VIRULENCE OF *BORDETELLA PARAPERTUSSIS*: A COMPARISON OF OVINE AND HUMAN ISOLATES

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ABSTRACT

Bordetella parapertussis is a cause of mild whooping cough in man and, in recent years, has been isolated from both healthy and pneumonic sheep. It has also been shown to interact synergistically with *Pasteurella haemolytica* in exacerbating ovine pasteurellosis. The relationship between ovine and human isolates of *B. parapertussis* has been examined at a taxonomic level and the presence of different types established. As the biological relevance of this differentiation is unknown, isolates of human and ovine origin were compared on the basis of their characteristics, including growth in ovine tracheobronchial washings (TBWs), their adherence to ovine and human cell lines and to ovine tracheal organ cultures, their interaction with ovine alveolar macrophages, their LPS production and LPS interaction with antisera.

TBW are dilutions of the fluids that bathe the respiratory mucosae and are therefore the fluids in which *B. parapertussis* survive *in vivo*. This study showed that ovine *B. parapertussis* had the potential to grow in ovine TBW and suggested that the ability of ovine and human *B. parapertussis* to grow in TBW was host specific. Growth in nutrient broth also showed that human isolates grew more slowly than ovine isolates thereby suggesting that ovine and human *B. parapertussis* have different nutritional requirements.

In the diseased host, *B. parapertussis* is thought to adhere specifically to ciliated respiratory epithelium. A comparison of ovine and human *B. parapertussis* in terms of adherence to and invasion of non-ciliated continuous cell lines showed no significant host cell preference. However, the adherence of ovine isolates to ovine

tracheal organ cultures, which contain viable ciliated cells, was greater than the adherence of human isolates and therefore, the adherence of *B. parapertussis* to ciliated epithelial cells may be host specific.

Investigation of the interaction between *B. parapertussis* and continuous respiratory cell lines as well as ovine alveolar macrophages revealed that the isolates which were more adherent to these cells were also more invasive. This study showed that both ovine and human *B. parapertussis* were capable of invasion and at least short-term survival within ovine alveolar macrophages.

Analysis of the LPS produced by ovine and human *B. parapertussis* revealed that all the ovine isolates tested produced a rough-type LPS, whereas, the majority of human isolates had a smooth-type LPS. The exception was the human isolate NCTC 5952 which produced a pattern on PAGE similar to those of ovine *B. parapertussis*. The use of PAGE for LPS analysis also revealed that the choice of bacterial growth medium affected the LPS pattern visualised on silver-stained gels. Western blots of ovine and human *B. parapertussis* LPS with rabbit sera raised against ovine and human isolates showed that the O-antigens of the human *B. parapertussis* LPS were immunodominant. These blots also showed that sera raised against an ovine isolate reacted with all the isolates tested whereas, sera raised against a human isolate reacted only with human *B. parapertussis*. In a bactericidal assay anti-human *B. parapertussis* serum killed a high proportion of human isolates but only a small percentage of ovine isolates. In comparison, anti-ovine *B. parapertussis* serum killed approximately the same number of ovine and human *B. parapertussis*. This bactericidal activity reflected the pattern of serum

interaction with *B. parapertussis* LPS on Western blots. It may be that the LPS of *B. parapertussis* cells is a target for the bactericidal activity of sera.

The use of LPS PAGE could not distinguish clearly between ovine and human isolates. However, the results of studies on growth, adherence to ovine tracheal organ culture and adherence to and invasion of ovine alveolar macrophages presented here strongly suggest that ovine and human *B. parapertussis* exhibit host-specific interactions.

DECLARATION

The work described in this thesis was conducted at the Bacteriology Division of Moredun Research Institute, Edinburgh. The work presented here was carried out by myself, except where stated.

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ABBREVIATIONS

ACT - Adenylate cyclase toxin

BG - Bordet-Gengou

Bvg - Bordetella virulence gene

BWB - Blot wash buffer

cfu - colony forming units

CL - cyclodextrin liquid

DNA - Deoxyribonucleic acid

DW - Distilled water

EDTA - Diaminoethanetetra-acetic acid disodium salt

EMEM - Eagle's minimum essential medium

FBS - Foetal bovine serum

FHA - Filamentous haemagglutinin

FLL - Foetal lamb lung

FLT - Foetal lamb turbinate

HEPES - N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]

HLT - Heat-labile toxin

HLY - Haemolysin

IgG - Immunoglobulin G

IgA - Immunoglobulin A

IS - Insertion sequence

kDa - Kilodalton

LPS - Lipopolysaccharide

LS - Lamb serum

MEE - Multilocus enzyme electrophoresis

MRI - Moredun Research Institute

NB - Nutrient broth

PAGE - Polyacrylamide gel electrophoresis

PBS - Phosphate buffered saline

PRN - Pertactin

PT - Pertussis toxin

SDS - Sodium dodecyl sulphate

SPF - Specific pathogen-free

TBS - Tris-buffered saline

TTBS - Tween tris-buffered saline

TBW - Tracheobronchial washings

TCF - Tracheal colonisation factor

TCT - Tracheal cytotoxin

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

TPB - Tryptose phosphate broth

TRIS - (TRIS [hydroxymethyl] amino-methane)

CHAPTER 1

INTRODUCTION

Much of the research on the genus *Bordetella* has been carried out on *Bordetella pertussis* and *B. bronchiseptica*. *B. parapertussis* and *B. avium* have not been widely studied and are usually included in experiments only as comparators with the other *Bordetella* species. The pathogenicity of *B. parapertussis* is assumed to be much the same as *B. pertussis* for they both cause whooping cough in man, although at different severities. However, the recent isolation of *B. parapertussis* from sheep has suggested this bacterium may be a more versatile pathogen than previously thought.

1.1. Definition

Bordetella species are aerobic, gram-negative cocco-bacilli (0.3-0.5 μm x 0.5-2.0 μm). Until recently, they were considered to be obligate respiratory tract pathogens but it has been shown that *B. bronchiseptica* and *B. avium* have the capacity to grow and survive outside their hosts in low nutrient conditions (Porter and Wardlaw, 1993). Moreover, the latest additions to the *Bordetella* genus, *B. hinzii*, *B. holmesii* and *B. trematum* have all been isolated from body sites other than the respiratory tract.

1.2. Discovery of *Bordetella*

Diseases that may have been caused by *Bordetella* species have been recognised for centuries but the causative organisms were not isolated until the 20th century. Bordet and Gengou first isolated *B. pertussis* in 1906 using potato-glycerol agar containing 50% blood. Since then, six other *Bordetella* species have been recognised.

In 1910, Ferry isolated *B. bronchiseptica* (originally *Bacillus bronchicanis*) from the respiratory tract of dogs with distemper. *B. bronchiseptica* is now recognised to be an important respiratory pathogen of many wild and domestic animals as well as an opportunistic pathogen of man (Mesnard *et al.*, 1993).

B. parapertussis (originally *Bacillus parapertussis*) was discovered by Eldering and Kendrick in 1938 and described as a species closely related to both *B. pertussis* and *B. bronchiseptica* and capable of causing mild whooping cough. Until 1987 it was thought that *B. parapertussis* was an obligate human pathogen but *B. parapertussis*-like organisms have since been isolated from the respiratory tract of sheep in New Zealand (Cullinane *et al.*, 1987) and Scotland (Porter *et al.*, 1994).

In 1967, Filion *et al.* isolated a *B. bronchiseptica*-like organism from respiratory disease in turkey poults. Hinz *et al.* (1978) reported similar findings and in 1984 *Bordetella avium* was proposed by Kersters *et al.* (1984) as the name of the causative organism. *Bordetella holmesii* was proposed as the name for a group of non-oxidising gram-negative bacteria isolated since 1983 from human blood cultures (Weyant *et al*, 1995). Some of these patients had underlying conditions that may have enhanced their susceptibility to infection. However, it is not yet

known whether *B. holmesii* is capable of infection in a healthy host or if it is an opportunistic pathogen.

Cookson *et al.* in 1994 proposed the name *Bordetella hinzii* for a bacterium isolated from an immunocompromised patient. Their investigations showed that this isolate was similar to, but not identical with, *B. avium*. In 1995, Vandamme *et al.* published a report on strains of *B. hinzii* isolated from the respiratory tracts of turkeys, chickens and humans. Experimental infection of young turkeys and chickens found the organism now known as *B. hinzii* to be non-pathogenic (Jackwood *et al.*, 1995).

The most recent addition to the *Bordetella* genus is *Bordetella trematum* (Vandamme *et al.*, 1996). This was isolated from wounds and ear infections in humans.

1.3. Taxonomy of the genus *Bordetella*

Since their discovery, *Bordetella* species have been allocated to many different genera. The various species have been members of the *Alcaligenes, Bacillus, Haemophilus* and *Brucella* genera. With the advance of molecular biology, studies have determined that *Bordetella* are most closely related to *Alcaligenes* and are now assigned to the beta-2 subdivision of *Proteobacteria* (Weyant *et al.*, 1995).

The first *Bordetella* species to be identified was *B. pertussis*. This was initially named *Bacillus pertussis*. When *B. parapertussis* was discovered in 1938 (Eldering and Kendrick, 1938) this was given the name *Bacillus parapertussis*. Later, both these organisms were assigned to the *Haemophilus* genus. *B.*

bronchiseptica was originally assigned to the genus *Alcaligenes* (Bergey, 1925). This was followed by a move to the genus *Brucella* (Topley and Wilson, 1929) and then to *Haemophilus* (Wilson and Miles, 1946, cited by Goodnow, 1980). It was some years later that this bacterium was renamed *B. bronchiseptica*. *B. avium* was originally assigned to the *Alcaligenes faecalis* species and was then described as a *B. bronchiseptica*-like organism until 1984 when it was given its current species designation. The more recently identified species have been assigned directly to the *Bordetella* genus.

Studies have shown the genus *Bordetella* to contain a closely related group of bacteria and there are still questions raised about the accuracy of having so many individual species rather than subspecies of a single species.

Johnson and Sneath (1973) analysed 134 features of *B. pertussis* and *B. parapertussis* and concluded, on the basis of a numerical taxonomy study, that they are the most closely related of the *Bordetella* species. However, by analysing the electrophoretic mobilities of certain metabolic enzymes, Musser *et al.* (1986) reported that *B. parapertussis* was more closely related to *B. bronchiseptica*. This was supported by analysis of insertion sequences by van der Zee *et al.* (1997) who suggested that *B. parapertussis* should be assigned as a subspecies of *B. bronchiseptica*. The similarity between *B. parapertussis* and *B. bronchiseptica* was further demonstrated by the finding that both have a transcriptionally-silent pertussis toxin gene (Arico and Rappuoli, 1987). The close relationship of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* was reported by De Ley *et al.* (1986) who found that these three species could not be differentiated from each other by the mean G+C contents of their genomic DNAs or by testing the thermal

stability of DNA-rRNA hybrids (using labelled rRNA from *B. bronchiseptica*).

In comparison, *B. avium* is more easily differentiated from *B. pertussis*. Its distinctiveness from *B. pertussis, B. parapertussis* and *B. bronchiseptica* is demonstrated by the lower G + C content of 61-62.6 mol% of its DNA compared with a range of 67.7%-69.5% for the other tested species (Kersters *et al.*, 1984). This difference is reinforced by the absence of a pertussis toxin gene (Arico and Rappuoli, 1987) and lack of production of adenylate cyclase toxin (Hewlett and Gordon, 1988).

Many studies have been carried out on the species designation of the genus *Bordetella* using a variety of methods: deoxyribonucleotide sequencing (Kloos *et al.*, 1981); cultural and biochemical characteristics (Hinz *et al.*, 1983); multilocus enzyme electrophoresis (Musser *et al.*, 1986); ribonucleic acid hybridisation (De Ley *et al.*, 1986); insertion sequence analysis (van der Zee *et al.*, 1996, 1997). Although various methods have been used, all the studies came to the conclusion that *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are a single genospecies and that *B. avium* is a true species. For convenience of presentation however, the specific names will be used in this thesis.

1.4. Isolation and nutrition

Bordet and Gengou were the first to achieve the growth of *B. pertussis* on solid media using a glycerol-potato extract medium containing 50% blood (Bordet and Gengou, 1906). Modified versions of this medium are still in use for the growth of *Bordetella* species. In 1977, Regan and Lowe developed a charcoal-based agar

supplemented with 10% defibrinated horse blood and 40 μg ml^{-1} cephalexin. This medium is still recommended today for the primary isolation and transport of *B. pertussis*.

Stainer and Scholte (Stainer and Scholte, 1971) developed a liquid medium containing glutamic acid and proline which enhanced the growth of *B. pertussis*. Although this was suitable for the growth of large quantities of *B. pertussis*, it only supported growth from a large inoculum. The addition of heptakis (2,6-*O*-dimethyl) β-cyclodextrin, which forms water soluble complexes with hydrophobic molecules such as fatty acids, enabled the growth of *B. pertussis* from a small inoculum. This also increased the production of PT, FHA and ACT, which were released into the culture supernate (Imaizumi *et al.*, 1983; Sato *et al.*, 1984). The increased production of virulence factors in this medium facilitated the production of acellular vaccines.

B. pertussis is the most fastidious of the *Bordetella* species. This species can only be grown on specialised media such as Bordet-Gengou agar, Regan-Lowe agar and cyclodextrin liquid. The other bordetellae are able to grow on blood agar, nutrient agar and MacConkey's agar. Growth of *B. pertussis* is inhibited by unsaturated fatty acids which are found in most media (Pollock, 1947), colloidal copper sulphide (Proom, 1955) and contaminating detergents from glassware. However, the addition of starch, albumin and charcoal reduces the effects of these inhibitors (Jebb and Tomlinson, 1957; Wilson, 1963) on *B. pertussis*. The growth characteristics of *Bordetella* species are detailed in Table 1.1.

Bordetella species have simple nutritional requirements. Proom (1955) studied

Table 1.1. Characteristics of *Bordetella* species

Characteristics	*B. pertussis*	*B.parapertussis*	*B.bronchiseptica*	*B. avium*	*B. hinzii*	*B holmesii*	*B. trematum*
Colonies visible on agar (days)	3	1-2	1	1	2	2-3	1
Tyrosine agar: Growth	-	+	+	+	+	+	+
Browning	-	-	-	-	-	+	-
Growth on MacConkey's agar	-	+	+	+	+	+	+
Motile	-	-	+	+	+	-	+
β-like haemolysis	+	+	V	V	?	-	?
Citrate utilisation	-	V	+	V	+	-	-
Nitrate reduction	-	-	+	-	-	-	V
Oxidase	+	-	+	+	+	-	-
Urease	-	+	+	-	V	-	-

+, positive; -, negative; V, variable; ?, unknown. Adapted from Parton, 1998.

B. pertussis, *B. parapertussis* and *B. bronchiseptica* and found that these species all had an absolute requirement for nicotinic acid. This was also found to be the case for *B. avium* (Kersters *et al.*, 1984). However, Porter *et al.* (1991) demonstrated that *B. bronchiseptica* could grow, to some extent, in phosphate-buffered saline and natural waters without the addition of nicotinic acid.

Amino acids are required as a carbon and nitrogen source for *B. pertussis, B. parapertussis*, *B. bronchiseptica* and *B. avium* (Proom, 1955; Kersters *et al.*, 1984). Glutamic acid is the preferred carbon and nitrogen source of *B. pertussis, B. parapertussis* and *B. bronchiseptica* and is the first to be utilised in simple amino acid mixtures but can be replaced by α-ketoglutaric acid (Proom, 1955; Jebb and Tomlinson, 1955, 1957; Rowatt, 1955). An organic form of sulphur such as cysteine, cystine or methionine is also required by *B. pertussis* (Proom, 1955; Jebb and Tomlinson, 1957). *B. bronchiseptica* has the simplest nutritional needs. It can grow in a mixture of glutamic acid, proline and leucine while *B. parapertussis* required added cystine and methionine and *B. pertussis* required, in addition, alanine, asparagine and serine (Proom, 1955). Goldner *et al.*, (1966), however, found that glutamic acid and proline were the only amino acids whose absence limited the growth of *B. pertussis*. *B. bronchiseptica* and *B. avium* can also utilise carbon sources such as lactate and citrate (Proom, 1955; Kersters *et al.*, 1984). No information is available on the nutritional requirements of the other *Bordetella* species.

1.5. Diseases

1.5.1. *Bordetella pertussis*

B. pertussis is the causative agent of whooping cough, a respiratory infection that mainly affects young children. This organism is transmitted by aerosol droplets and has an initial incubation period of about 7 days before the onset of symptoms (Wardlaw, 1990). The typical clinical features of this disease include fever and a paroxysmal cough that is often followed by the distinctive inspiratory whoop. This may result in vomiting and apnoea that in turn can lead to respiratory complications and CNS disturbances (Parton, 1998). In some countries, including the UK, epidemics of whooping cough have been reported to occur in cycles with the epidemic peak occurring every 3-4 years (Lautrop, 1971).

Before vaccines were developed, whooping cough was a major cause of infant death across the globe. Since vaccination was introduced, the mortality rate has fallen considerably and, in developed countries with an extensive vaccination programme, the number of whooping cough cases are at a low level (Willems and Mooi, 1996). However, in unvaccinated populations, whooping cough is responsible for severe morbidity and mortality with an estimated 60 million cases and >0.5 million deaths globally per annum (Muller *et al.*, 1986).

1.5.2. *Bordetella parapertussis*

B. parapertussis is generally considered to cause a milder form of whooping cough

than *B. pertussis*. Despite the absence of pertussis toxin (PT), *B. parapertussis* is capable of causing a severe disease (Heininger *et al.*, 1994) and in some cases death (Zuelzer and Wheeler, 1946; Linneman and Perry, 1977). This suggests that PT is not necessary for severe cases of whooping cough.

In the USA and Europe, *B. parapertussis* has been reported to cause 5% of *Bordetella* infections (Willems and Mooi, 1996). However, in Eastern Europe the figure is as high as 30% (Neimark *et al.*, 1961) despite the use of whooping cough vaccines in these countries. This supports the findings of Khelef *et al.* (1993a) who demonstrated that there is no cross protection between *B. pertussis* and *B. parapertussis* antigens.

The milder disease most often associated with *B. parapertussis* may result in a sub-clinical infection and will often go unnoticed. A study of the incidence of *B. parapertussis* infection in children in Moscow in 1959 found that 50% of the children tested were asymptomatic carriers of *B. parapertussis* (Neimark *et al.*, 1961). The same study also observed that 30% of bacteria isolated from suspected cases of pertussis were wrongly identified as *B. pertussis* when in fact the causative agent was *B. parapertussis*. However, the true figure representing *B. parapertussis* infection is not known. The closest indication of the prevalence of *B. parapertussis* infection is given by the results of serological studies carried out in Europe (Vysoka-Burianova, 1963) where more than 50% of the adult population had antibodies to *B. parapertussis*, and the USA (Flosdorf *et al.*, 1942) which found that 91% of the adults tested had antibodies to *B. parapertussis*. This suggests that *B. parapertussis* is circulating throughout most of the population and thus creating a potential reservoir of infection.

It is also possible that *B. pertussis* and *B. parapertussis* cause a co-infection. Kawai *et al.* (1996) reported the persistent colonisation of the lungs and trachea of mice by *B. parapertussis* when these mice had been infected with both *B. pertussis* and *B. parapertussis*. A study in the USA (Linnemann and Perry, 1977) documented cases of *B. parapertussis* infection with lymphocytosis. However, *B. parapertussis* does not produce PT, which is the lymphocytosis-promoting factor. Some of the patients family were tested for the culture of *B. pertussis* and two-thirds tested positive. This suggests that the patients had come into contact with *B. pertussis*, which facilitated the infection of the host by *B. parapertussis*. Further studies have also reported dual infections or simultaneous outbreaks of these two organisms (Eldering and Kendrick, 1938; Lautrop, 1958; Donchev and Stoyanova, 1961; Taranger *et al.*, 1994).

B. parapertussis was previously thought to be an obligate human respiratory tract pathogen. However, in 1987 Cullinane *et al.* isolated *B. parapertussis* in New Zealand from nasal swabs, bronchial washings and lung tissue of both healthy and pneumonic lambs but not from adult sheep. They suggested that the failure of others to isolate this bacteria from lambs may have been because lung tissue is the material usually investigated from pneumonic lambs, yet this tissue provided a low rate of recovery of *B. parapertussis*. Also, an incubation time of up to seven days was required and if other bacteria are present, persistent subculturing may be necessary before a pure culture can be obtained for identification. The identification of these isolates by Cullinane *et al.* (1987) was based on biochemical reactions. They found that these ovine isolates were indistinguishable from known *B. parapertussis* except for the speed of the urease reaction and lack of pigment

production on peptone agar, which are more characteristic of *B. bronchiseptica*.

Porter *et al.* (1994) reported the isolation of *B. parapertussis* from the lungs of both healthy and pneumonic adult sheep in Scotland. As with Cullinane *et al.* (1987), most of their ovine isolates produced a fast urease reaction. However, all of the Scottish ovine isolates did produce browning on tyrosine agar (except batch K). Porter *et al.* (1994) suggested that the isolates of batch K were more *B. bronchiseptica*-like than *B. parapertussis*-like based on the results of their biochemical analysis and the finding that these isolates were motile whereas all the other *B. parapertussis* isolates tested (both human and ovine) were non-motile. Porter *et al.* (1991) reported that *B. bronchiseptica* was capable of growth in phosphate-buffered saline (PBS). However, like the *B. parapertussis* isolates tested, the isolates of batch K did not grow in PBS and they did not reduce nitrate, whereas, the majority of the *B. bronchiseptica* reference strains did.

Multilocus enzyme electrophoresis (MEE) separated the human and ovine *B. parapertussis* and isolates of *B. bronchiseptica* into different groups (Porter *et al.*, 1994). On the basis of these analyses they concluded that the bacteria isolated from the lungs of adult sheep were *B. parapertussis*, except batch K which required more study, and suggested the potential for a possible zoonotic infection of man. Study of the LPS, fatty acid patterns and antibiotic sensitivity (Porter *et al.*, 1995c) failed to differentiate clearly between isolates from different host species. Interestingly, analysis of the LPS and antibiotic sensitivity found the human isolate NCTC 5952 to be more similar to ovine than human isolates. This result is reflected in the pulsed-field gel electrophoresis analysis of Porter *et al.* (1996) where banding pattern of NCTC 5952 was identical to some of the ovine rather

than human isolates. The rest of the *B. parapertussis* isolates tested indicated that human and ovine isolates of *B. parapertussis* are genetically distinct and that variation exists within isolates from the same host species. The idea of two distinct populations of *B. parapertussis* is supported by the work of van der Zee *et al.* (1996) who, by DNA fingerprint analysis using insertion sequence (IS) element IS*1001*, revealed that ovine *B. parapertussis* are distinct from human *B. parapertussis*. They also examined the presence of IS*1002* within *Bordetella* species and found that IS*1002* was present in both *B. pertussis* and human *B. parapertussis*. In contrast, IS*1002* was absent from ovine *B. parapertussis*. Also, IS-associated restriction fragment length polymorphism analysis revealed that ovine *B. parapertussis* are genetically more polymorphic than human *B. parapertussis*. This suggests that human *B. parapertussis* diverged from a single clone more recently than ovine *B. parapertussis*. van der Zee *et al.* (1996) concluded that ovine and human *B. parapertussis* appeared to have evolved independently from *B. bronchiseptica*. Further study by van der Zee *et al.* (1997) using MEE and analysis of IS distribution suggested that human *B. parapertussis* derived from a pig strain of *B. bronchiseptica* and that ovine *B. parapertussis* probably evolved from *B. bronchiseptica* earlier than human *B. parapertussis*.

Studies on the New Zealand ovine isolates of *B. parapertussis* demonstrated their pathogenicity and ability to cause pneumonia in mice (Chen *et al.*, 1989) and colostrum-deprived lambs (Chen *et al.*, 1988a and 1988b). These experiments demonstrated that ovine *B. parapertussis* targeted the alveolar macrophages and alveolar epithelium and could cause pulmonary lesions in both sheep and mouse models. This could compromise the pulmonary defence mechanisms and allow the

invasion of the lower respiratory tract by other pathogens such as *Pasteurella haemolytica*, which is capable of causing a more severe pneumonia. Chen *et al.* (1990) also showed that cell-free extracts of ovine *B. parapertussis* could cause pneumonia in mice. This suggests that the extracelluar virulence factors of ovine parapertussis may be involved in causing the lesions associated with the type of pneumonia seen using whole cells. These findings also suggest that the toxins of *B. parapertussis* compromise the host and therefore enable secondary infections to take place.

