relies on either streptomycin or oxytetracycline administrated by the intramuscular route at fairly high dosage (Benkirane and De Alwis 2002). Antibiotic resistance has been reported for streptomycin and sulphonamide in some strains of *P. multocida*, but it was not reported for the HS serotypes (De Alwis, 1992).

1.5.4.7 Prevention and control

The best way to prevent HS in cattle and buffaloes is vaccination. It is a major control measure and various vaccine types have been developed, including broth bacterin, oil adjuvanted vaccines, alum-precipitated vaccines, the double emulsion vaccine and a live vaccine (Verma and Jaiswal, 1998). The most effective bacterin is the oil-adjuvant vaccine where one dose provides protection for 9-12 month so it needs to be administered annually (Verma and Jaiswal, 1998; Tabatabaei *et al.*, 2002). The alum-precipitated bacterin needs to be given twice yearly and this vaccine appears to be the

most popularly used in Asia. Both bacterins have the disadvantage of conferring an immunity of short duration, and the potential to cause endotoxic shock when high density bacterins are used (Wijewardana, 1992).

The immunoprotective efficacy of *P. multocida* 6:B OMPs was examined in the mouse model. Prototype vaccines were prepared using OMPs with adjuvants including dioleoyl phosphatidyl choline-based liposomes and Montanide ISA206 water-in oil-in water emulsion. Antibody response to the vaccine was monitored using an indirect ELISA test. This study showed that immunized mice had a high titre of antibodies with both of the formulations. The vaccinated mice were able to survive a live virulent bacterial challenge. Based on the findings of the study it can be inferred that OMPs are important determinants of immunoprotection, hence can serve as vaccine candidates against haemorrhagic septicaemia (Basagoudanavar *et al.,* 2006).

A number of attempts have been made to create live, attenuated vaccines for HS, including a number of streptomycin-dependent (Str^D) mutants of HS-causing *P. multocida* B:2 obtained from Sri Lanka. These mutants were avirulent for mice, when inoculated alone, but some mutants killed mice when inoculated with streptomycin. Although most of the mutants were stable, some of them produced streptomycin-independent revertants. It was shown that the rate of reversion varied between different mutants and most of them were highly virulent for mice (De Alwis *et al.,* 1980). A *P. multocida* serotype B:3,4 was isolated from a fallow deer (Dama dama) in England after an outbreak of septicaemic disease (Jones and Hussaini, 1982). It was reported to have a low virulence for cattle possibly due to the lack of transferrin-binding proteins (Veken *et al.*, 1994). It has also been used as an exprimental live vaccine, containing 2 x 10^7 viable

organisms per dose, in cattle and buffaloes. The strain was able to raise antibodies in cattle that protected mice against serotype B infections. Six months after immunization, the vaccine was reported to be more efficacious than an alume-precipitated vaccine in protecting calves against B:2 challenge (Myint *et al.*, 1987). A capsular mutant (AL18) of *P. multocida* B:2 has been constructed by inactivation of the *bcb*H gene (a gene predicted to be involved in polysaccharide biosynthesis). Immunisation of mice with the mutant was able to confer significant protection against wild-type challenge, while immunisation with similar doses of either killed wild-type or killed mutant failed to confer protection (Boyce & Adler, 2001).

Other live vaccines have been based on *aro*A mutants of *P. multocida* strains. The *aro*A gene encodes 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, which is involved in the conversion of shikimic acid to chorismic acid, an intermediate common to the biosynthesis of aromatic amino acids L-tryptophan, L—tyrosine and L-phenylalanine. Mutation in the *aro*A gene creates dependence for growth on aromatic compounds that are not available in the host, as this pathway is not operative in mammalian cells (Tabatabaei *et al.*, 2002). The *aro*A strains derived from *P. multocida* serotype A:1 (PMP1) and *P. multocida* serotype A:3 (PMP3), when tested for virulence in a mouse model, both showed high attenuation and were able to protect mice against a lethal parental strain challenge (Homchampa *et al.*, 1992). These strains were also shown to provide protection against challenge in chickens (Scott *et al.*, 1999). A marker free *aro*A deletion derivative (strain JMRT12) of a virulent field isolate of *P. multocida* B:2 (strain 85020) obtained from Sri Lanka was created which was highly attenuated in a mouse model of HS (Tabatabaei *et al.,* 2002). Mice infected with HS-associated wild-type *P. multocida* B:2 showed that the LD_{50} varied according to the different routes of

challenge. The dose of *P. multocida* 85020 which killed 50% of inoculated animals were given by the intraperitoneal (IP) route was <20 CFU/ mouse, whereas when delivered by the intranasal IN route there was 10^3 CFU/mouse (Tabatabaei et al., 2002). Intraperitoneal injection of mice with 1 CFU per mouse of the *P. multocida* 85020 parent strain killed 2 of 3 mice but 2.6 x 10^7 CFU of the mutant did not kill any of them (Tabatabaei *et al.*, 2002). It was shown that a single immunization by the intramascular route with the JMRT12 *aro*A strain was able to create complete protection against subsequent homologous challenge in the mouse model with up to 1000 LD_{50} of the parent strain. Further work with the *aro*A strains undertaken in calves (Hodgson *et al.*, 2005; Dagleish *et al.*, 2007) showed that intramuscular injection twice of 10^7, 10^8 or 10^9 CFU twice at a 4-week interval of the *aro*A strain JRMT12 completely protected calves against challenge with the virulent parent 85020 strain.

1.6 Host defences

The phagocytes of the innate immune system provide a first line of defence against many common microorganisms and are essential for the control of common bacterial infections (Leon and Ardavin, 2008). However they cannot always eliminate infectious organisms, and lymphocytes of the adaptive immune system have evolved to provide a more versatile means of defence that, in addition, provides an increased level of protection from a subsequent re-infection with the same pathogen. The cells of the innate immune system play a crucial part in the initiation and subsequent direction of the adaptive immune responses. Moreover, because there is a delay of 4-7 days before the initial adaptive immune response takes effect, the innate immune response has a critical role in controlling infections during this period (Charles *et al.*, 1999).

Natural acquired immunity to HS was described in 1980 (De Alwis and Carter 1980). In Sri Lanka, where high, moderate and low HS incidence areas can be identified, the naturally acquired immunity comes from sub-clinical infection. De Alwis (1982) reported that when susceptible buffalo calves were exposed to natural infection with the HS agent, some calves succumbed to disease while the others developed varying degrees of immunity which in most instances is considerably higher than that induced by vaccination. Immunity was attributed to protective antibodies that develop following non-fatal exposure and can persist for more than one year in some animals.

1.7 Aim of this study

The goal of the present study was to study the internalization of HS strains of *P. multocida* B:2 by mammalian cells and to determine the fate of intracellular *P. multocida* B:2. If *P. multocida* B:2 can survive in J774.2 macrophage like cell lines or BL-3 bovine lymphoma cell lines, can they proliferate within these cell lines and destroy the cells?

CHAPTER 2

Materials and methods:

2.1 Bacterial strains

Two strains of *Pasteurella multocida* serotype B:2 were used: strain 85020 wild type was originally imported from Sri Lanka under licence PO/95/22, and an *aro*A deletion derivative 85020 of this strain was created by allelic exchange (Tabatabaei *et al.*, 2002). *Escherichia coli* XL1-Blue (Stratagene), and *Pasteurella multocida* serogroup A:3 (Supplied by Dr. J.C. Hodgson, Moredun Research Institute, Edinburgh) were also used (Table 2).

2.1.1 Growth media and sterilisation

Bacteria were grown on brain heart infusion broth (BHIB, Oxoid) (Appendix 5.1), brain heart infusion agar (BHIA) or BHIA supplemented with 5% (v/v) defibrinated sheep blood (E&O Laboratories, Scotland) (SBA) (Appendix 5.1). All the culture media were sterilised by autoclaving at 15 p.s.i (121°C) for 15 min.

2.1.2 Storage of isolates

All strains were stored frozen at -80°C in BHI containing 50% (v/v) glycerol. *P. multocida* strains from the frozen glycerol stocks were subcultured routinely on SBA at 37ºC.

Table 2: Bacterial strains used to investigate the adherence to and invasion of mammalian cells.

Strain	Origin	Source
Pasteurella multocida serotype B:2 (85020)	Bovine haemorrhagic septicaemia Sri Lanka	Division of Infection and Immunity, University of Glasgow (Dr. Coote).
JRMT12 (an *aro*A derivative of *Pasteurella multocida* B:2 85020).	Created by allelic exchange (Tabatabaei *et al.,* 2002).	Division of Infection and Immunity, University of Glasgow (Dr. Coote).
Pasteurella multocida serotype A3 (MRI, 671/90)	Bovine pneumonia Scotland	Moredun Research Institute (Dr. J. Hodgson).
Escherichia coli XL1-Blue genotype: endA1, gyrA96 (Nal^R), thi-1, recA1, relA1, lac, glnV44, F'[:Tn10 $proAB^+$ $lacI^q$ Δ(lacZ)M15] hsdR17(r_K^- m_K^+)		Stratagene.

2.1.3 Culture purity checks

Bacterial colonies from all agar plates and broth cultures were subjected to purity checks by Gram staining and by culture on SBA or BHIA and, after incubation at 37ºC for 24 h, checked for purity of colony growth.

2.2 Antibiotics

The following antibiotics were used: polymyxin B sulphate (PB) (Sigma) and gentamicin (GM) (Sigma) at a final concentration of (350 μg/ml) each. Antibiotics were dissolved in distilled water at 350 mg/ml as stock solutions, sterilised by passage through a 0.2 μm membrane (Sartorius, Germany) and stored frozen at -20°C.

2.3 Preparation of bacterial inocula and growth curves

P. multocida B:2 85020 wild type and the *aro*A derivative of this strain (JRMT12) were used. A loopful of bacteria was taken from a stock culture of *P. multocida* that had been maintained at -80°C and plated onto SBA. After incubation overnight at 37°C, a single bacterial colony was added to 25 ml of BHIB and grown overnight at 37°C in an orbital incubator set at 150 r.p.m. Then, 0.5 ml of this culture was inoculated into 25 ml of pre-warmed BHIB and the OD at 600 nm measured at intervals.

2.4 Bacterial viability measurements

It was necessary to confirm that the conditions used in the antibiotics assay for intracellular survival were sufficient to eliminate the extracellular bacteria. A 1.0 ml inoculum from an overnight culture in BHIB was introduced into a flask containing 20

ml of BHIB and incubated for 4 h (log-phase) at 37°C in an orbital incubator at 150 r.p.m. After centrifugation at 3,000 x g for 30 min, the cells were resuspended in 10 ml of RPMI 1640 medium (Gibco) containing 1 ml L-glutamine (Gibco), 20 ml of foetal calf serum (Gibco) and diluted in the same medium to give a bacterial concentration of 10^5 CFU/ml. Gentamicin and polymyxin B sulphate (Appendix 5.6) were added to a final concentration of 350 μg/ml, either separately or together, and, after 0, 60, and 120 min of incubation, samples were removed and dilutions were plated onto BHIA and the number of colonies was determined after 24 h of incubation at 37°C.

2.5 Mammalian cell lines

J774.2 murine macrophage like cells and BL-3 bovine leukaemia-derived lymphoma cells were used in this work (provided by Dr John Coote, University of Glasgow, Infection and Immunity Division).

2.5.1 J774.2 cells

J774.2 cells were maintained in 20 ml of RPMI 1640 (Gibco) medium supplemented with 1 ml L-glutamine (Gibco), 1 ml foetal calf serum (Gibco) and 1 ml antibiotic solution (Gibco) (complete medium) (Appendix 5.4) at 37°C, in an air atmosphere of 5% CO_2, 95% under controlled humidity. One flask was used to seed three flasks and this produced confluent growth within 48-72 h. The numbers of viable cells was determined by counting trypan blue-excluding cells in a haemocytometer (Section 2.6). Loss of cell viability was estimated microscopically by noting the number of dead cells taking up the dye, expressed as a percentage of the total cell count (section 2.6).

2.5.2 BL-3 cells

BL-3 cells were grown in suspension culture in a medium (BL-3 medium) (Appendix 5.5) containing 50 ml L-15 Leibovitz medium (Gibco), 50 ml RPMI 1640 medium (Gibco) with 20 ml foetal bovine serum (Gibco), 1 ml L- glutamine (Gibco), and 1 ml penicillin and streptomycin solution (Gibco) at 37ºC in an atmosphere of 5% CO_2 95% air under controlled humidity. Cells were split 1:5 in the above medium and grown for a further 4 days. Cultures were centrifuged at 1000-x g at room temperature for 5 min, the supernatant was discarded and the cells were resuspended in 5 ml of fresh complete BL-3 medium, using 1 ml of cells to 10 ml of fresh medium. Cells were then incubated for a further 72 h at 37ºC in a 5% CO_2 incubator. Viable cell numbers were determined by trypan blue exclusion (see section 2.6).

2.6 Assessment of mammalian cell viability by trypan blue method

For counting cell suspensions, a mixture containing 10 µl of trypan blue (0.4% w/v) (Sigma), and 10 µl of the cell suspension was placed in a improved Neubauer (1/400 mm^2 x 0.1 mm depth) counting chamber. After 10 min, the chamber was viewed by light microscopy and examined under 10 X objective lens. For each sample, the number of cells in 4 squares was determined, the average number of cells per single square was determined and multiplied by 2 x 10^4 to give the number of cells per ml. Viability of cells was determined by blue stained cells being counted as dead cells while clear cells were counted as viable.

2.7 Assessment of viability of J774.2 cells by MTT assay

A colorimetric assay that determined the activity of mitochondrial dehydrogenases was the second method used to assess the viability of J774.2 cells. Live cells reduce [3-(4, 5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) by mitochondrial dehydrogenases to a purple formazan crystalline form in proportion to the number of viable cells. To 100 µl of cell culture in a well of a 96-well cell culture plate (Costar, 3799), 50 µl of MTT at a final concentration of 1.5 mg/ml in RPMI 1640 (Gibco) without phenol red were then added. Cells were incubated for 40 min at 37ºC in the 5% CO_2 atmosphere and then 50 µl of solubilization stop solution (0.5% w/v SDS, 25mM HCl in 90% v/v isopropanol) were added. Plates were incubated for 5 min on a rotating plate at 37ºC, each sample was mixed and the absorbance at 600nm was measured on a micro-plate reader (FLUO star OPTIMA, Germany). The % of dead cells was determined by:

% Dead cells = 1- (OD (test)/ OD (-ve)) X 100

test = cells treated with bacteria

-ve = cells only.

2.8 Adhesion and invasion assay

2.8.1 J774.2 assay

To determine if *P. multocida* strain B: 2 is able to invade mammalian cells and is capable of intracellular survival and growth, two cell types were used; J774.2 macrophage-like cells and BL-3 bovine lymphoma cells. J774.2 macrophages cultured in RPMI complete medium were removed by scraping and were then washed with RPMI 1640 basal medium by centrifugation at 1000 x g for 5 min and resuspended in RPMI

supplemented with L-glutamine and foetal bovine serum (i.e. no antibiotics). Viable cell numbers were determined by trypan blue exclusion. Approximately 10^6 viable cells per ml were seeded into each well of a 6-well tissue culture plate. Actual numbers of viable cells were determined for each experiment. Macrophages were infected with 0.5 ml of a suspension of bacteria at 10^9 CFU/ml to give a multiplicity of infection (MOI) of 500:1 and the plate incubated for 2 h at 37ºC, in 5% CO_2, to allow bacterial adherence and/or invasion. In some experiments the 6-well plates were centrifuged at 1000 x g for 5 min to ensure close contact between the bacteria and mammalian cells. After incubation for 2 h, the sample was centrifuged for 5 min at 1000 x g and the culture medium was discarded; the infected cells were carefully washed twice more by centrifugation at 1000 x g for 5 min and resuspension in RPMI 1460 basal medium to remove extracellular bacteria and then resuspended in RPMI complete medium with gentamicin and polymyxin B sulphate, each at the final concentration of 350 µg/ml, to kill any remaining extracellular bacteria. Incubation was continued for 2 h. Cells were then washed 3 times as above with RPMI 1460 basal medium and lysed by addition of digitonin (Sigma) at a final concentration 100 µg/ ml for 30 min. The average number of viable intracellular bacteria was determined after plating 10-fold dilutions of the lysate in duplicate on BHI agar.

2.8.2 BL-3 assay

Cells were grown in complete BL-3 medium containing L-15 Leibovitz, RPMI 1640 medium, L- glutamine, fetal bovine serum and penicillin, streptomycin solution. For the infection assay, the BL-3 cells were washed three times with fresh BL-3 medium to remove antibiotics and then resuspended in 5 ml of BL-3 medium. Approximately 10^6 cells per ml were then infected with bacteria at a MOI 500:1 and the same procedure

used as for J774.2 cells was followed, except that BL-3 medium was used in place of RPMI (Section 2.8.1). The average number of viable intracellular bacteria was determined after plating 10-fold dilutions of the lysate in duplicate on BHI agar.

2.9 Transmission electron microscopy

Cell samples were fixed with 2.5 % (v/v) glutaraldehyde in 0.1 M PO_4 buffer pH 7.4 for 1 h, then rinsed 3 times with buffer containing 2% (w/v) sucrose for 5 min and stored in this solution at 4ºC overnight. Samples were then fixed with 1% (w/v) osmium tetroxide for 1 h at room temperature and rinsed 3 times for 20 min each with distilled H_2O. An ethanol series containing 30%, 50%, 70%, 90% (v/v) ethanol was then applied for 10 min each, followed by three changes at 100% ethanol for 10 min. Once sufficiently dehydrated, 3 time washes with 1:1 propylene oxide and resin were conducted overnight at room temperature on a rotator in a fume hood. The pellet was then embedded in fresh resin plus accelerator in a mould and resin polymerised at 60ºC for 48 h. Finally, 70-80 nm sections were cut and stained with 2% (w/v) methanolic uranyl acetate and Reynolds lead citrate stain for 5 min. Sections were examined with a LEO 912AB transmission electron microscope operating at 80 KV, 1000x magnification.

2.10 Statistical evaluation

The results were assessed statically by student's t-test.

CHAPTER 3

Results:

3.1 Growth curves

The growth curves of *P. multocida* B:2 strains 85020 wild type and JRMT12 are shown in Figure 3. Both strains grew equally well in BHI medium.

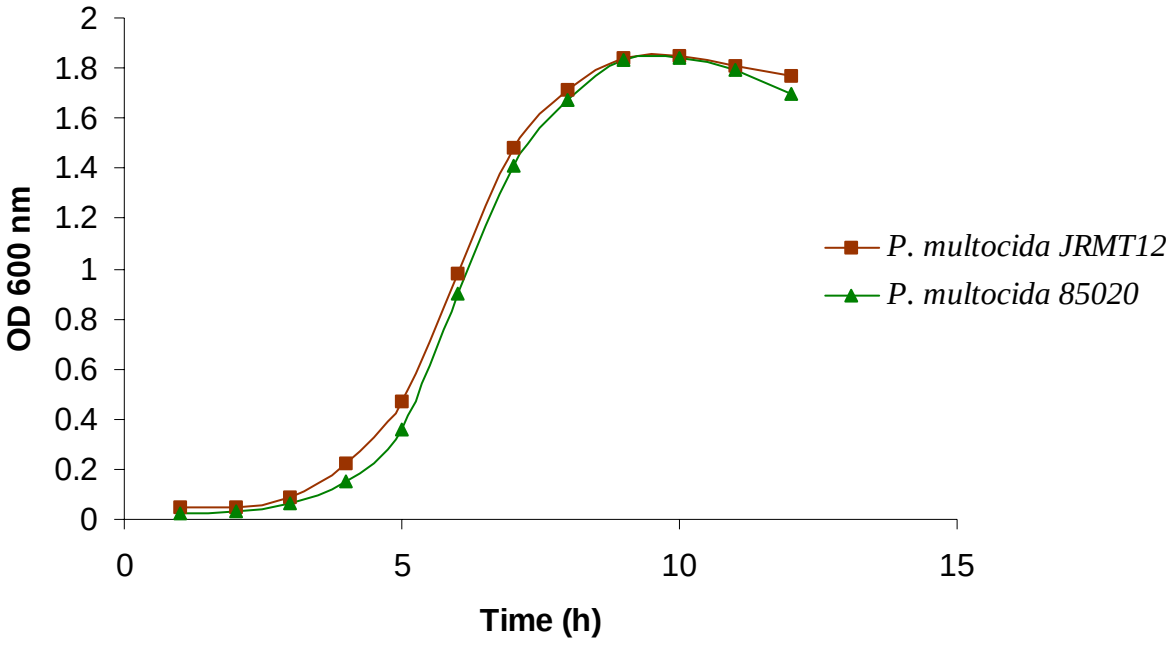


Figure 3. Growth curves of *P. multocida* B: 2 strains.
25 ml of BHIB was inoculated with 0.5 ml of overnight cultures of the strains 85020 and JRMT12 and incubated at 37°C with shaking. The OD at 600 nm was measured at intervals.