Experiments using mice showed that the combined administration of ovine *B. parapertussis* and *P. haemolytica* produced a more severe bronchopneumonia than when either agent is given alone. The results suggest either a synergy between the two organisms (Jian *et al.*, 1991) or that *B. parapertussis* predisposes the host to *P. haemolytica* infection (Porter *et al.*, 1995b). Further work has shown the same predisposition effect using SPF lambs (Porter *et al.*, 1995a).

1.5.3. *Bordetella bronchiseptica*

Unlike *B. pertussis* and *B. parapertussis*, *B. bronchiseptica* has a broad host range. This bacterium has been isolated from many different mammals including dogs, cats, horses, monkeys and humans but it is the role of *B. bronchiseptica* in atrophic rhinitis of pigs that has received the most interest.

Atrophic rhinitis in swine causes a major economic loss worldwide and may be found in 40% or more of swine herds in the USA (Harris and Switzer, 1972). *B. bronchiseptica* alone can cause a mild turbinate atrophy in pigs (Cross and Claflin,

1962; Brassine *et al.*, 1976) but the more severe disease is caused by a synergy with *Pasteurella multocida*. *B. bronchiseptica* produces toxins that can damage the respiratory tract of pigs. This may then predispose them to *P. multocida* (Rutter, 1985) and possibly result in a more severe infection.

B. bronchiseptica also causes infectious tracheobronchitis in dogs (Goodnow, 1980; Bemis, 1992) which is a highly contagious disease that affects all ages of dogs and is commonly found in dogs kept in close confinement (Bemis *et al.*, 1977). This bacterium has also been reported as the cause of 10% of all feline respiratory infections (Snyder *et al.*, 1973). *B. bronchiseptica* has occasionally been associated with human infection, mostly through contact with an infected animal, and in these cases it is usually an opportunistic infection in an immunocompromised host (Ghosh and Tranter, 1979; Woolfrey and Moody, 1991; Gueirard *et al.*, 1995).

B. bronchiseptica is a somewhat unusual *Bordetella* species in that it can cause disease in a wide range of animals. This species has also been reported to survive in nutritionally-deficient environments. Porter and Wardlaw (1993) noted the long-term survival and growth of *B. bronchiseptica* in PBS and lakewater at 10°C for up to 24 weeks. This suggests that there may be reservoirs of infection in the environment and has implications for the source of *B. bronchiseptica* infections.

1.5.4. *Bordetella avium*

This is the causative agent of turkey coryza which is a highly contagious disease of turkey poults and secondary infections that arise can result in a mortality rate of

75% (Simmons *et al.*, 1983). Although *B. avium* is usually associated with respiratory infections of turkeys it has also been isolated from other avian species including chickens (Jackwood *et al.*, 1995; Kersters *et al.*, 1984). Porter *et al.* (1991) briefly mentioned that *B. avium* is also capable of growing in PBS and natural fresh waters. These are low nutrient environments and the survival of *B. avium* suggests a possible environmental source of infection.

1.5.5. *Bordetella holmesii*

B. holmesii is one of the newer species of the genus *Bordetella*. It has been isolated from blood cultures of mostly young adults (Weyant *et al.*, 1995) and has been attributed as the cause of sepsis in an asplenic adolescent (Lindquist *et al.*, 1995). Most of the subjects from whom this bacterium was isolated had an underlying condition that may have rendered them susceptible to an opportunistic infection.

1.5.6. *Bordetella hinzii*

This species has been isolated from both humans and poultry (Cookson *et al.*, 1994; Vandamme *et al.*, 1995; Funke *et al.*, 1996). Two strains were isolated repeatedly from a cystic fibrosis patient over a 3 year period (Funke *et al.*, 1996). Another strain was the cause of bacteraemia in an AIDS patient (Cookson *et al.*, 1994).

1.5.7. *Bordetella trematum*

Little is known about the most recently discovered member of the *Bordetella* species, *B. trematum*. To date, Vandamme *et al.* (1996) have reported the isolation of the bacterium from wounds and ear infections in humans and presented the reasons for determining it to be a *Bordetella* species based on analysis using both phenotypic and genotypic methods.

1.6. Interaction with *Pasteurella* species

Two *Pasteurella* species have been associated with *Bordetella* infections. *B. bronchiseptica* and *P. multocida* have a synergistic relationship in atrophic rhinitis in pigs (Rutter, 1985) and *B. parapertussis* is reported to predispose sheep to *P. haemolytica* infection (Jian *et al.*, 1991; Porter *et al.*, 1995a).

P. haemolytica is a major cause of pneumonia in sheep worldwide and its impact has resulted in economic losses. This bacterium is found in the upper respiratory tract of clinically healthy sheep and causes disease, usually when the host is predisposed. Two possible causes of predisposition are environmental factors and other infectious agents. Some outbreaks have been associated with changes in weather and dipping but there have been no epidemiological surveys on the effect of external factors on incidence of disease (Gilmour, 1992). Experimental investigations have produced disease in sheep when they have been pre-infected with a range of viruses (Gilmour and Gilmour, 1989). More recently, experiments have demonstrated that *B. parapertussis* predisposes specific-pathogen-free lambs

to *P. haemolytica* infection and induces a more severe disease than when either agent is given alone (Porter *et al.*, 1995a). It is suggested that, as in atrophic rhinitis due to *B. bronchiseptica*, *B. parapertussis* produces a tracheal cytotoxin that damages the cilia of the respiratory epithelium thereby creating an accumulation of mucus. This could allow the establishment of *P. haemolytica* infection. In addition, the adenylate cyclase toxin of *B. parapertussis* may interfere with macrophage function enabling *P. haemolytica* to overcome the host defences.

1.7. Virulence factors

The members of the genus *Bordetella* produce a variety of virulence factors including toxins and adhesins that are utilised in causing a respiratory tract infection. Not all of these factors are produced by all *Bordetella* species. Table 1.2 is a summary of virulence factors produced by the different species. No information is published on the virulence factors, if any, produced by *B. hinzii*, *B. holmesii* or *B. trematum*.

Most of what is known of these virulence factors has been taken from studies carried out on *B. pertussis*. There may be subtle differences in the factors produced by the various *Bordetella* species but their functions are assumed to be the same for all species.

Table 1.2. Virulence factors produced by *Bordetella* species.

Virulence factor	*B. pertussis*	*B. parapertussis*	*B. bronchiseptica*	*B. avium*
Lipopolysaccharide	+	+	+	+
Tracheal cytotoxin	+	+	+	+
Heat-labile toxin	+	+	+	+
Fimbriae	+	+	+	+
Filamentous haemagglutinin	+	+	+	-
Adenylate cyclase toxin	+	+	+	-
Pertactin	+	+	+	-
BrkA	+	?	?	-
Pertussis toxin	+	-	-	-
Tracheal colonisation factor	+	-	-	-
Osteotoxin	-	-	-	+

+, present; -, absent; ?, unknown. Adapted from Parton, 1998.

1.7.1. Lipopolysaccharide (LPS)

This is produced by all gram-negative bacteria. Like LPS produced by other bacteria, the LPS of bordetellae have the properties of pyrogenicity, adjuvanticity, stimulation of IL-1 and TNF, macrophage activation and mitogenicity (Chaby and Caroff, 1988; Watanabe *et al.*, 1990).

The LPS of *B. pertussis* and ovine *B. parapertussis* are of the rough type which has no O-antigen, whereas the LPS of human *B. parapertussis* and *B. bronchiseptica* are both smooth types which have the O-antigen (Peppler, 1984; Amano *et al.*, 1990; Brodeur *et al.*, 1993; van den Akker, 1998). The LPS of *B. bronchiseptica* has been reported to have structures in common with both *B. pertussis* and human *B. parapertussis* (Amano *et al.*, 1990). This was based on the fact that LPS from *B. bronchiseptica* reacted with antisera raised against *B. pertussis* and *B. parapertussis* while the *B. pertussis* and human *B. parapertussis* LPS's were found to be serologically different from each other. In addition, SDS-PAGE revealed that the LPS of all three species tested contained low molecular weight bands.

LPS is not included in the acellular vaccine preparations because of its endotoxic effects and it is generally not considered to be a protective antigen (Pittman, 1984). However, boiling whole-cell pertussis vaccine destroyed all but the heat-stable LPS yet the vaccine still protected mice against intranasal challenge with *B. pertussis* (Standfast, 1958) suggesting that the LPS does play a role in protective immunity.

1.7.2. Tracheal Cytotoxin (TCT)

TCT is known to be produced by at least 4 of the *Bordetella* species, *B. pertussis, B. parapertussis, B. bronchiseptica and B. avium* (Goldman, 1988). This toxin is derived from the peptidoglycan of the bacterial cell envelope and is released into the culture medium during the logarithmic phase of growth (Cookson *et al.*, 1989). TCT activity has been demonstrated in hamster tracheal organ cultures where it caused ciliostasis and ciliated cell extrusion as well as inhibition of DNA synthesis in hamster tracheal epithelial cell cultures (Goldman and Herwaldt, 1985). Considering that the ciliated cells of the airways are responsible for the clearance of tracheal mucus and entrapped microorganisms, the loss of ciliary activity will result in the accumulation of mucus in the respiratory tract and enable the multiplication of bacteria. *B. pertussis* infection could therefore promote secondary infections of the respiratory tract by this mechanism.

The toxic activity of TCT is due to its stimulation of IL-1 production by respiratory epithelial cells (Heiss *et al.*, 1993) which triggers the synthesis of nitric oxide in the respiratory epithelium. Nitric oxide can be cytotoxic or cytostatic to cells including those of the respiratory epithelium (Heiss *et al.*, 1994). TCT has also been shown to be toxic for human neutrophils and may therefore contribute to the survival of *B. pertussis* in the airways of the host (Cundell *et al.*, 1994).

1.7.3. Heat-Labile Toxin (HLT)

The HLT is a cytoplasmic protein that is produced by *B. pertussis, B.*

parapertussis, B. bronchiseptica and *B. avium*. It has yet to be determined whether the other *Bordetella* produce this toxin. The role of HLT in disease is unknown yet experiments in animals have shown it to be lethal, dermonecrotising (Iida and Okonogi, 1971) and vasoconstrictive (Endoh *et al.*, 1986). However, the HLT of *B. bronchiseptica* is suggested to have a role in atrophic rhinitis of pigs (Hanada *et al.*, 1979; Horiguchi *et al.*, 1994).

HLT is inactivated by heating at 56°C for 30 min and, as a result, it is destroyed during whole-cell vaccine preparation. The mouse-toxicity test for whole-cell pertussis vaccine was introduced to confirm that the HLT had been destroyed (Pittman, 1952). These vaccines are effective against whooping cough, which suggests that antibodies to HLT are not required for protective immunity. Indeed, *B. pertussis* mutants deficient in HLT are as virulent as the wild-type strains in a mouse model (Weiss and Goodwin, 1989).

The HLT produced by *B. pertussis, B. parapertussis* and *B. bronchiseptica* are identical genetically and biologically whereas there are differences between them and the HLT of *B. avium* (Walker and Weiss, 1994).

1.7.4. Fimbriae

The fimbriae are produced by *B. pertussis, B. parapertussis, B. bronchiseptica* and *B. avium*. Three types of fimbriae are produced by *Bordetella* and are encoded by the genes *fim*2, *fim*3 and *fim*X (Cuzzoni *et al.*, 1990). *fim*2 and *fim* 3 are expressed by all of the species whereas *fim*X is silent in *B. pertussis* but is expressed in *B. bronchiseptica* (Rappuoli, 1994).

Fimbriae of bacteria usually have a role in adhesion. However, this has not been clearly defined for the *Bordetella* species. Antibodies to fimbriae have been shown to block the adherence of *B. pertussis* to non-ciliated tissue culture cells but they do not inhibit the adherence to ciliated cells which are the natural targets (Tuomanen, 1988).

Fimbriae have been included in some of the acellular vaccine preparations (Willems and Mooi, 1996) although they have only been shown to protect mice against *B. pertussis* infection and their role in human disease is still not clearly defined.

1.7.5. Filamentous Haemagglutinin (FHA)

The FHA of *B. pertussis* is a 220kDa secreted protein that acts as one of the major adhesins. There are three binding domains of the FHA which determine the different adherence mechanisms of *B. pertussis*. The carbohydrate-binding site enables the bacteria to adhere to the ciliated respiratory cells (Tuomanen *et al.*, 1988) whilst the heparin-binding domain allows the adherence to non-ciliated cells (Menozzi *et al.*, 1991; Locht *et al.*, 1993). These two binding sites enable *B. pertussis* to bind to and colonise the respiratory tract of the host before establishing an infection. A third domain is an Arg-Gly-Asp (RGD) region which promotes adherence to the macrophage integrin CR3 (Relman *et al.*, 1990). Intracellular uptake via CR3 does not trigger an oxidative burst (Wright and Silverstein, 1983). Therefore, internalisation into the macrophage by this route may promote the intracellular survival of *B. pertussis* and possibly create a reservoir of infection.

The role of FHA as a major virulence factor of *B. pertussis* has promoted its inclusion in some acellular vaccine preparations. FHA immunisation of mice provided significant protection against aerosol challenge with *B. pertussis* (Cahill *et al.*, 1995) and reduced the levels of *B. pertussis* recovered from the lungs and trachea (Locht *et al.*, 1993).

B. parapertussis and *B. bronchiseptica* produce similar FHA proteins which are surface-associated whereas the FHA of *B. pertussis* can be found secreted in the culture supernatant (Locht *et al.*, 1993). However, there is a lack of antigenic cross-reactivity between the FHA produced by the different species. Immunisation with FHA from *B. pertussis* does not protect mice from lung colonisation by *B. parapertussis* (Khelef *et al.*, 1993a) and although antibodies against the FHA of *B. pertussis* recognise whole cell lysates of *B. pertussis* they do not recognise whole-cell lysates of *B. parapertussis* or *B. bronchiseptica* (Domenighini *et al.*, 1990).

1.7.6. Adenylate Cyclase Toxin (ACT)

The ACT of *B. pertussis* is a bifunctional protein with both adenylate cyclase and haemolysin activity that can be found associated with the bacterial cell envelope and also in the supernatant of bacterial cultures (Wolff *et al.*, 1980). It is a member of the RTX toxin family and like all of these it is pore-forming and cytolytic. The C-terminal portion of the toxin confers the haemolytic activity and this penetrates the target eukaryotic cell membrane and facilitates the transport of the N-terminal portion into the target cell. The N-terminal portion is then cleaved, releasing the adenylate cyclase enzymic moiety which is then activated by the eukaryotic protein

calmodulin. The active adenylate cyclase causes unregulated synthesis of cAMP and is cytotoxic to a range of cells including the immune-effector cells (Confer and Eaton, 1982; Hewlett and Gordon, 1988; Hewlett *et al.*, 1991; Rogel and Hanski; 1992). This enables *B. pertussis* to survive in the host and cause infection. The immuno-compromising action of the ACT could also result in the host becoming more susceptible to secondary infections (Confer and Eaton, 1982).

Studies on ACT in mice (Guiso *et al.*, 1989) and *in vitro* (Hewlett and Gordon, 1988) have confirmed that it plays an important role in *B. pertussis* infection. *B. pertussis* mutants lacking ACT have been shown to be less virulent than the wild-type strain (Weiss *et al.*, 1983) and displayed a decreased survival rate within human macrophages (Masure, 1992). Further work showed that mutants lacking adenylate cyclase or haemolytic activities were unable to initiate infection (Khelef *et al.*, 1992).

The important role of ACT as a virulence factor of *B. pertussis* suggests that it would be useful as a component of acellular vaccines. Active immunisation of mice with purified adenylate cyclase-haemolysin protects mice against *B. pertussis* infection by significantly enhancing the clearance of the bacteria from the respiratory tract (Guiso *et al.*, 1991). However, it has been reported that the adenylate cyclase from *B. pertussis* may cross react with the adenylate cyclase produced in the human brain and this has raised doubts about its suitability as a vaccine component (Parton, 1998).

B. parapertussis and *B. bronchiseptica* produce ACT similar to *B. pertussis* but they are antigenically distinct (Hewlett and Gordon, 1988).

1.7.7. Pertactin (PRN)

PRN is a surface-associated protein that is produced by three of the *Bordetella* species. A form of pertactin designated as P.69 is produced by *B. pertussis* and has an apparent molecular weight of 69kDa in SDS-PAGE. *B. parapertussis* produces P.70 and *B. bronchiseptica* produces P.68 which, although of slightly different sizes, are homologous proteins (Li *et al.*, 1991, 1992).

In vitro studies have demonstrated that eukaryotic cells can bind to purified PRN and that like FHA, PRN mediates adhesion by a RGD sequence (Leininger *et al.*, 1991). The same study showed that mutants lacking PRN have reduced adherence properties but can still adhere which suggests that more than one adhesin is required for the binding of bacteria to host cells.

The P.68 protein protects pigs from *B. bronchiseptica* infection (Kobisch and Novotny, 1990) and the P.69 protects mice against aerosol challenge with *B. pertussis* (Shahin *et al.*, 1990). These experiments and the role of pertactin as an adhesin have prompted the inclusion of this protein in some of the new acellular vaccines (Willems and Mooi, 1996).

1.7.8. BrkA

Serum resistance is a newly-described virulence factor associated with *B. pertussis*. This locus is encoded by two open reading frames, BrkA and BrkB (Fernandez and Weiss, 1994), both of which are needed for serum resistance. BrkB is thought to be cytoplasmic and is used to transport BrkA to the outer-membrane. BrkA has

two RGD sequences and is involved in adherence and invasion, as well as in serum resistance.

The importance of Brk was shown by Fernandez and Weiss (1994) who demonstrated that a mutant in the Brk locus was more susceptible to serum killing, less virulent in mice and displayed less adherence and invasion of HeLa cells.

Although *B. parapertussis* and *B. bronchiseptica* contain *brk* sequences, the genes may be transcriptionally silent because the antigens could not be detected with an antibody to BrkA (Fernandez and Weiss, 1994).

1.7.9. Pertussis Toxin (PT)

PT is produced by only one *Bordetella* species, *B. pertussis*. It is the most studied toxin produced by a member of the *Bordetella* and, in toxoided form, is included in all acellular vaccine preparations. The toxin is composed of an A subunit and a B subunit (Tamura *et al.*, 1982). It is the B subunit that binds to cell surfaces and facilitates the transport of the A subunit which causes the toxic activity. The A subunit has ADP-ribosylating activity which acts upon the G proteins of target eukaryotic cells causing alteration in the response of these cells to external stimuli (Rappuoli and Pizza, 1991). This can result in a variety of consequences including: adhesion to eukaryotic cells (Tuomanen and Weiss, 1985) and macrophages, promoting leukocytosis, increased vascular permeability, anaphylaxis (Munoz and Bergman, 1977) and histamine sensitisation.

Although *B. pertussis* is the only member of the *Bordetella* to produce PT, both *B. parapertussis* and *B. bronchiseptica* contain transcriptionally silent PT genes

(Arico and Rappuoli, 1987). The PT gene in these bacteria is not expressed because of a defective promoter region. Monack *et al.* (1989) showed that the insertion of a functional PT operon into *B. parapertussis* leads to the synthesis of active PT and resulted in an increase in the pathogenic potential of the strain. However, *B. parapertussis* can still cause disease despite the absence of PT, which suggests that PT is not a necessity for causing whooping cough (Linneman and Perry, 1977; Heininger *et al.*, 1994; Wirsing von Konig and Finger, 1994).

1.7.10. Tracheal Colonisation Factor (TCF)

This protein is unique to *B. pertussis* and is found both on the surface of bacterial cells and released in the culture supernate. TCF has an RGD binding sequence and is suggested to function as an adhesin. This is supported by experiments which demonstrated that mutants lacking TCF had a decreased ability to colonise the trachea of mice (Finn and Stevens, 1995). TCF has only recently been discovered and its role in pathogenesis and immunity associated with *B. pertussis* is unknown.

1.7.11. Osteotoxin

This is produced only by *B. avium*. It is cytotoxic for mammalian cells, including osteogenic and tracheal cells (Gentry-Weeks *et al.*, 1993). It is possible that *B. avium* produces this protein to compensate for the lack of virulence factors it possesses when compared with other *Bordetella* species.

1.7.12. Piracy of adhesins

The piracy of adhesins phenomenon has been reported by Tuomanaen (1986) on studies using *B. pertussis*. The two main adhesins of *B. pertussis*, PT and FHA, are secreted into the surrounding medium and these were shown to bind to the surfaces of other bacteria such as *Haemophilus influenzae*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. This enhanced the adherence of these bacteria to ciliated cells and such an event could contribute to the secondary infections that are often associated with *B. pertussis* infection.

1.8. Adherence

Bordetella species mostly cause lower respiratory tract infections and therefore the ability to colonise and produce infection depends upon the production of a variety of adhesins. By producing adherence factors, such as FHA, PRN and PT, the *Bordetella* can adhere to the cilia of the tracheal epithelial cells thereby preventing their clearance from the respiratory tract. The adherence to, and subsequent colonisation of, ciliated epithelial cells plays a major role in the pathogenicity of *Bordetella* species and as such, a variety of assays has been used to observe the interaction of these bacteria with the cells of the host respiratory tract.

1.8.1. Adherence assays

The adhesion of *Bordetella* species to cells has been observed using many types of

assays including: tracheal organ culture, ciliated epithelial cells and established cell lines.

Tracheal organ culture most closely reflects the situation *in vivo*. Investigation of the interaction of *B. pertussis* with tracheal organ culture has been carried out using hamster (Collier *et al.*, 1977; Muse *et al.*, 1977) and baboon (Funnell and Robinson, 1993) trachea. Collier *et al.* (1977) showed that *B. pertussis* adhered only to ciliated cells resulting in damage and ultimately the loss of these cells. The non-ciliated cells of the same trachea were uninfected, which demonstrated the selective attachment of *B. pertussis* to ciliated cells. Further investigation showed that *B. pertussis* mutants deficient in FHA and those deficient in the production of fimbriae had reduced adherence to baboon tracheal rings (Funnell and Robinson *et al.*, 1993). The use of tracheal rings for investigation into the adherence mechanisms of *Bordetella* allows the study of the interaction between bacteria and the ciliated cells of the trachea. However, the systems discussed here do not study the interaction of *B. pertussis* with the trachea of the host species. Such a system would possibly be more relevant than the interaction with tracheal epithelial cells of a species that does not suffer from a *Bordetella* infection.

Bordetella species adhere to the ciliated respiratory epithelium (Yokomizo and Shimizu, 1979; Marks *et al.*, 1980) therefore the use of ciliated respiratory epithelial cells provides an opportunity to study the interaction of these bacteria with host cells. A study of *B. bronchiseptica* revealed that it attached preferentially to the cilia of ciliated epithelial cells (Jaques *et al.*, 1988) and a study of *B. pertussis* revealed that adherence was specific for the ciliary tuft close to the cell body (Tuomanen and Hendley, 1983). Ciliated respiratory epithelial cells have been

used to investigate the species specificity of *Bordetella* (Tuomanen *et al.*, 1983) and showed that *B. pertussis* and *B. parapertussis* adhered best to human ciliated cells whereas *B. bronchiseptica* adhered best to non-human mammalian cells. *B. avium* has been shown to adhere specifically to turkey ciliated epithelial cells (Tuomanen, 1988).

The use of ciliated cells allows study of the interaction of *Bordetella* with their natural target cells. However, these cells do not survive long in tissue culture (Tuomanen, 1988). Therefore, non-ciliated cell lines provide an alternative method for investigating the adherence of these bacteria.

A variety of cell lines has been used in adherence assays for *B. pertussis* including: human buccal epithelial cells (Plotkin and Bemis, 1984), Vero cells (Gorringe *et al.*, 1985; Funnell and Robinson, 1993), HeLa cells (Sato *et al.*, 1981), HEp-2 cells (Geuijen, 1996) and WiDr cells (Urisu *et al.*, 1986). Different mechanisms of *B. pertussis* adherence have been demonstrated using HeLa, Vero, and WiDr cell lines. The adherence of *B. pertussis* to Vero cells involves FHA, PT and fimbriae (Gorringe *et al.*, 1985), whereas, the adherence to HeLa cells utilises FHA and fimbriae (Sato *et al.*, 1981; Redhead, 1985), and the adherence to WiDr cells utilises only FHA (Urisu, 1986). Although these cell lines are of use to observe the adherence of *B. pertussis* they are not ciliated and therefore do not reflect the situation which occurs *in vivo*. In addition, none of the cell lines mentioned are natural target cells for the *Bordetella* species so the relevance of using such cell lines is both limited and questionable.

1.9. Invasion

Bordetella species were thought to be non-invasive pathogens (Preston, 1988). However, there has been a number of reports recently on the invasive potential of these species. HeLa cells have been used in a number of studies to demonstrate the invasive capabilities of *B. pertussis* and *B. parapertussis* (Ewanowich *et al.*, 1989; Lee *et al.*, 1990). Ewanowich *et al.* (1989) reported the invasive capacities of their strains of *B. parapertussis* were comparable with those of *Salmonella* and *Shigella*, which are invasive as part of their disease process. By using cytochalasins B and D, which inhibit microfilaments, Ewanowich *et al.* (1989) indicated that the uptake of *B. parapertussis* is via an endocytic process. The same study showed that hyperimmune sera raised against FHA did not inhibit invasion by *B. parapertussis*, therefore, suggesting that FHA is not essential for the invasion of HeLa cells by *B. parapertussis*. Lee *et al.* (1990) suggested that FHA and PRN were both necessary for the invasion of *B. pertussis* in HeLa cells and it is therefore possible that these species have slightly different invasion mechanisms. The possibility of different invasion mechanisms by *Bordetella* species is further supported by Schipper *et al.* (1994) who used human colon carcinoma Caco-2 and lung carcinoma cells in their assays. They reported that a significant proportion of *B. bronchiseptica* could be found free in the cytoplasm of infected cells where they persisted, whereas, *B. pertussis* remained inside vacuoles and the number of viable cells decreased over time. Guzman *et al.* (1994) used mouse dendritic cells to investigate the invasive ability of *B. bronchiseptica*. Using transmission electron microscopy, they revealed that *B. bronchiseptica* grew and replicated intracellulary and suggested

that this may be due to *bvg*-down-regulated products. It has already been established that, unlike other *Bordetella* species, *B. bronchiseptica* has *bvg*-downregulated genes that enable its survival outside its natural host environment (Yuk *et al.*,1996). Therefore, it is possible that this process enables its multiplication inside host cells when the other species have yet to be shown to replicate in such circumstances.

Regardless of the mechanisms employed by *Bordetella* species to invade cells, the ability to invade host cells provides these organisms with a definite survival advantage and could contribute to a carrier status.

1.10. Interaction with host immune cells

Macrophages are an important part of the host immune system and as such, the ability to survive phagocytosis and remain viable within the cell provides a bacterium with a significant survival advantage.

Cheers and Gray (1969) were the first to observe that *B. pertussis* could persist intracellularly within murine alveolar macrophages experimentally. Further experimental investigations showed that *B. pertussis* could survive *in vitro* within rabbit alveolar macrophages for 4 h (Saukkonen *et al.*, 1991) and human macrophages for 3 d (Friedman *et al.*, 1992). However, the first report of intracellular *B. pertussis* occurring naturally was by Bromberg *et al.* (1991) who detected *B. pertussis* within the pulmonary alveolar macrophages of children with human immunodeficiency virus infection. *B. bronchiseptica* has been reported to survive *in vitro* within murine macrophages (Banemann and Gross, 1997) and

murine macrophage-like cell lines (Forde *et al.*, 1998).

Using macrophages and polymorphonuclear leucocytes, possible mechanisms employed by *B. pertussis* and *B. bronchiseptica* for the uptake and survival within phagocytes have been suggested. *B. pertussis* can adhere to macrophages via PT and FHA (Relman *et al.*, 1990; Saukkonen *et al.*, 1991; van't Wout *et al.*, 1992). PT carries carbohydrate-recognition domains which can mediate attachment of *B. pertussis* to carbohydrates present on the macrophage cell surface (Saukkonen *et al.*, 1991). Relman *et al.* (1990) determined that the RGD sequence of the *B. pertussis* adhesin FHA interacts with the integrin CR3 on the macrophage surface. Saukkonen *et al.* (1991) showed that this interaction promoted the phagocytosis of *B. pertussis* into mammalian macrophages. The importance of the presence of FHA is demonstrated by Friedman *et al.* (1992) who found that FHA mutants resulted in decreased internalisation within macrophages. Intracellular uptake via CR3 does not trigger an oxidative burst (Wright and Silverstein, 1983) and this may promote intracelluar survival of *B. pertussis* within the macrophage. Interaction with macrophage CR3 to induce phagocytosis is common among intracellular pathogens such as *Legionella pneumophila* (Payne and Horwitz, 1987) and *Leishmania donovani* (Wilson and Pearson, 1988) therefore this mechanism suggests an important role for intracellular persistence during *B. pertussis* infection.

The survival of *B. pertussis* within macrophages is suggested to be due to the production of AC which increases the intracellular cAMP concentration thereby preventing the fusion of the *B. pertussis*-containing phagosome and the lysosome (Masure, 1993). This is supported by evidence that AC mutant strains had a

decreased ability to enter and survive intracellularly (Friedman *et al.*, 1992) and drug-induced elevation of cAMP in macrophages complemented the survival of AC mutants (Masure, 1993). The AC-HLY (adenylate cyclase-haemolysin) of *B. pertussis* also induces host macrophage apoptosis (Khelef *et al.*, 1993b; Khelef and Guiso, 1995) and AC and PT inhibit the oxidative burst of macrophages (Confer and Eaton, 1982). This could allow prolonged intracellular survival of the bacteria. The production of virulence factors appears to increase the uptake and survival of *B. pertussis* within macrophages (Friedman *et al.*, 1992). This is in contrast with the method of macrophage entry and intracellular survival employed by *B. bronchiseptica*. The expression of *bvg*-activated virulence factors is not necessary for the uptake and intracellular survival of *B. bronchiseptica*. Intracellular uptake and survival of *B. bronchiseptica* within macrophages was found to increase in the absence of *bvg*-dependent virulence factors. Phagosome-lysosome fusion occurred when *B. bronchiseptica* was within the phagosome (Banemann and Gross, 1997). This is in contrast to *B. pertussis* which inhibits the fusion of the phagosome and lysosome (Steed *et al.*, 1991; 1992). The fusion would expose *B. bronchiseptica* to attack by reactive oxygen metabolites and lysosomal material, yet *B. bronchiseptica* can survive within murine macrophages (Banemann and Gross, 1997) suggesting that negatively-regulated virulence factors may contribute to the intracellular survival. These findings suggest that there may be an intracellular phase during *B. bronchiseptica* and *B. pertussis* infections.

1.11. Genetic regulation

The expression of *Bordetella* virulence factors is regulated by the *Bordetella* virulence gene (*bvg*) locus which is composed of two genes, *bvg*A and *bvg*S. The BvgS protein is located in the cytoplasmic membrane and acts as a sensor of environmental signals. This passes messages across the membrane to the BvgA protein which is found in the cytoplasm (Stibitz and Yang, 1991). BvgA then activates the transcription of the virulence factors encoded by the virulence-activated genes (*vag*) and the repression of the virulence-repressed genes (*vrg*) (Weiss *et al.*, 1983).

BvgAS positively regulates most of the virulence factors presumed to be required for the initiation and establishment of infection including, PT, FHA, ACT, HLT, PRN and fimbriae. These are encoded by the *vag* genes. The motility of *B. bronchiseptica* and *B. avium* is negatively regulated by the BvgAS system (Akerley *et al.*, 1992; Yuk *et al.*, 1996) and is encoded by the *vrg* genes. This provides these species with a possible mechanism of survival outwith the host.

1.11.1. Antigenic modulation

Bordetella species have the ability to switch between virulent (Bvg^+) and avirulent (Bvg^-) phases. This is called antigenic modulation and occurs in response to changes in the environment such as low temperatures and the presence of modulators such as NaCl, $MgSO_4$ and nicotinic acid (Lacey, 1960). Antigenic modulation is freely reversible and affects all of the cells in the population.

1.11.2. Phase variation

Phase variation also involves the switch from the Bvg^+ phase to Bvg^- phase, however, it is rarely reversible. This occurs when there is frameshift mutation or a deletion in the *bvg*S gene (Stibitz *et al.*, 1989) and arises at a frequency of 1 per 10^3-10^6 organisms.

1.12. Vaccines

In 1974-1975 whooping cough vaccine uptake in the UK decreased from around 80% to 40% because of concern about the possible harmful side effects of vaccination. The decrease in immunisation of the susceptible population resulted in an increase in incidence of whooping cough in 1978-1979 when the next peak of the epidemic cycle occurred (Noble *et al.*, 1987).

In general, vaccination programmes against whooping cough have been very effective in reducing the incidence of this disease but there are concerns that, although the current whole-cell vaccines protect against disease, they do not protect against infection (Willems and Mooi, 1996). This can lead to adults with waning immunity becoming an important reservoir of infection for those who are susceptible (Aoyama *et al.*, 1992). It could also explain why, in many countries with high uptake of the vaccine, there are still peaks in the incidence of pertussis every 3-4 years (Fine, 1988). It also provides evidence that the whole-cell vaccines have not significantly affected the circulation of *B. pertussis* in the human population (Fine and Clarkson, 1987; Grenfell and Anderson, 1989).

Whole-cell vaccines do give good protection against whooping cough but the concerns about the safety of these vaccines and their reactogenicity prompted the search for safer vaccines (Parton, 1998). The extensive knowledge of pertussis antigens enabled the production of acellular vaccines which contain what are considered to be the major virulence factors of *B. pertussis* involved in infection: pertussis toxin in toxoided form (PTd), filamentous haemagglutinin (FHA), fimbriae and pertactin (PRN) (Willems and Mooi, 1996). Acellular vaccines have been used in Japan since 1981 and have shown to be effective and resulted in a lower rate of side effects compared to whole-cell vaccines (Kimura and Kuno-Sakai, 1990). However, as with the whole-cell vaccines, the acellular vaccines appear to protect only against the disease and not infection, which still leaves the problem of an adult reservoir of infection (Aoyama *et al*, 1993). This finding is supported by one of the first field trials of two acellular vaccines, during 1986-1987 in Sweden. The vaccines used were a monocomponent PTd vaccine and a two-component PTd and FHA vaccine (Ad Hoc Group, 1988). The reactogenicity of these vaccines was low but the efficacy was less than would have been expected for whole cell vaccines. However, both these vaccines gave reasonable protection against the disease and provide a safer alternative to the whole-cell vaccines. Further testing of other acellular vaccine formulations has supported the earlier findings that they have a reduced reactogenicity and are safer than whole-cell vaccines. These vaccine trials have shown that the most efficacious acellular vaccines contained components such as FHA, PRN or agglutinogens (fimbriae) in addition to PTd (Plotkin and Cadoz, 1997).

Although the monocomponent PTd vaccines did give good protection against *B.*

pertussis, these vaccines would not protect against *B. parapertussis* infections because *B. parapertussis* does not produce PTd. *B. parapertussis* also causes whooping cough albeit usually less severe and less frequently than *B. pertussis* but, it still has the potential to cause a severe infection. Therefore, more antigens than PTd alone need to be components of an acellular vaccine if it is to be completely successful against whooping cough. Studies have shown a lack of cross protection between *B. pertussis* and *B. parapertussis* due to subtle differences in their antigens (Khelef *et al.*, 1993a). However, a recent vaccine trial comparing a whole-cell vaccine to an acellular vaccine containing PT, FHA, PRN and fimbriae demonstrated that the acellular vaccine provided better protection against *B. parapertussis* infection (Heininger *et al.*, 1998).

1.13. Respiratory tract

1.13.1. Structure of the respiratory tract

The respiratory tract of all mammals includes the nasal cavities, which extend through the nasopharynx and oropharynx to the trachea. The trachea divides into two bronchi that extend into the lungs. Inside the lungs, the bronchi are subdivided into bronchioles, which branch into the alveoli.

Several mechanisms protect the respiratory tract from infection. The nasal turbinates have ciliated cells and a mucus lining. Bacteria which become trapped in the mucus are expelled from the nasal cavity by ciliary activity and swallowing or by sneezing. The same protection mechanism exists in the trachea, which is lined with ciliated epithelial cells. Here, the coughing reflex may help to expel any

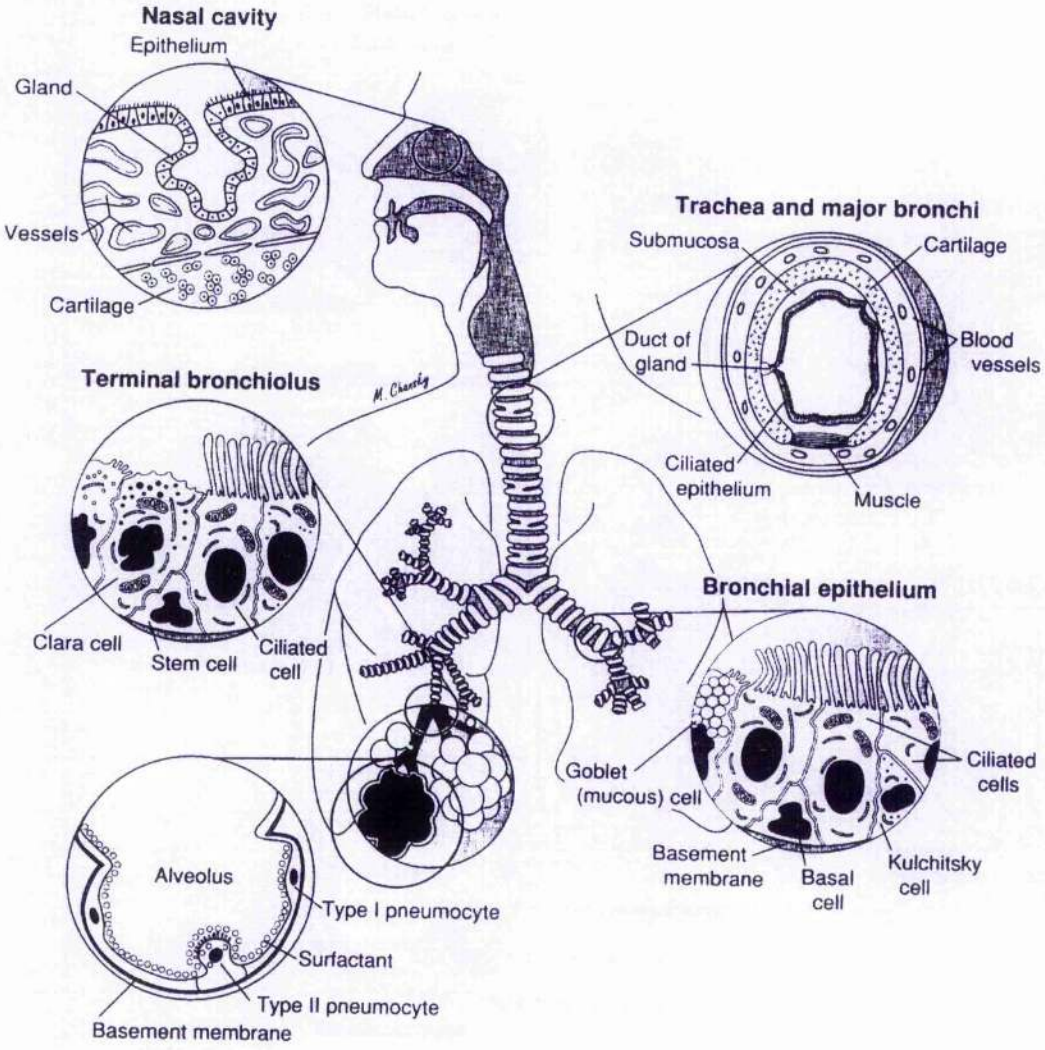


Fig 1.1. Diagram of human respiratory tract. Damjanov (1996).

trapped bacteria. Respiratory secretions contain many factors which have either antimicrobial properties or prevent the establishment of bacterial infection in the respiratory tract. These include lung surfactant, lysozyme, lactoferrin and secretory IgA. If these defence mechanisms are evaded, the bacteria finally enter the alveoli where they are ingested by alveolar macrophages. Once ingested, bacteria are either killed or persist intracellulary.

1.13.2. Composition of respiratory fluids

Respiratory tract secretions from humans contain approximately 95% water, proteins and glycoproteins (2-3%), lipids (1%) and minerals (1%) (Matthews *et al.*, 1963). There are few reports on the contents of respiratory secretions and those that are available present different amounts. The amounts given here are for human secretions.

The protein content of respiratory secretions has been reported to be between 4.2 mg ml^{-1} and 0.068 mg ml^{-1} (Low *et al.*, 1978; Kaliner, 1991). Albumin is the major serum protein of the secretions and is reported to be present in respiratory secretions at a concentration of 1.42 mg ml^{-1} (Reynolds and Newball, 1974) and in TBW, which are more dilute, at 17.4 μg ml^{-1} (Low *et al.*, 1978). Brogan *et al.* (1975) reported the concentration of lysozyme to be 0.16-0.7 mg ml^{-1} and lactoferrin to be 0.05-0.5 mg ml^{-1}. Other protein constituents include immunoglobulins. Reynolds and Newball (1974) measured the IgA concentration at 0.91 mg ml^{-1}, IgG 0.17 mg ml^{-1} and IgE 73.2 ng ml^{-1}.

The carbohydrate concentration of respiratory secretions has been reported to be

9.51mg ml^{-1} (Matthews *et al.*, 1963) and 0.008 mg ml^{-1} in TBW (Low *et al.*, 1978). Matthews *et al.* (1963) measured the total lipid concentration of respiratory secretions to be 8.4 mg ml^{-1}. The lipid content of human TBW was measured in three parts by Low *et al.* (1978). Non-polar lipids were present at a concentration of 77.8 μg ml^{-1}, polar lipids at 44.1 μg ml^{-1} and phospholipid at 1.09 μg ml^{-1}. The same study found the phospholipid of human TBW to be 83.8% phosphatidylcholine which is the lung surfactant providing surface tension in the lung to prevent collapse. The amino acid content of human respiratory secretions has been reported by Potter *et al.* (1967) to be 31.8 mM. The analysis of human respiratory secretions by Matthews *et al.* (1963) determined the chloride concentration to be 1.62 mg ml^{-1}, sodium 1.65 mg ml^{-1}, potassium 0.132 mg ml^{-1} and calcium 0.062 mg ml^{-1}.

Low *et al.* (1978) also measured the different types of immune cells in human TBW. Of these, 94.8% were macrophages, 3.5% were lymphocytes and polymorphonuclear leukocytes accounted for 1.7%.

The composition of respiratory secretions is known to change when the subject is in the disease state. Matthews *et al.* (1963) reported that patients with cystic fibrosis had higher concentrations of protein, lipid and DNA in respiratory secretions when compared with healthy people. It has also been reported that the amino acid content of washings increases with the severity of infection (Potter *et al.*, 1967).

1.14. Aims of Thesis

Previous studies have shown that ovine and human isolates of *B. parapertussis* are genetically distinct and that variation exists within isolates from the same host species (Porter *et al.*, 1994 and 1996; van der Zee *et al.*, 1996). However, there has been little investigation into the virulence capabilities of these isolates. The aims of this study were to compare the virulence-related properties of ovine and human *B. parapertussis* isolates and to determine if there was any host specificity.

The survival of *B. parapertussis* in the respiratory tract requires the ability to adhere to the respiratory mucosa and to grow in the respiratory fluid. In addition, *B. parapertussis* also encounters the alveolar macrophages of the respiratory tract and the ability to remain viable within these cells would provide *B. parapertussis* with a significant survival advantage. The aim, therefore, was to study these factors using *in vitro* assays designed to demonstrate any host specificity of ovine and human *B. parapertussis*.

Porter *et al.* (1995c) showed that ovine and human *B. parapertussis* reacted differently to monoclonal antibodies specific for LPS. This may be reflected by different LPS patterns on PAGE and this was also investigated.

Using multilocus enzyme electrophoresis, Porter *et al.* (1994) showed that ovine *B. parapertussis* isolates could be separated into two electrophoretic types. A representative from each of these groups (isolates H1 and K2) and two human isolates (B285 and NCTC 10520) were used throughout this study.

CHAPTER 2

MATERIALS AND METHODS

2.1. General methods

2.1.1. Bacteria and culture conditions

Throughout this work, the following bacterial strains were used.

Table 2.1. List of bacterial isolates.

Strains	Source	Reference
B. parapertussis		
H1, K2, C, J1	Ovine lung	Moredun Research Institute (Porter *et al.*, 1994)
NZ928	Ovine lung	Prof. B. Manktelow, Massey University, New Zealand
B285, B268, B271	Human	Dr. A. van der Zee, National Institute for Public Health and Environmental Protection, The Netherlands
NCTC 10520, NCTC 5952	Human	Dr. R. Parton, University of Glasgow
P. haemolytica		
A2 (124/92)	Ovine	Moredun Research Institute
Corynebacterium bovis		Moredun Research Institute

All strains were stored on glass beads using the CRYOBANK system (Mast Diagnostics) where tubes containing glass beads and a hypertonic cryopreservative solution were inoculated with bacterial cultures then stored at –70°C. When required, the strains were recovered from storage and plated onto Bordet-Gengou

agar (Difco) supplemented with 15% sheep blood and incubated at 37°C for 2-3 days in a moist atmosphere. Further culture of *B. parapertussis* was either in nutrient broth (Difco) or cyclodextrin liquid medium (Imaizumi *et al.*, 1983). *P. haemolytica* was grown overnight at 37°C on agar containing 5% sheep blood and *C. bovis* was grown on 5% sheep blood agar for 2 days at 37°C.

2.1.2. Haemagglutination assay

This was based on a haemagglutination assay developed by Ishikawa and Isayama (1988). Briefly, bacteria were resuspended in phosphate-buffered saline (PBS, pH 7.4) and standardised to an $A_{680} = 1.0$. Serial doubling dilutions were made in U-well microtitre plates, each well containing 50 µl of a bacterial suspension. 50 µl of 1% sheep blood in PBS was added to each well. The plates were incubated at 37°C for 1 h. The end point was taken as the first dilution where a button of red blood cells appeared at the bottom of a well. The greater the dilution at the end point, the more filamentous haemagglutinin produced.

2.2. Growth studies

2.2.1. Collection of lung washings

Ovine tracheobronchial washings (TBW) were collected and prepared as described by Sutherland (1989). Briefly, the lungs and tracheas were excised from sheep and washed with 500 ml PBS. The lungs were massaged and the fluid poured into a

container through a gauze-covered filter. This was repeated once and a total volume of approximately 500 ml was recovered. The washings were centrifuged at 500 x g to pellet any cellular debris and the supernatant collected. The supernate was further centrifuged at 2000 x g for 30 min to pellet any bacteria present then filtered through a 0.45 μm filter. The TBW were plated onto blood agar overnight at 37°C to check for sterility then aliquoted and stored at -70°C.

2.2.2. Growth experiments

The method was based on that used by Porter and Wardlaw (1994). Bacteria were recovered from storage at –70°C and grown for 3 days on Bordet-Gengou agar at 37°C. The cultures were resuspended in PBS and the bacterial suspensions were standardised to A_{540} = 0.45 which gave a concentration of approximately 1 x 10^8 cfu ml^{-1}. The bacteria were pelleted at 11600 x g for 10 min then washed three times and resuspended in PBS. 25 μl of bacterial suspension was added to 975 μl of test fluid to give a bacterial concentration of approximately 1 x 10^7 cfu ml^{-1}. The number of viable bacteria was confirmed by plate counts on blood agar. The inoculated fluids were incubated for 72 h at 37°C on a shaker at 135 rpm. Counts of viable bacteria were taken every 24 h by plating dilutions onto blood agar.

2.2.3. Purification of IgG

IgG was purified from TBW 5 using a protein G column (MAb Trap GII, Pharmacia) according to the manufacturer's instructions. Briefly, the column was washed with 5ml of distilled water to remove the ethanol preservative. From this

point, all fractions were collected in volumes of 1ml and the absorbance of each fraction was measured at 280 nm. The column was equilibrated with 4ml of the supplied binding buffer then 1ml of the sample TBW was applied. The column was then washed with 7–10 ml of binding buffer until no absorbance was measured in the effluent. This was followed by elution with 3–5 ml of the provided elution buffer. At this point, the IgG was eluted from the column and the column was reconditioned with 5 ml of binding buffer.

The concentration of IgG present in TBW 5 and the amount purified was analysed in the Biochemistry Department, MRI by J. Small.

2.2.4. SDS-PAGE

Samples were dissolved in sample buffer (2% w/v sodium dodecyl sulphate (SDS), 5% w/v glycerol, 2% v/v 2-mercaptoethanol, 0.002% bromophenol blue and 125mM Tris/HCl, pH 6.8) and heated in a boiling water bath for 5 min. Samples were then resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% (w/v) acrylamide gel using the discontinuous buffer system of Laemmli (1970). Proteins were detected by Coomassie blue and/or silver staining. (All reagents for SDS-PAGE are detailed in Appendix I).