3.2 Rate of bacterial killing by antibiotics

Strains 85020 and JRMT12 were grown for 4 h in BHIB with shaking at 37°C to give log phase bacteria. Then, 10^5 CFU/ml were transferred to RPMI supplemented with the antimicrobials gentamicin and polymyxin B sulphate, separately or together, at 350 μg/ml final concentration at 37ºC with shaking. At 0, 60 and 120 min, samples were removed for dilution and plating onto BHIA. According to the data in Table 3 (A and B), viability of the *P. multocida* B:2 strains decreased rapidly with both gentamicin and polymyxin used singly or together. Gentamicin and polymyxin together resulted in significantly reduced numbers of viable bacteria in comparison to each antibiotic used singly. In all cases, complete loss of CFU was observed only at 120 min, when no colonies of bacteria were detected. Thus, for both *P. multocida* B:2 85020 and JRMT12, a 120 min incubation period with both antibiotics was considered necessary to ensure elimination of all extracellular bacteria. It can be concluded that gentamicin and polymyxin exhibited bactericidal activity against *P. multocida* B:2 and, over 120 min, produced >99.9% killing of both strains if both antibiotics were used together.

3.3 Mammalian cell viability

3.3.1 Assessment of mammalian cell viability by trypan blue method

To examine the effect of *P. multocida* on viability of J774.2 cells, the cells were scraped from the tissue culture plates at 0, 2 and 4 h post-infection with bacteria at a MOI of 500:1 and resuspended in pre-warmed RPMI complete medium (without antibiotics) and cell viability was determined by trypan blue staining. Macrophage viability in the presence of *P. multocida* B:2 was compared to the control where no bacteria were present throughout the experiment. There were approximately 14% dead cells at 0 time in all three samples (Figure 4, A, B, C). At 2 h and 4 h, the percentage of

Table 3: Effect of antibiotics on the viable counts of the two strains of *P. multocida* B:2. Bacteria were diluted to 10^5 CFU/ml in RPMI medium containing a final concentration of 350 µg/ml each of gentamicin or polymyxin, alone or in combination. Cells were incubated at 37ºC and, at 0, 60, and 120 min, viable *P. multocida* B:2 were enumerated by plating 100 µl samples onto BHIA in triplicate and incubation at 37ºC overnight.

A: Viable counts of *P.multocida* wild type 85020.

B: Viable counts of *P. multocida aro*A JRMT12

A:

Time (min) measure	Polymyxin (350µg/ml) CFU/100 µl at Dilution:			Gentamicin (350µg/ml) CFU/100 µl at Dilution:			Polymyxin and Gentamicin (350µg/ml of each) CFU/100 µl at Dilution:		
	Neat	10^{-1}	10^{-2}	Neat	10^{-1}	10^{-2}	Neat	10^{-1}	10^{-2}
0	ND	~1950	210	ND	~1900	210	ND	~1800	220
60	100	0	ND	70	0	ND	9	0	ND
120	0	0	ND	0	0	ND	0	0	ND

B:

Time (min) measure	Polymyxin (350µg/ml) CFU/100 µl at Dilution:			Gentamicin (350µg/ml) CFU/100 µl at Dilution:			Polymyxin and Gentamicin (350µg/ml of each) CFU/100 µl at Dilution:		
	Neat	10^{-1}	10^{-2}	Neat	10^{-1}	10^{-2}	Neat	10^{-1}	10^{-2}
0	ND	~2600	290	ND	~2500	300	ND	~1900	210
60	60	0	ND	120	0	ND	10	0	ND
120	0	0	ND	0	0	ND	0	0	ND

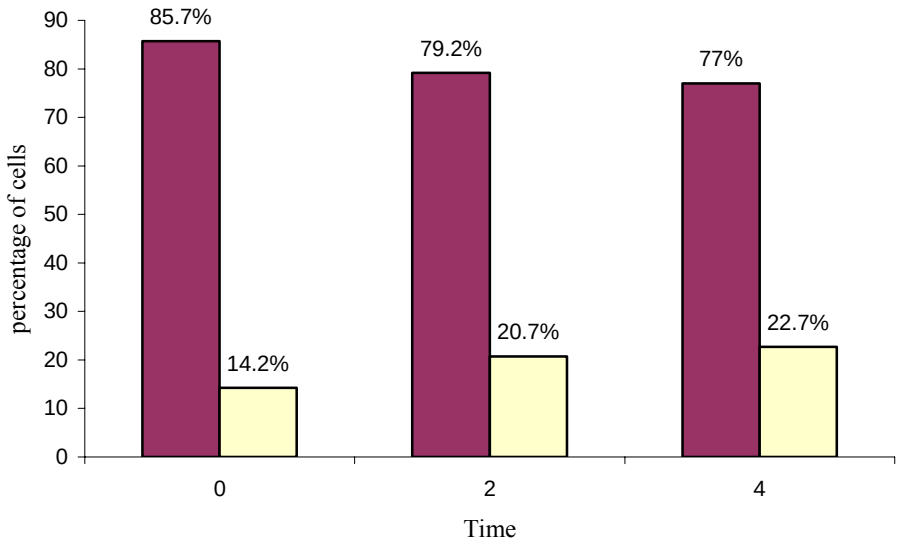
ND: not done

0 : no colony detected

Figure 4: Assessment of viability of J774.2 cells by trypan blue method. Approximately 10^6 cells were infected with log-phase *P. multocida* B:2 strains in RPMI complete medium (without antibiotics) at a ratio of 500:1 bacteria: J774.2 cell. The cells were centrifuged in 1000 x g for 5 min and incubated in 5% CO_2 at 37ºC. The numbers of viable J774.2 cells were estimated at 0, 2 and 4 h by using trypan blue exclusion.

A. Percentages of live and dead J774.2 cells in uninfected controls (average of 2 determinations).
B. Percentage of live and dead J774.2 cells with wild-type *P. multocida* B:2 strain 85020 (average of 2 determinations).
C. Percentage of live and dead J774.2 cells with the *aro*A derivative of *P. multocida* B:2 strain JRMT12 (average of 2 determinations).

A



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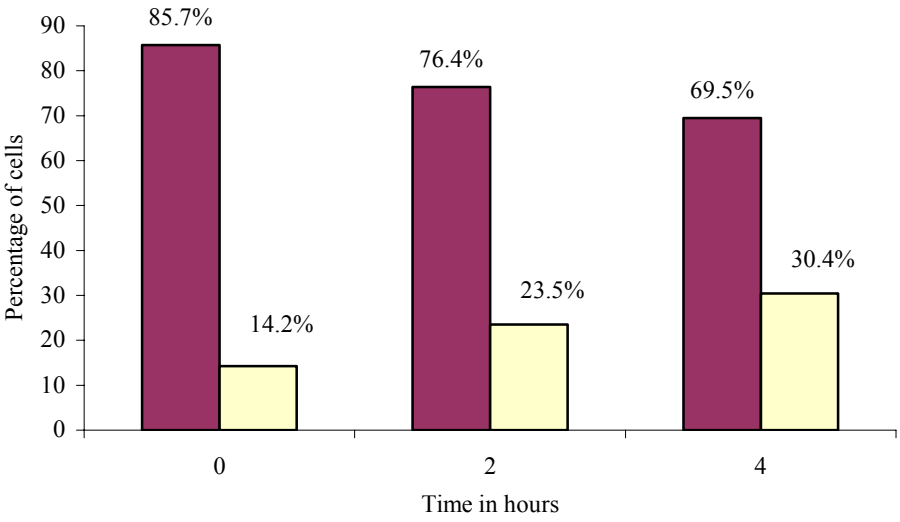

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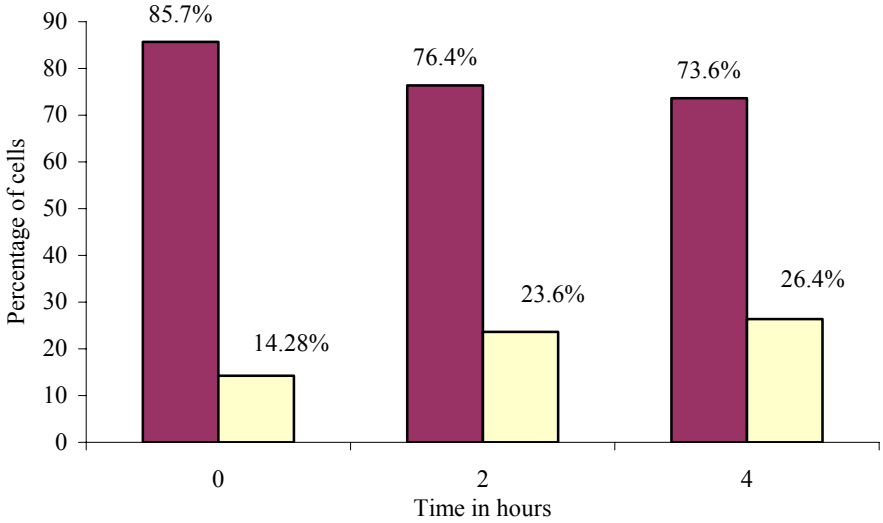


% Live Cells % Dead Cells

3.3 Mammalian cell viability

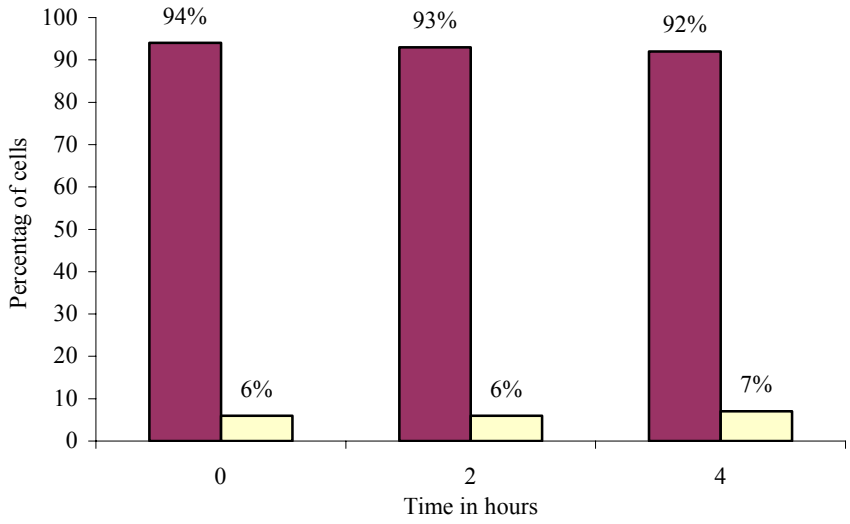
3.3.1 Assessment of mammalian cell viability by trypan blue method

To examine the effect of *P. multocida* on viability of J774.2 cells, the cells were scraped from the tissue culture plates at 0, 2 and 4 h post-infection with bacteria at a MOI of 500:1 and resuspended in pre-warmed RPMI complete medium (without antibiotics) and cell viability was determined by trypan blue staining. Macrophage viability in the presence of *P. multocida* B:2 was compared to the control where no bacteria were present throughout the experiment. There were approximately 14% dead cells at 0 time in all three samples (Figure 4, A, B, C). At 2 h and 4 h, the percentage of dead cells had increased slightly in the untreated control (Figure 4 A). However, there were no significant differences in the number of dead cells in cultures with either strain of *P. multocida* B:2 compared to the uninfected control (Figure 4, B, C), although incubation in the presence of the two *P. multocida* B:2 strains reduced the percentage viability of J774.2 cells to values that were slightly lower than the control values at 2 h for both strains. The loss in viability was more apparent with the wild-type 85020 at 4 h than with JRMT12 (Figure 4 B and C). This small difference may have been due to variations in the experimental procedure and may not have been significant. Overall our data showed a slight decrease in viability of J774.2 in presence of bacteria when compared to the control, suggesting that *P. multocida* B:2 had some slight toxicity to host cells. However, when the viability of BL-3 cells was determined with the same assay, the cells were not damaged by exposure to *P. multocida* B:2 (Figure 5). In this experiment, fewer dead cells were observed for the BL-3 cells compared to the J774.2 cells, and there were no significant differences in cell viability in the presence of either strain of *P. multocida* B:2 compared to the control.

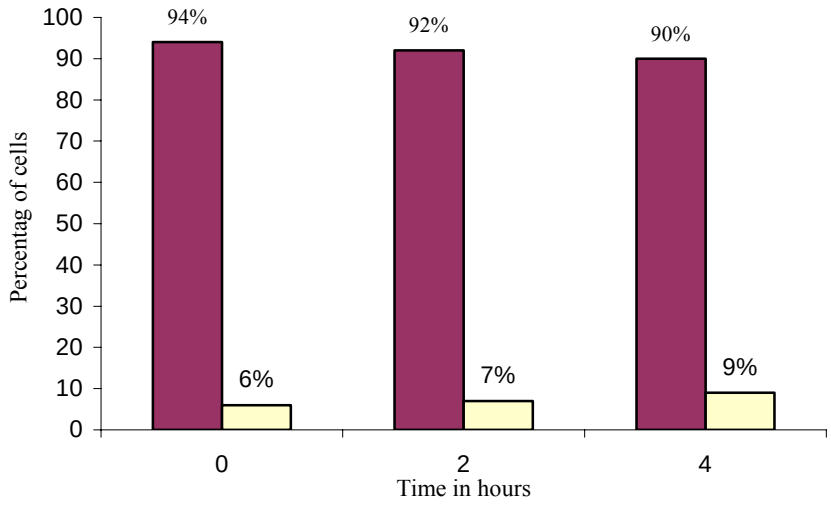
Figure 5: Assessment of viability of BL-3 cells by trypan blue method. Approximately 10^6 cells were infected with log-phase *P. multocida* B:2 strains in BL-3 medium free of antibiotics at a ratio of 500:1 bacteria: BL-3 cell. The cells were centrifuged in 1000 x-g for 5 min and incubated in 5% CO_2 at 37ºC. The numbers of viable cells were estimated at 0, 2 and 4 h by using trypan blue exclusion.

A. Percentages of live and dead BL-3 cells in uninfected controls (average of 2 determinations).
B. Percentages of live and dead BL-3 cells with wild-type *P. multocida* B:2 strain 85020 (average of 2 determinations).
C. Percentages of live and dead BL-3 cells with the *aro*A derivative of *P. multocida* B:2 strain JRMT12 (average of 2 determinations).

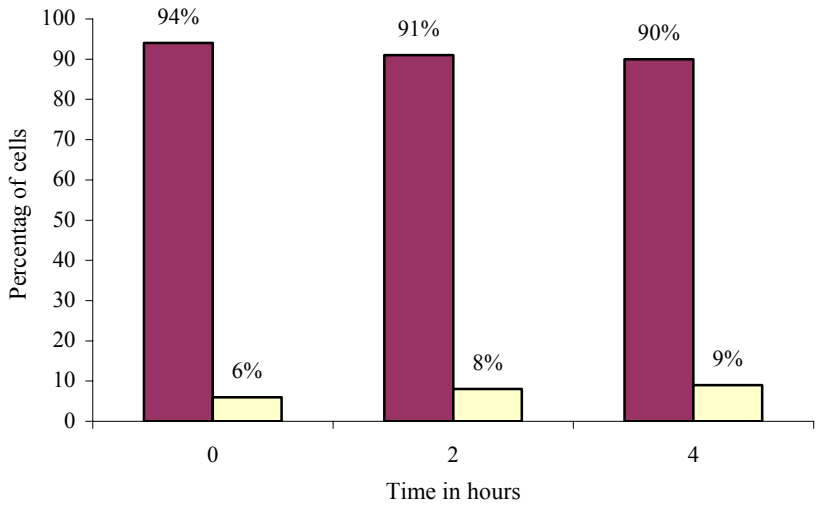
A



B



C



% Live Cells

% Dead Cells

3.3.2 Assessment of changes in the J774.2 cell viability by MTT assay

The data above suggested *P. multocida* B:2 failed to kill J774.2 or BL-3 cells. A colorimetric assay that determines the activity of mitochondrial dehydrogenases was the second method used to assess the viability of J774.2 cells exposed to *P. multocida* B:2 strains and other bacteria. Approximately 10^6 J774.2 cells were resuspended with RPMI complete medium (without antibiotics) without phenol red in 6-well cell culture plates and mixed bacteria at ratio of 500:1 bacteria: J774.2 cells. Samples were then centrifuged at 1000 x g for 5 min at room temperature. Following an invasion incubation period of 2 h, 100 µl of mixture transferred to a 96-well cell culture plate and 50 µl of MTT dye at a concentration of 1.5 mg/ml in RPMI without phenol red were added, cells were incubated for 40 min at 37ºC at 5 % CO_2. Without washing or removing the supernatant, 50 µl of solubilization stop solution were added to dissolve the purple formazan product to give a coloured solution. After 5 min incubation, plates were read at a wavelength 600 nm with any automated micro-plate reader that determines the extent of reduction MTT. The result indicated that, in general, greater values were obtained at 4 h than at 2 h, which would perhaps be expected with a greater period of metabolic activity. However, the reduction of MTT was higher for cells that harboured bacteria, due presumably to the activity of to bacterial dehydrogenises (Table 4 A and B). This was reflected in the high values obtained after incubation of MTT with bacteria alone. Any loss of viability of J774.2 cells upon exposure to the bacteria was, therefore, being masked by the bacterial activity. This assay was not therefore considered suitable for assessing mammalian cell viability in presence of live bacteria.

Table 4: Total effect of bacteria on the J774.2 cells by MTT assay. Approximately 10^6 cells were infected with *P. multocida* B:2 strains, *P. multocida* A:3 and *E. coli* XL1-Blue at a ratio 500:1 bacteria to J774.2 cells. After 2 h incubation, 100 µl of infected cells transferred to the 96- well plate. 50 µl of MTT at a final concentration 1.5 mg/ml were added and cells were incubated for 40 min. cells were then treated with 50 µl stop solution and left for 5 min. sample were then read at 600 nm wave length.

A. The average of 4 determination absorbance of infected J774.2 cells with or without bacteria after 2 h incubation.
B. The average of 4 determination absorbance of infected J774.2 cells with or without bacteria after 4 h incubation.

A:

Cell treatments	**Average absorbance at 600 nm**
J774.2 cells without treatment (-ve control)	0.57
J774.2 cells with Triton X-100 (+ve control, 100% killing)	0.16
P.m 85020 +J774.2 cells	2.47
P.m JRMT12+J774.2 cells	2.15
P.m A:3+J774.2 cells	2.8
E. coli +J774.2 cells	1.9
P.m 85020 alone	1.7
P.m JRMT12 alone	1.35
P.m A:3 alone	1.7
E. coli alone	1.3
MTT alone	0.1

P. m 85020: *P. multocida* B:2 strain 85020

P. m JRMT12: *P. multocida* B:2 strain JRMT12

P. m A:3 : *P. multocida* A:3

E. coli : *E. coli* XL1-Blue

B:

Cell treatments	**Average absorbance at 600 nm**
J774.2 cells without treatment (-ve control)	1.0
J774.2 cells with Triton X-100 (+ve control, 100% killing)	0.18
P.m 85020 +J774.2 cells	1.25
P.m JRMT12+J774.2 cells	1.75
P.m A:3+J774.2 cells	1.8
E. coli +J774.2 cells	1.9
P.m 85020 alone	1.1
P.m JRMT12 alone	1.04
P.m A:3 alone	0.79
E. coli alone	2.0
MTT alone	0.19

P. m 85020: *P. multocida* B:2 strain 85020

P. m JRMT12: *P. multocida* B:2 strain JRMT12

P. m A:3 : *P. multocida* A:3

E. coli : *E. coli* XL1-Blue

3.4 Uptake and intracellular survival of bacteria

3.4.1 J774.2 Assay

Intracellular detection of the parent 85020 strain and the JRMT12 *aro*A *P. multocida* B:2 strain within cultured macrophage-like cells was determined by infecting J774.2 cells with *P. multocida* strains at a ratio of 500: 1 bacteria: J774.2 cell. In some experiments, the effect of close cell-to-cell contact was examined by centrifugation of the tissue culture plate in order to directly deposit the bacteria onto the macrophages (Table 5 Exp. 1A, 1B, 2A, 2B, Table 6 Exp. 1B, 2B, 3A, 3B, Table 7 Exp. 1, 2, Table 8 Exp. 1). After exposure of the bacteria to the macrophages for 2 h, washing was done to remove the majority of extracellular bacteria and then residual extracellular bacteria were killed with a combination of gentamicin and polymyxin B sulphate for 2 h (Table 5 Exp. 1A, 2A, Table 6 Exp 1A, 1B, 2A, 2B, 3A, 3B, Table 7 Exp. 1A, 1B, 2A and Table 8 Exp. 1A, 1B, 1C). In some assays, the antibiotics were omitted in order to measure both adherence and invasion (Table 5 Exp.1B, 2B, Table 6 Exp. 1B, 2B, 3B, 3D and Table 7 Exp 2B, 2D, 2F). A further 3 washes to remove antibiotics was followed by exposure to digitonin for 30 min at a final concentration of 100 μg/ml to lyse the mammalian cells and release intracellular bacteria. Serial 10-fold dilutions were prepared from each sample in PBS and 100 μl portions spread on BHI agar and incubated at 37°C for 48 h. The viable count results are shown in Tables 5, 6, 7 and 8.

We first compared the number of intracellular bacteria after treating J774.2 cells with bacteria without centrifugation and with and without treatment with antibiotics. Bacterial counts made after treatment with and in the absence of antibiotics would represent intracellular and adherent plus intracellular bacteria, respectively (Table 5 Exp. 1A and

1B). The counts from the 10^{-2} dilution were used to calculate intracellular bacteria and 10^{-3} dilution to calculate adherent bacteria per cell. The average number of intracellular wild-type 85020 strain was determined to be ~0.03 per J774.2 cell while 0.22 bacteria per J774.2 cell were detected without antibiotics, indicating a combination of binding and invasion. For the JRMT12 strain under the same conditions, an intracellular count of ~0.09 bacteria/ cell was detected with 0.41 bacteria/ cell without antibiotics (Table 5 Exp1A, 1B and 2A, 2B). There was an indication, therefore; that following infection of macrophage cells for 2 h, strain 85020 showed a slightly lower invasive capacity compared with strain JRMT12 which may have been a reflection of greater adherence by JRMT12. Similar results were obtained when a centrifugation step was included: fewer invasive bacteria were detected with the wild-type strain compared with JRMT12 strain (0.19 versus 0.31 bacteria/ cell) (Table 6 Exp 1, 2). When invasion capacity was compared when cells and bacteria were centrifuged together and those not, a clear increase in intracellular bacteria was obtained after centrifugation (Table 6 Exp 1A and 2A, compared with Table 5 Exp 1A and 2A). The centrifugation assay also increased the numbers of bacteria judged to be adherent or intracellular, to 3.45 and 5.6 bacteria cell with strain 85020 and strain JRMT12, respectively with (Table 6 Exp. 1B and 2B). These data indicated that a greater number of bacteria were able to adhere to and invade cells after centrifugation.

As expected, if antibiotics were maintained in the culture medium and cells were then exposured to digitonin, no bacterial colonies were detected, presumably because the suspending medium would rapidly kill them (Table 6, Exp. 3A, 3C).

Table 5: Adhesion and intracellular survival of *P. multocida* B:2 strains with J774.2 macrophage-like cells. J774.2 cells and *P. multocida* were mixed at a MOI of 1:500, left 2 h and, after removal of most of the extracellular bacteria by centrifugation and washing, either the J774.2 cells were treated with antibiotics (gentamicin and polymyxin, each at 350μg/ml final concentration) or left untreated for 2 h. After further centrifugation and washing, J774.2 cells were lysed with digitonin and viable bacteria recovered after dilution and plating (see section 2.7.1). The number of intracellular bacteria was calculated using the counts from the 10^{-2} dilution and numbers of adherent bacteria using the 10^{-3} dilution (see section 2.8.1).

Experiment 1

A. P.m 85020 + J774.2 cells: No centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m 85020 + J774.2 cells: `` `` `` no antibiotics `` `` `` `` `

	Treatments	CFU/ 100 µl at dilution 10^{-2}	10^{-3}	10^{-4}	10^{-5}	Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
A	P.m 85020+ cells	440	0			$\simeq$ 0.03	
B	`` `` ``	2640	342	0			0.22

Experiment 2

A. P.m JRMT12+ J774.2 cells: No centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m JRMT12+ J774.2 cells: `` `` `` no antibiotics `` `` `` `` ``

	Treatment	CFU/ 100 µl at dilution 10^{-2}	10^{-3}	10^{-4}	10^{-5}	Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
A	P.m JRMT 12 + cells	650	3	0		$\simeq$ 0.09	
B	`` `` ``	1620	310	0			0.413

Table 6: Adhesion and intracellular survival of *P. multocida* B:2 strains with J774.2 macrophage-like cells. J774.2 cells and *P. multocida* were mixed at a MOI of 1:500, and centrifuged at 1000 x g for 5 min (Exp. 1 and 2) or left non-centrifuged (Exp. 3). Cells were then incubated for 2 h followed by removal of most of the extracellular bacteria by centrifugation and washing. The J774.2 cells were treated with antibiotics (gentamicin and polymyxin, each at 350μg/ml final concentration) or left untreated for 2 h. After further centrifugation and washing (Exp. 1 and 2), J774.2 cells were lysed with digitonin and viable bacteria recovered after dilution and plating (see section 2.7.1). In Exp. 3, no washes step to remove antibiotics was done. The number of intracellular bacteria was calculated using the counts from the 10^{-3} dilution and numbers of adherent bacteria using the 10^{-5} dilution. The numbers of adherent bacteria for Exp. 3B and 3D were calculated using the 10^{-4} dilution (see section 2.8.1).

Experiment 1

A. P.m 85020 + J774.2 cells: Centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m 85020 + J774.2 cells: `` `` `` no antibiotics `` `` `` `` ``

	Treatment	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-2}	10^{-3}	10^{-4}	10^{-5}		
A	P.m 85020+ cells	1860	210	0	0	0.19	
B	`` `` ``	NC	3850	410	38		3.45

Expriment 2

A. P.m JRMT12+ J774.2 cells: Centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min plate out

B. P.m JRMT12+ J774.2 cells: `` `` `` no antibiotics `` `` `` `` ``

	Treatment	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-2}	10^{-3}	10^{-4}	10^{-5}		
A	P.m JRMT 12+cells	1610	341	31	0	0.31	
B	`` `` ``	NC	4400	630	56		5.6

NC non countable.

Experiment 3

A. P.m 85020 + J774.2 cells: No centrifugation, left 2h, 2x wash, antibiotics added, left 2h, no wash, digitonin added, left 30 min, plate out

B. P.m 85020 + J774.2 cells: `` `` `` no antibiotics `` `` `` `` ``

C. P.m JRMT12+ J774.2 cells: `` `` `` antibiotics added `` `` `` `` ``

D. P.m JRMT12+ J774.2 cells: `` `` `` no antibiotics `` `` `` `` `` ``

	Treatments	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-1}	10^{-2}	10^{-3}	10^{-4}		
A	P.m 85020 + cells	0	0			0	
B	`` `` ``	NC	NC	719	58		1.36
C	P.m JRMT12+ cells	0	0			0	
D	`` `` ``	NC	NC	1815	118		2.8

NC non countable

When no antibiotics were employed together with no washing after 2 h (Table 6, Exp. 3A, 3D), the number of adherent bacteria higher than when washing was included (Table 5, Exp. 1B, 2B) which would indicate that the further washing step removed loosly attached bacteria. Table 7 summarises three further experiments to confirm the data on invasion of the parent 85020 and *aro*A strain JRMT12 with and without initial centrifugation. In two of the experiments, JRMT12 showed greater invasive capacity than 85020 (Table 7, Exp 1A compared to Exp 2A and Exp 3 compared to Exp. 3B), but in the other (Table 7, Exp 1B compared to Exp 2B) the reverse was true. Overall, the combined data did indicate that JRMT12 had a slightly better invasive capacity than the parent 85020 strains and perhaps the best comparison is Table 7, Exp. 3 where the two strains were compared during the same experiment.

Comparisons were made between the *P. multocida* B:2 strains and *E. coli* XL 1-Blue and *P. multocida* serogroup A:3 (Table 8 Exp. 1 and 2). The data in Table 8, experiment 1, revealed that the 85020 serotype B:2 strain had a significantly higher (~100-fold) capacity for invasion than the *P. multocida* A:3 strain which was in turn 10-20 fold better than *E. coli* XL1-Blue, which exhibited a very low number of intracellular bacteria. Essentially, similar results were obtained in a repeat experiment (Table 8 Exp. 2) where *P. multocida* B:2 JRMT12 was used instead of 85020. It should be noted that adhesion of the A:3 and *E. coli* XL1-Blue strains were ~ 50% of that of the *P. multocida* B:2 strains but this would only in part account for the much lower invasive capacity. In conclusion, the results indicated that both wild type strain 85020 and an *aro*A mutant strain JRMT12 could enter mammalian cells and survive for at least 2 h (Tables 5, 6, 7 and 8). Without antibiotics, ~10-fold more bacteria were shown to be adherent to J774.2 cells. The capacity of *P. multocida* A:3 and *E. coli* XL1- Blue to adhere to J774.2 cells

was only slightly lower than that of *P. multocida* B:2 strains, but their invasive capacity was markedly less.

3.4.1.1 Intracellular growth

To determine if intracellular growth and replication of internalised *P. multocida* B:2 occurred within J774.2 cells, the cells were incubated for a further 2 h (total of 6 h) after the extracellular bacteria had been removed from the medium by treatment with antibiotics. After this extra period of time, more intracellular bacteria were recovered (Table 9, Exp. 1A) compared with the situation if this 2 h extension was not included (Table 9, Exp. 1B). However, if cells were incubated with fresh RPMI medium containing 50 μg/ml final concentration each of polymyxin and gentamicin for the extra 2 h, considerably fewer intracellular bacteria were recovered (Table 9 Exp. 1C). These data might suggest that the apparent increase in the number of intracellular bacteria per J774.2 cell between 4 and 6 h resulted from increased growth of surviving extracellular bacteria rather than division of bacteria within the cells. However, it is also possible that this extended time period allowed the antibiotics to penetrate the J774.2 cells and start to kill the intracellular bacteria.

Table 7: Summary of three different experiments on intracellular survival of *P. multocida* B:2 strains with J774.2 macrophage-like cells. In some experiments, J774.2 cells were centrifuged at 1000 x g for 5 min immediately after exposure to bacteria (Exp. 1B, 2B, and 3A, 3B), while in others the centrifugation step was omitted (Exp. 1A and 2A). Antibiotics (gentamicin and polymyxin, each at 350μg/ml final concentration) were added. Number of intracellular bacteria was calculated using the count from the 10^{-3} dilution except for Exp.3A where the 10^{-2} dilution was used.

Experiment 1

A. P.m 85020 + J774.2 cells: No centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m 85020 + J774.2 cells: Centrifugation `` `` `` `` `` `` `` ``

	Treatments	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-2}	10^{-3}	10^{-4}	10^{-5}		
A	P.m 85020+ cells	1045	126	0	0	0.21	
B	P.m 85020+ cells	2460	371	0	0	0.64	

Experiment 2

A. P.m JRMT12+ J774.2 cells: No Centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m JRMT12+ J774.2 cells: Centrifugation `` `` `` `` `` `` `` ``

	Treatments	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-2}	10^{-3}	10^{-4}	10^{-5}		
A	P.m JRMT 12+ cells	1106	224	0		0.28	
B	P.m JRMT 12+ cells	1214	252	0		0.315	

Experiment 3

A. P.m 85020+ J774.2 cells: Centrifugation left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m JRMT12+ J774.2 cells: `` `` `` `` `` `` `` `` ``

	Treatments	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-1}	10^{-2}	10^{-3}	10^{-4}		
A	P. m 85020 + cells	NC	214	0	0	0.06	
B	P. m JRMT12 + cells	NC	570	128	0	0.178	

NC non countable

Table 8: Adhesion and intracellular survival of *P. multocida* B:2 strains compared to *P. multocida* serogroup A:3 and *E. coli* XL1-Blue with J774.2 macrophage-like cells. Cells were centrifuged at 1000 x g for 5 min immediately after exposure to bacteria. In some assays, antibiotics (gentamicin and polymyxin, each at 350μg/ml final concentration) were added (Exp. 1A, 1C, 1E and 2A, 2B, 2C), while in others (Exp. 1B, 1D, 1F), the antibiotic treatment was omitted. Number of intracellular bacteria were calculated using the counts from the 10^{-3} dilution for Exp.1A and Exp. 2A, 2D, 2F and 10^{-1} dilution for Exp. 1B, 1C and Exp 2C and 2F. The numbers of adherent bacteria were calculated using the 10^{-4} dilution for Exp 2B and the 10^{-3} dilution for Exp.2 A, 2D and 2F.

Experiment 1

A. P.m 85020 + J774.2 cells: Centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m A:3 + J774.2 cells: `` `` `` `` `` `` `` `` ``

C. *E. coli* + J774.2 cells : `` `` `` `` `` `` `` `` ``

	Treatments	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-1}	10^{-2}	10^{-3}	10^{-4}		
A	P. m 85020 + cells	NC	NC	128	0	0.2	
B	P. m A3 + cells	211	0			0.0034	
C	*E. coli* + cells	103	0			0.0016	

P. m *P. multocida* *E. coli* *Escherichia coli* *P.m.* A:3 *P.multocida* A:3 NC non countable

Experiment 2

A. P.m JRMT12 + J774.2 cells: Centrifuged, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m JRMT12 + J774.2 cells: `` `` `` no antibiotics `` `` `` `` `` ``

C. P.m A:3 + J774.2 cells: `` `` `` antibiotics add `` `` `` `` `` ``

D. P.m A:3 + J774.2 cells: `` `` `` no antibiotics `` `` `` `` `` ``

E. *E. coli* + J774.2 cells: `` `` `` antibiotics add `` `` `` `` `` ``

F. *E. coli* + J774.2 cells: `` `` `` no antibiotics `` `` `` `` `` ``

	Treatments	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-1}	10^{-2}	10^{-3}	10^{-4}		
A	P. m JRMT12 + cells	NC	NC	108		0.24	
B	P. m JRMT12 + cells	NC	NC	NC	116		2.57
C	P. m A3 + cells	480	0			0.01	
D	P. m A3 + cells	NC	NC	418	0		1.6
E	E. coli + cells	83	0			0.00014	
F	E. coli + cells	NC	NC	614	0		1.36

P. m *P. multocida* *E. coli* *Escherichia coli* *P.m.* A:3 *P.multocida* A:3 NC non countable

Table 9: Intracellular survival of *P. multocida* B:2 85020 strains within J774.2 macrophage-like cells. Cells were centrifuged at 1000 x g for 5 min immediately after exposure to bacteria. Antibiotics (gentamicin and polymyxin, each at 350μg/ml final concentration) were added for 2 h. Cells were then washed 3 times to remove the antibiotics and then incubated for 2 h (Exp. 1A) before digitonin treatment or the cells were treated directly with digitonin (Exp. 1B) or antibiotics (gentamicin and polymyxin, each at 50μg/ml final concentration) were added with a further incubation for 2 h before treatment with digitonin (Exp. 1C). The number of intracellular bacteria for Exp. 1A was calculated using the counts from the 10^{-3} dilution and in Exp. 1A and 1B from the 10^{-2} dilution.

Experiment 1

A. P.m 85020 + J774.2 cells: Centrifugation, left 2h , 2x wash, antibiotics added, left 2h, 3x wash, left 2h, digitonin added, left 30 min, plate out

B. P.m 85020 + J774.2 cells: `` `` `` antibiotics added `` `` digitonin added, left 30 min ``

C. P.m 85020 + J774.2 cells: `` `` `` `` `` `` antibiotics added 50µg/ml, left 2h, digitonin added, left 30 min Plate out

	Treatments	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-1}	10^{-2}	10^{-3}	10^{-4}		
A	P. m 85020 + cells	NC	NC	101		1.629	
B	P. m 85020 + cells	NC	415	0		0.092	
C	P. m 85020 + cells	778	198	0		0.04	

NC non countable

3.4.2 BL-3 cell assay

P. multocida B:2 strains were also tested for their ability to invade and survive in BL-3 cell cultures. After an infection period of 2 h, the BL-3 cells were washed and resuspended in fresh BL-3 medium with polymyxin and gentamicin each at a final concentration of 350 μg/ml, to kill extracellular bacteria. As in the experiment with the J774.2 cells, viable intracellular bacteria of both strains were recovered from lysed cells to quantify the invasion ability by counting of intracellular CFU/ml on BHI agar. The data in Table 10 (Exp. 1 and 2) showed that the JRMT12 strain was able to invade cells slightly better than the parent strain 85020, as was seen with J774.2 cells. However, the invasive capacity of the *P. multocida* B:2 strains for BL-3 cells was less than that for J774.2 cells (section 3.3.1, Tables 5, 6, 7 and 8) even when initial centrifugation was employed to encourage contact between cells.

Again, the invasive capacity of *P. multocida* A:3 and *E. coli* XL1-Blue were compared with the *P. multocida* B:2 strains (Table 11, Exp. 1 and 2). As noted for J774.2 cells (Table 8), the invasive capacity of the *P. multocida* A:3 and *E. coli* XL1-Blue were markedly poorer than both 85020 and JRMT12 *P. multocida* B:2 strains. As with J774.2 cells, the numbers of bacteria judged to be adherent and or invasive was similar for all three strains (Table 11, Exp. 1). Thus, as with the J774.2 cells, the B:2 strains showed ~ 100 –fold greater invasive capacity for BL-3 cells than the A:3 and *E. coli* XL1-Blue strains, even though all three strains showed a similar attachment capacity. However, the invasive capacity of the *P. multocida* B:2 strains for BL-3 cells was ~5-fold less efficient than with J774.2 cells.

Table 10: Adhesion and intracellular survival of *P. multocida* B:2 strains with BL-3 cells. Bacteria were mixed with BL-3 cells at a MOI of 500:1 and centrifuged at 1000 x g for 5 min immediately after exposure of BL-3 cells to the bacteria. In some assays, antibiotics (gentamicin and polymyxin, each at 350 μg/ml final concentration) were added (Exp. 1A and 2A), while in others (Exp. 1B and 2B), the antibiotic treatment was omitted. The number of intracellular bacteria was calculated using the counts from the 10^{-2} dilution and numbers of adherent bacteria using the 10^{-3} dilution.

Experiment 1

A. P.m 85020 + BL-3 cells: Centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m 85020 + BL-3 cells: `` `` `` no antibiotics `` `` `` `` ``

	Treatments	CFU/ 100 µml at dilution				Number of intracellular bacteria/BL-3 cell	Number of intracellular + adherent bacteria / BL-3 cell
		Neat	10^{-1}	10^{-2}	10^{-3}		
A	P.m. 85020 +cells	NC	2840	216	0	0.045	
B	P.m. 85020 +cells	NC	N.C	N.C	401		0.84

Experiment 2

A. P.m JRMT12+ BL-3 cells: Centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min, plate out

B. P.m JRMT12+ BL-3 cells: `` `` `` no antibiotics `` `` `` `` ``

	Treatments	CFU/ 100 µl at dilution				Number of intracellular bacteria/BL-3 cell	Number of intracellular + adherent bacteria / BL-3 cell
		Neat	10^{-1}	10^{-2}	10^{-3}		
A	P.m. JRMT12 +cells	N.C	N.C	416	0	0.067	
B	P.m. JRMT12 +cells	NC	N.C	2428	303		0.48

NC: non countable

Table 11: Adhesion and intracellular survival of *P. multocida* B:2 strains, *P. multocida* serogroup A:3 and *E. coli* XL1-Blue with BL-3 cells. Cells were centrifuged at 1000 x g for 5 min immediately after exposure to bacteria. In some assays, antibiotics (gentamicin and polymyxin, each at 350 μg/ml final concentration) were added (Exp. 1A, 1C, 1E and 2A, 2B, 2C), while in others (Exp. 1B, 1D, 1F), the antibiotic treatment was omitted. The number of intracellular bacteria were calculated using the counts from the 10^{-2} dilution Exp.1A and 10^{-1} dilution for Exp. 1C and 1E. The numbers of adherent bacteria were calculated from the 10^{-3} dilution, apart from Exp. 1F where the 10^{4} dilution.