2.2.5. Coomassie blue staining of SDS-PAGE gels for protein

Coomassie blue stain was added to a gel and shaken for a minimum of 2 h at room temperature. The gel was then placed in destaining solution and shaken at room

temperature until the required destaining was achieved.

2.2.6. Silver staining of SDS-PAGE gels for proteins

Silver staining of gels was achieved using the method of Morrisey (1981).

Gels were incubated for 30 min in 50% v/v methanol and 10% v/v acetic acid followed by incubation in 5% v/v methanol and 7% v/v acetic acid for 30 min and 10% v/v glutaraldehyde for a further 30 min. Following an overnight wash in distilled water the gel was added to 0.005 mg ml^{-1} dithiothreitol for 30 min then incubated in 0.1% w/v silver nitrate for 30 min and rinsed once in a small amount of distilled water. The gel was developed in 100 ml of 3% sodium carbonate solution with 500 µl of 37% formaldehyde and the reaction stopped by the addition of 7 ml of 2.3 M citric acid.

2.2.7. Western blotting for proteins

SDS-PAGE gels were electroblotted onto nitrocellulose (Schleicher & Schuell) by the method of Towbin *et al.* (1979). Nitrocellulose was blocked using 10 mg ml^{-1} BSA in blot wash buffer (BWB) for 1 h at room temperature with gentle agitation then washed three times in BWB. For detection, the nitrocellulose was incubated at room temperature for 1 h with a test fluid then washed as described above. Nitrocellulose was then incubated for 1h at room temperature with horseradish-peroxidase conjugated to donkey anti-sheep IgG (Scottish Antibody Production Unit) and again washed in BWB. Substrate colour development was carried out at

room temperature in 100 mM Tris pH 7.0, 0.5 mg ml^{-1} diaminobenzidine and 2 µl ml^{-1} of 30% hydrogen peroxide. The reaction was terminated by the addition of water. (All reagents detailed in Appendix 1).

2.2.8. Analysis of TBW

Analysis of TBW was carried out in the Biochemistry Department, MRI by J. Small.

Albumin, calcium, cholesterol, glucose, lipase, magnesium, phosphorus and total protein were analysed using commercial kits adapted for a Monarch 2000 centrifugal analyser. Sodium, potassium and chloride were measured using an ion-specific electrode on a Monarch 2000 centrifugal analyser. IgG was estimated turbidmetrically using the method of Seneviratne and Moores (1980) which was adapted to a Monarch 2000 centrifugal analyser. The iron present in TBW was analysed using a PU9200X atomic absorption spectrophotometer.

2.2.9. API analysis

API ZYM (bioMerieux) was used to measure the production of enzymes by the different *B. parapertussis* isolates. Bacterial cultures were suspended in sterile distilled water to give a suspension with turbidity between a McFarland No 5 and No 6 standard (bioMerieux). The API ZYM strips were inoculated with the different bacterial suspensions and incubated for 4 hours at 37°C. After incubation, ZYM A and ZYM B reagents were added to each strip and a colour

reaction developed. The colour intensity was proportional to the amount of enzyme produced. This system allowed the measurement of the following enzymes: alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphthol-ASBI-phosphohydrolase, α galactosidase, β galactosidase, β glucoronidase, α glucosidase, β glucosidase, N-acetyl-β glucosaminidase, α mannosidase, α fucosidase.

2.3. Bacterial adherence

2.3.1. Adherence assay using cell lines

The cells were obtained from the Tissue Culture Department, MRI. Foetal lamb turbinate cells, foetal lamb lung cells and human foetal lung cells (MRC-5) were provided in 8-well Permanox chamber slides (Lab-Tek) at a concentration of 1 x 10^5 cells ml^{-1}. The foetal lamb cells were grown in 199 medium (Difco) supplemented with 15 % tryptose phosphate broth and 0.65% sodium bicarbonate. Before use, 10% heat-inactivated foetal bovine serum (FBS) and 1% glutamine was added to the medium (199S). The MRC-5 cells were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 1% basic amino acid solution and 10% FBS. Bacteria were taken from 72 h cyclodextrin liquid cultures. The bacteria were pelleted at 11600 x g for 10 min then washed three times in 199S. The bacterial suspensions were then standardised to give an infectivity ratio of approximately 30 bacteria per cell. The cells were washed three times with Hank's balanced salt solution (Hanks) and 250 µl of a bacterial suspension was added to each well. The slides were incubated for 1 h at 37°C in 5% CO_2. After

incubation, the slides were washed three times in Hanks, to remove non-adherent bacteria, then fixed with 1% formol saline for 30 min. The slides were then Gram-stained and the number of bacteria adhering to 100 cells was counted.

2.3.2. Adherence assay using tracheal organ culture

This was based on the method described by Funnell and Robinson (1993). Trachea were excised from sheep and stored in Hanks solution during transportation. The trachea were then cut into rings and placed in wells of a 6-well tissue culture dish (Life Technologies) where they were held in place with molten 1.5% w/v bacteriological agar (Difco). A bacterial suspension of approximately 3 x 10^6 cfu ml^{-1} in EMEM supplemented with 10% FBS and 1% HEPES was added to the inside of each ring. The rings were then incubated for 1 hour at 37°C in 5% CO_2. The rings were washed three times in Hanks and the inside of the rings scraped using a sterile scalpel blade and this was resuspended in EMEM. Serial dilutions of this were plated onto sheep blood agar to determine the number of bacteria that had adhered to the tracheal rings.

B. parapertussis H1 culture supernate was obtained from cultures in cyclodextrin liquid medium that had grown to a concentration of approximately 1x10^8 cfu ml^{-1}. The cultures were centrifuged at 11600 x g for 5 min to pellet the bacterial cells and the supernate was filtered through a 0.45 μm filter.

2.4. Invasion

The ability of different isolates of *B. parapertussis* to invade cell lines was

investigated using a method based on that described by Ewanowich *et al.* (1989). The cell lines and conditions were the same as for the adherence assay using cell lines except that the bacteria and cells were incubated together for 5 h at 37°C in 5% CO_2. The cells were washed three times in Hanks then incubated in 100 μg ml^{-1} gentamicin in 199S medium for 90 min at 37°C in 5% CO_2. Following a further washing step, the cells were lysed with 1% saponin in PBS containing 2% FBS. From this, serial dilutions of the lysate were plated onto sheep blood agar to determine the number of bacteria that had invaded the cell lines and remained viable.

2.5. Macrophage interaction

2.5.1. Collection and culture of alveolar macrophages

Collection was as previously described in collection of lung washings but instead of discarding the pelleted cells they were kept. The remaining steps were as described by Sutherland (1989). If contaminating red blood cells were present they were removed by resuspending the pellet in 10 ml of 1x PBS. Double-distilled water (20 ml) was added and mixed quickly for 30 sec. This was followed by the addition of 10x PBS (2 ml). The suspension was then centrifuged at 800 x g for 5 min and the supernate, containing the cells, was removed. Cells were washed in Hanks balanced salt solution and resuspended in RPMI 1640 supplemented with 150 IU ml^{-1} nystatin, 50 IU ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Viability was determined with nigrosin (0.1%) and macrophages were counted using an improved Neubauer haemocytometer. Macrophage concentration was adjusted to

$1x10^6$ cells ml^{-1} and 100 µl added to 96-well tissue-culture plates. These were incubated at 37°C in 5% CO_2 until required.

2.5.2. Phagocytosis assay

This was based on the method described by M°Neil *et al.* (1994). Bacterial cultures were adjusted to approximately $3x10^6$ cfu ml^{-1}, washed and resuspended in RPMI without antibiotics. The macrophage cultures in 96-well tissue-culture plates were washed three times in Hanks, the bacteria added and incubated at 37°C. After incubation for 90 min, macrophages were washed three times in Hanks then RPMI containing 100 µg ml^{-1} of gentamicin was added to kill extracellular bacteria and the plates were incubated for a further 60 minutes. The cells were then washed three times with Hanks and macrophages were lysed by the addition of 100µl of 1% saponin in PBS containing 2% FBS. From this lysate, serial dilutions were plated onto sheep blood agar to determine the numbers of surviving intracellular bacteria.

For opsonisation, washed bacteria were incubated in antiserum at room temperature for 15 min.

2.5.3. Adherence to macrophages

This was based on the method described by Hondalus and Mosser (1994). Macrophages ($1x10^5$ ml^{-1}) and bacteria ($3x10^6$ cfu ml^{-1}) were prepared as above except that the macrophage culture was carried out in 8-chamber Permanox slides

(Lab-Tek). Each bacterial isolate was added to one chamber of a slide. After incubation for 90 min, the slides were washed and 3% formaldehyde was added to each chamber. The slides were incubated for 30 min and washed with Hanks then Gram-stained and left to dry. Using 1000x magnification and oil-immersion, the number of bacteria associated with 100 macrophages was counted.

2.5.4. Intracellular bacteria within macrophages

The assay was performed as above except that following the 90 min incubation, the slides were washed, then 100% methanol was added to each chamber. The number of bacteria associated with 100 macrophages was counted. Methanol permeabilises the macrophage cell membrane allowing the visualisation of both extracellular and intracellular bacteria. In order to calculate the number of intracellular bacteria within 100 macrophages, the number of bacteria adhering to 100 macrophages (as described in 2.5.3) was subtracted from the number of bacteria associated with 100 macrophages using methanol fixation.

2.5.5. Intracellular survival of bacteria in macrophages

The same methods were used as for the phagocytosis assay and for calculation of the number of adherent and intracellular bacteria except, after the addition of gentamicin for 60 minutes, the cells were washed three times in Hanks and 10 μg ml^{-1} polymyxin in RPMI was added to each well/chamber. The plates/slides were then incubated for a total of either 24h or 48h. The cells were then washed three

times with Hanks and the cells on 96-well plates were lysed as before and dilutions plated onto blood agar. The cells on chamber slides were fixed and stained as before.

2.6. Lipopolysaccharide (LPS) analysis

2.6.1. LPS preparation

Bacterial LPS was prepared using the micro phenol method of Fomsgaard *et al.* (1993). Bacteria were removed from Bordet-Gengou agar and resuspended in PBS then added to 2 ml of either nutrient broth or cyclodextrin liquid medium to give a final concentration of approximately 1 x 10^6 cfu ml^{-1}. The broths were incubated for 3 days at 37°C. 1.5 ml of the cultures was transferred to an Eppendorf tube. The cells were pelleted at 11600 x g, washed twice and resuspended in 500 µl of pyrogen-free water. Aqueous phenol (500 µl of 90% v/v) was added and the tube vortexed for 10 sec followed by placement in a water bath at 70°C for 10 min with vortexing at intervals. The tube was immediately placed on ice for a minimum of 2 min and centrifuged at 2900 x g for 4 min at room temperature. The upper clear layer was removed carefully with a pastette and stored at -20°C.

2.6.2. PAGE for LPS

Using the method of Hancock and Poxton (1988), LPS samples were boiled in sample buffer (0.125M Tris/HCl pH 6.8, 4% SDS, 20% glycerol, 2% 2-

mercaptoethanol and 0.002% bromophenol blue) for 5 min. The samples were resolved using 12% or 14% (w/v) gels using the discontinuous buffer system of Laemmli (1970). LPS was visualised using silver stain (see below). (Reagents for LPS PAGE detailed in Appendix I).

2.6.3. LPS silver stain

Using the method of Hancock and Poxton (1988), the gel was placed into a container with 200 ml of 25% v/v propan-2-ol and 7% v/v acetic acid overnight. The solution was discarded and replaced with 1.05 g of periodic acid in 150 ml of distilled water and 4 ml of the above solution for 5 min. The gel was then washed in at least 4 changes of distilled water for over 4 h. This was followed by incubation for 15 min in a solution consisting of 21 ml of 0.36% sodium hydroxide, 1.4 ml of ammonia solution (0.88 SG), 4 ml of 19.4% silver nitrate solution (which had been added slowly) topped up to 100ml with water. The gel was again washed in 4 changes of distilled water for over 40 min. The water was replaced with a freshly prepared solution of 0.005% citric acid in 0.019% formaldehyde and left to develop. When the desired staining was reached, the gel was repeatedly rinsed in large volumes of distilled water.

2.6.4. Western blotting of LPS

LPS PAGE gels were electroblotted onto nitrocellulose (Schleicher & Schuell) by the method of Hancock and Poxton (1988). After blotting overnight at 10-12 V,

the nitrocellulose was washed in Tris-buffered saline (TBS) for 10 min then blocked in 3% w/v gelatin in TBS for 30 min at room temperature. The nitrocellulose was incubated for 3 h with antiserum diluted in TBS followed by a brief rinse in distilled water and washed for two 10 min periods in Tween Tris-buffered saline (TTBS). The nitrocellulose was then incubated with horse-radish peroxidase conjugated to goat anti-rabbit (Bio-Rad) or donkey anti-sheep (Sigma) IgG diluted to a working concentration in 1% gelatin in TBS. Following a final washing step, the nitrocellulose was developed in 100 mM Tris pH 7.0, 0.5 mg ml^{-1} diaminobenzidine and 2 µl ml^{-1} of 30% v/v hydrogen peroxide. The reaction was terminated by the addition of water. (Reagents for western blotting of LPS PAGE are detailed in Appendix I)

2.7. Bactericidal assay

Bactericidal assays were carried out based on the method of Sutherland (1988), as adapted by M. Maley (personal communication). The assays were performed in flat-bottomed 96-well microtitre plates (Life Technologies). Bacteria were removed from Bordet-Gengou agar and resuspended in PBS to give a concentration of approximately 1 x 10^3 cfu ml^{-1}. 10 µl amounts of antiserum (heat inactivated at 56°C for 30 min) and 50 µl of bacterial cell suspension were incubated together in triplicate wells for 15 min at room temperature. 40 µl of SPF lamb serum was added to each well as a source of complement. The plates were sealed and incubated at 37°C for 0 and 45 min. The controls used were: 50 µl of bacterial suspension + 50 µl of PBS, to observe the effect of the diluent alone; 50 µl of

bacterial suspension + 10 µl of PBS + 40 µl of complement, to see the effect of the complement alone; 50 µl of bacterial suspension + 10 µl of antiserum + 40 µl of heat inactivated complement source, to observe the effect of antibody in the absence of complement. After incubation, 10 µl from each well was removed and inoculated onto sheep blood agar plates, which were incubated at 37°C for 3 days. The percentage of bacterial inoculum killed (%K) in each suspension was calculated using the following formula:

$\%K = 1 - (\text{mean cfu ml}^{-1} \text{ at } T_{45}/\text{mean cfu ml}^{-1} \text{ at } T_0) \times 100.$

2.8. Statistical analysis

Results were analysed for statistical significance using the Mann-Whitney test and repeated measures of analysis of variance (where mentioned).

CHAPTER 3

GROWTH OF OVINE AND HUMAN *BORDETELLA PARAPERTUSSIS* IN OVINE TRACHEOBRONCHIAL WASHINGS (TBW).

3.1. Introduction

B. parapertussis is a respiratory tract pathogen of both humans and sheep. Cullinane *et al.* (1987) and Porter *et al.* (1994) isolated this bacterium from the tracheobronchial washings (TBW) of both lambs and adult sheep. TBW are dilutions of the fluids that bathe the respiratory mucosae and therefore the fluids in which *B. parapertussis* grows *in vivo*. The isolation of *B. parapertussis* from two different host species suggests a potential for transfer between humans and sheep. If this is the case, then *B. parapertussis* should grow in both human and ovine TBW regardless of which species the bacterium was isolated from. This study monitored the growth of ovine and human isolates of *B. parapertussis* in TBW from 5 different sheep to determine if there was any host-specificity of the different isolates. Nutrient broth was used as a positive control and PBS (a non-nutrient fluid) was the negative control.

3.2. Materials and methods

Ovine TBW were used to monitor the growth of ovine and human *B. parapertussis*. The methods used for the collection of TBW, growth studies, IgG

purification, SDS-PAGE, Western blotting, TBW analysis and API tests are detailed in section 2.2

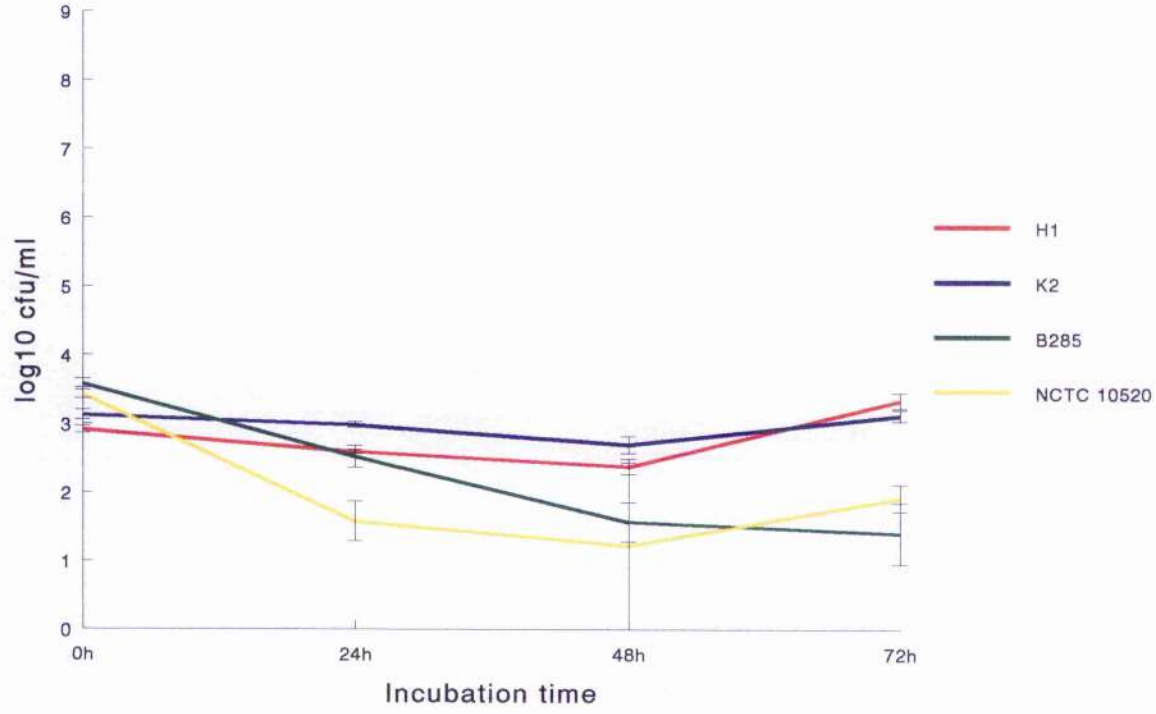
3.3. Growth in ovine TBW monitored by viable counts

B. parapertussis ovine isolates (H1 and K2) and human isolates (NCTC 10520 and B285) were inoculated into ovine TBW and incubated for 72 h at 37°C. Samples were taken every 24 h and plated onto blood agar to determine the number of viable bacteria. The growth of the different *B. parapertussis* isolates in the various fluids is represented in Fig. 3.1-3.7.

TBW 1 (Fig. 3.1) and 2 (Fig. 3.2) demonstrated little difference between the growth of ovine and human *B. parapertussis* isolates. The number of viable ovine *B. parapertussis* remained approximately the same as at the time of inoculation. This was also the case for the human isolates tested in TBW 2. In TBW 1, the number of viable human *B. parapertussis* cells declined over 72 h by one log unit. With TBW 3, 4 and 5 (Fig. 3.3-3.5), there was a steady increase in the number of ovine *B. parapertussis* and a decrease in the number of viable human *B. parapertussis*. The human isolates in TBW 3 declined in numbers in the first 24 h then slowly increased. The human strain B285 died in both TBW 4 and 5. NCTC 10520 died in TBW 5 and decreased in viable numbers in the initial 24 h in TBW 4 followed by a slow increase in numbers. In contrast, the ovine *B. parapertussis* grew slightly in TBW 4 whereas better growth was demonstrated in TBW 3 and 5. All the isolates grew in nutrient broth (Fig. 3.6). However, the ovine strains had a greater rate of growth than the human strains. PBS did not support the growth of

Fig. 3.1. Growth of ovine (H1 and K2) and human (B285 and NCTC 10520) *B. parapertussis* in ovine TBW 1 over 72 h at 37°C.

Fig. 3.2. Growth of ovine (H1 and K2) and human (B285 and NCTC 10520) *B. parapertussis* in ovine TBW 2 over 72 h at 37°C.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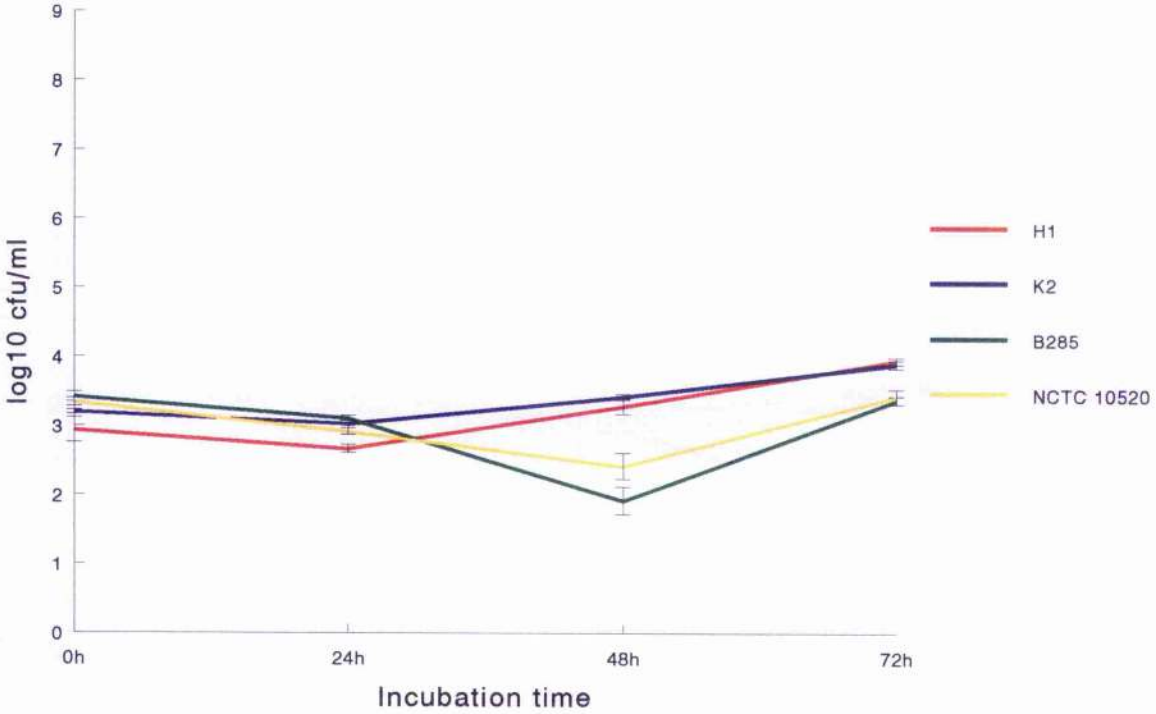
48h
72h
Incubation time
H1
K2
B285
NCTC 10520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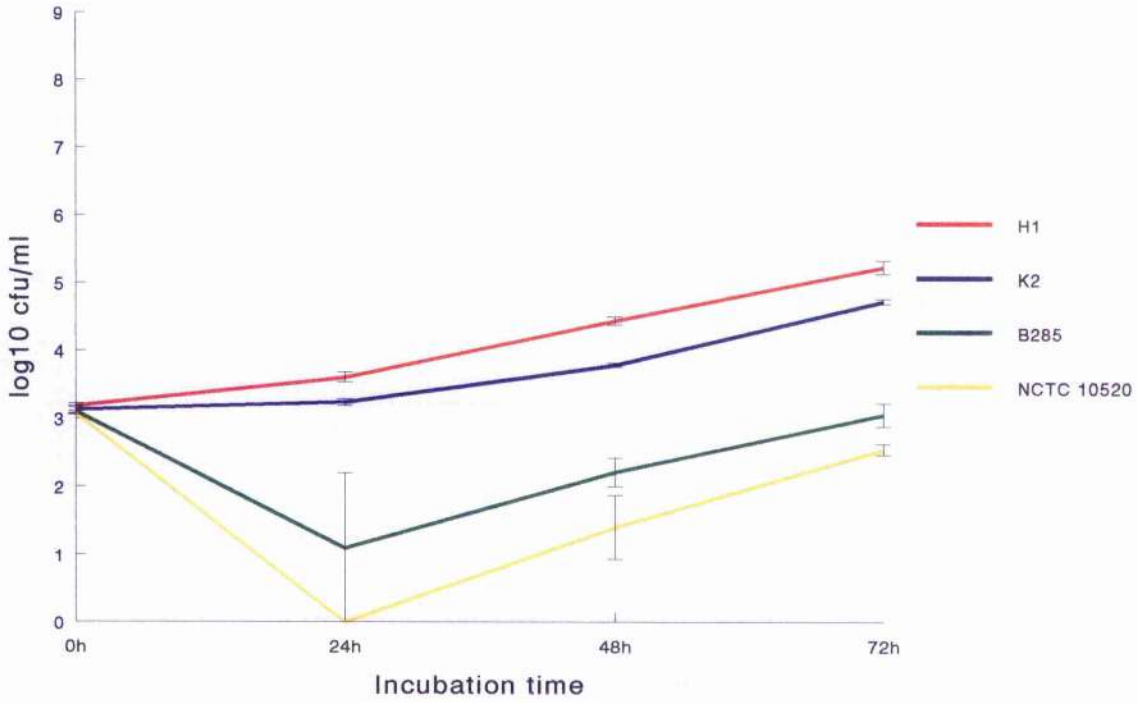
0h
24h
48h
72h
Incubation time
H1
K2
B285
NCTC 10520

Fig. 3.3. Growth of ovine (H1 and K2) and human (B285 and NCTC 10520) *B. parapertussis* in ovine TBW 3 over 72 h at 37°C.

Fig. 3.4. Growth of ovine (H1 and K2) and human (B285 and NCTC 10520) *B. parapertussis* in ovine TBW 4 over 72 h at 37°C.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Incubation time
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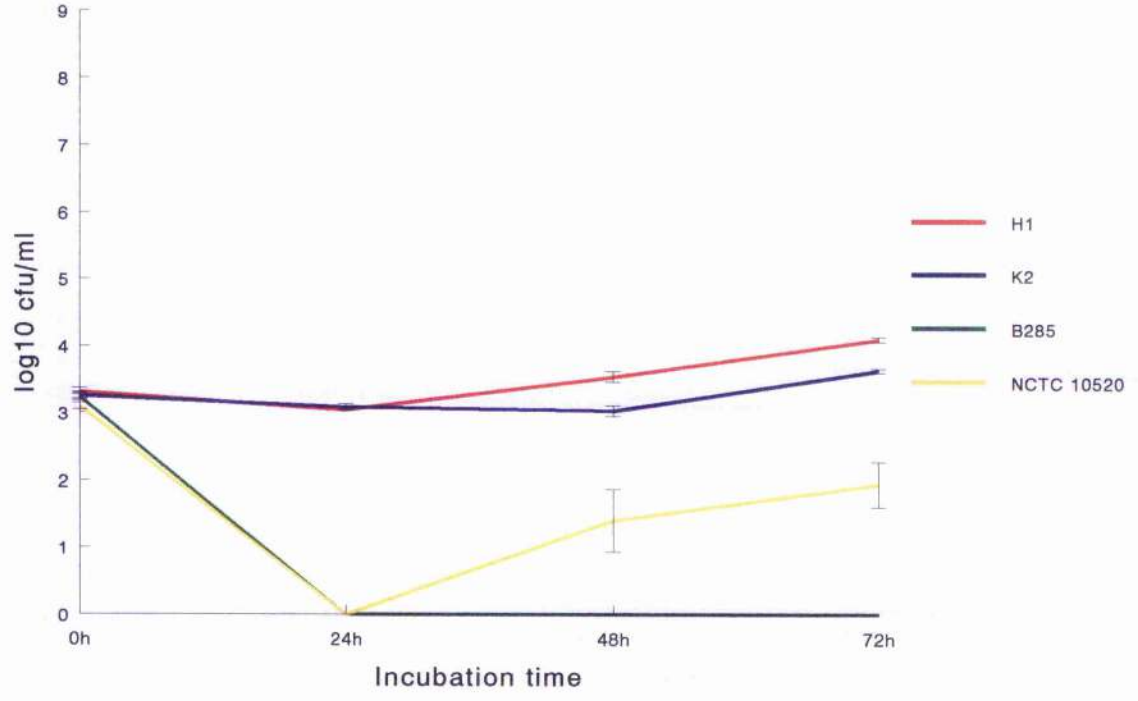
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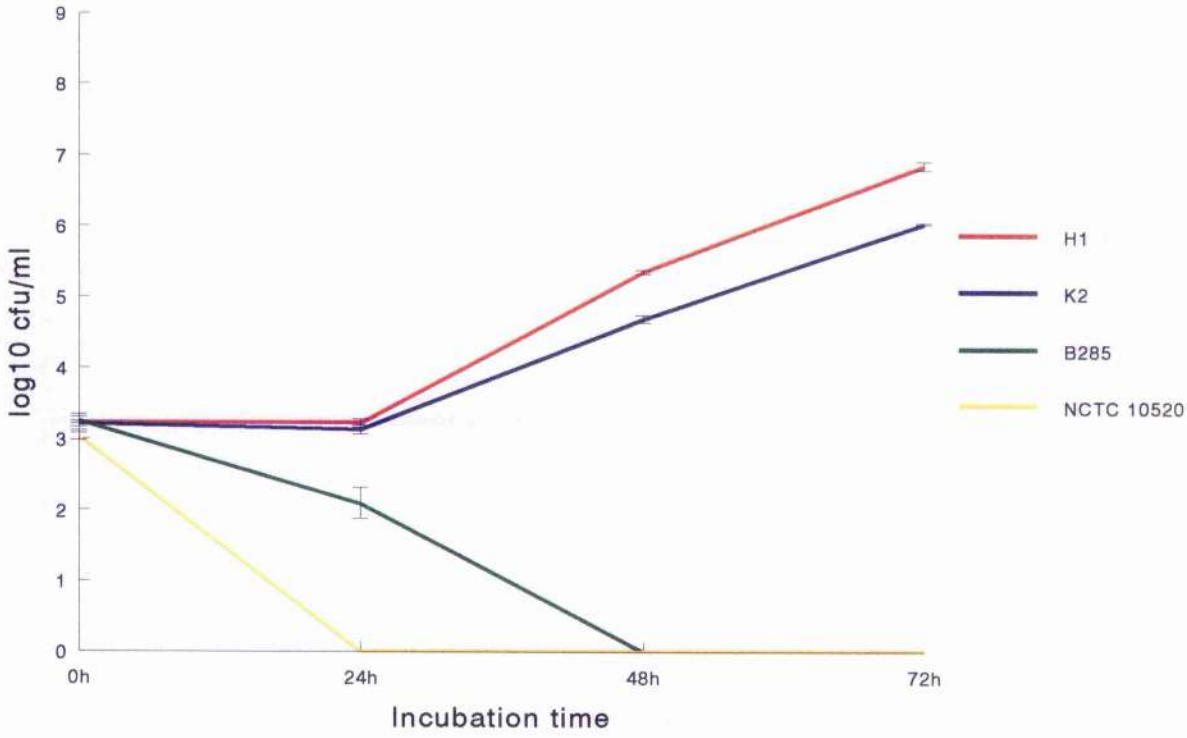
24h
48h
72h
Incubation time
H1
K2
B285
NCTC 10520

Fig. 3.5. Growth of ovine (H1 and K2) and human (B285 and NCTC 10520) *B. parapertussis* in ovine TBW 5 over 72 h at 37°C.


log10 cfu/ml
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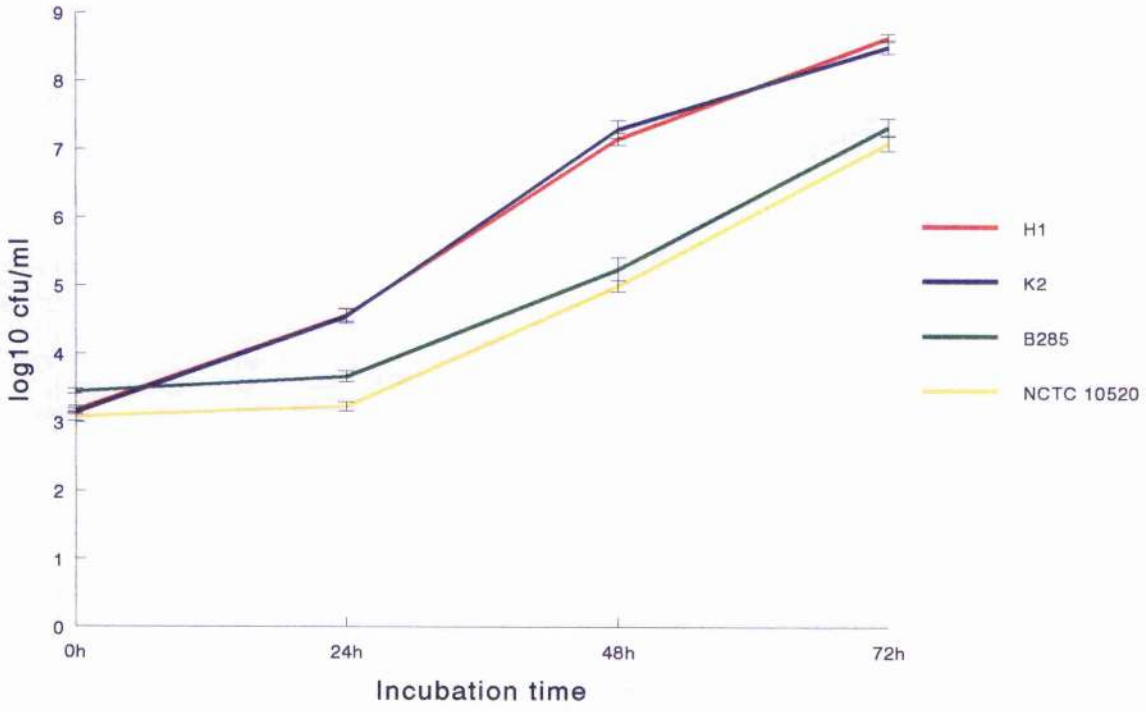
0h
24h
48h
72h
Incubation time
H1
K2
B285
NCTC 10520

Fig. 3.6. Growth of ovine (H1 and K2) and human (B285 and NCTC 10520) *B. parapertussis* in nutrient broth over 72 h at 37°C.

Fig. 3.7. Decline of viable ovine (H1 and K2) and human (B285 and NCTC 10520) *B. parapertussis* in PBS over 72 h at 37°C.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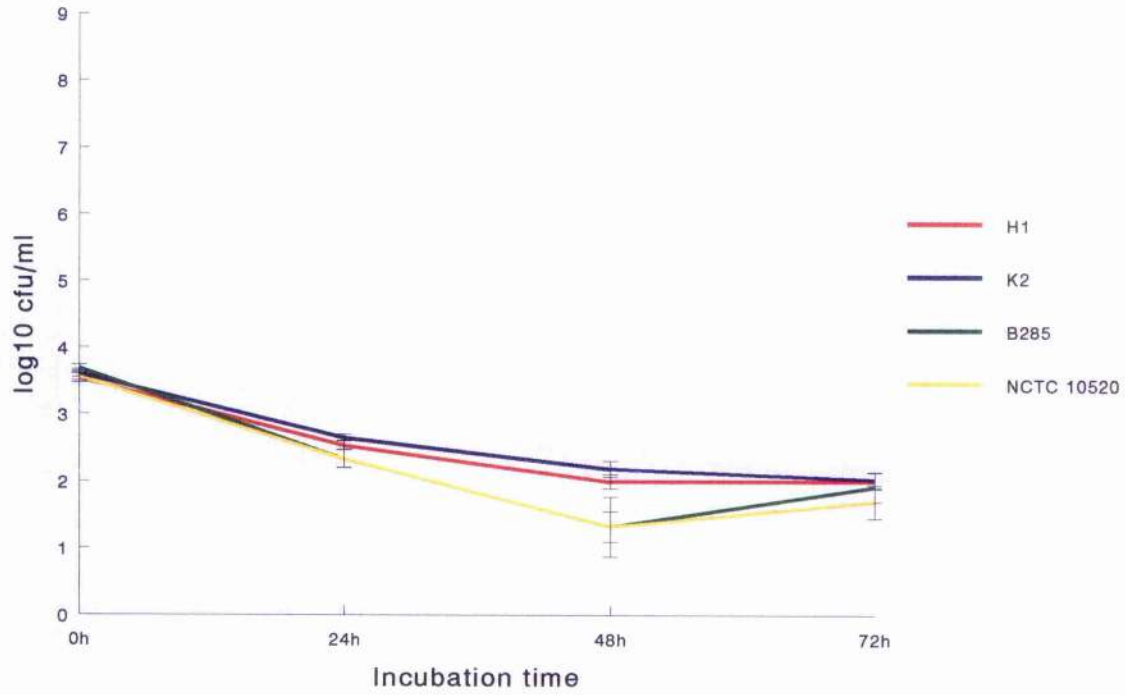
Incubation time
H1
K2
B285
NCTC 10520


log10 cfu/ml
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0h
24h
48h
72h
Incubation time
H1
K2
B285
NCTC 10520

any of the *B. parapertussis* isolates tested (Fig. 3.7). All of the isolates demonstrated a slow decline in viable numbers over 72 h.

3.4. Statistical analysis of growth of *B. parapertussis*.

The Mann-Whitney test was used to determine if the growth of human *B. parapertussis* at each time point was significantly different from the growth of ovine *B. parapertussis*.

Table 3.1. Significance of comparison of human vs. ovine *B. parapertussis* growth in various fluids at each time point using the Mann-Whitney test.

	Significance of difference between ovine and human *B. parapertussis*		
Growth fluid	24 h	48 h	72 h
TBW 1	0.006	0.003	0.002
TBW 2	0.13 *	0.001	0.001
TBW 3	0.0005	0.0007	0.0008
TBW 4	0.0003	0.0006	0.0006
TBW 5	0.0007	0.0003	0.0003
NB	<0.0001	<0.0001	<0.0001
PBS	<0.0001	<0.0001	0.04

This test confirmed that, in all but one comparison (*), the growth of the two ovine *B. parapertussis* isolates was significantly greater than that of the two human isolates.

Repeated measures of analysis of variance were used to determine if the overall growth of human *B. parapertussis* in each of the fluids tested was significantly lower than the growth of ovine *B. parapertussis*.

Table 3.2. Significance of the comparison of ovine and human *B. parapertussis* growth in fluids using repeated measures analysis of variance.

Growth fluid	Significant difference between ovine and human isolates
TBW 1	<0.001
TBW 2	0.001
TBW 3	<0.001
TBW 4	<0.001
TBW 5	<0.001
NB	<0.001
PBS	<0.001

Using repeated measures of analysis of variance, it was found that there was a statistically significant difference between the survival of ovine and human *B. parapertussis* in all TBW, nutrient broth and PBS.

3.5. Analysis of TBW

The 5 TBW were analysed for the presence of various nutrients and proteins. The results are presented in Table 3.3.

There was little difference in the concentration of components tested for between all the TBW. The most notable exception was TBW 5 which contained the highest concentration of most substances tested for, particularly total protein, magnesium, potassium, but it contained the lowest amount of glucose.

Table 3.3 Analysis of ovine TBW for potential nutrients (results in mmol l^{-1} unless stated).

	Concentration of components in:					
Component	TBW 1	TBW 2	TBW 3	TBW 4	TBW 5	PBS
Total Protein (mg ml^{-1})	1.21	0.76	1.26	0.58	1.76	ND
IgG (mg ml^{-1})	0.42	0.43	0.83	0.40	0.98	ND
Albumin (mg ml^{-1})	0.33	0.30	0.40	0.18	0.68	ND
Cholesterol	0.15	0.12	0.19	0.14	0.16	ND
Glucose	0.14	0.05	0.14	0.07	0.02	ND
Calcium	0.20	0.11	0.13	0.08	0.25	1.05
Chloride	140	141	137	139	141	124
Iron (µmol l^{-1})	5.00	5.30	5.80	5.90	6.00	ND
Magnesium	0.13	0.08	0.10	0.08	0.26	ND
Phosphorus	8.87	8.74	8.73	8.55	8.82	7.97
Potassium	5.03	4.73	4.78	4.69	8.02	4.05
Sodium	157	158	155	156	156	148

ND – not detectable.

3.6. Further analysis of TBW 5

The death of human isolates and growth of ovine isolates in TBW 5 revealed a difference between ovine and human *B. parapertussis*. It is possible that IgG was responsible for the death of the human isolates. IgG was purified from TBW 5 using a protein G column. TBW 5 was applied to the column where the IgG bound to protein G. The fractions collected contained TBW 5 minus IgG. During the elution step, purified IgG was collected.

Some of the fractions collected were run on SDS-PAGE and visualised using a silver stain to determine which fractions contained IgG. Fig. 3.8 clearly shows that IgG is purified using this column. Lanes 6-9 show the presence of both a heavy (approximately 55 kDa) and a light (approximately 25kDa) chain of IgG whereas lanes 2-5 do not contain IgG. However, the amount of IgG in the 1 ml sample

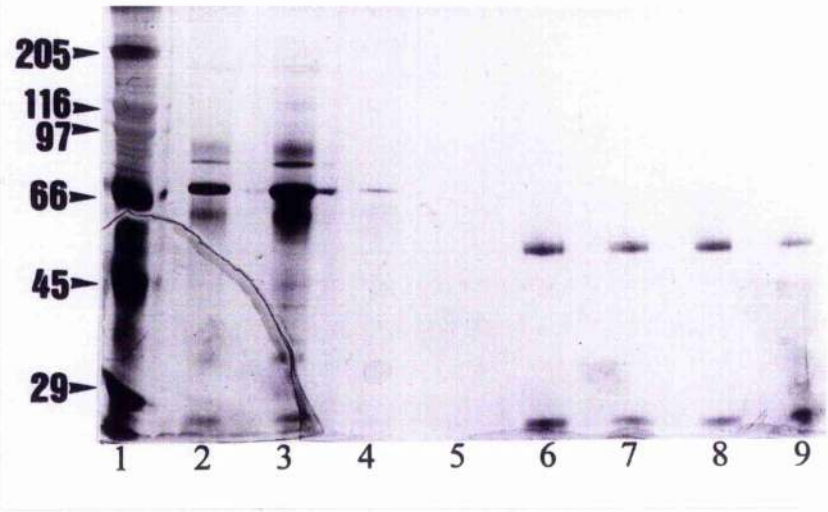


Fig. 3.8. IgG purified from TBW 5 visualised using silver stain. Lane order: (lane 1) High molecular weight markers, (lane 2-4) TBW 5 - IgG (fractions 4-6), (lanes 5-9) purified IgG (fractions 10-14).

applied to the column is eluted over a number of fractions therefore diluting the concentration of the IgG present in TBW 5.

TBW 5 and the fractions which gave the highest absorbance readings at A_{280} during the application of the sample (TBW 5 – IgG) and elution of IgG were analysed for the presence or absence of IgG.

The concentration of IgG present in TBW 5 was 0.98 mg ml^{-1} whereas IgG purified from TBW 5 (using the fraction which gave the highest absorbance reading) contained 0.23 mg ml^{-1} and none was detected in the fluid collected following application of the sample (TBW 5 - IgG).

The three fractions mentioned above were tested for their ability to react with ovine and human *B. parapertussis* on a western blot but neither TBW 5, TBW 5 – IgG nor purified IgG recognised the human or ovine isolates (results not shown).

3.7. API analysis

The differences between the growth of ovine and human *B. parapertussis* in TBW and NB suggested the possibility of different nutritional requirements. The API ZYM kit was used to measure the production of a variety of enzymes by the different isolates. The results are presented in Table 3.4.

API ZYM did not detect any significant difference in the production of these enzymes by ovine and human *B. parapertussis*. It is possible that ovine and human *B. parapertussis* do have different nutritional requirements and produce different enzymes or different quantities of enzymes that were not assayed for.

Table 3.4. API ZYM results for ovine and human *B. parapertussis*.

	Concentration of enzymes (nmol) produced by isolate:			
Enzyme tested for	H1	K2	B285	NCTC 10520
Alkaline phosphatase	0	0	0	0
Esterase	5	20	20	20
Esterase lipase	5	5	5	5
Lipase	0	0	0	0
Leucine arylamidase	≥40	≥40	≥40	≥40
Valine arylamidase	5	5	5	5
Cystine arylamidase	5	5	5	5
Trypsin	0	0	0	0
Chymotrypsin	10	5	5	5
Acid phosphatase	5	5	5	5
Naphthol-AS-BI-phosphohydrolase	5	5	5	5
α galactosidase	0	0	0	0
β galactosidase	0	0	0	0
β glucuronidase	0	0	0	0
α glucosidase	0	0	0	0
β glucosidase	0	0	0	0
N-acetyl-β glucosaminidase	0	0	0	0
α mannosidase	0	0	0	0
α fucosidase	0	0	0	0

3.8. Discussion

This study was designed to compare the growth of ovine and human *B. parapertussis* in ovine TBW to determine if there was any host specificity of these isolates. The results indicated that ovine *B. parapertussis* isolates were capable of growth in ovine TBW. As sheep are the natural host of these isolates, their growth in these TBW was predictable. However, the rates of growth in the different TBW were not consistent. In TBW 1, 2 and 4 there was not a great deal of growth of ovine *B. parapertussis* whereas TBW 3 and 5 showed greater rates of growth.

In comparison, the human isolates either died or struggled to maintain their numbers. This is in contrast to the findings of Porter and Wardlaw (1994) who reported that their human strain NCTC 10520 grew in sheep TBW. However,

NCTC 10520 was one of the human isolates tested in this study. These conflicting results and the differences in the growth rates of ovine *B. parapertussis* in ovine TBW suggests that the differences in growth rates were due to the fact that the TBW came from different sheep. Therefore, the varying growth rates observed may be due to differences in the composition of the TBW.

There were obvious differences between the growth of ovine and human *B. parapertussis* in TBW 3, 4 and 5. This was most noticeable in the death of B285 in TBW 4 and the death of both human isolates tested, B285 and NCTC 10520, in TBW 5. The death of the human isolates in two of the TBW and the fact that the difference between growth of the human isolates in all 5 TBW compared to ovine isolates is statistically significant suggests some host specificity of the isolates. It would have been useful to compare the growth of ovine and human *B. parapertussis* in human TBW to investigate further any possible host specificity but these were not available.

Both ovine and human *B. parapertussis* demonstrated a slow decline in the number of viable cells when incubated in PBS over 72 h. This contrasts somewhat with the findings of Porter and Wardlaw (1994) who found that their *B. parapertussis* isolate (NCTC 10520) had died in PBS by day 3.

Nutrient broth was used as a positive control as it is known to support the growth of *B. parapertussis*. Observation of the growth of ovine and human *B. parapertussis* revealed that the human isolates grew more slowly than ovine isolates in nutrient broth. The difference showed a clear distinction between ovine and human *B. parapertussis* that was statistically significant. While the ovine isolates demonstrated a steady initial growth rate over the first 24 h, the human

isolates went into a lag phase. Following the lag phase, the human isolates grew at an increased rate. However, the number of viable bacteria did not reach the same concentration as the ovine isolates over the duration of the experiments. This suggests the possibility that human *B. parapertussis* are more fastidious than ovine *B. parapertussis*. It would be interesting to continue this experiment for a longer period of time to see if the human isolates were capable of reaching the same concentration of viable cells as the ovine isolates.

The analysis of TBW revealed that all the TBW had similar values for the proteins and nutrients tested. The exception was the concentration of potassium in TBW 5, which was higher than in the other TBW. The ovine isolates grew in this TBW whereas the human isolates died. However, it is unlikely that the higher potassium concentration was responsible for the death of the human strains in TBW 5. The potassium concentration was lowest in TBW 4 yet the human isolate B285 died and the other TBW contained slightly higher potassium levels but neither of the human isolates died. It is possible that, because the TBW are washings of the lungs, some of them may be more dilute than others. However, as mentioned above, all TBW had similar values for the proteins and nutrients tested for. It may be that these TBW differed in factors that were not assayed for. It must be noted that TBW are washings and therefore are dilutions of the fluid normally found in lungs. By collecting washings, factors essential for the growth of *B. parapertussis* may be diluted to below a critical concentration.