Experiment 1

A. P.m JRMT12 + BL-3 cells: Centrifugation, left 2h, 2x wash, antibiotics added, left 2h, 3x wash, digitonin added, left 30 min plate out

B. P.m JRMT12 + BL-3 cells: `` `` `` no antibiotics `` `` `` `` ``

C. P.m A:3 + BL-3 cells: `` `` `` antibiotics added `` `` `` `` ``

D. P.m A:3 + BL-3 cells: `` `` `` no antibiotics `` `` `` `` ``

E. E. coli + BL-3 cells: `` `` `` antibiotics added `` `` `` `` ``

F. E. coli + BL-3 cells: `` `` `` no antibiotics `` `` `` `` ``

	Treatments	CFU/ 100 µl at dilution				Number of intracellular bacteria/J774.2 cell	Number of intracellular + adherent bacteria / J774.2 cell
		10^{-1}	10^{-2}	10^{-3}	10^{-4}		
A	P. m JRMT12 + cells	NC	314	0		0.066	
B	P. m JRMT12 + cells	NC	NC	467	0		0.3
C	P. m A3 + cells	117	0			0.0023	
D	P. m A3 + cells	NC	NC	651	0		0.39
E	E. coli + cells	11	0			0.0002	
F	E. coli + cells	NC	NC	NC	43		0.686

P. m *P. multocida* *E. coli* *Escherichia coli* *P.m.* A:3 *P.multocida* A:3 NC non countable

3.5 Electron microscopy

To study further the fate of intracellular bacteria, TEM studies were performed (Figures 6 and 7). J774.2 macrophage cells and BL-3 cells were treated with *P. multocida* serogroup B:2 strain 85020, *P. multocida* serogroup A:3 and *E. coli* XL1-Blue. Cells were infected at a MOI of 500:1 in 6-well tissue culture plates. Fixation of samples was done with glutaraldehyde and osmium tetroxide after an incubation period of 4 h (Figures 6 A-F, and 7 A-F) and 6 h (Figures 6 G-L, and 7 G-L). After dehydration via graded alcoholic steps, the samples were embedded in epon and serial ultra thin sections were stained with uranyl acetate and lead citrate before examination with the LEO 912AB transmission electron microscope.

3.5.1 J774.2 cell micrography

With the *P. multocida* B:2 85020 strain, J774.2 cells with intracellular bacteria were observed (Figures 6 A, C, H) but extracellular bacteria were also seen in close association with the plasma membrane (Figure 6 H). Intracellular bacteria were not seen with *P. multocida* A:3 and *E. coli* XL1-Blue, although J774.2 cells showed cytoplasmic extensions around the bacteria which could be interpreted as being the first step in the engulfment process (Figures 6 E and K). An assessment of the morphological features seen by TEM after infection of J774.2 cells with *P. multocida* B:2, *P. multocida* A:3 and *E.coli* XL1-Blue is summarized in Table 12. Vacuolation was observed at both 4 h or 6 h postinfection (Figures 6 B, C, G and I), with *P. multocida* B:2, and approximately 10% of the J774.2 cells showed this feature at 4 h (Table 12A). The intracellular bacteria (seen in 28% of cells examined, Table 12A) were generally situated within membrane-bound vacuoles (Figures 6 A, C, H), although occasionally a bacterium free in the

Figure 6: TEM of J774.2 cells infected with bacteria. J774.2 cells were infected at a MOI of 500:1 with *P. multocida* B:2 strains 85020, *P. multocida* serogroup A:3 or *E.coli* XL1-Blue. Cells were centrifuged at 1000 x g for 5 min and incubated for 2 h. Removal of most extracellular bacteria by centrifugation and washing was then performed. The J774.2 cells were treated with antibiotics (gentamicin and polymyxin, each at 350 μg/ml final concentration) for 2 h, then antibiotics were removed by washing and samples taken for TEM at 4 h (A-F),or 6 h (G-L). The main features to note are cytoplasmic vacuoles and localization of intracellular bacteria. Non-internalised bacteria were also detected.

A- *P. multocida* B:2 (arrowed) within phagosomes (4 h incubation).

B- J774.2 cell treated with *P. multocida* B:2. A number of vacuoles are visible (4 h incubation).

C- Vacuoles and a *P. multocida* B:2 cell within a phagosome (arrow 1) and a bacterial cell free within the cytoplasm (arrow 2) (4 h incubation).

D- Normal appearance of a J774.2 cell without bacteria (4 h incubation).

E- *P. multocida* A:3 cells associated with the extended (arrowed) surface of a J774.2 cell (4 h incubation).

F- A J774.2 cell after incubation with *E. coli* XL-Blue (4 h incubation).

G- Vacuolation of a J774.2 cell treated with *P. multocida* B:2 strain (6 h incubation).

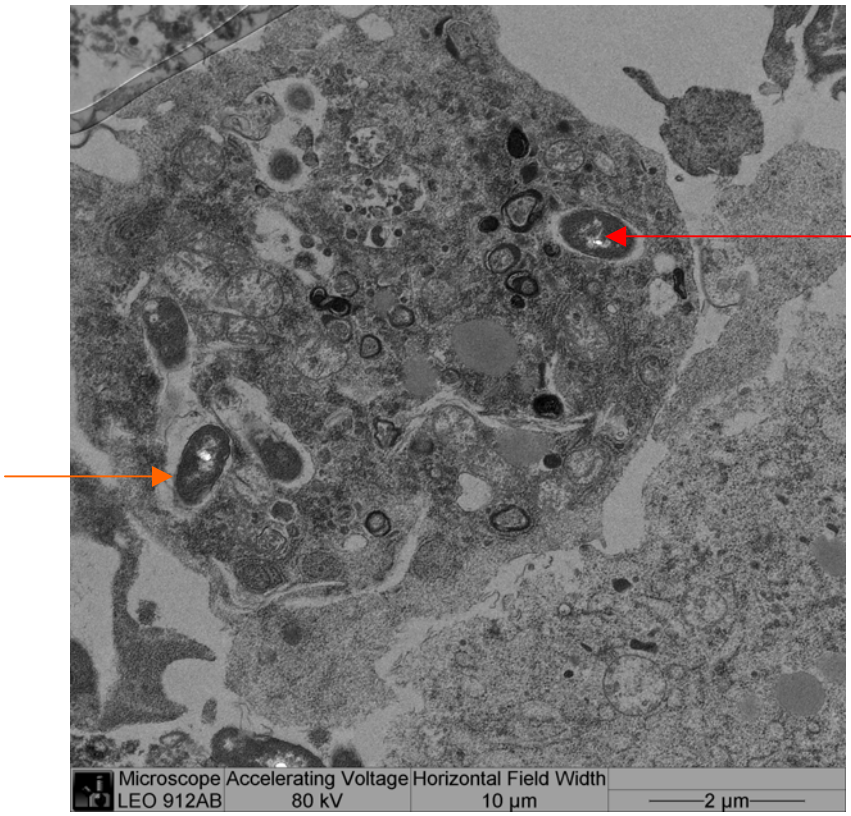
H- Intracellular *P. multocida* B:2 cells within a J774.2 cell and associated with the extended cell membrane (arrowed). (6 h incubation).

I- Damaged J774.2 cell after treatment with *P. multocida* B:2 strain (6 h incubation).

J- Untreated J774.2 cell (6 h incubation).

K- A J774.2 cell treated with *P. multocida* A:3 (6 h incubation), showing extended membrane projections (arrowed).

L- A J774.2 cell after incubation with *E. coli* strain XL1-Blue (6 h incubation).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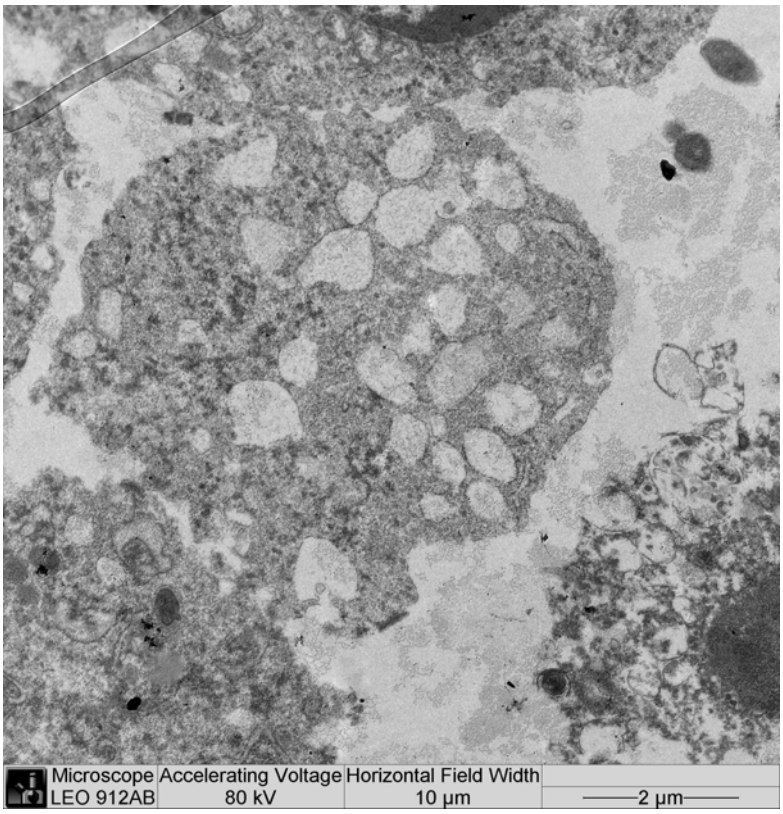
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

A


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
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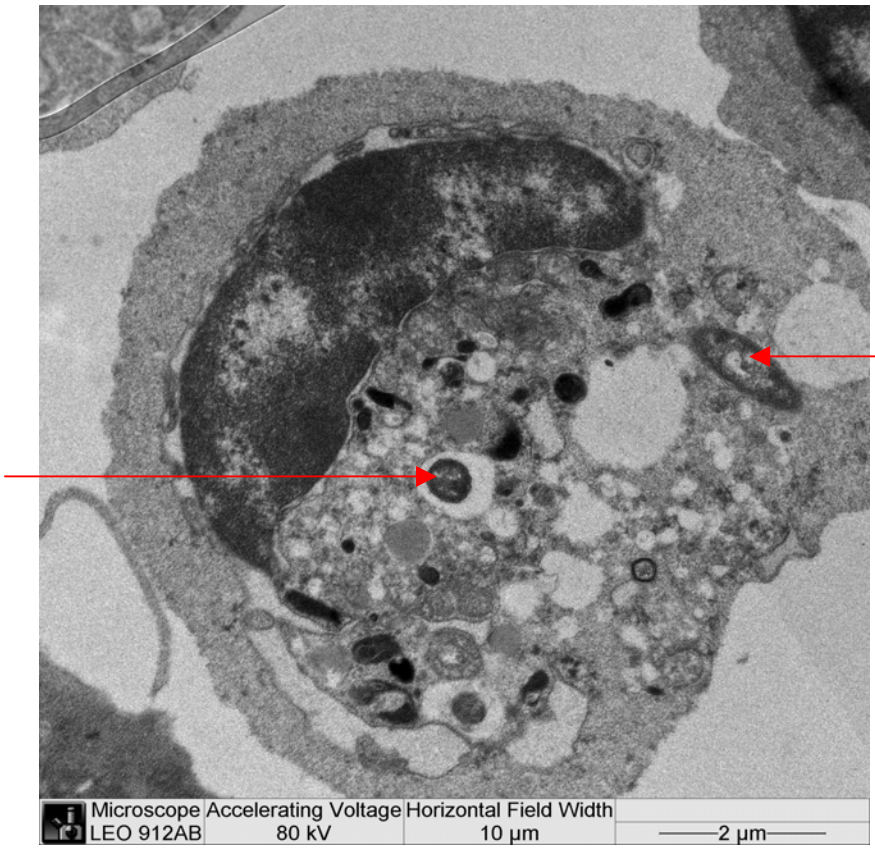
Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
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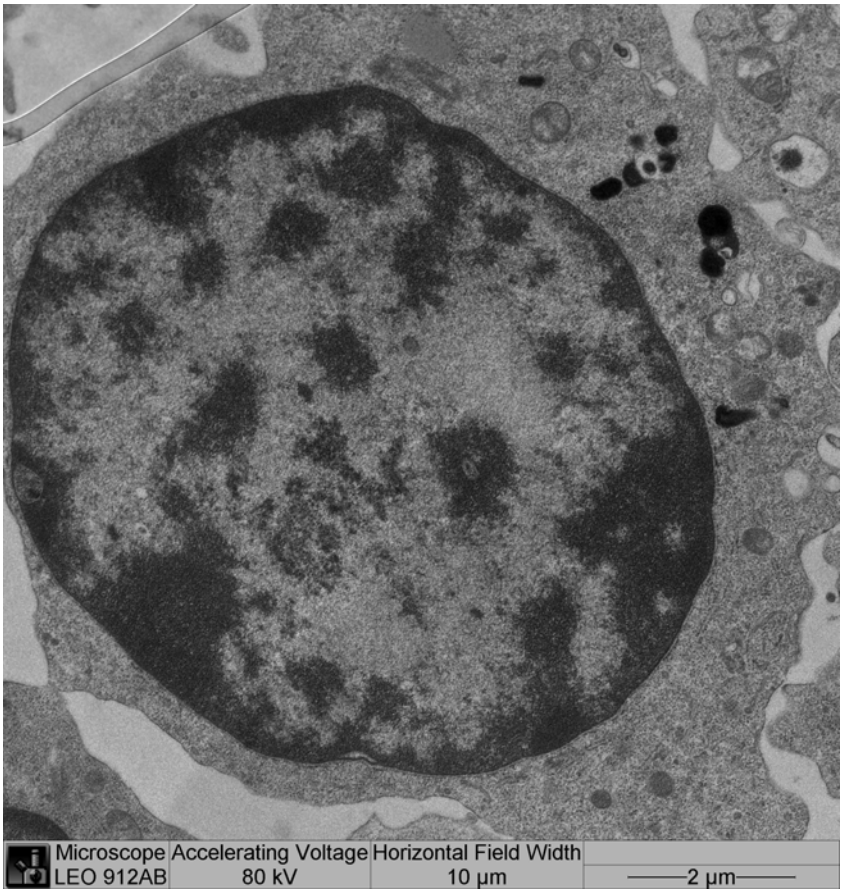
2 µm

C: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LEO 912AB
Accelerating Voltage
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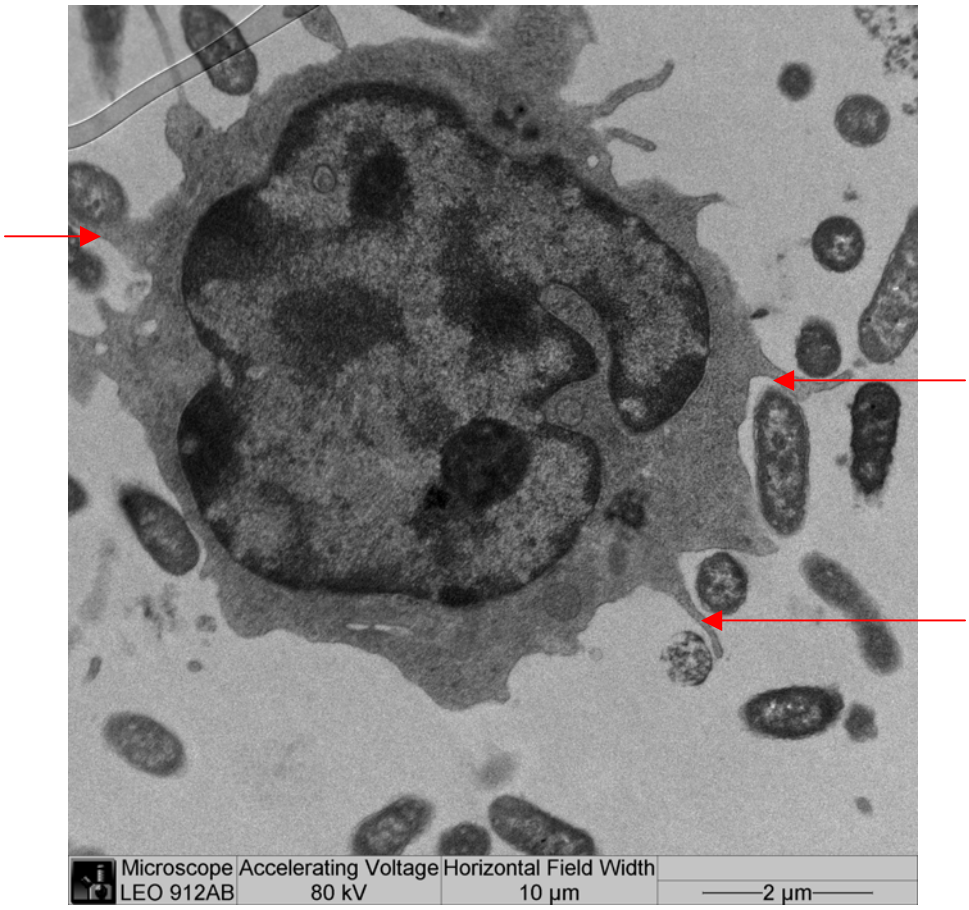
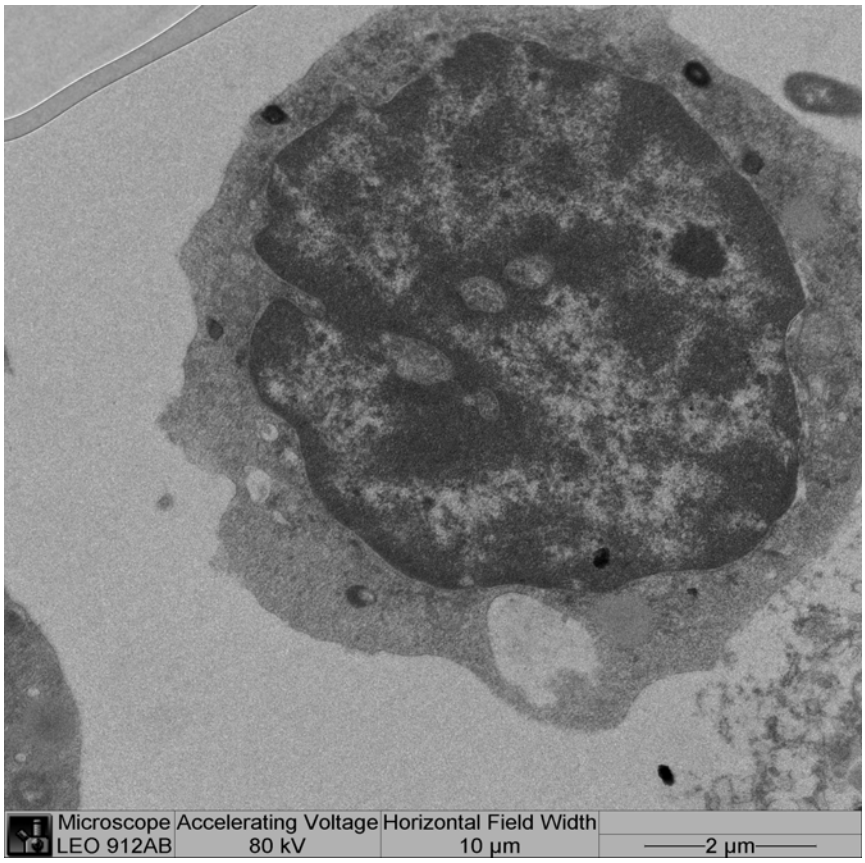
2 µm

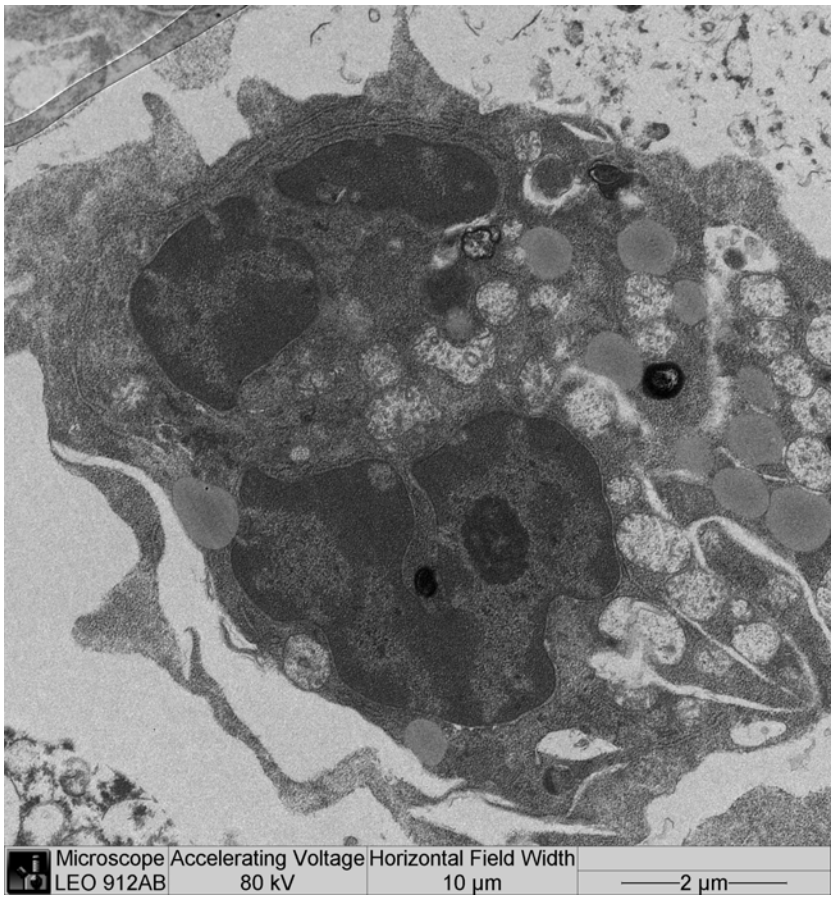
D:


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

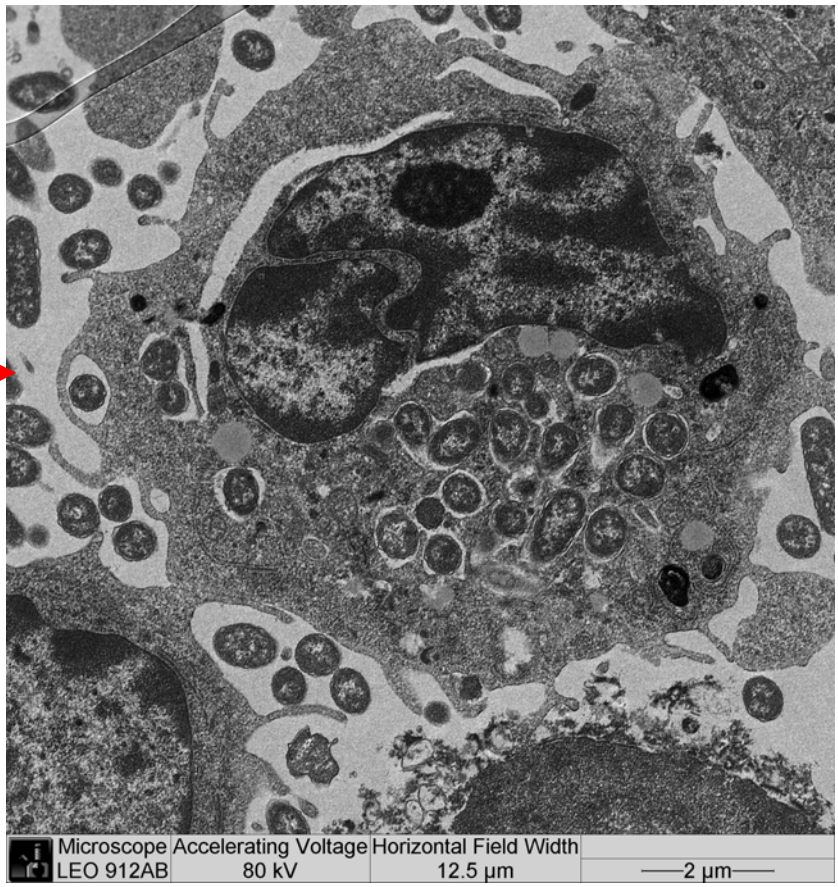
E:


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

F:


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

G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LEO 912AB
Accelerating Voltage
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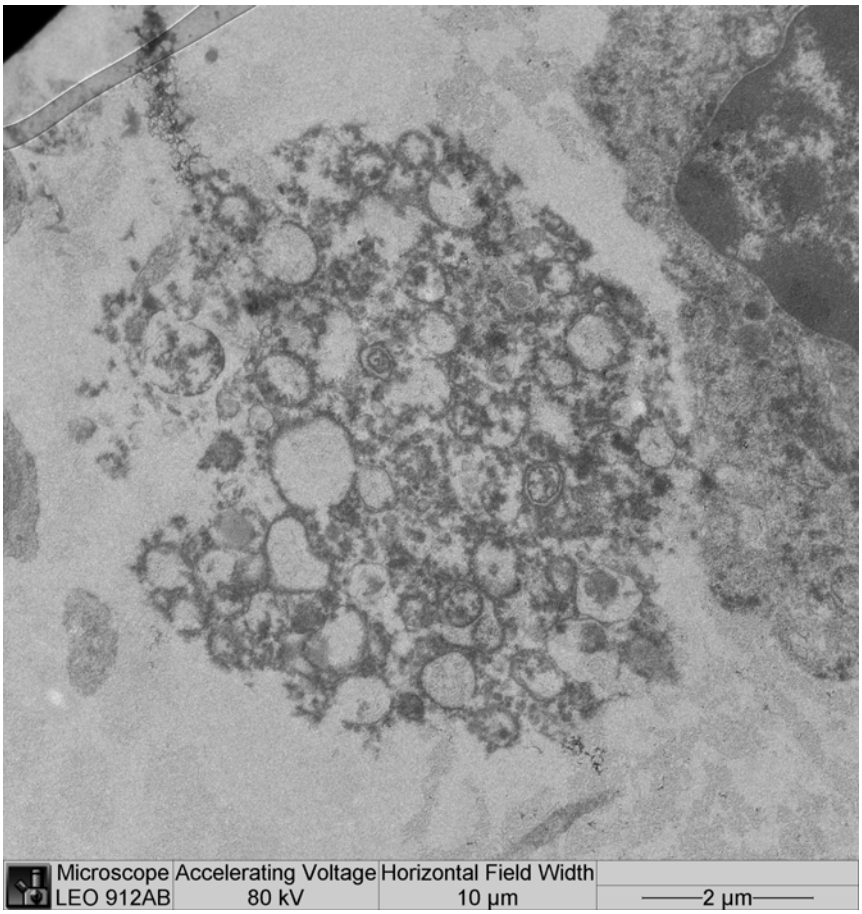
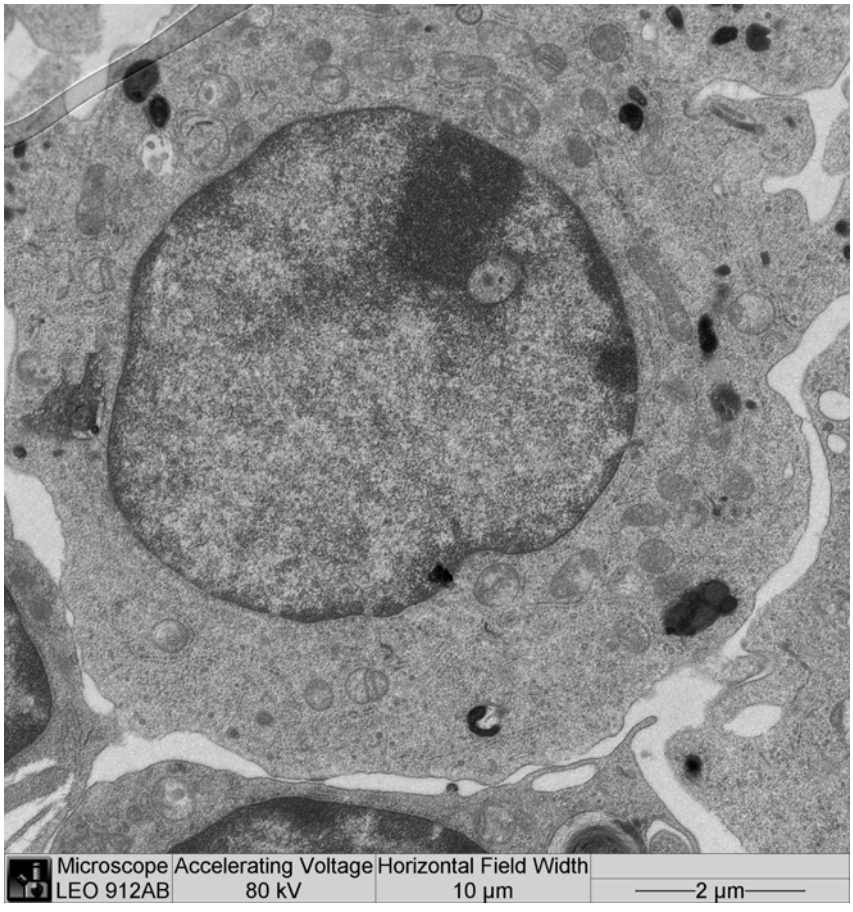
2 µm

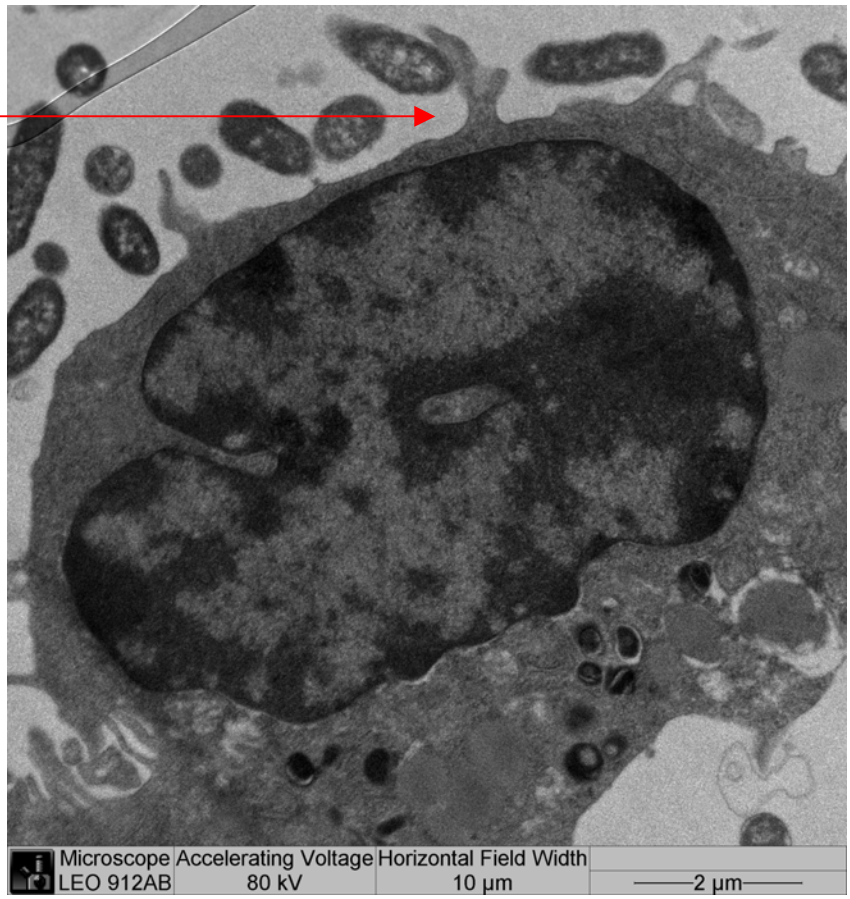
H


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

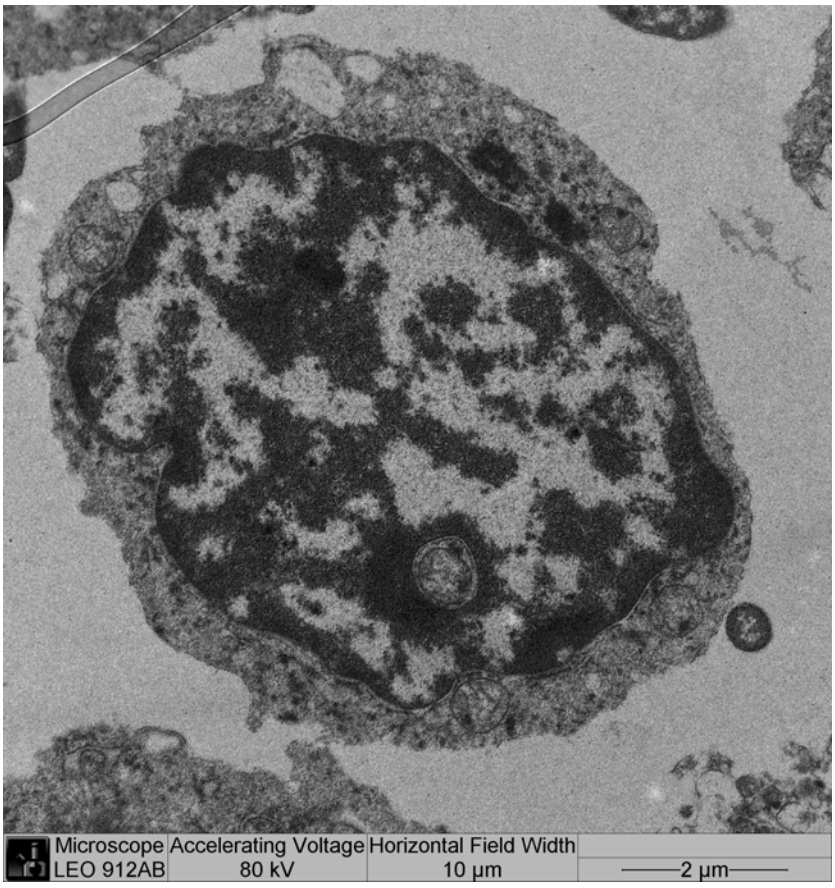
I


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

J


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

K


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

L

Table 12: Assessment of the morphological features seen by TEM after infection of J774.2 cells with different bacteria. J774.2 cells were mixed with bacterial strains at a MOI of 1:500. Cells were then centrifuged at 1000 x g for five min immediately after exposure to bacteria and incubated for 2 h. After removal of most of the extracellular bacteria by centrifugation and washing, the J774.2 cells were resuspended in fresh RPMI complete medium and incubated for a further 2 h before fixation of samples for TEM. For each strain, 100 individual J774.2 cells were examined. Cells were assessed to be dead if the cell membrane was discontinuous or ruptured.

A. Percentage of cells showing vacuoles, intracellular bacteria, dead cells and bacteria free in the cytoplasm of J774.2 cells with different strains of bacteria.
B. Break down of numbers of intracellular *P. multocida* B:2 strain 85020 bacteria within 100 individual J774.2 cells at 4 h after challenge.

A:

Treatments	Vacuolation of J774.2 cells	Percentage of cells showing vacuolation	Intracellular survival	Percentage of J774.2 cells with intracellular bacteria	Dead cells	Percentage of dead cells	Percentage of cells with free bacteria in the cytoplasm
P. m B+J774.2 cells	Observed	10%	Observed	28%	Observed	11%	8%
P. mA+J774.2 cells	----	----	----	----	----	----	----
E. coli +J774.2 cells	----	----	----	----	----	----	----
RPMI medium+J774.2	----	----	----	----	----	----	----

---- not observed *P. m* B *Pasteurella multocida* B:2 strain 85020 P. mA : *Pasteurella multocida* A :3 *E. coli* XL1-Blue

B:

No of *P. multocida* B:2 85020 bacteria within individual J774.2 cells	Total number of cells
1	9
2	2
3	6
4	1
5	2
6	3
8	1

cytoplasm was observed (Figure 6C) which might have escaped from the vacuoles. Approximately 8% of the J774.2 cells examined showed intracellular bacteria free in the cytoplasm (Table 12A). Sometimes vacuoles were seen to contain multiple bacteria (Figures 6 A, H, Table 12B), which may have been the result of several bacteria being engulfed at the same time. Therefore, after 6 h of infection, consistent numbers of bacteria were found within phagosomes (Figure 6 H) and vacuolation was a prominent feature with J774.2 cells after treatment with *P. multocida* B:2 (Figures 6, B, C, G). This features were but was not apparent following treatment with *P. multocida* A:3 or *E. coli* XL1-Blue.

3.5.2 BL-3 cell micrography

As can be seen in the TEM analysis presented in Figure 7, *P. multocida* B:2 was located within BL-3 cells after 4 or 6 h. In similar experiments with *P. multocida* A:3 and *E. coli* XL1-Blue, attached bacteria were observed, but no intracellular bacteria were seen. Many extracellular bacteria were found, most of them being associated with the cell membrane and, in some instances, extracellular bacterial division was observed (Figures 7 B and G). An assessment of the morphological features is given in Table 13. Vacuolation was not seen to any significant extend with BL-3 cells after treatment with any of the bacteria but, instead evidence of a more general damage to the cytoplasm, creating large vacuole-like areas was more apparent (Figure 7 A, B, C, E, F, G, K, L) with all bacteria. Clear membrane disintegration of these mammalian cells was found (Figure 7, B, H, I). This was not seen with BL-3 cells that had been exposed to the bacteria (Figure 7D, H). Thus, TEM observations indicated that exposure of BL-3 host cells to all three bacterial species was associated with extensive cell damage.

Figure 7: Electron micrographs of BL-3 cells infected at a MOI of 500:1 with *P. multocida* B:2 strains 85020, *P. multocida* serogroup A:3 or *E.coli* XL1-Blue. Cells were centrifuged at 1000 x g for 5 min and incubated for 2 h. Removal of most extracellular bacteria by centrifugation and washing was then performed. The BL-3 cells were treated with antibiotics (gentamicin and polymyxin, each at 350 µg/ml final concentration) for 2 h then antibiotics were removed by washing and samples taken for TEM at 4 h (A-F),or 6 h (G-L). The main features to note are localization of intracellular bacteria and apparent extensive cell damage. Non-internalised bacteria were also detected.

A- P. *multocida* B:2 strain 85020 within vacuoles of BL-3 cells (4 h incubation). Note the appearance of BL-3 cell damage.

C- BL-3 cells treated with *P. multocida* B:2 showing a damaged cell (4 h incubation) and evidence of dividing bacteria (arrowed).

D- *P. multocida* B:2 strain 85020 (arrowed) free within cytoplasm (4 h incubation). The BL-3 cell appears damaged.

E- Normal appearance of BL-3 cell without bacteria (4 h incubation).

F- BL-3 cell treated with *P. multocida* A:3 (4 h incubation). The BL-3 cell appears damaged

G- BL-3 cell treatment with *E. coli* XL1-Blue (4 h incubation). The BL-3 cell appears damaged

H- Localization of *P. multocida* B:2 cell (arrow 1) within a BL-3 cell (6 h incubation). Extracellular bacteria were also present (arrow 2).

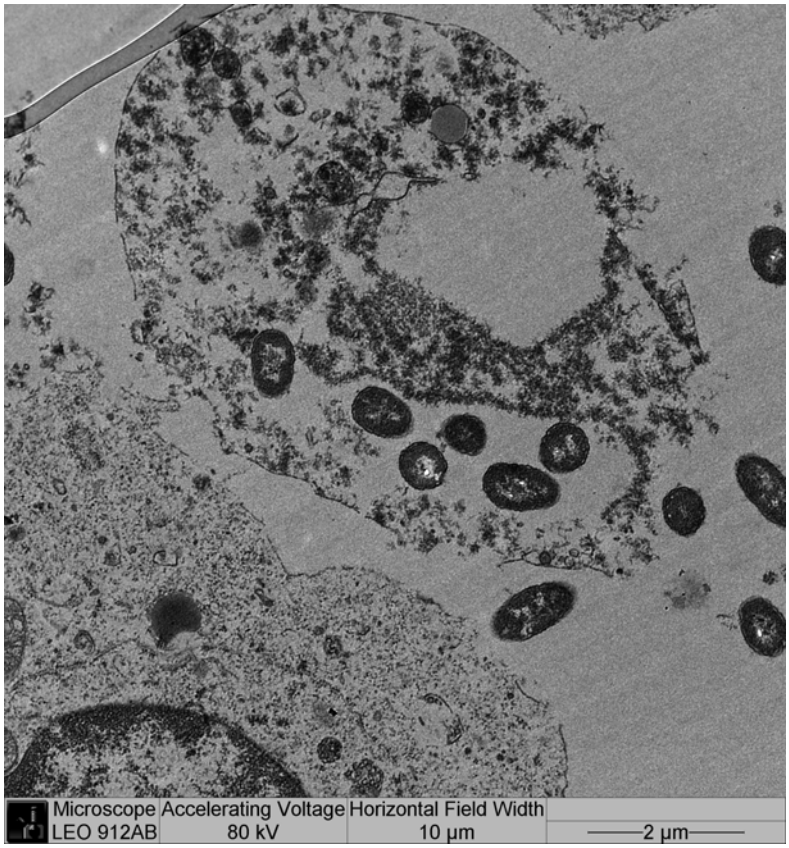
I- Damaged BL-3 cell, treated with *P. multocida* B:2 (6 h incubation).

J- Damaged BL-3 cell after treatment with the *P. multocida* B:2 (6 h incubation).

K- Untreated BL-3 cell (6 h incubation).

L- BL-3 cell after treatment with *P. multocida* A:3 (6 h incubation).