It has been reported that all the bordetellae have an absolute requirement for nicotinic acid and that amino acids are required as a carbon and nitrogen source. The concentration of these nutrients was not assayed for. It is possible that the

different concentrations of nicotinic acid and amino acids could account for the growth differences of *B. parapertussis*. Although, Porter (1991) reported that the number of viable *B. parapertussis* surviving in a TBW did not vary according to the amino acid or nicotinic acid content of the fluid. However, it remains possible that different nutritional requirements account for the death of human *B. parapertussis* and survival of ovine *B. parapertussis* in ovine TBW.

The concentration of IgG purified from TBW 5 was lower than the IgG present in whole TBW 5. A possible explanation for this is that the IgG is eluted from the column throughout a series of fractions thereby diluting the IgG concentration. Although TBW 5 did contain more IgG than the other TBW, Western blots of TBW 5 and purified IgG from TBW 5 did not react with any of the *B. parapertussis* tested. It is therefore unlikely that IgG is responsible for the death of the two human isolates tested in TBW 5. It is unlikely that IgA caused the death of human *B. parapertussis* in TBW 5 as the main function of secretory IgA (sIgA) in the respiratory tract is not as an opsonin but to cross-link target bacteria and trap them in the mucus thereby facilitating their clearance by the mucociliary escalator (Kraehenbuhl and Neutra, 1992)

The death of human *B. parapertussis* and survival of ovine *B. parapertussis* in ovine TBW could be due to the production or absence of metabolic enzymes. However API ZYM, which measures the production of some enzymes, did not reveal any difference between the ovine and human isolates. It is possible that other differences in enzyme production could be responsible for the death of the human isolates and that these enzymes were not tested for using the API ZYM.

Another possiblity, which could account for the growth differences of human and

ovine *B. parapertussis* in ovine TBW, is that the human and ovine isolates may have different susceptibilities to antibacterial factors which may be present in TBW. Such factors may include lung surfactant, lysozyme, defensins, fatty acids and lactoferrin.

In conclusion, the different growth patterns of ovine and human *B. parapertussis* in ovine TBW did suggest some host specificity of the isolates. Moreover, the use of TBW from different sheep revealed that the growth rate of *B. parapertussis* in ovine TBW was dependent on the host. The different growth rates of ovine and human *B. parapertussis* in ovine TBW and the slower growth of the human isolates in nutrient broth suggest that there are nutritional differences and that the human isolates may be more fastidious.

CHAPTER 4

ADHERENCE OF OVINE AND HUMAN *BORDETELLA PARAPERTUSSIS* TO CELL LINES AND OVINE TRACHEAL ORGAN CULTURES.

4.1. Introduction

In order to establish an infection in the respiratory tract, *B. parapertussis* has to overcome several host defence mechanisms including the mucociliary clearance mechanism which entraps microorganisms and other foreign particles in the mucus layer. The cilia then sweep these trapped particles up from the lower respiratory tract and back from the nasal passages where they are either swallowed or expelled from the respiratory tract. To avoid clearance, *B. parapertussis* adheres to the ciliated epithelial cells by producing a variety of adhesins, thereby enabling the bacteria to colonise the respiratory tract. The ability to adhere to host cells is thus an essential step for the establishment of *B. parapertussis* infection and causation of disease.

In this chapter, the aims were to observe the adherence of ovine and human *B. parapertussis* to ovine and human cells including, respiratory cell-culture lines and ovine tracheal organ culture. The invasion of the cell lines by *B. parapertussis* was also investigated. The assays were designed to determine any host specificity of the ovine and human *B. parapertussis* isolates tested.

4.2. Materials and methods

The materials and methods used in this chapter include adherence assays using cell lines and ovine tracheal organ culture, which can be found in section 2.3. The haemagglutination assay is detailed in section 2.1.2 and the method used for the invasion assay is in section 2.4.

4.3. Haemagglutination assay

One of the major adhesins of *B. pertussis* is filamentous haemagglutinin (FHA) (Tuomanen and Weiss, 1985). It is known that *B. parapertussis* produces a similar protein (Locht *et al.*, 1993) therefore, it is possible that this FHA-like molecule also functions as an adhesin.

Serial dilutions of cyclodextrin liquid cultures of ovine and human isolates were tested for the production of FHA by comparing their ability to agglutinate sheep red blood cells. The results are shown in Table 4.1 where the end point was taken as the reciprocal of the highest dilution at which no more agglutination occurred.

Table 4.1. Haemagglutination of sheep red blood cells by ovine and human *B. parapertussis*.

Type	Strain	End point
Ovine	H1	128
	K2	4
Human	B285	32
	NCTC 10520	32

4.4. Adherence to ovine and human cell lines

The adherence of ovine and human *B. parapertussis* to ovine and human non-ciliated respiratory epithelial cell lines was observed. The cell lines used were foetal lamb turbinate (FLT), foetal lamb lung (FLL) and human foetal lung (MRC-5). *B. parapertussis* isolates from cyclodextrin liquid cultures were incubated with cells on chamber slides for 60 min at a ratio of approximately 30 bacteria per cell. The slides were washed then gram-stained and the number of bacteria adhering to 100 cells were counted.

Fig. 4.1 displays the adherence of ovine and human *B. parapertussis* to the cell lines and shows that the ovine isolate H1 was more adherent to these cells than the other isolates. H1 adhered to the ovine cell lines (FLT and FLL) better than ovine isolate K2 and both the human isolates. H1 also adhered to the human cell line (MRC-5) better than K2 and the human isolate B285 but slightly less than the other human isolate, NCTC 10520. Although H1 adhered to the ovine cell lines more than to MRC-5, the difference was not statistically significant ($p>0.05$, using the Mann-Whitney test) and therefore did not demonstrate any host specificity of this isolate.

Ovine *B. parapertussis* K2 did not adhere to the cells as well as H1 and demonstrated no preference for ovine cells in comparison with human cells.

Interestingly, the human isolate B285 appeared to adhere better to both the ovine cell lines than to MRC-5, whereas, the other human isolate, NCTC 10520, adhered better to MRC-5. However, neither of the human isolates specifically

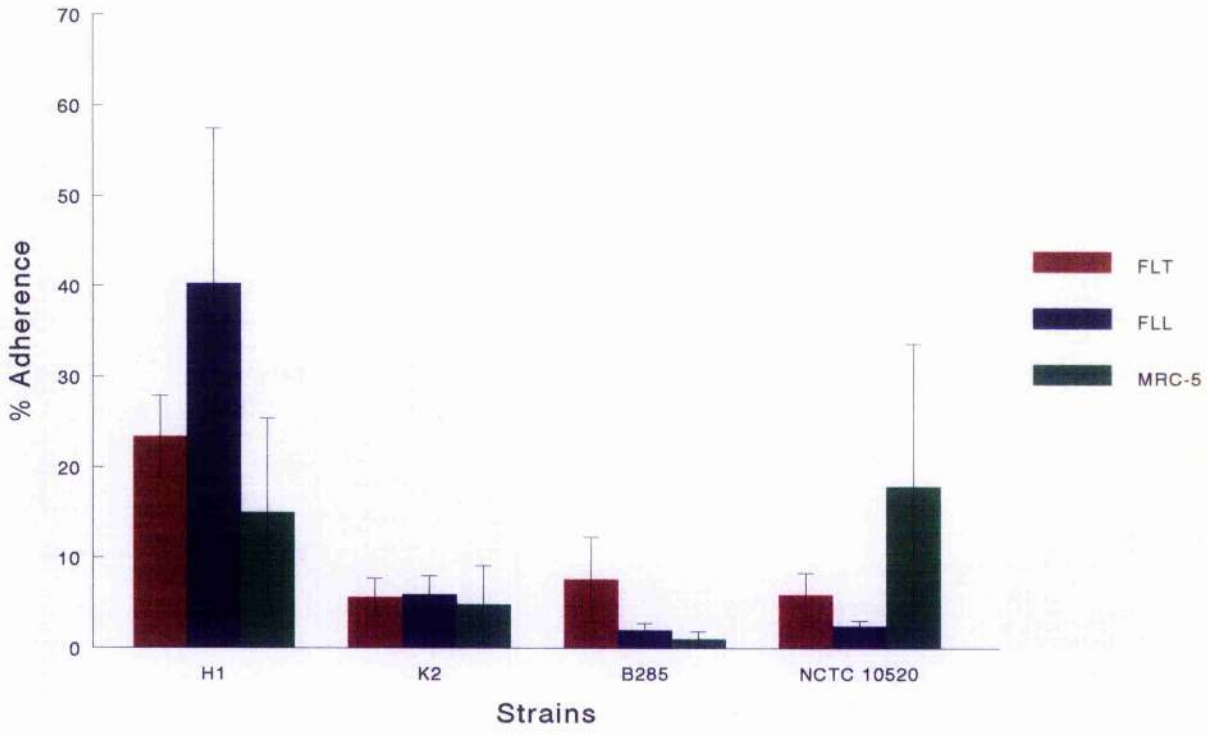


Fig. 4.1. Percentage adherence of ovine and human *B. parapertussis* to ovine (FLT and FLL) and human (MRC-5) cell lines.

adhered to one cell type.

4.5. Invasion of cell lines

For this assay, the cell lines used were the same as for the adherence assay (FLT, FLL and MRC-5). Bacteria were added to the cells at a concentration of approximately 30 per cell and incubated for 5 h, washed, then incubated in gentamicin (100 $\mu g\ ml^{-1}$) for 2 h. The cells were then lysed and plated onto blood agar to determine the number of viable intracellular bacteria. To ensure that the concentration of gentamicin used killed the bacteria, gentamicin was added to the bacterial inoculum for 2 h, in the absence of cells, resulting in a viable count of zero. The percentage invasion of the cell lines by ovine and human *B. parapertussis* is shown in Fig. 4.2.

From the results, it is clear that all the isolates tested invaded the human cell line (MRC-5) more than the ovine cell lines (FLT and FLL). The invasion of the human cell line compared to the ovine cell lines is statistically significant with $p = 0.002$ for human isolates and $p = 0.0008$ for ovine isolates. There was no host specificity for the invasion of cell lines and no difference between the invasive capacity of ovine and human *B. parapertussis* for all the cell lines. Overall, the invasion of the human cell line by ovine and human isolates was significantly greater ($p = 0.001$) than the invasion of ovine cell lines.

The human cell line (MRC-5) was grown in EMEM whereas the ovine cell lines (FLT and FLL) were grown in 199S medium. It is possible that this could cause the human cell line to become more susceptible to invasion by *B. parapertussis.*

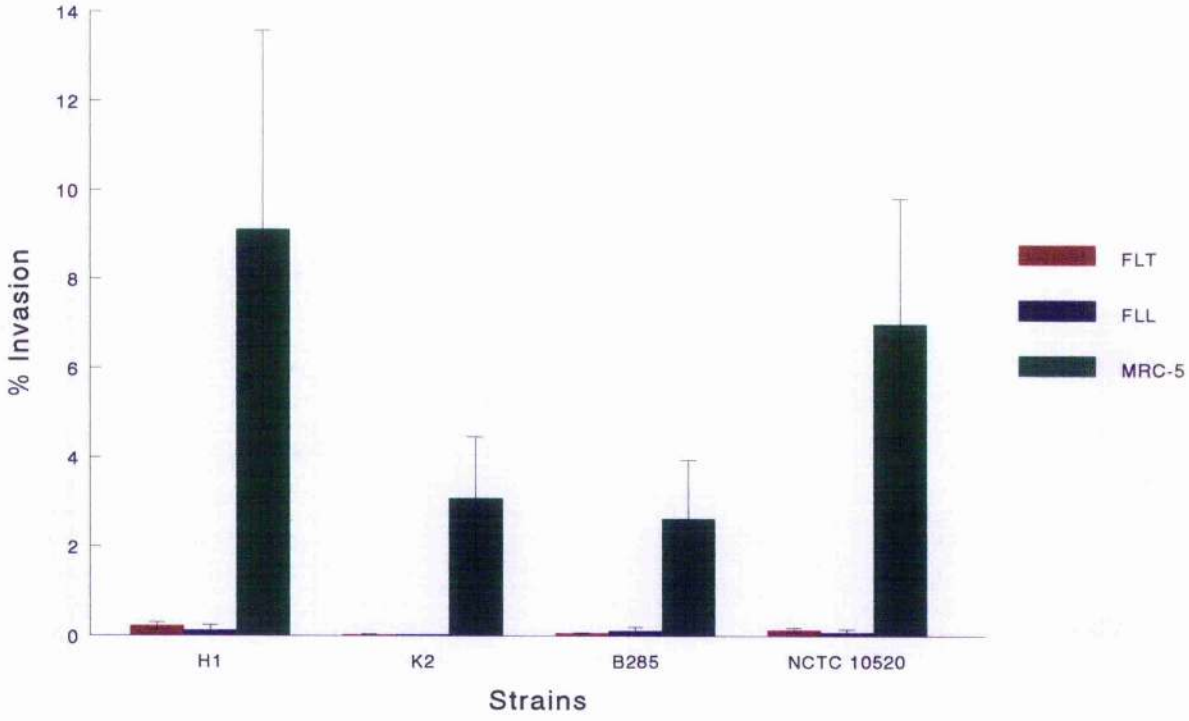


Fig. 4.2. Percentage invasion of ovine (FLL and FLT) and human (MRC-5) cell lines by ovine and human *B. parapertussis*.

FLT were grown in EMEM and the invasion by ovine and human *B. parapertussis* was again determined.

Table 4.2. *B. parapertussis* invasion of FLT cells grown in different media.

Isolate	199S	EMEM
H1	0.13 %	0.20 %
K2	0.01 %	0.02 %
B285	0.05 %	0.04 %
NCTC 10520	0.05 %	0.09 %

The results showed that the change of growth media only marginally increased the invasion of three of the isolates. It is therefore unlikely that the growth medium affects the invasion of the cell lines. A comparison of the ovine and human cell lines grown in 199S was not possible because the MRC-5 could not grow in this medium.

4.6. Adherence to ovine tracheal organ culture

The adherence of ovine and human *B. parapertussis* to ovine tracheal rings was compared. *Pasteurella haemolytica* A2, an ovine respiratory pathogen, was included to determine if this bacterium could adhere to ovine trachea as efficiently as *B. parapertussis* and also to investigate a possible interaction between these two species in the ovine respiratory tract.

Tracheal rings were infected with approximately 3 x 10^6 cfu ml^{-1} of bacterial suspension and incubated for 1 h. The inside of the rings were then washed and scraped to remove adherent bacteria. The number of viable adherent bacteria was determined by viable counts. Fig. 4.3. is a photograph of ovine *B. parapertussis*

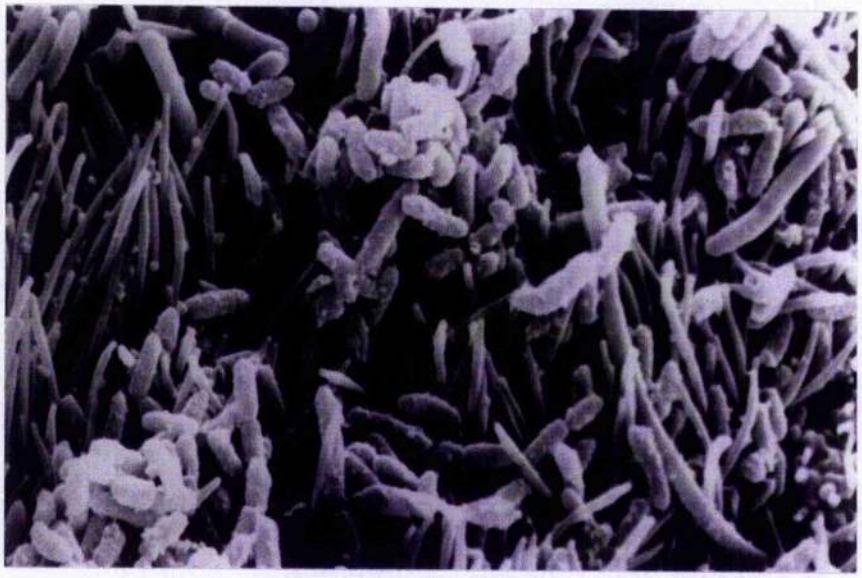


Fig. 4.3. Scanning electron micrograph of ovine *B. parapertussis* isolate C adhering to the cilia of ovine tracheal organ culture (experiment carried out by J.F.Porter).

isolate C adhering to the tip of the cilia of an ovine tracheal organ culture. A comparison of the adherence of ovine and human *B. parapertussis* and *P. haemolytica* A2 is shown in Fig. 4.4.

The ovine strains adhered to ovine tracheal rings at a rate that was significantly different from the human strains ($p < 0.03$), suggesting that ovine *B. parapertussis* adhere better than human *B. parapertussis* to ovine tracheal rings. *P. haemolytica* A2 also adhered better than the human *B. parapertussis* isolates to ovine tracheal rings. There was no significant difference between the adherence of the ovine isolates H1 and K2 which suggests little variation within the ovine *B. parapertussis* in ability to adhere to ovine tracheal organ. This was also the case for the human isolates.

4.6.1. Interaction of H1 supernate and *P. haemolytica* A2

B. pertussis culture supernate, which contains the adhesins PT and FHA, has been reported to enhance the adherence of other respiratory pathogens (Tuomanen, 1986). The supernate of a 72 h shaking culture of *B. parapertussis* H1 in cyclodextrin liquid was used to try to enhance the adherence of *P. haemolytica* A2 to ovine tracheal rings. This supernate was assayed for the presence of FHA using the haemagglutination assay detailed in section 2.1.2. The assay showed that FHA was present in the supernate (titre = 4). The results of adherence of *P. haemolytica* A2 with *B. parapertussis* H1 culture supernate to ovine tracheal rings are shown in Fig. 4.5. A control experiment replacing the H1 supernate fluid with cyclodextrin liquid (the media used for the growth of *B. parapertussis* H1) was also carried out

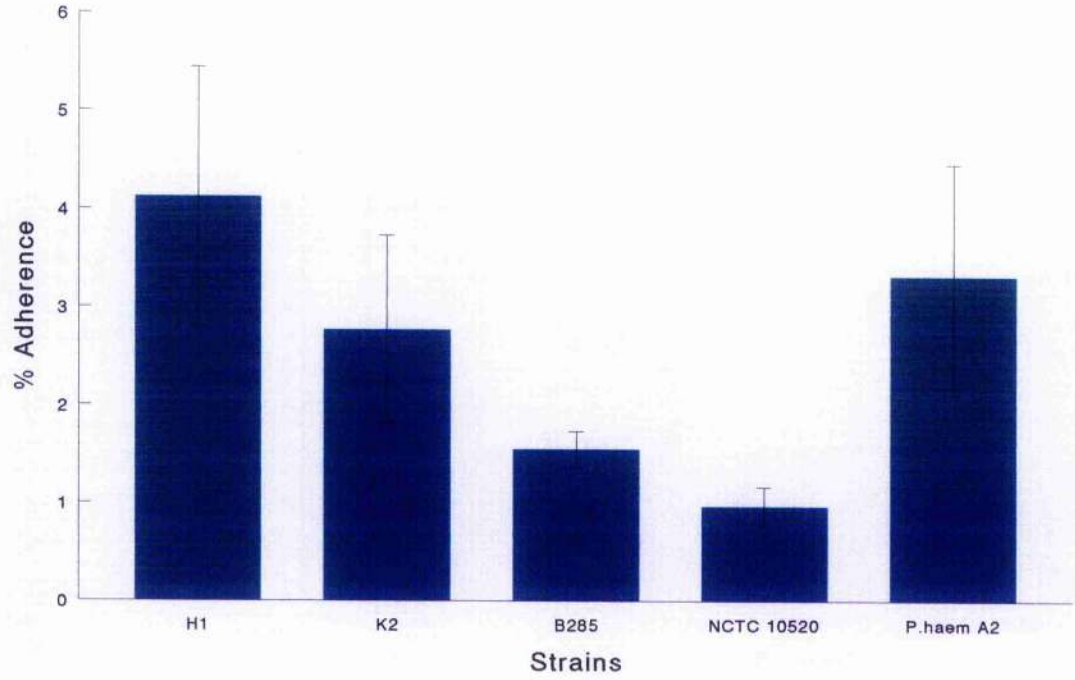


Fig. 4.4. Percentage adherence of ovine and human *B. parapertussis* and *P. haemolytica* A2 to ovine tracheal ring organ culture.

Fig. 4.5. Percentage adherence of *P. haemolytica* A2 to ovine tracheal ring organ culture following incubation with *B. parapertussis* H1 culture supernatant.

Methods used to alter the adherence of *P. haemolytica* A2 to ovine tracheal rings:

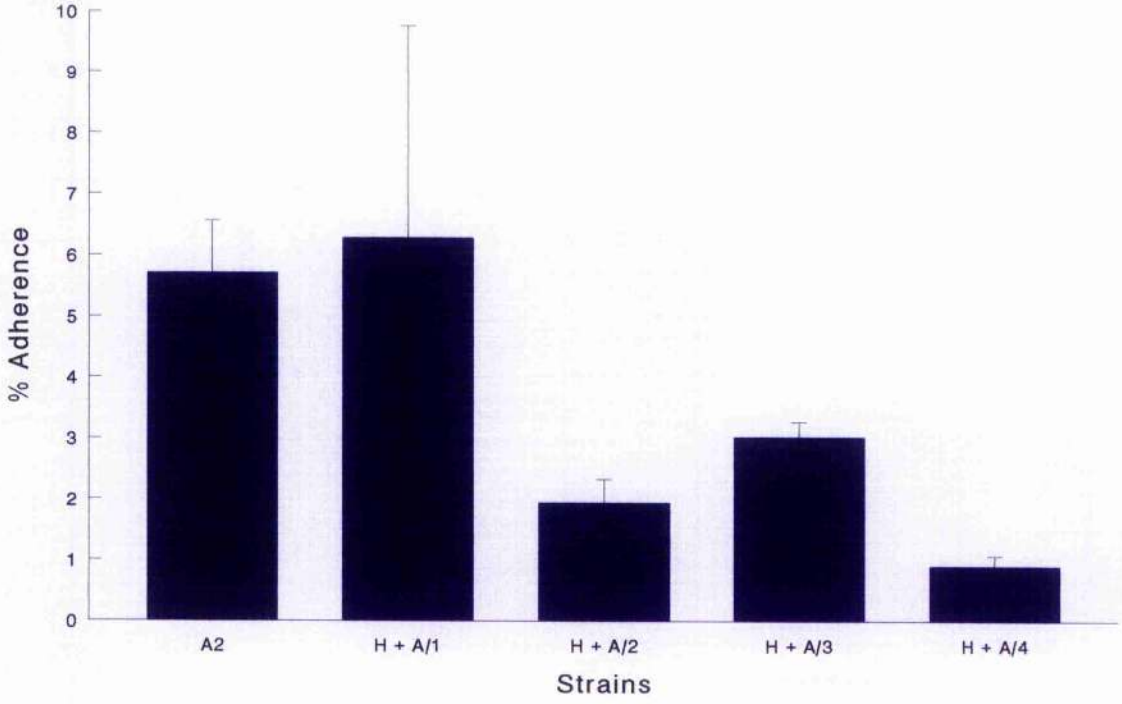
H + A/1 - H1 supernate added to rings for 1 h then removed and A2 added to rings.

H + A/2 - H1 supernate added to rings for 3 h then removed and A2 added to rings.

H + A/3 - A2 incubated in H1 supernate for 1 h at 37°C then washed and added to rings.

H + A/4 - A2 suspension prepared in H1 supernate.

Fig. 4.6. Control experiment - percentage adherence of *P. haemolytica* A2 to ovine tracheal ring organ culture following incubation with cyclodextrin liquid medium. See legend for Fig. 4.5 for an explanation of the abbreviations used.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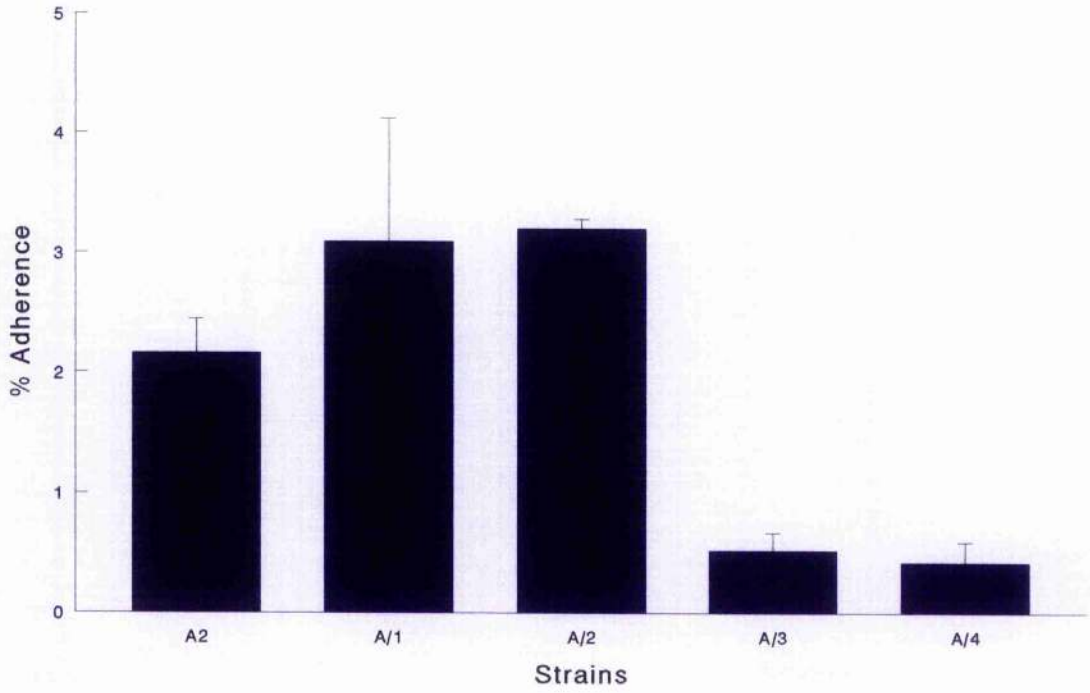
Strains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Strains

and these results are displayed in Fig. 4.6.

Fig. 4.5. clearly shows that H1 culture supernatant decreased the adherence of *P. haemolytica* A2 to tracheal organ culture with the exception of H + A/1 where the adherence of A2 was marginally, but not significantly, increased. However, the graph of the controls (Fig. 4.6.) shows the same pattern for the adherence of A2 when H1 culture supernatant is replaced with cyclodextrin liquid, except for A/2 where the adherence of *P. haemolytica* A2 increased. A comparison of A2 versus A/2 and H+A/2 versus A/2 revealed that neither were statistically significant.

4.7. Discussion

The adherence of *B. parapertussis* to cell lines revealed that the isolates tested were capable of adhering to cell lines within an hour of incubation and that an inoculum of approximately 30 bacteria per cell was sufficient for observing the bacterial adherence. This contrasts with previous studies of *Bordetella pertussis* where an inoculum of approximately 1000-10000 bacteria per cell and an incubation time of up to 5 h were required (Funnell and Robinson, 1993; Redhead, 1985). These assays resulted in percentage adherence of up to 11% (Redhead, 1985). In comparison, the adherence assays presented here resulted in adherence of up to 40%. However, the difference in percentage adherence may be because a higher inoculum was used by Redhead (1985) and although 11% adherence appears low, it represents a much higher number of bacteria per cell than the assays presented in this chapter.

The adherence assay using continuous cell lines did not show any host specificity

of the ovine and human isolates. Although the ovine isolate H1 appeared to adhere to the ovine cell lines better than to the human cell line, this difference was not statistically significant. This was also the case for the human isolate NCTC 10520 which adhered better to the human cell line than to the ovine cell lines. The lack of host specificity of the isolates suggests the possibility of a potential transfer of *B. parapertussis* infection between humans and sheep.

The invasion assay used in this chapter was based on that of Ewanowich *et al.* (1989) who studied the invasion of HeLa cells by human *B. parapertussis*. Their findings suggested that *B. parapertussis* was as invasive as *Salmonella* and *Shigella* which are able to invade host epithelial cells as part of their disease cycle. The results presented here show that both the ovine and human *B. parapertussis* isolates did not invade the ovine cell lines particularly well (Fig. 4.2). However, both the ovine and human isolates invaded the human cell line. The invasion of cell lines was not host specific, in that the human isolates did not invade the human cell line better than the ovine isolates.

It is interesting that both ovine and human *B. parapertussis* could invade the human cell line (MRC-5) much more than the ovine cell lines. However, the ovine and human cell lines were grown in different media but when the ovine cell lines were grown in the same medium as MRC-5 there was little change in the invasion of these cells by *B. parapertussis*. MRC-5 were not able to grow in 199S which suggests that these cells require a more specialised medium than ovine cells. It is possible that because the human cell line is more difficult to grow in tissue culture it is weaker than the ovine cells and therefore more susceptible to invasion by *B. parapertussis*. Alternatively, both ovine and human *B. parapertussis* may

recognise specific receptors on the human cells which enable the invasion of these cells but the same receptors may not be present on the ovine cells.

It was expected that as adherence is considered necessary for invasion, the ovine isolates would invade the ovine cell lines at a higher rate than the human cell line since they adhered preferentially to ovine cells. Yet, none of the isolates invaded the ovine cell line particularly well despite adhering to these cells. This is notable in the case of H1 which demonstrated good adherence to the ovine cell lines but very little invasion of these cells. However, the ovine isolate H1 and the human isolate NCTC 10520 adhered to and invaded the human cell line better than the other isolates. This does suggest that binding of the bacteria to cells may enhance any subsequent invasion of cells by both ovine and human *B. parapertussis*.

The ovine isolate H1 was overall more adherent and invasive than any of the other isolates tested (Fig. 4.1 and Fig. 4.2). This is possibly due to the higher production of the adhesin FHA by H1 compared with the amount produced by the other isolates. NCTC 10520 and B285 produced equal amounts of FHA (Section 4.3). However, NCTC 10520 adhered to and invaded the human cell line (MRC-5) better than B285. This suggests a possible role for another adhesin, possibly pertactin (PRN), in the adherence to and invasion of cell lines by *B. parapertussis*. It may be that PRN plays a role in the ability of NCTC 10520 to invade the human cell line by enhancing the adherence of this isolate to the cell and thus increasing the chance of uptake into the cell. It would have been interesting to compare the amount of PRN produced by B285 and NCTC 10520 to see if different concentrations of this protein were produced and if it affected the ability of these bacteria to adhere to and invade cells.

Previous studies have shown that PRN plays an important role in the adherence of *B. pertussis* to various cell lines e.g. Chinese hamster ovary cells and HeLa cells (Leininger *et al.*, 1991; Arico *et al.*, 1993). Leininger *et al.* (1991) found that the absence of PRN resulted in 30-40% less adherence of these bacteria to cells. The role of PRN in the invasion of cell lines by *B. pertussis* has already been suggested by Lee *et al.* (1990) and Everest *et al.* (1996). *B. pertussis* and *B. parapertussis* produce pertactins which are not identical but are very similar (Li *et al.*, 1991). Therefore, it is possible that the PRN of *B. parapertussis* may also play a role in adherence and invasion.

B. parapertussis also possess agglutinogens (Eldering *et al.*, 1957) and some of them may be located on fimbriae. The precise role of *B. parapertussis* fimbriae has not been defined. It may be that they are also involved in the adherence of *B. parapertussis* to target cells. However, the fimbriae of *B. pertussis* do not appear to play a role in attachment to the cilia or respiratory epithelial cells which are the natural location of this species (Tuomanen, 1988). Yet, they do enable the organism to bind to other mammalian cells including Vero cells (Gorringe *et al.*, 1985) and HeLa cells (Sato *et al.*, 1981). It is possible that the fimbriae of *B. parapertussis* also play a role in adherence to continuous cell culture lines, such as those used in this chapter. Any adherence caused by fimbriae could be detected by examining the interaction of the bacteria and cells using electron microscopy or by the use of monoclonal antibodies to specific fimbriae which may block the adherence of *B. parapertussis* to the cells.

Cell lines are convenient *in vitro* systems that can be used to investigate the adherence of bacteria. However, these cells cannot accurately reflect an *in vivo*

situation because the cells will have adapted to a different environment and cellular structures such as cilia are missing. *B. pertussis* has been shown to attach primarily to the cilia of human respiratory epithelial cells (Tuomanen and Hendley, 1983) and it is logical to assume that *B. parapertussis* also adheres preferentially to cilia.

Tracheal organ culture allows study of the adherence of *Bordetella* species to ciliated host epithelial cells and the use of ovine tracheal organ culture revealed possible host specificity of *B. parapertussis*. The adherence of ovine isolates to ovine tracheal rings was significantly different to the adherence of human isolates to these rings ($p < 0.03$). An interesting comparison would be to measure the adherence of ovine and human *B. parapertussis* to human or primate tracheal rings to see if there is indeed some host specificity of the interaction between these different isolates with tracheal organ cultures. The possibility of host specific adherence of *Bordetella* species has already been investigated. Using human and animal ciliated cells, Tuomanen *et al.* (1983) demonstrated that the human *B. parapertussis* isolate tested adhered better to human ciliated cells than to those from rabbits, mice or hamsters. There was no significant difference between the adherence of the ovine isolates H1 and K2 to tracheal rings which suggests little variation within ovine *B. parapertussis* in the ability to adhere to ovine tracheal organ culture. Similarly, there was little difference in adherence between the two human isolates to the ovine tracheal rings.

P. haemolytica is a respiratory tract pathogen of sheep and a major cause of ovine pneumonia. This species is usually found in the upper respiratory tract of clinically healthy sheep and causes disease when the host is predisposed. It has been

suggested that *B. parapertussis* colonises the respiratory tract of sheep and this predisposes the host to a secondary infection caused by *P. haemolytica* (Jian *et al.*, 1991; Porter *et al.*, 1995a).

The adherence of *P. haemolytica* A2 to ovine tracheal organ culture was also demonstrated. Using this assay, *P. haemolytica* was more adherent to tracheal rings than one of the ovine and both of the human isolates of *B. parapertussis* (Fig. 4.4). In fact, *P. haemolytica* adhered to the tracheal rings almost as well as the ovine *B. parapertussis* H1. It has been reported that *P. haemolytica* have fimbriae (Morck *et al.*, 1987; Morck *et al.*, 1988) but the adherence mechanisms of *P. haemolytica* are unknown.

Tuomanen (1986) reported that adhesins secreted by *B. pertussis* (FHA and PT) could be used by other pathogenic bacteria, such as *Streptococcus pneumoniae, Haemophilus influenzae* and *Staphylococcus aureus*, to enhance their adherence to ciliated cells. It was suggested that such piracy of adhesins may contribute to secondary infections during whooping cough. It is possible that piracy of *B. parapertussis* adhesins aids the establishment of *P. haemolytica* infection in sheep. However, the supernate of ovine *B. parapertussis* H1 increased or decreased the adherence of *P. haemolytica* A2 depending on the method used (Fig. 4.6.). Moreover, a control experiment using uninfected growth medium instead of culture supernatant resulted in the same findings (Fig. 4.7.), with one exception. When the H1 supernatant was incubated with tracheal rings for 3 h prior to infection with *P. haemolytica* A2, the adherence of *P. haemolytica* A2 to the rings decreased. The control experiment showed the uninfected growth medium to increase the adherence of *P. haemolytica* A2. All the other methods in this experiment

involved an incubation of *P. haemolytica* A2 and H1 supernatant of 1 h. It may be that a longer incubation time of 3 h resulted in *B. parapertussis* adhesins binding to the cells thereby preventing the binding of *P. haemolytica*. This suggests that the two microorganisms are competing for the same receptors.

B. parapertussis may allow the establishment of *P. haemolytica* infection of the respiratory tract by the production of *B. parapertussis* toxins which damage host cells thereby enabling *P. haemolytica* to cause disease. It is likely that ovine *B. parapertussis* produce tracheal cytotoxin (TCT) and the heat-labile toxin (HLT). TCT is produced by human *B. parapertussis* and causes damage to the ciliated epithelium of the trachea leading to sloughing of the cilia (Goldman, 1988). It has already been suggested that TCT is responsible for the damage of ciliated epithelial cells observed in lambs and mice infected with ovine *B. parapertussis* (Chen *et al.*, 1990). This would prevent efficient clearance of an infectious agent such as *P. haemolytica* which could then establish an infection. A similar interaction has been suggested in swine atrophic rhinitis. Chanter *et al.* (1989) proposed that the TCT of *B. bronchiseptica* caused ciliostasis and this allowed colonisation of the nasal passages by *Pasteurella multocida*. Ovine *B. parapertussis* may also produce HLT, a toxin which is known to be produced by human *B. parapertussis* (Walker and Weiss, 1994). The HLT of *B. bronchiseptica* may have a role in producing the turbinate atrophy associated with swine atrophic rhinitis (Rutter and Mackenzie, 1984). These actions of HLT could result in a secondary infection of the host respiratory tract.

The adherence assays presented here have suggested some host specificity in the adherence of ovine and human *B. parapertussis* to ovine tracheal organ culture. It

would have been useful to compare the adherence of the different isolates to human tracheal organ culture to clarify this point. Also, the invasion of the human cell line by *B. parapertussis* indicated the importance of adherence as a prerequisite for invasion. It would be interesting to look further into the role of FHA and PRN in the adherence of both the ovine and human isolates to tracheal rings and to determine if the differences in the adherence and invasion of the two human strains were caused by the production of PRN. This could be accomplished by the use of monoclonal antibodies to FHA or PRN or by the addition of FHA or PRN to organisms deficient in the production of these proteins. The adherence of *P. haemolytica* A2 to tracheal rings and a possible inhibition of adherence by the actions *B. parapertussis* virulence factors warrants further investigation.

CHAPTER 5

INTERACTION OF OVINE AND HUMAN *BORDETELLA PARAPERTUSSIS* WITH ALVEOLAR MACROPHAGES

5.1. Introduction

Alveolar macrophages are the first line of host defence against bacterial invasion of the lower respiratory tract. Studies have shown that *B. pertussis* can adhere to pulmonary macrophages and also invade and survive intracellularly (Saukkonen *et al.*, 1991; Friedman *et al.*, 1992). *B. pertussis* is also capable of multiplying within macrophages thereby providing a possible reservoir of viable bacteria capable of causing further infection (Guierard *et al.*, 1998). There are no reports of the interaction of *B. parapertussis* with macrophages and whether these bacteria can survive phagocytosis although their ability to invade and survive within other cell types, such as HeLa cells has been shown (Ewanowich *et al.*, 1989). This study compared the interaction of ovine and human *B. parapertussis* with ovine alveolar macrophages and the survival of these bacteria once internalised within the cells.

5.2. Materials and methods

The interaction of ovine *B. parapertussis* H1 and K2 and human *B. parapertussis* B285 and NCTC 10520, *Pasteurella haemolytica* A2 and *Corynebacterium bovis* with alveolar macrophages was studied in this chapter. Bacterial adherence,

intracellular uptake and survival within macrophages was studied using the assays detailed in section 2.5.

5.3. Adherence of *B. parapertussis* to ovine alveolar macrophages

B. parapertussis isolates were incubated for 90 min with freshly prepared ovine alveolar macrophages at a ratio of approximately 30 bacteria per cell. The slides were then washed, fixed with formol saline and the number of bacteria adhering to 100 macrophages was counted. The percentage adherence to ovine alveolar macrophages is shown in Fig. 5.1.

As with the adherence to cell lines and tracheal organ culture (chapter 4), the ovine isolate H1 adhered to the macrophages better than the other isolates tested. K2, an ovine isolate, adhered to the ovine alveolar macrophages better than both the human isolates and B285 adhered to the macrophages more than NCTC 10520. Both *P. haemolytica* A2 and *C. bovis* adhered to the ovine alveolar macrophages.

5.4. Macrophage uptake of *B. parapertussis*

The use of a differential fixation technique allowed calculation of the macrophage uptake of ovine and human *B. parapertussis*. Fixing the cells with methanol permeabilises the macrophage cell membrane and allows the visualisation of both intracellular and extracellular bacteria. In comparison, fixation with formol saline allows only extracellular bacteria to be observed. Therefore, the difference

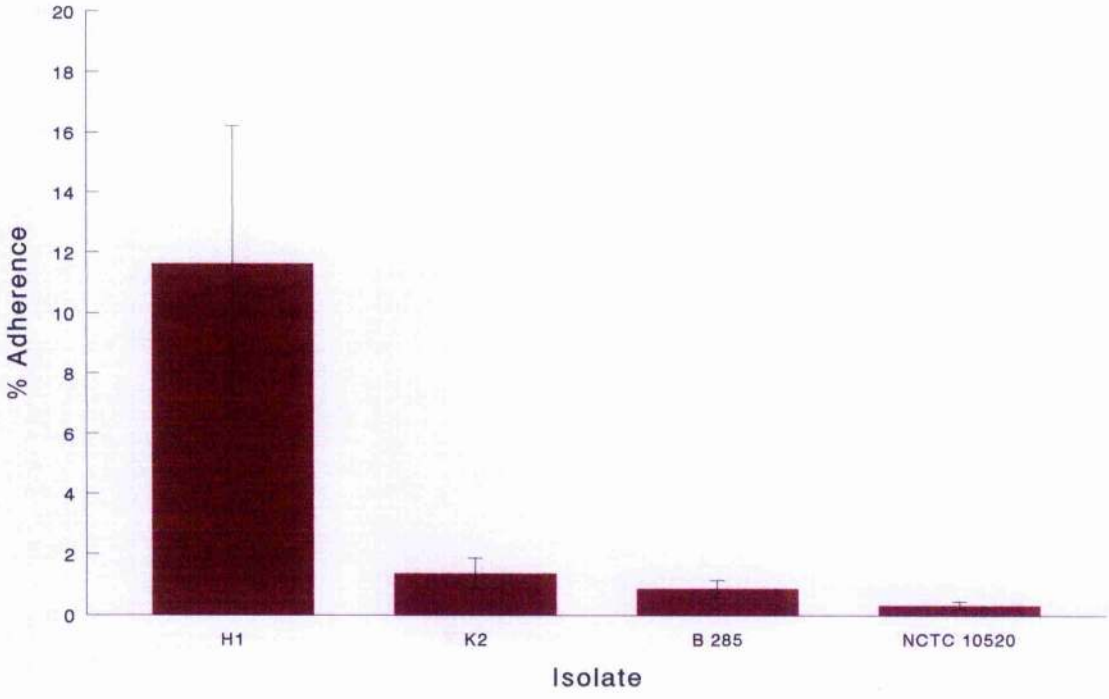


Fig. 5.1. Adherence of ovine (H1 and K2) and human (B 285 and NCTC 10520) *B. parapertussis* to ovine alveolar macrophages following 90 min incubation.

between these two methods enables the calculation of the number of intracelluar bacteria. *Pasteurella haemolytica* A2 was used as a positive control as it has been shown to destroy macrophages with 90 min of incubation (Rowe, 1997) and *Corynebacterium bovis* was used as a negative control.

Fig 5.2 A shows the interaction of ovine *B. parapertussis* H1 with the macrophages as visualised using formol saline as a fixative. Here, the bacteria can be seen adhering to the cell. Methanol fixation was used in Fig. 5.2. B and this shows both extracellular and intracellular bacteria associated with ovine alveolar macrophages.

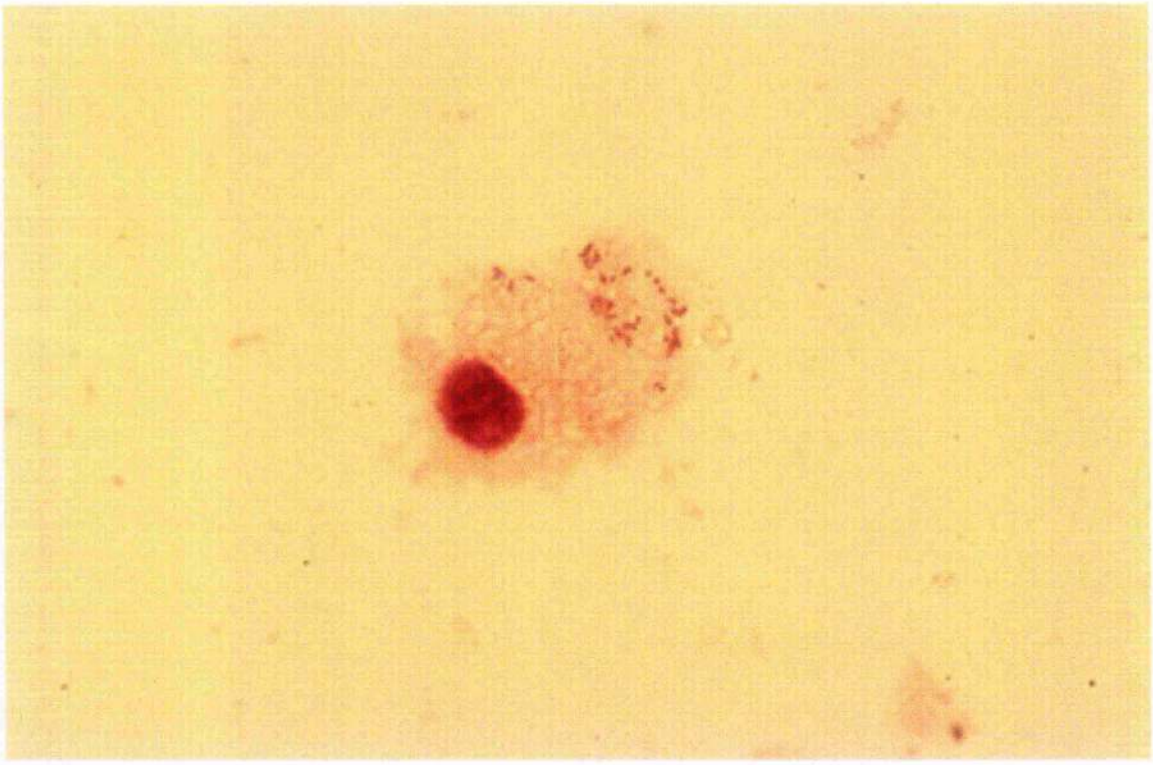
Table 5.1. Comparison of ovine macrophage uptake of ovine and human *B. parapertussis* after incubation for 90 min.

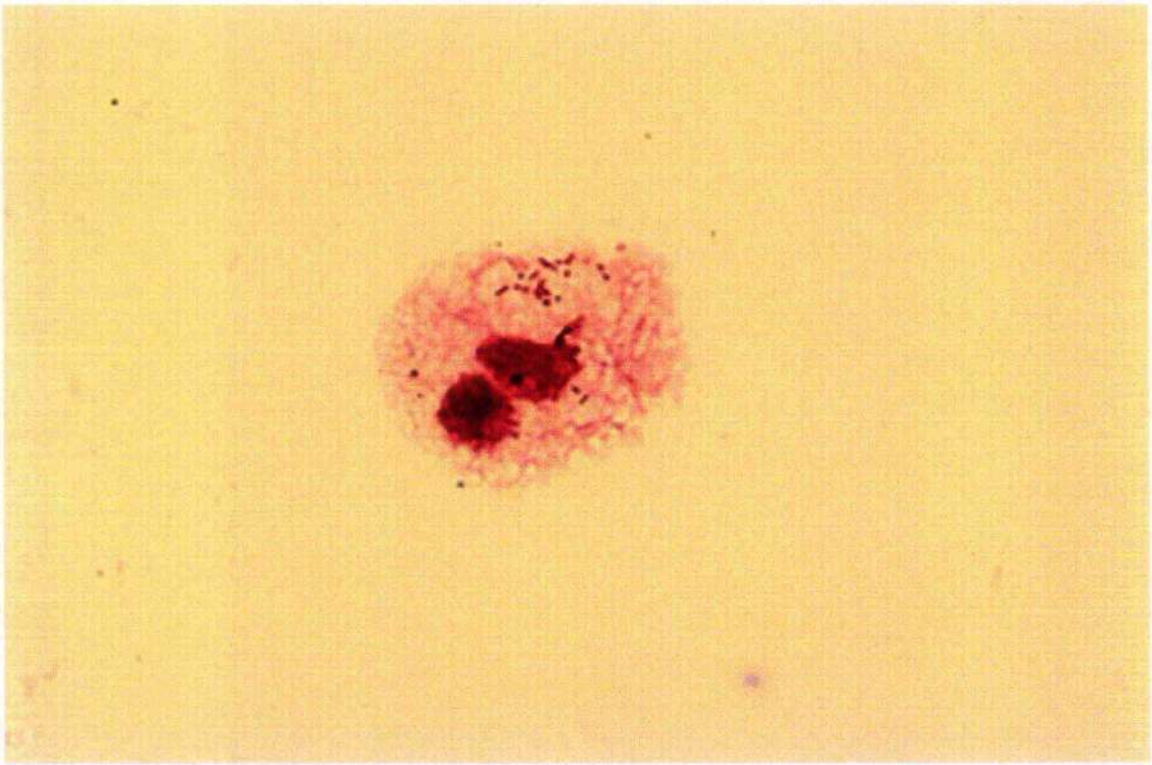
Isolate	% Intracellular
H1	14.57 ± 9.44
K2	0.63 ± 0.63
B 285	0.13 ± 0.13
NCTC 10520	0.12 ± 0.09
P. haemolytica A2	0.00 ± 0.00
C. bovis	0.31 ± 0.31

Table 5.1. shows that the uptake of ovine isolate H1 into ovine alveolar macrophages is higher than the uptake of the other isolates. This correlates with the comparison of adherence of the ovine and human isolates where H1 adhered to the macrophages greater than the other isolates. *P. haemolytica* A2 was used as a positive control because it acts quickly on the macrophage, possibly through the action of its leukotoxin, and at 90 min,

Fig. 5.2A. Photographic representation of *B. parapertussis* H1 interaction with an ovine alveolar macrophage using methanol fixation where both intracellular and extracellular bacteria can be visualised. x 1000 magnification.