L- BL-3 cell after treatment with of *E. coli* strain XL1-Blue (6 h incubation).


Microscope
LEO 912AB
Accelerating Voltage
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Horizontal Field Width
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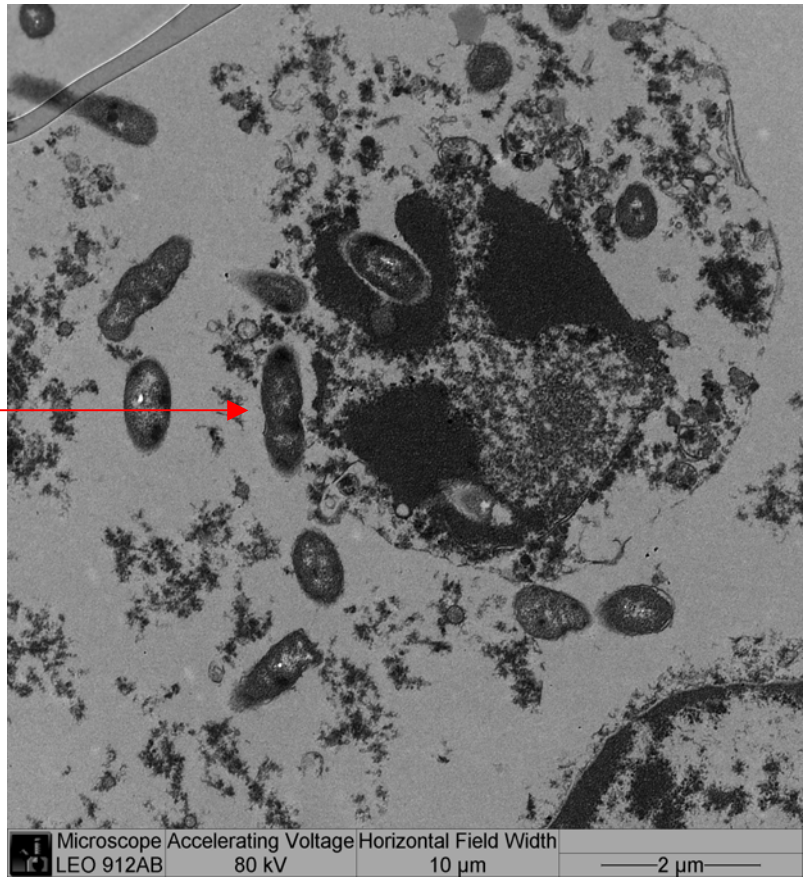
2 µm

A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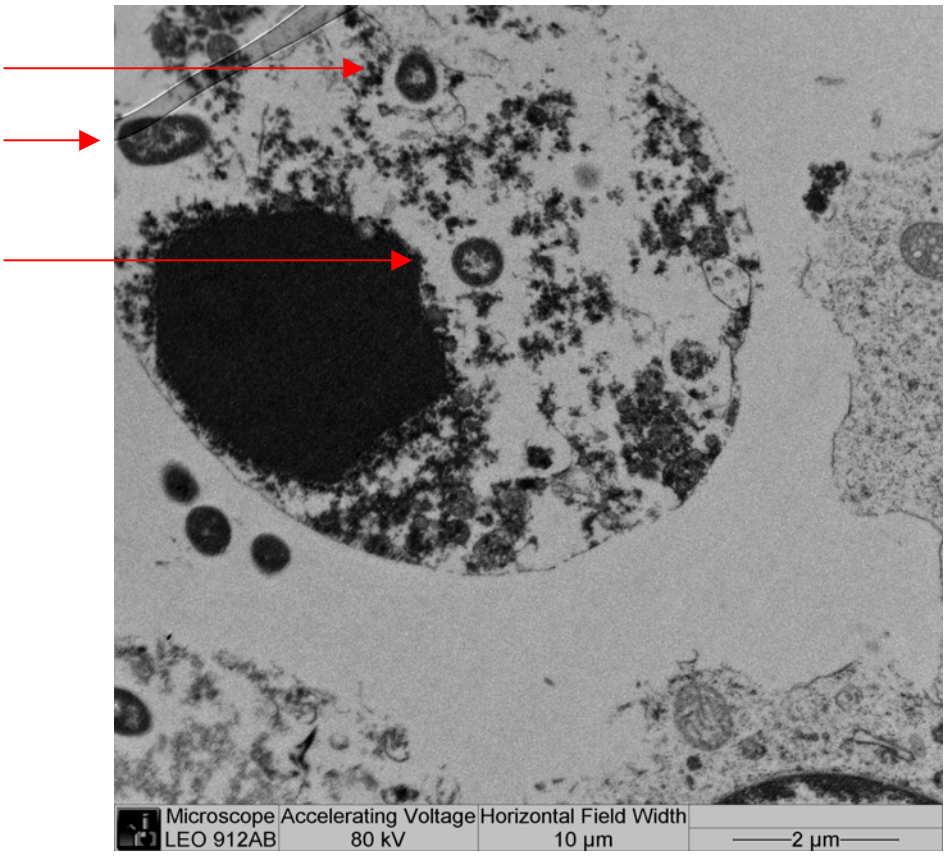
B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LEO 912AB
Accelerating Voltage
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Horizontal Field Width
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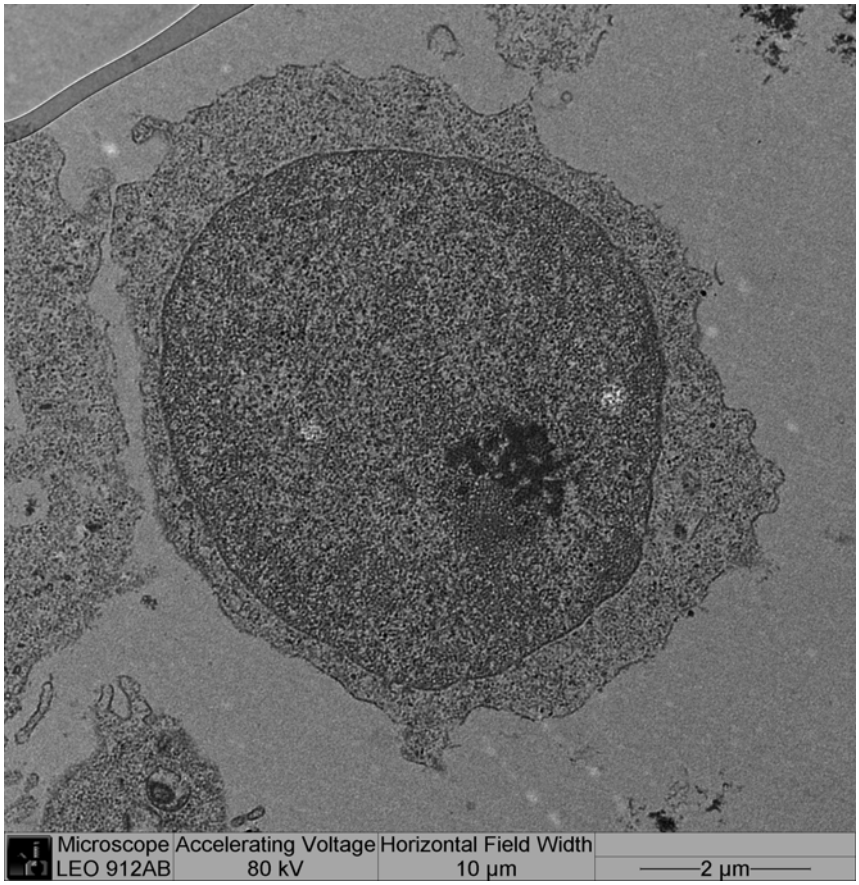
C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LEO 912AB
Accelerating Voltage
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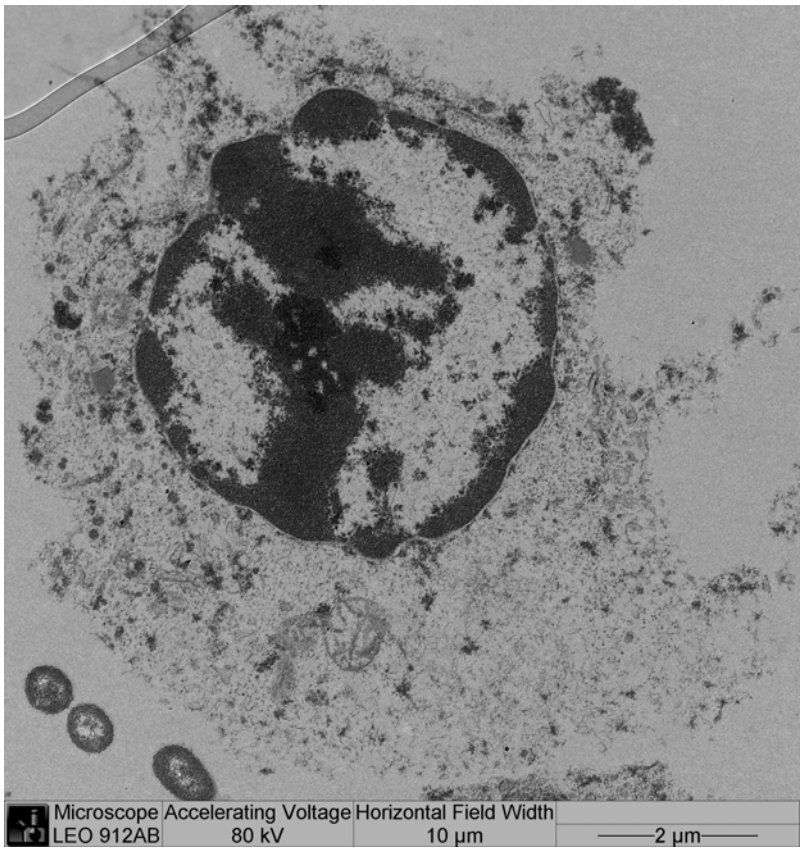
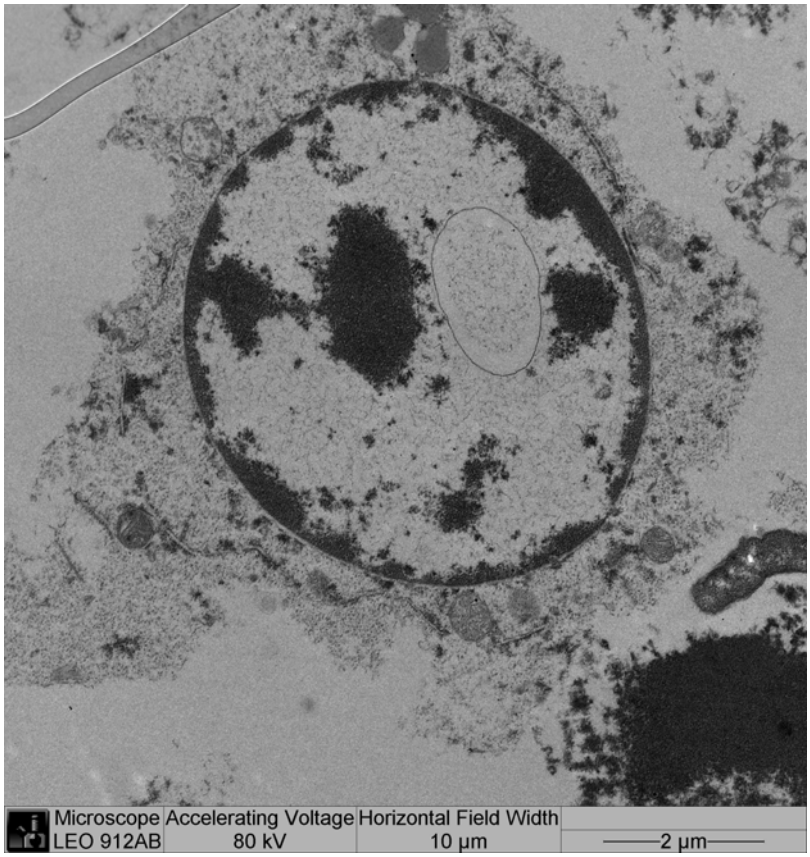
D


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

E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LEO 912AB
Accelerating Voltage
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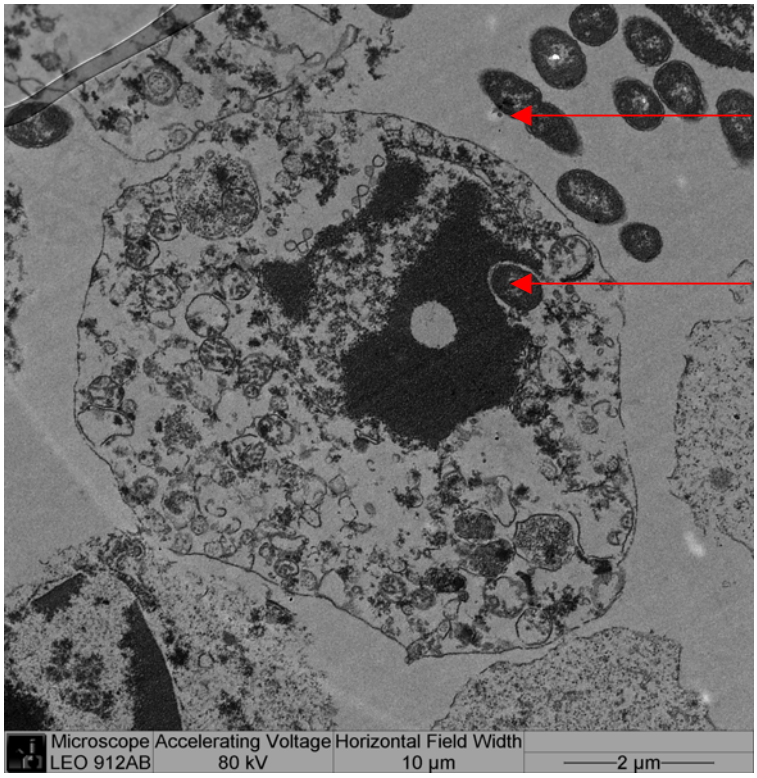
Horizontal Field Width
10 µm
2 µm

F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LEO 912AB
Accelerating Voltage
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Horizontal Field Width
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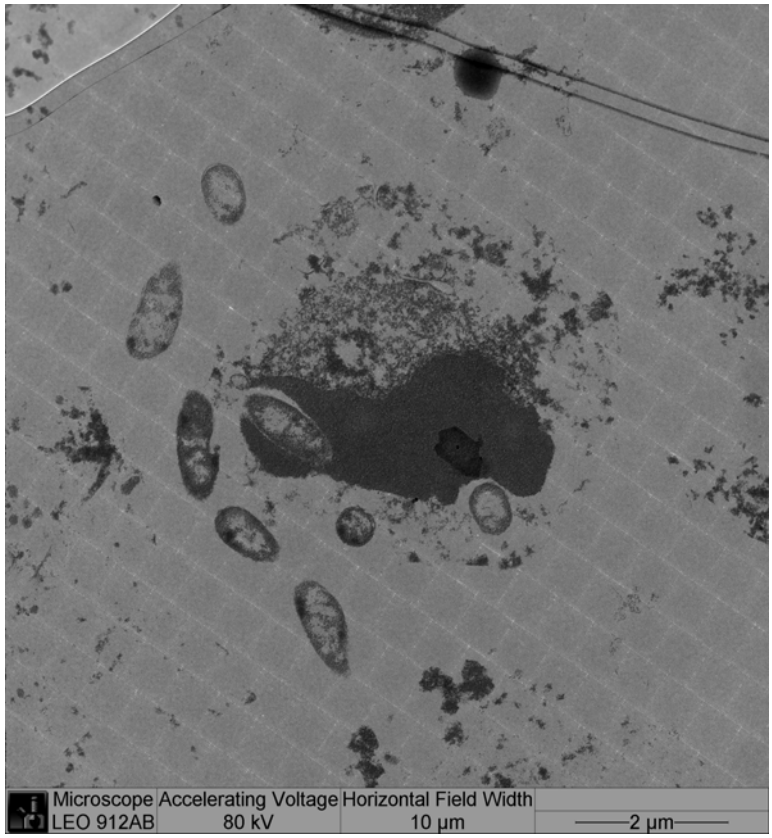
2 µm

G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LEO 912AB
Accelerating Voltage
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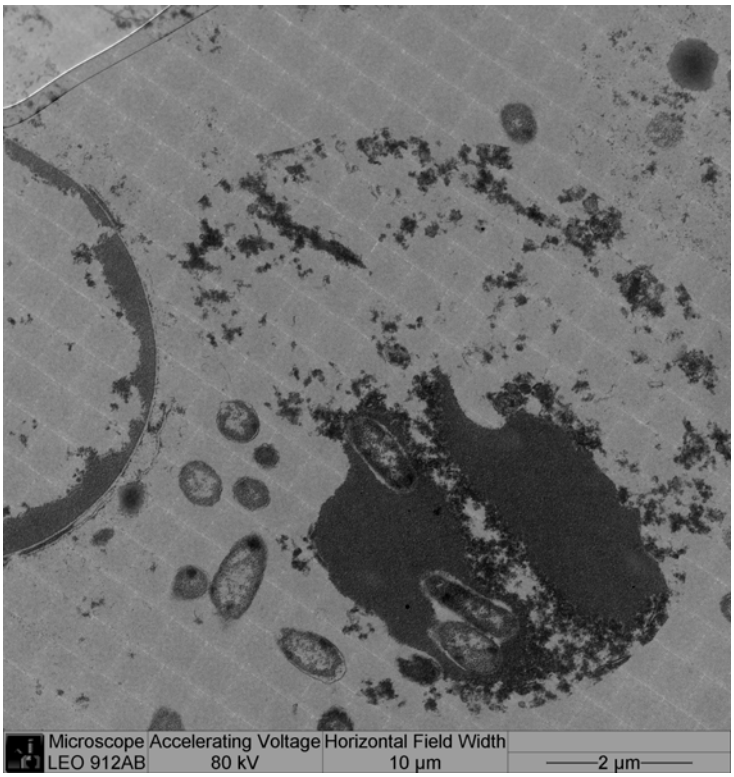
Horizontal Field Width
10 µm
2 µm

H


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
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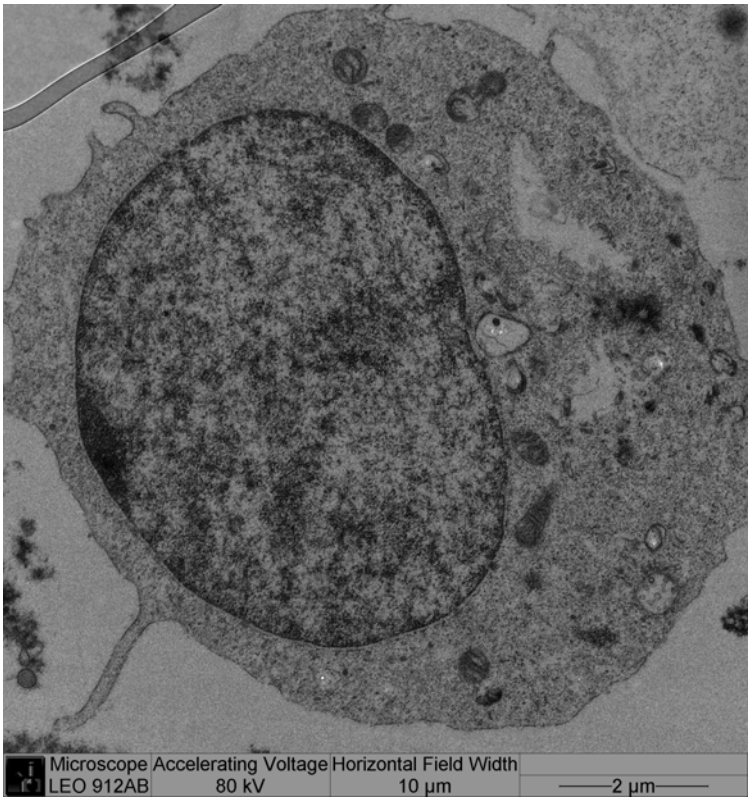
2 µm

I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LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
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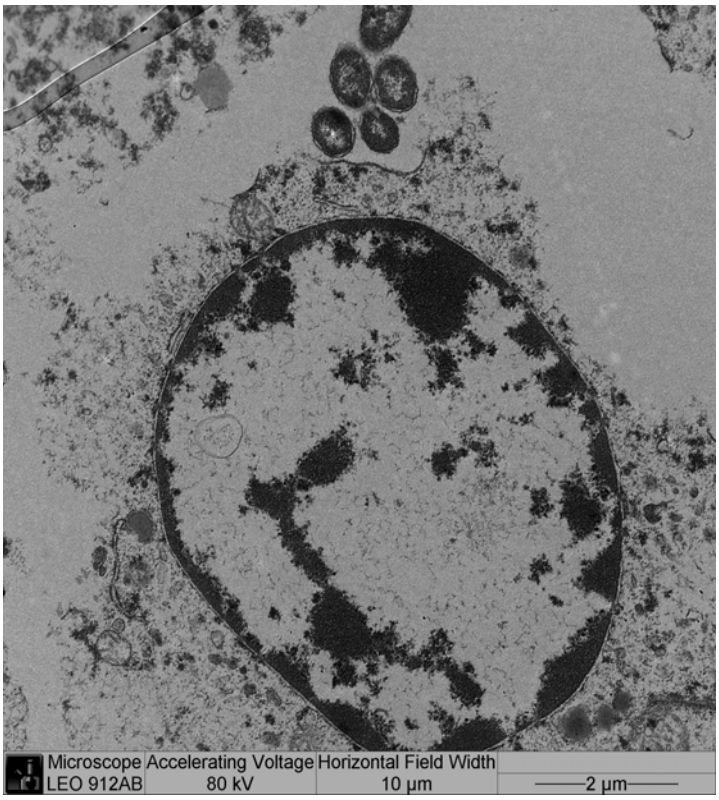
2 µm

J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LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
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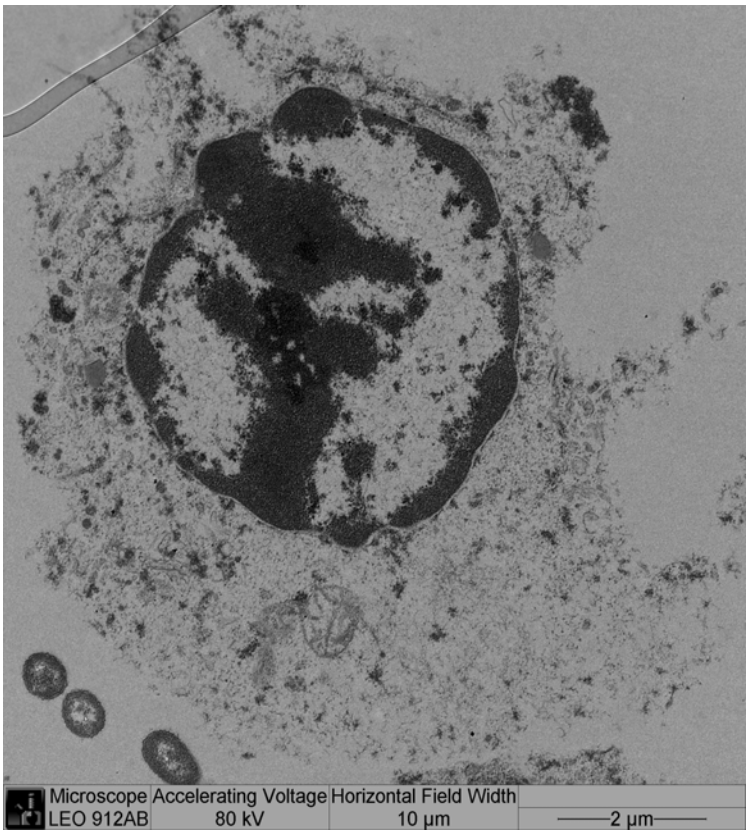
2 µm

K


Microscope
LEO 912AB
Accelerating Voltage
80 kV
Horizontal Field Width
10 µm
2 µm

L

In conclusion, the action of *P. multocida* B:2 on both cell types resulted in some morphological changes with apparent cell damage particularly seen in the BL-3 cells (Table 13). Vacuolation was a prominent feature with J774.2 cells. Electron microscopy appeared to confirm the ability of *P. multocida* B:2 to survive intracellularly in that the morphology of the intracellular bacteria was normal. The results obtained with the control *P. multocida* A:3 and *E. coli* XL1-Blue suggested that they not capable of invasion, but merely attach to the eukaryotic cell membrane in high numbers (Tables 12 and 13).

CHAPTER 4

4. Discussion:

Although *P. multocida* B:2 has been identified to cause HS in cattle or buffaloes, the manner in which *P. multocida* B:2 causes HS disease remains unclear. In particular, the rapid spread of infecting bacteria from the respiratory tract to the blood and lymph, characterized by oedematous swelling of the head and neck and swollen haemorrhagic lymph nodes, is a notable feature of HS. An important stage in the pathogenesis of infection by *P. multocida* is the translocation through epithelial tissue to the pulmonary interstitium. The potential to adhere to and invade cells may constitute a mechanism that enables the bacteria to invade the blood stream. A marker-free *aro*A deletion derivative (strain JRMT12) was constructed from an HS-associated *P. multocida* serotype B:2 strain 85020 obtained from Sri Lanka. Tabatabaei *et al.* (2002) suggested that the *aro*A derivative of the *P. multocida* B:2 strain may be an excellent candidate for an attenuated live vaccine against HS. It was subsequently shown that intramuscular injection in calves of 10^7, 10^8 or 10^9 CFU twice at a 4-week interval of the *aro*A strain JRMT12 completely protected calves against challenge with the virulent parent 85020 strain (Hodgson *et al*., 2005; Dagleish *et al*., 2007). In this study, it was shown that *P. multocida* B:2 85020 and JRMT12 strains could enter and survive for at least 2 h in macrophage-like J774.2 and bovine lymphoma cells BL-3. The fact that, in our experiments, the HS strains of *P. multocida* could invade and survival in mammalian cells whereas *P. multocida* A:3 and *E. coli* XL1-Blue, although able to adhere to macrophages and BL-3 cells to a similar extent as the *P. multocida* B:2 strains, had a much reduced invasive capacity, is in accordance with the manner in which the *P. multocida* B:2 strains become rapidly bacteraemic in the host whereas *P. multocida* A:3

serotype disease is not characterized by this property. Whether this has any safety implication for the *aro*A vaccine strain remains to be determined. The attenuated strain is cleared from calves rapidly, but the possibility of intracellular survival for a longer period requires further investigation.

4.1 Bacterial adherence and entry into the host cells

4.1.1 Adherence of bacteria

Attachment is a primary prerequisite for bacterial infection of a host and ligands that are involved in such adherence are potential virulence factors. Several studies have examined the adherence of different *P. multocida* isolates to different cell types; tissues or organs and this often reflected serogroup-specific host preference and pathogenicity.

P. multocida serotype A:3 was found in association with the mucosal epithelium of the nasopharynges of rabbits with respiratory tract infections (Glorioso *et al.*, 1982). The bacteria specifically attached to squamous epithelial cells of the pharyngeal mucosa both *in vivo* and *in vitro* and to some tissue culture cell lines such as HeLa cells. The attachment to pharyngeal and HeLa cells was inhibited by N-acetyl-D-glucosamine. These findings suggested that this amino sugar may be a component of the receptor on both animal cell surfaces and that the fimbriae may be the adhesins (Glorioso *et al.*, 1982). Esslinger *et al*. (1994) reported that *P. multocida* strains of serotype A adhered strongly to HeLa cells and to alveolar macrophages from rabbits, rats, mice, cattle and horses. The adhesions were markedly reduced by pre-treatment of the *Pasteurella* with hyaluronidase or pre-incubation of the HeLa cells with hyaluronic acid. This indicated a possible role of hyaluronic acid (contained in the capsules of serotype A strains) in adhesion and subsequent colonization of the bacteria (Esslinger *et al.*, 1994).

In *vitro* experiments were undertaken to study the adhesion to tracheal mucosa, lung and aorta explants from freshly killed rabbits of two different strains of *P. multocida*. Serotype A:3 and serotype D:1 strains isolated from a dead rabbit with septicaemia, were used (Al-Haddawi *et al.*, 2000). When the explants were observed under the scanning electron microscope, the type D strain was more highly adhering to trachea and aorta explants compared to the type A strain. Adhesion to lung explants was best achieved by the type A strain after incubation for 45 min but, after incubation for 2 h, no significant difference was observed between the strains. The capsular material of *P. multocida* type A strain and the toxin of the type D strain were thought to influence the adherence to lung tissue in the rabbit. Adhesion of strain D to aorta may indicate the expression of receptors on the endothelium to that strain and may also explain the ability of certain strains to cause septicaemia (Al-Haddawi *et al.*, 2000).

P. multocida serogroups A, D and B:2 strains are known to possess type IV fimbriae (designated PtfA (Ruffolo *et al.*, 1997; Dabo *et al.*, 2007). Under microaerophilic conditions *P. multocida* showed an increased expression of the fimbriae, which were observed to form bundles. Fimbriae purified by high-performance reverse-phase liquid chromatography constituted a single 18-kDa subunit, the first 21 amino acids of which shared very high similarity with the N-terminal amino acid sequence of type 4 fimbrial subunits from other bacteria. Antiserum against the *P. multocida* 18-kDa protein immuno-stained the type 4 fimbrial subunit of *Moraxella bovis* and *Dichelobacter nodosus*. Based on these observations, *P. multocida* possessed type 4 fimbriae and the *P. multocida* fimbrial subunit was designated *Ptf*A. A later study was carried out to isolate and characterize the gene *(ptf*A) encoding type 4 fimbrial protein from *P. multocida* serogroup B:2 (Siju *et al.*, 2007). Among the surface components of *P. multocid*a, type 4

fimbriae are considered important because they frequently mediate colonization of host surfaces. Thus, attachment by fimbriae to host surfaces is usually correlated with virulence (Siju *et al.*, 2007).

E. coli has been shown to adhere to human epithelial HEp-2 cells (Zepeda-Lopez and Gonzalez-Lugo, 1995) and *E. coli* strains K12, MS101 and HB101 exhibited adhesion to *in vitro*-cultivated HEp-2 cells but did not have the capacity for invasion (Stehling *et al.*, 2008).

Many pathogens including members of the family *Pasteurellaceae* exploit host extracellular matrix (ECM) molecules for virulence through adherence and colonization. The adherence of clinical *Haemophilus influenzae* strains to immobilized extracellular matrix components has been examined. The strains showed a very high level of adherence to laminin and type I collagen, as well as adhesion to fibronectin (Bresser *et al.*, 2000). Another study demonstrated that the interaction of *Streptococcus pyogenes* fibronectin-binding protein (SfbI) with fibronectin on non-phagocytic human epithelial HEp-2 cells triggered bacterial internalization (Molinari *et al.*, 1997). Blocking of the Sfbl adhesin by antibodies either against the whole protein or against the fibronectin-binding domains of SfbI, as well as pre-treatment of HEp-2 cells with purified SfbI protein, prevented both *S. pyogenes* attachment and internalization. It was also demonstrated that SfbI was enough to trigger the internalization process, as inert latex beads pre-coated with the purified SfbI protein were ingested. Experiments performed with a recombinant SfbI protein encompassing the two fibronectin-binding regions of the SfbI molecule demonstrated that these binding regions are essential and sufficient to activate uptake by HEp-2 cells. These results demonstrated that the fibronectin-binding protein SfbI was involved in both *S. pyogenes* attachment to and ingestion by HEp-2

cells and contributed to the elucidation of the underlying molecular events leading to eukaryotic cell invasion by *S. pyogenes* (Molinari *et al.*, 1997).

The broad host range characteristics of *P. multocida* A:3 suggests recognition of host cell surface components (ECM molecules) common to multiple animal species and tissue types. Recent studies investigated the binding of *P. multocida* A to ECM molecules and showed that bovine *P. multocida* isolates bind to several ECM molecules including fibronectin (Fn) (Dabo *et al.*, 2005). Bacteria bound both soluble and immobilized Fn and preferentially bound the N-terminal heparin-binding fragment (Hep-1) of Fn, similar to that described for other bacterial pathogens (Dabo *et al.*, 2005). The *P. multocida* A proteins identified as putative Fn-binding proteins include *P. multocida* OmpA, the TonB-dependent receptor HgbA (hemoglobin-binding protein A) and the transferrin-binding protein A (TbpA). *P. multocida* OmpA bound Madin–Darby bovine kidney (MDBK) via heparin and/or Fn bridging (Dabo *et al.*, 2003). *P. multocida* A regulatory genes have also been reported as potential virulence factors: DNA adenine methylase (*Dam*) and the Rci recombinase (*rci*) gene. In *Salmonella* typhi, Rci protein inverted the DNA in the C-terminal region of the type IV pili, resulting in the inhibition of the minor pilus protein expression (Morris *et al.*, 2003). Whether Rci of *P. multocida* has a similar action on the bacterium type IV pili is unknown. The *dam* gene regulates the expression of virulence genes in several Gram-negative bacteria including *P. multocida* (Heusipp *et al.*, 2007).

Two serotype A:3,4 strains of *P. multocida* that differed in virulence in turkeys were examined for their ability to invade epithelial cell monolayers grown in tissue culture (Lee *et al.,* 1994). Both organisms were comparably adherent to cells of turkey kidney origin. However, the virulent strain (86-1913) penetrated primary turkey kidney epithelial cell monolayers at 10 times the level of the low-virulence vaccine strain. The

virulent strain was also able to invade porcine epithelial cells (PK15) and feline epithelial cells (CRFK) in cell culture. Neither organism invaded rabbit epithelial cells (RK13). Invasion of turkey cells was prevented by inhibition of bacterial protein or RNA synthesis but not by pre-treatment of the monolayers with periodate, trypsin, or neuraminidase. Invasion might be a mechanism of pathogenicity for this organism, contributing to colonization or virulence (Lee *et al.,* 1994).

Adherence studies with a capsulated strain of *P. multocida* A:3 and turkey air sac macrophages in culture showed that the bacteria were capable of adhering in large numbers to the macrophages but were not internalized (Pruimboom *et al.,* 1996). A noncapsulated variant of the serotype A:3 strain showed little or no adherence and was not internalized. These data indicated that the adhesive properties were caused by the presence of the capsule on the bacteria. The role of capsular hyaluronic acid in this adherence to macrophages was investigated. Depolymerization of the bacterial capsule with hyaluronidase increased phagocytosis by macrophage cultures, and addition of hyaluronic acid to the macrophages inhibited bacterial adherence. However, exposure of macrophages to chondroitin sulphate B, an anionic polysaccharide similar to hyaluronic acid, did not affect the adhesive properties and resistance to phagocytosis of capsulated organisms. Treatment of macrophages with sodium metaperiodate or trypsin suppressed bacterial binding. Collectively, these data indicate that *P. multocida* adhesion to air sac macrophages, but not internalization, was mediated by capsular hyaluronic acid and suggested that recognition of this bacterial polysaccharide is a result of a specific glycoprotein receptor.

In a study by Galdiero *et al.* (2001), confluent bovine aortic endothelial cells (BAEC) were infected with *P. multocida* serotype B *and P. haemolytica* serotype A:1 at ratios of

infection of 10, 50, 100 or 1000 bacteria/cell. After keeping cells and bacteria in contact for 30 or 60 min at 4ºC, an association assay was carried out. The infected cells were fixed with methanol and cell association of bacteria was visualized by Giemsa staining. The results indicated no significance differences in adherence between the two species of absence at the different bacteria/cell ratios (Galdiero *et al*., 2001). In our studies, in the observe of killing of extracellular bacteria (by omitting gentamicin and polymyxin) the number of intracellular plus adherent bacteria of different strains of *P. multocida* B:2, *P. multocida* A:3 and *E coli* XL1-Blue with J774.2 and BL-3 cells could be determined. By comparison with the number of intracellular bacteria (by using gentamicin and polymyxin) the number of adherent bacteria could then be assessed. After incubation for 2 or 4 h with J774.2 cells, the number of adherent *P. multocida* B:2 JRMT12 was consistently approximately 2-fold higher than *P. multocida* B:2 85020 (Tables 5 and 6) with or without a centrifugation step to bring the bacteria and mammalian cells into close contact. When the adherence of *P. multocida* B:2 JRMT12 was compared with that of *P. multocida* A:3 and *E. coli* XL1-Blue, it was again approximately 2-fold higher (Table 8). With BL-3 cells, the number of adherent bacteria were lower, and *P. multocida* B:2 strain 85020 appeared to adhere better than the mutant strain (Table 10). Adherence of strain JRMT12 to BL-3 cells was comparable to that of *P. multocida* A:3 whereas *E. coli* XL1-Blue appeared to adhere better (Table 11). However, these experiments would need to be repeated to confirm these differences. Therefore, BL-3 cells generally showed a reduced adherence of bacteria as compared to J774.2 cell lines. This may be explained due to the fact that the J774.2 is a macrophage like cell line and may be more able to attach and ingest bacteria, as compared to BL-3 cells, which is a lymphoma cell line. These results suggested that variation in the number of the adherent bacteria in the *vitro* system depends on the surface characteristics of both the mammalian cell lines and the bacteria. With regard to the two

P. multocida B:2 strains, the difference in adhesion reflect some modification in the surface properties associated with altered aromatic acid biosynthesis.

For many pathogens, adherence and/or invasion involve association with host extracellular matrix molecules such as fibronectin (Fn) (Dabo *et al.,* 2005). Fn bound to *P. multocida* A and inhibited its adherence to Madin-Darby bovine kidney cells, suggesting the involvement of Fn in the adherence of the bacterium to host cells. By comparison, soluble Fn bound less to the *E. coli* JM 109 control. In further experiment with the *P. multocida* B:2 strain, it would be of interest to determine the effect of Fn on their adherence to J774.2 and HeLa cells.

4.1.2 Invasion of J774.2 cells.

Internalisation of *P. multocida* B:2 was detected 4 or 6 h after infection of mammalian cells by TEM and viable bacteria could be recovered from within these cells, by plate counts. Pathogenic bacteria such as *Listeria monocytogenes* and species of *Salmonella*, *Shigella* and *Yersinia* are capable of inducing non-phagocytic cells to internalise them by a process termed invasion. This process involves adherence of the pathogen to the host cell followed by bacterial internalization into a membrane bound vacuole. The mechanisms of internalization are supplied by the host cell and involve cytoskeletal rearrangements and engulfment of the attached bacteria. (see interaction section 1.4). Internalization does not necessarily follow bacterial adherence to the host cell, as many bacteria which are capable of adherence to host cell surfaces are not invasive (Rosenshine and Finlay, 1993). However, in bacteria such as *Salmonella* and *Shigella flexeneri,* invasiveness is encoded by multiple gene systems which promote invasion by interacting with eukaryotic transmembrane proteins (Rosenshine and Finlay, 1993). Also *Salmonella* invasion is associated with a transient increase of the inositol

phosphate concentration in the host cell cytoplasm (Ruschkowski *et al.*, 1992; Brett and Cossart, 1997). These results indicate that *S. typhimurium* may activate host cell phospholipase C activity to form inositol phosphates which in turn stimulate release of intracellular calcium stores to facilitate bacterial uptake. With HeLa cells, *S. typhimurium* invasion is initiated shortly after bacterial addition to the cells, reaching a maximal invasion rate after about 10 min, and saturating the uptake capacity of the host cells after 30 min. Invading salmonellae induce extensive rearrangement of host actin filaments and other cytoskeletal proteins, including actin, tallin, ezrin, α-actinin and tubulin (Rosenshine and Finlay, 1993). Invasion is associated with significant rearrangement of the host cytoskeleton, indicating that signals are transduced between the bacterium and the host cell cytoplasm, across the eukaryotic cell membrane.

Our study showed that *P. multocida* B:2 has the ability to adhere to and invade J774.2 cells, using an invasion assay previously employed by Tabatabaei (2000), where a MOI of 500:1 was used. Using gentamicin and polymyxin, we showed in this study that *P. multocida* B:2 can invade and survive within both J774.2 and BL-3 cell lines. Much of the knowledge regarding bacterial internalisation and intracellular survival has been gained from studies using an assay in which cultured mammalian cells are infected with bacteria for a pre-determined time. The cells recovered after antibiotic treatment to kill extracellular bacteria are presumed to be internalised within the cells. The preliminary killing assay with gentamicin and polymyxin at concentration of 350 μg/ml of each antibiotic revealed very similar killing profiles for both *P. multocida* B:2 strains with a rapid and significant decline in bacteria over a 2 h period. A combination of gentamicin and polymyxin together at the concentration of 350 μg/ml of each reduced the number of viable *P. multocida* B:2 bacteria by >99.9% and essentially killed all bacteria. There was no significant difference in the susceptibility between two *P. multocida* B:2 strains

tested. Although the antibiotic assay seems to be a valuable test for measuring internalisation, it might not be suitable for long-term observation following internalisation as the antibiotics may be slowly taken up by the mammalian cells and, if they achieve sufficient concentration, may kill intracellular bacteria (see below).

Our data also demonstrated that *P. multocida* B:2 strains displayed an invasive ability for J774.2 cells that increased by up to an order of magnitude when bacteria cells were brought together by centrifugation, compared to when bacteria and cells were left simply to interact after mixing. This is likely to be due to the increased contact between the *P. multocida* B:2 cells and the surface receptors. The different invasion efficiencies of the *P. multocida* B:2 strains for J774.2 and BL-3 cells were likely due to a number of possible reasons including the fact that J774.2 cells are potentially phagocytic and the possibility of different interactions with cell surface receptors present on J774.2 and BL-3 cells. *P. multocida* B:2 fimbriae have been reported to serve as the initial attachment point to host cells (Siju *et al.*, 2007). However, after internalization, some pathogenic bacteria like *P. multocida* B:2 strains remain viable within eukaryotic vacuoles at least for 4 h post-infection. In contrast, other bacteria such as *L. monocytogenese* and *S. flexneri* lyse the vacuole membrane shortly after internalization, thereafter residing and multiplying in the cytoplasm. In the cytoplasmic vacuole, nutrients for bacteria may be limited. Moreover, in phagocytic cells such as macrophages, fusion of phagosomes with lysosomes exposes the intracellular bacteria to antibacterial factors such as proteolytic enzymes, low pH, oxidative radicals and antibacterial peptides (Rosenshine and Finlay, 1993). *S. flexneri* has several genes that are needed for protection against these factors (Brett and Cossart, 1997). In addition, *S. typhimurium* can inhibit phagosome lysosome fusion in macrophages (Buchmeier and Heffron, 1991). Although the mechanism responsible for inhibitiing phagosome-lysosome is unknown, this study suggested that

the phagosome lysosome fusion was not dependent on either opsonization or the O side chain of LPS but required viable salmonellae. Intracellular survival of *P. multocida* B:2 within macrophages and the fusion levels of phagocytic vesicles with lysosomes is recommended for future study.

With the intracellular assay using *P. multocida* B:2 strains, numbers of intracellular bacteria recovered at 4 h and at 6 h showed that, after washing J774.2 cells free of antibiotics, an apparent increase in bacterial counts was observed but, when a low level of antibiotics is maintained, fewer bacteria were recovered. It is difficult to interpret these data. If it is assumed that treatment with antibiotics at 350 μg/ml kills all extracellular bacteria, then the increase in intracellular bacterial counts between 4 and 6 h in the absence of antibiotics would represent growth of the intracellular bacteria. If, however, it is assumed that the antibiotics at 350 μg/ml did not kill all extracellular bacteria and a small number remained viable, then the increase may simply have been due to the replication of these residual extracellular bacteria. In the presence of a low level of antibiotics (50 μg/ml) the intracellular count declined between 4 and 6 h. This could be interpreted in two ways. First, the low level of antibiotics prevented replication of residual extracellular bacteria and those within the cell did not replicate and slowly lost viability; second, antibiotics leakage into the mammalian cells began to kill intracellular bacteria and stopped their replication. In the first case, it could be concluded that the bacteria do not replicate within the mammalian cells and could survive intracellularly for a period of only a few hours. In the second case a capacity to replicate within the mammalian cells (indicated by an increase in intracellular bacteria counts in the absence of antibiotics) was interfered with by leakage of the antibiotics into the cells.

Several groups working with other micro-organisms such as *Rhodococcus equi* (Hondalus and Mosser, 1994), *Klebisella pneumoniae* (Oelschlaeger and Tall, 1997), and *Mycobacterium tuberculosis* (Mehta *et al.*, 1996), have reported a continual decrease in recovered intracellular bacteria despite microscopic observations suggesting bacterial replication. Although aminoglycoside antibiotics such as gentamicin are generally thought to be unable to penetrate eukaryotic cell membranes, study showed they can enter macrophages and inactivate intracellular bacteria (Crowle *et al.*, 1984; Drevets *et al.*, 1994). For example, Drevets *et al*. (1994) reported that gentamicin caused normally non-bactericidal macrophages to kill *Listeria monocytogenes* and gentamicin caused listericidal cells to kill significantly more bacteria. This study also investigated whether lysates of macrophage hybrids cultured for 72 h in gentamicin-containing medium and then washed could kill *Listeria* cells. When cultured with 50 to 100 micrograms of gentamicin per ml, cell lysates were extremely listericidal and showed the presence of intracellular gentamicin. It was believed that gentamicin does not penetrate cell membranes, and hypothesized that it can be internalized by the cell through pinocytosis and can enter the same intracellular compartment as does phagocytosed *L. monocytogenes*. Macrophages, which had phagocytosed *L. monocytogenes*, were incubated with the fluorochrome lucifer yellow to trace pinocytosed medium. About half of the *Listeria* cells within the macrophages were surrounded by lucifer yellow, indicating delivery of pinocytosed fluid, which could contain antibiotics, to phagosomes containing bacteria. This experiment described that membrane-impermeant antibiotics can enter macrophages and kill intracellular bacteria. Thus, the use of gentamicin in macrophage bactericidal assays can interfere with the results and interpretation of experiments designed to study macrophage bactericidal activity (Drevets *et al.*, 1994). Furthermore, bacterial infection with *P. multocida* B:2 strains could cause mammalian cell lysis and release of intracellular bacteria into the

medium. These microorganisms would be subsequently killed by any extracellular antibiotics and thus, despite their intracellular origin, they would not contribute to the recovered intracellular pool of bacteria. This is of particular concern where low level invasion or internalisation is being assayed using plate counts in the absence of antibiotics, as those bacteria designated as intracellular may in fact represent any extracellular survivors. This aspect is highlighted by the observation that internalised *Actinobacillus actinomycetemcomitans* bacteria could exit KB (human epidermoid carcinoma) cells and were found in the external environment soon after entry (Meyer *et al.*, 1996).

Interestingly, the *P. multocida* B:2 85020 wild-type strain had an apparently decreased ability to be internalised by J774.2 cells compared to the *P. multocida* B:2 JRMT12 (*aro*A) strain (Table 5). The reason for this is not clear, as both strains grew equally well in BHIB, but the colony morphology of the 85020 and JRMT12 strains differed slightly on BHIA. This may indicate some alteration in surface properties related to the defect in aromatic amino acid biosynthesis, and could be responsible for the differences in internalization. This possibility is all the more likely because, in general, JRMT12 was able to adhere to J774.2 cells better than the parent 85020 strain (Table 5).

The number of *P. multocida* B:2 85020 wild type and JRMT 12 recovered after internalization was 50-100 fold greater than those recorded for the *P. multocida* serotype A:3 strain and this in turn exhibited 10-20 fold greater recovery than for *E coli* XL1-Blue. It is clear from these data that the *P. multocida* B:2 serotype strains 85020 and JRMT12 had an invasive capacity that exceeded that of the *P. multocida* serotype A:3 stain. This invasive capacity may be a virulence property which could potentially

explain the enhanced capacity of *P. multocida* B:2 strains to translocate through the respiratory epithelium into the blood stream. Taking the *E. coli* XL1-Blue strain used in this study as a non-invasive control, it was clear also that the *P. multocida* A:3 strain had a better invasive capacity than *E. coli* XL1-Blue, although this was markedly reduced compared to the *P. multocida* B:2 strains. This may be relevant to the more non-invasive nature of disease caused by the *P. multocida* A:3 strains. The capacity of *P. multocida* to invade mammalian cells is in agreement with previous reports (see section 1.4.2), but the markedly better capacity of the *P. multocida* B:2 compared to the *P. multocida* A:3 serotype has not been previously noted and is a novel finding. Although the J774.2 cell line is a mouse macrophage-like cell line, it dos not appear to be actively phagocytic since the *P. multocida* A:3 and *E. coli* XL1-Blue strains were only poorly taken up, according to the invasion assay, and not seen intracellulary by TEM. These finding support the suggestion that *P. multocida* B:2 is an invasive organism.

4.1.3 Invasion of BL-3 cells

A previous study investigated the association and the invasion of a bovine aortic endothelial cell (BAEC) line by a *P. multocida* serotype B strain as a model for the possible intracellular survival during *Pasteurella* infections (Galdiero *et al.*, 2001). The data suggested that *P. multocida* B was able to adhere to and to invade BAECs. Confluent BAEC monolayers were infected with bacteria at a ratio of infection of 10, 50, 100 or 1000 bacteria per cell. The optimal bacteria/cells ratio was found to be 100/1, while the optimal infection time was approximately 4 h of incubation. In addition, bacterial internalization was dependent on microfilament and microtubule stability (Galdiero *et al.*, 2001). The study used cytochalasin D, an inhibitor of actin polymerization, colchicine and nocodazole, inhibitors of microtubule formation, and taxol, which stabilizes the microtubules, to determine the involvement of microfilaments

and microtubules in these processes. In the presence of cytochalasin D invasion was reduced by 60%; in the presence of colchicine it was reduced by 97% and in the presence of nocodazole it was reduced to 95%. It can be concluded that invasion by *P. multocida* serotype B:2 involves active host cell involvement and demands further study.

The fate of *P. multocida* B:2 after interaction with BL-3 cells was determined in this study. As shown in Tables 8 and 9, both *P. multocida* B:2 85020 and its *aro*A mutant, strain JRMT12 were able to enter BL-3 cells and survive for at least 2 h. As with J774.2 cells, the invasive capacity of the *P. multocida* B:2 85020 appeared to be slightly lower than the JRMT12 strain, but this difference was not significant. The invasive capacities of the *P. multocida* A:3 and *E coli* XL1-Blue strains were markedly lower than that of the *P. multocida* B:2 strains. In fact, the invasion capacity of both *P. multocida* B:2 strains for BL-3 cells was noticeably 20-30 fold higher than for *P. multocida* A:3 which was in turn 3-10 fold higher than for *E. coli* XL1-Blue. Generally, the invasion capacity of the bacteria for BL-3 cells was lower than that for J774.2 cells and the difference was probably a reflection of the reduced adherence of the bacteria to the BL-3 cells compared to the J774.2 cells. This is perhaps to be expected as J774.2 cells are a macrophage-like cell line and would be expected to attach to and ingest the bacteria more readily than the bovine lymphoma cell line

4.2 TEM and mammalian cells

We found that *P. multocida* B:2 cells could be detected intracellularly in J774.2 and BL-3 cells at 4 h and 6 h post-invasion by transmission electron microscopy (TEM). The *P. multocida* B:2 strain was almost always observed as individual cells or occasionally as group of cells inside membrane-bound cytoplasmic vacuoles and rarely in the cytoplasm of both cell lines. Electron micrographs of J774.2 cells often showed the

presence of pseudopod-like elongations that appeared to extend around the bacteria and engulf them. The J774.2 and BL-3 cells were extensively altered by *P. multocida* B:2. In particular, J774.2 cells exhibited extensive vacuolation and, although some vacuoles contained bacteria, vacuolation was apparent without the presence of bacteria (Figure 6 B, C, G, I). Vacuolation was less apparent with BL-3 cells, but in this case cell damage was more obvious (Figure 7 A, B, C, E, H, I) than with J774.2 cells (Figure 6 I). *P. multocida* A:3 and *E. coli* XL 1-Blue did not induce vacuolation in J774.2 cells, but appeared to induce cell damage to BL-3 cells in a manner similar to the *P. multocida* B:2 strains (Figure 7 E, F, K, L). This was apparently associated with exposure to bacteria as cells appeared normal without exposure to bacteria. The TEM data, which showed apparent damage to BL-3 cells, was at variance to trypan blue exclusion data which showed that less of viability of BL-3 cells upon exposure to B:2 strains was minimal, loss than that observed for J774.2 cells (Figures 4 and 5). This is difficult to explain, although in the trypan blue experiments, cells were not exposed to antibiotics and there is the possibility that polymyxin and gentamicin, at the concentrations used, had detrimental effects on BL-3 cells in particular. In a previous study, dramatic effects on the appearance and viability of macrophages were observed (Shah *et al.*, 1996). Macrophage were infected with *P. multocida* B:2, at an MOI of 10:1 bacteria: cells and macrophages were examined for cytotoxicity by EM (Shah *et al.*, 1996). We believe that additional studies will be necessary to establish if *P. multocida* B:2 strains have cytotoxic activity towards mammalian cells. For example, J774A.1 macrophages were examined with *Salmonella typhimurium* and cytotoxicity was assessed by DNA staining with ethidium homodimer-1 which distinguishes; live from dead cells after incubation for 20 min. Ethidium homodimer-1 is a high affinity, membrane-impermeant dye that can only stain the DNA of the nucleus of dead cells (Chen *et al.*, 1996). We failed to determine the cytotoxicity of bacteria to the J774.2 macrophage cells by MTT assay

because the bacteria also contributed to the reduction of the tetrazolium dye. Galdiero *et al.* (2001) assessed cytotoxicity by measuring release of lactic dehydrogenase from BAEC cells, but reported little or no cytotoxicity of their B serotype strain. However, Shah *et al.* (1996) reported cytotoxicity of *P. multocida* B:2,5 strain and indicated that this was due to the action of a toxin that caused vacuolation in RAW 264 macrophage cells. Our data also showed extensive vacuolation of J774.2 cells at 4 h post-infection with our B:2 85020 strain and is agreement with Shah *et al.* (1996). This was not apparent with the A:3 serotype strain (or *E. coli*) which is also is agreement with Shah *et al.* (1996) who showed vacuolation only occurred with their B:2 serotype.

Our study indicated that *P. multocida* B:2, like *Salmonella flexneri* and *Listeria monocytogenes,* could occasionally escape from the macrophage vacuoles and was found free in cytoplasm but more work would be needed to establish if this was an important feature of *P. multocida* behaviour.

Although vacuolation can be associated with phagocytic activity, a vacuolating toxin has been described in *Helicobacter pylori* (Cover *et al.*, 1992). Vacuolation is first detectable in the cytoplasm of cells about 90 min after the addition of vacuolating cytotoxin (Cover *et al.*, 1992). With *H. pylori* as the vacuoles increased in size, the vacuole membrane contained increasing quantities of rab7, a small cellular GTPase normally localized to late endosomal compartments. This suggested that the membrane of cytotoxin-induced vacuoles was derived at least in part from late endosomes. Total cellular levels of rab7 did not increase in vacuolated cells and, therefore, vacuolation was associated with redistribution of rab7 rather than increased production (Cover, 1996). This finding suggested that alteration of membrane traffic within the cells may be important in the process of vacuole formation. Vacuolated cells were capable of

excluding trypan blue, but prolonged exposure to high concentration of cytotoxin resulted in cell death (Cover, 1996). To assess whether *P. multocida* might encode a similar vacuolating toxin, homology was sought by alignment of protein sequences using BLAST (Basic Local Alignment Search Tool) for protein sequences between *P. multocida* A strain Pm70 genome sequences and vacuolating cytotoxin (VacA) of *H. pylori* strain J99 (see appendix 5.8). The database alignment measured a number of hits in the database of random sequences with the same distribution of amino acid as VacA. However, the score (bits) values indicated a poor alignment overall, with only a few short sequences showing some homology. These aligned regions were located mainly in the C-terminal region of VacA and it is possible that the B:2 strain produces one or more proteins with some homology to VacA in this region.

We compared interaction of *P. multocida* A:3 and *E. coli* XL1-Blue with both mammalian cell types, and found them to be less susceptible to these bacteria. Incubation of J774.2 cells with these bacteria did not produce any visible changes. No bacteria were detected inside the cells by TEM but many extracellular bacteria were detected which might have represented bacteria adherent to these cells. Our data demonstrated that *E coli* XL1-Blue adherence to these cells was low even compared to *P. multocida* A:3. No intracellular *P. multocida* A:3 or *E. coli* XL1-blue were detected intracellularly with BL-3 cells, but there was evidence of extensive cell damage. As discussed earlier, this may have been an artefact of BL-3 cell treatment.

In conclusion, this study explored whether *P. multocida* B:2 strains were internalized and could persist in mammalian cells. We used the ratio 500:1 *P. multocida* B:2 strains to J774.2, according to previous work by Tabatabaei (2000). Further work should establish the optimum ratio of bacteria: cells in order to achieve maximal invasion with

minimal cell damage. Insufficient time prevented the use of different ratios of bacteria to cells. This would be particularly important if, for example, a bovine epithelial cell line was studied in future work as being more relevant to the *in vivo* situation. Electron microscopy confirmed the presence of intracellular *P. multocida* B:2 bacteria. The number of internalized *P. multocida* B:2 bacteria was greater with J774.2 cells than with BL-3 cells. *P. multocida* A:3 and *E. coli* XL 1-Blue were found to bind significantly to J774.2 and BL-3 cells but were not internalised to any significant extent. The pathogenesis of HS strains is very complex and further investigations are necessary to improve our understanding of it. In addition, the recognition that the B:2 strains express the capacity to adhere and invade mammalian cells suggest that molecular studies to identify the components involved in these processes and the relevance of the vacuolating activity to pathogenesis may help to improve our understanding of the pathogenicity of *P. multocida* B:2 strains.

CHAPTER 5

5. Appendix:

5.1 Brain heart infusion broth or agar

Brain heart infusion (Oxoid)	37.0 g
Distilled water	1000 ml

The medium was dissolved in distilled water and sterilised by autoclaving for 20 min at 121°C. For solid medium, 1.2% (w/v) agar was added before autoclaving. Occasionally, 5% (v/v) sheep blood supplement was added (see below) to solid medium after it was allowed to cool to 50°C.

5.2 Sheep blood agar (SBA)

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

The medium was dissolved in distilled water and brought to the boil to dissolve completely, then sterilised by autoclaving at 121ºC for 15 min, then 5% (v/v) sterile defibrinated sheep blood (B&E laboratories) was added after the medium was cooled to 50ºC. The medium was mixed and poured into sterile petri dishes.

5.3 Phosphate-buffered saline (PBS)

NaCl	80 g
KCl	2 g
$Na_2HPO_4.12H_2O$	14.3 g
KH_2PO_4	2 g

Made up into 1000 ml of distilled H_2O, adjusted to pH 7.2. The solution was autoclaved at 121°C for 15 min and kept at 4°C.

5.4 RPMI complete medium

Heat-inactivated foetal bovine serum (FBS) (Gibco)	20 ml
L-Glutamine (Gibco)	1 ml

Constituents were dissolved in up to 200 ml RPMI 1640 medium (Gibco) and stored at 4°C. Antibiotics (penicillin and streptomycin) solution (Sigma) 1 ml was added as required.

5.5 BL-3 medium

L-15 Leibovitz medium (Gibco)	50 ml
Heat-inactivated FBS (Gibco)	20 ml
L-Glutamine (Gibco)	2 ml

Constituents were stored at 4°C. Antibiotics (penicillin and streptomycin) solution (Sigma) 1ml were added as required.

5.6 Preparation of antibiotics solutions for invasion assay.

Stock solutions of antibiotics (gentamicin 350mg/ml and polymyxin 350mg/ml)(Sigma) were dissolved in distilled H_2O and filter sterilised by passing the solution through a filter of 0.2 μm pore size (Sartorius). All antibiotics solutions were stored at -20°C.

5.7 Preparation of MTT dye solution

To prepare MTT dye solution, 1.5 mg/ ml of MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma) were dissolved in RPMI 1640 (Sigma) without phenol red and filter sterilized by passing to solution through a filter of 0.2 µm pore size just prior to adding it to the cells. The MTT solution was warmed to 37ºC for 10 min before use.

5.8 Basic local alignment search tool (BLAST)

Query ID : lcl|1970 **(**VacA of *Helicobacter Pylori***)**

Description None

Molecule type Amino acid

Query Length 2902

Database Name Microbial/272843

Description Completed *Pasteurella multocida* subsp. *multocida* strain. Pm70 proteins

Program BLASTP 2.2.18+ Citation

Database	Description	Posted Date

Reference

Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schäffer, Jinghui Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402.

Description

Sequences producing significant alignments:	Score (Bits)	EValue
ref|NP_246509.1| Hsf [*Pasteurella multocida* subsp. *multocida* ……	32.0	0.32
ref|NP_244996.1| PfhB2 [*Pasteurella multocida* subsp. *multocida*…….	29.3	2.1
ref|NP_245651.1| Hsf [*Pasteurella multocida* subsp. *multocida* ……. …….	28.9	2.7
ref|NP_245470.1| IspA [*Pasteurella multocida* subsp. *multocida*... …. ……	28.5	3.5
ref|NP_244994.1| PfhB1 [*Pasteurella multocida* subsp. *multocida*... ……. ..	28.5	3.5
ref|NP_246656.1| hypothetical protein PM1717 [*Pasteurella multocida*…..	28.1	4.6
ref|NP_245936.1| hypothetical protein PM0999 [*Pasteurella multocida* …..	27.7	6.0

CHAPTER 6

6. References

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