Fig. 5.2B. Photographic representation of *B. parapertussis* H1 adhering to an ovine alveolar macrophages using formol saline fixation. x 1000 magnification.





as expected, some of the macrophages were destroyed and no bacteria were intracellular. In comparison, internalisation of *C. bovis* appears to take longer.

5.5. Survival of *B. parapertussis* within ovine alveolar macrophages

To determine the number of viable intracellular bacteria, a phagocytosis assay was performed as above except, following the 90 min incubation, the cells were incubated in gentamicin for 60 min to kill any extracellular bacteria. The macrophages were then washed, lysed and dilutions of the resulting suspension were plated onto blood agar to determine the number of viable intracellular bacteria. The resulting number of viable bacteria were expressed as a percentage of the intracellular bacteria calculated in the preceding section. The results are shown in Table 5.2.

Table 5.2. Survival of phagocytosed *B. parapertussis*, expressed as % of intracellular bacteria.

Isolate	% Survival of intracellular bacteria
H1	10.70 ± 7.61
K2	0.298 ± 0.298
B285	1.44 ± 1.44
NCTC 10520	8.85 ± 6.43
P. haemolytica A2	0 ± 0
C. bovis	0 ± 0

A proportion of all the *B. parapertussis* isolates survived phagocytosis however, it is clear that the ovine isolate H1 survived phagocytosis better than the other isolates, although this was not significant. The survival of ovine isolate K2 was lower than that of H1 and both of the human isolates.

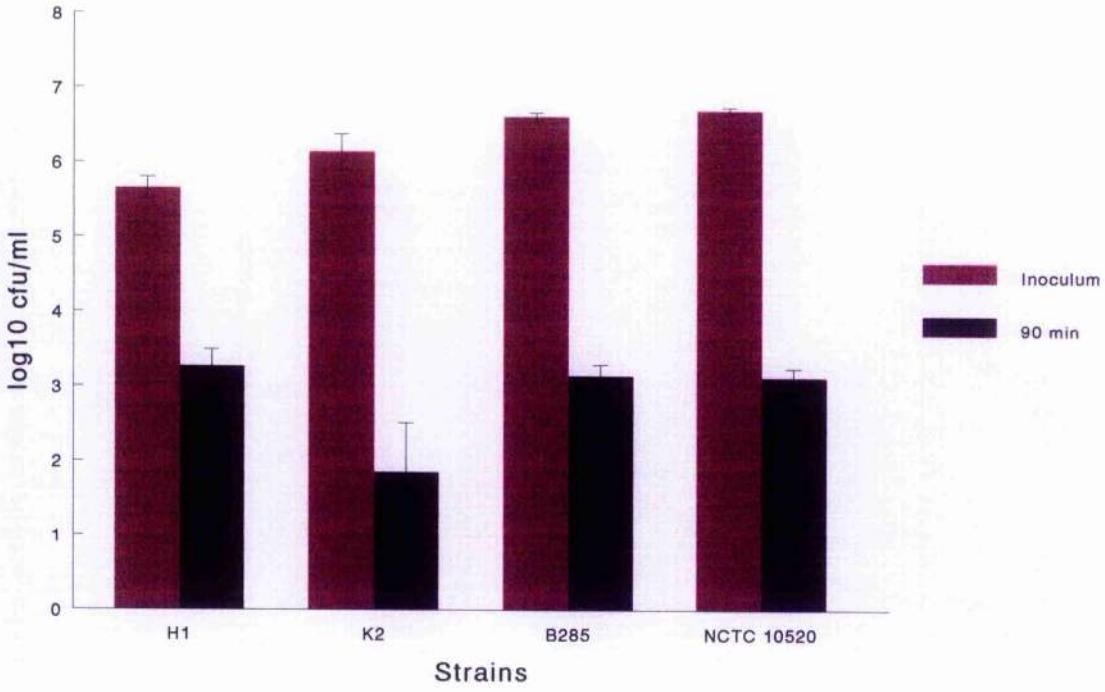


Fig. 5.3. Phagocytic survival of *B. parapertussis* after 90 min incubation compared with the inoculum used.

Fig. 5.3 shows the phagocytic survival of *B. parapertussis* compared to the inoculum. Here, a higher proportion of H1 survived phagocytosis compared to the other isolates tested. This also reinforces the findings above which stated that compared with the other *B. parapertussis*, K2 did not survive phagocytosis as well as the others.

5.6. Effect of opsonisation on phagocytosis

Bacteria were pre-treated with sera and TBWs to determine if opsonisation by any of these fluids affected bacterial survival following phagocytosis.

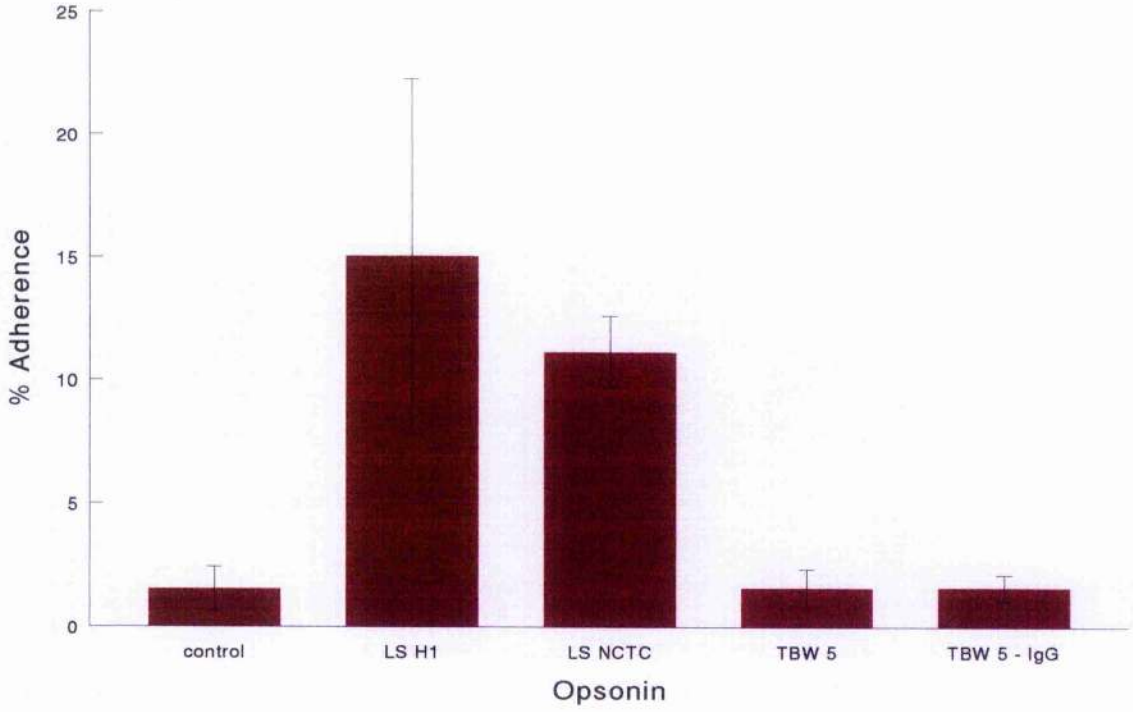
5.6.1. Bacterial adherence following opsonisation

Bacteria were opsonised by incubation with antisera or TBW for 15 min at room temperature. Following opsonisation, the bacteria were washed and added to the macrophages. The assay was then carried out as for section 5.3. Lamb antisera against ovine *B. parapertussis* H1 and human *B. parapertussis* NCTC 10520, TBW 5 and TBW 5 - IgG were used as opsonins.

The adherence of ovine and human *B. parapertussis* to ovine alveolar macrophages following opsonisation is shown in Fig. 5.4-5.7. Opsonsisation with lamb sera raised against H1 and NCTC 10520 increased the adherence of ovine isolate H1 and both of the human isolates. However, this resulted in little difference to the adherence of K2 to the macrophages. In all cases, opsonisation with TBW 5 - IgG

Fig. 5.4. Percentage adherence of ovine *B. parapertussis* H1 to macrophages following opsonisation with LS H1 (lamb serum raised against H1), LS NCTC (lamb serum raised against NCTC 10520), TBW 5 and TBW 5 - IgG.

Fig. 5.5. Percentage adherence of ovine *B. parapertussis* K2 to macrophages following opsonisation with LS H1 (lamb serum raised against H1), LS NCTC (lamb serum raised against NCTC 10520), TBW 5 and TBW 5 - IgG.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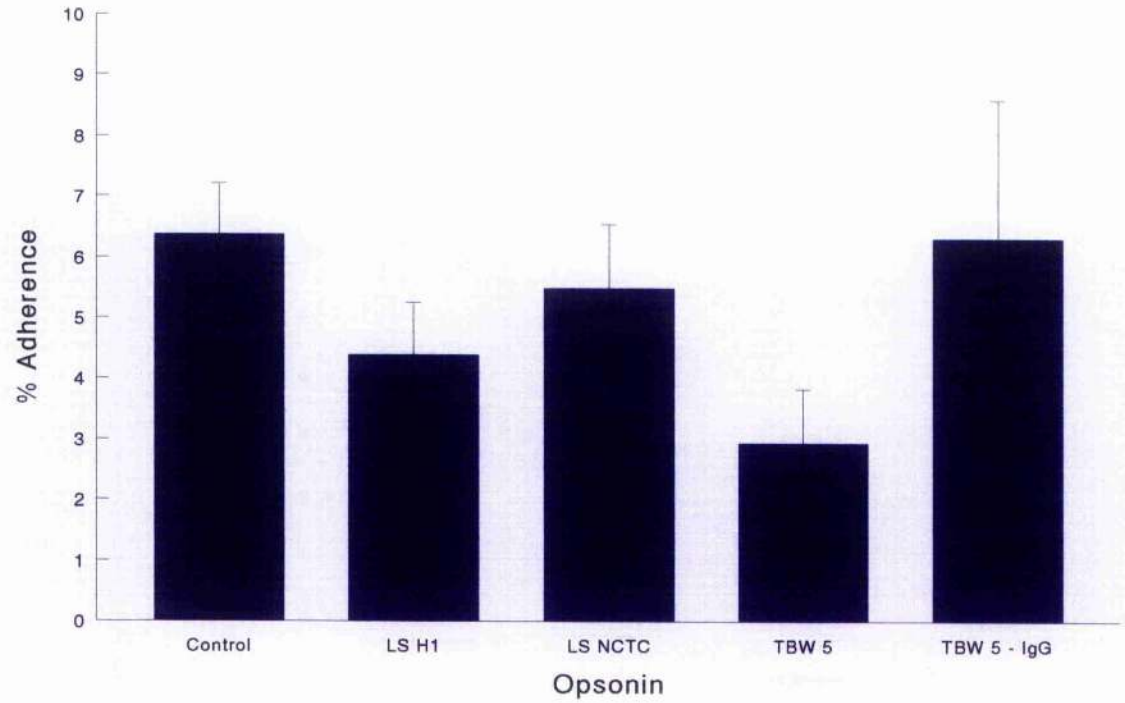
control
LS H1
LS NCTC
TBW 5
TBW 5 - IgG
Opsonin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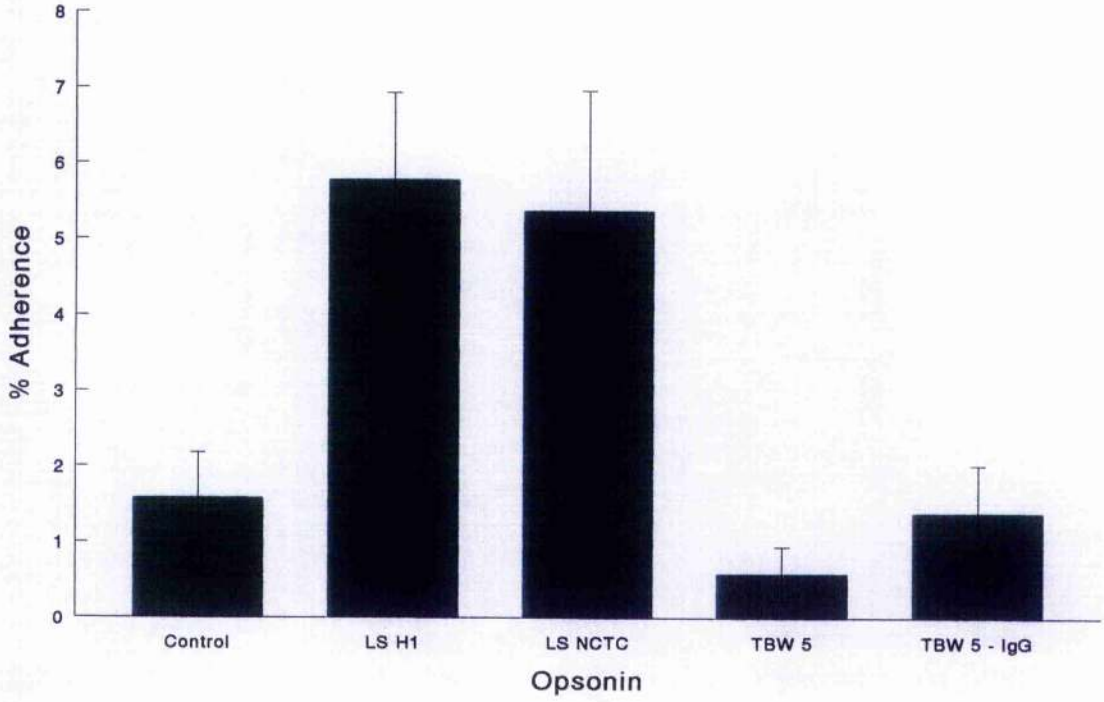
% Adherence
Control
LS H1
LS NCTC
TBW 5
TBW 5 - IgG
Opsonin

Fig. 5.6. Percentage adherence of human *B. parapertussis* B285 to macrophages following opsonisation with LS H1 (lamb serum raised against H1), LS NCTC (lamb serum raised against NCTC 10520), TBW 5 and TBW 5 - IgG.

Fig. 5.7. Percentage adherence of human *B. parapertussis* NCTC 10520 to macrophages following opsonisation with LS H1 (lamb serum raised against H1), LS NCTC (lamb serum raised against NCTC 10520), TBW 5 and TBW 5 - IgG.


% Adherence
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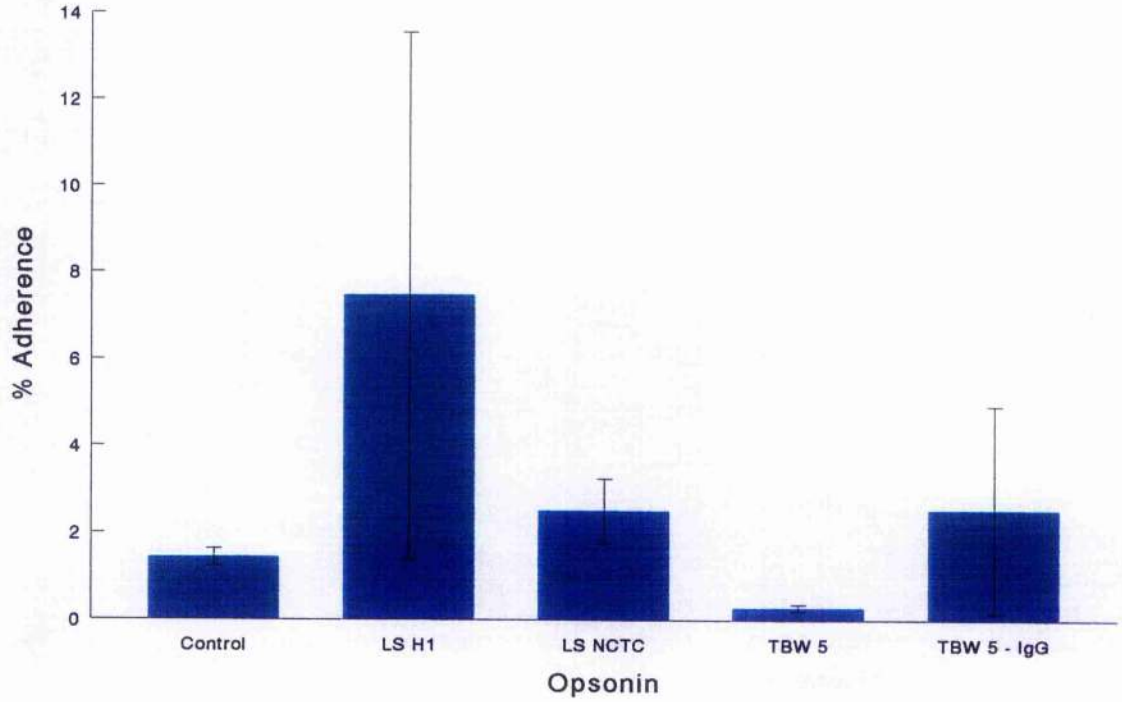
0
Control
LS H1
LS NCTC
TBW 5
TBW 5 - IgG
Opsonin


% Adherence
14
12
10
8
6
4
2
0
Control
LS H1
LS NCTC
TBW 5
TBW 5 - IgG
Opsonin

did not significantly affect the adherence of ovine and human *B. parapertussis* to macrophages. Opsonisation with TBW 5 had little effect on the adherence of H1, B285 and NCTC 10520 to ovine alveolar macrophages but, this fluid did decrease the adherence of K2, although not significantly.

5.6.2. Bacterial uptake following opsonisation

Table 5.3. % Intracellular bacteria within ovine macrophages following opsonisation with the following sera and TBWs.

	% Intracellular				
Isolate	Control	Lamb serum α-H1	Lamb serum α-NCTC 10520	TBW 5	TBW 5 - IgG
H1	0.64 ± 0.39	1.88 ± 1.88	2.30 ± 1.70	0.16 ± 0.15	0.34 ± 0.34
K2	0.00 ± 0.00	2.41 ± 2.41	3.75 ± 3.43	0.00 ± 0.00	0.52 ± 0.40
B 285	0.10 ± 0.10	0.80 ± 0.68	2.83 ± 2.66	0.13 ± 0.11	1.85 ± 1.03
NCTC 10520	0.26 ± 0.26	0.03 ± 0.03	3.71 ± 1.88	0.07 ± 0.01	0.64 ± 0.62

As shown above, opsonisation of all the isolates with lamb serum raised against NCTC 10520 increased the macrophage uptake of the bacteria. However, when the bacteria were pre-incubated with lamb serum raised against H1 the intracellular count of all the isolates except NCTC 10520 was increased. Opsonisation with TBW 5 only increased the uptake of B285 whereas opsonisation with TBW 5 - IgG increased the uptake of all the isolates except H1.

5.6.3. Survival within macrophages following opsonisation

Table 5.4. % Survival of phagocytosed *B. parapertussis* following opsonisation with the following fluids and sera. The survival is expressed as % of intracellular bacteria.

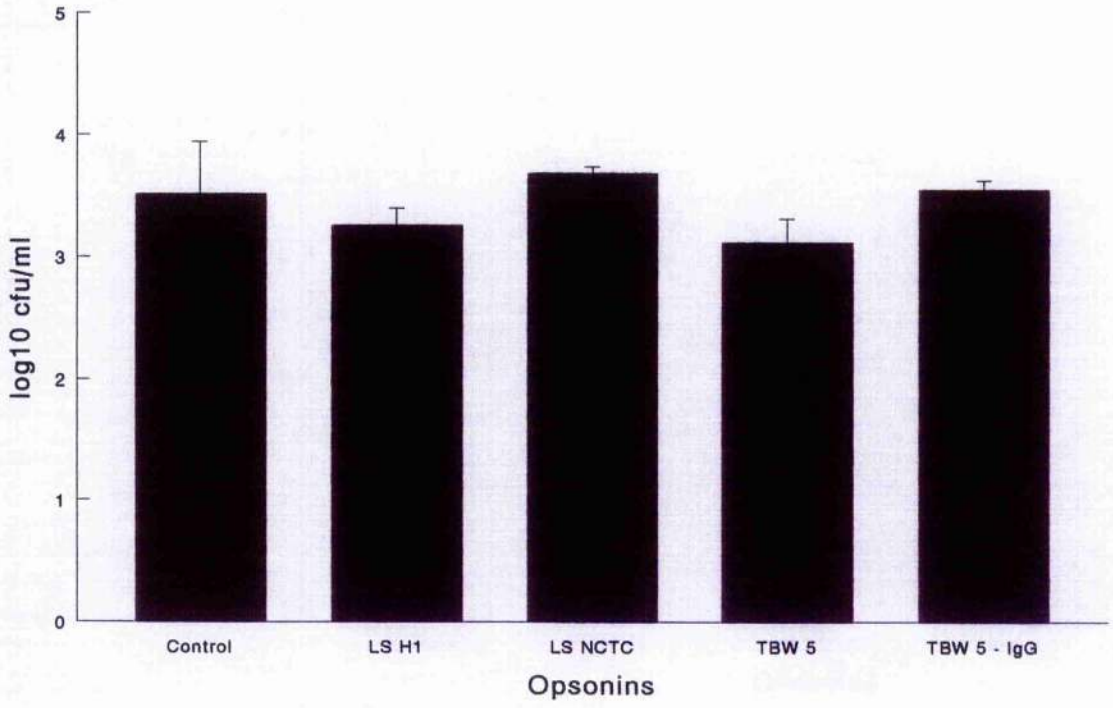
	% Survival				
Isolate	Control	Lamb serum α-H1	Lamb serum α-NCTC 10520	TBW 5	TBW 5 - IgG
H1	2.7 ± 1.54	0.48 ± 0.48	3.12 ± 2.07	33.8 ± 26	3.93 ± 3.93
K2	0 ± 0	0.317 ± 0.317	2.64 ± 2.32	0 ± 0	16.35 ± 8.53
B285	7.78 ± 7.78	35.3 ± 32.4	11.4 ± 10.7	37.9 ± 31.3	3.16 ± 1.80
NCTC 10520	6.41 ± 6.41	33.3 ± 33.3	0.547 ± 0.289	58.3 ± 21.1	2.59 ± 2.59

From the data above opsonisation with anti-H1 lamb serum increased the intracellular survival of B285, NCTC 10520 and K2 but decreased the survival of H1. However, opsonisation with anti-NCTC 10520 lamb serum increased the survival of intracellular H1, K2 and B285 but decreased the survival of NCTC 10520. The use of TBW 5 as an opsonin either increased or had no effect on the survival of intracellular *B. parapertussis*. In comparison, opsonisation with TBW 5 - IgG resulted in the increased survival of ovine *B. parapertussis* but the survival of human *B. parapertussis* decreased.

The survival of opsonised *B. parapertussis* within ovine alveolar macrophages was monitored as in section 5.5. The number of viable bacteria recovered upon lysis of the macrophages are shown in Fig. 5.8 - 5.11. These graphs show that the number of viable bacteria recovered from the macrophages was not affected by opsonisation. This contrasts with the data in Table 5.4. which showed that the survival of *B. parapertussis*, when expressed as a % of intracellular bacteria, varied when the bacteria were opsonised.

Fig. 5.8. Survival of ovine *B. parapertussis* H1 within ovine alveolar macrophages following opsonisation. Opsonins used were LS H1 (lamb serum raised against H1), LS NCTC (lamb serum raised against NCTC 10520), TBW 5 and TBW 5 - IgG.

Fig. 5.9. Survival of ovine *B. parapertussis* K2 within ovine alveolar macrophages following opsonisation. Opsonins used were LS H1 (lamb serum raised against H1), LS NCTC (lamb serum raised against NCTC 10520), TBW 5 and TBW 5 - IgG.


log10 cfu/ml
5
4
3
2
1
0
Control
LS H1
LS NCTC
TBW 5
TBW 5 - IgG
Opsonins

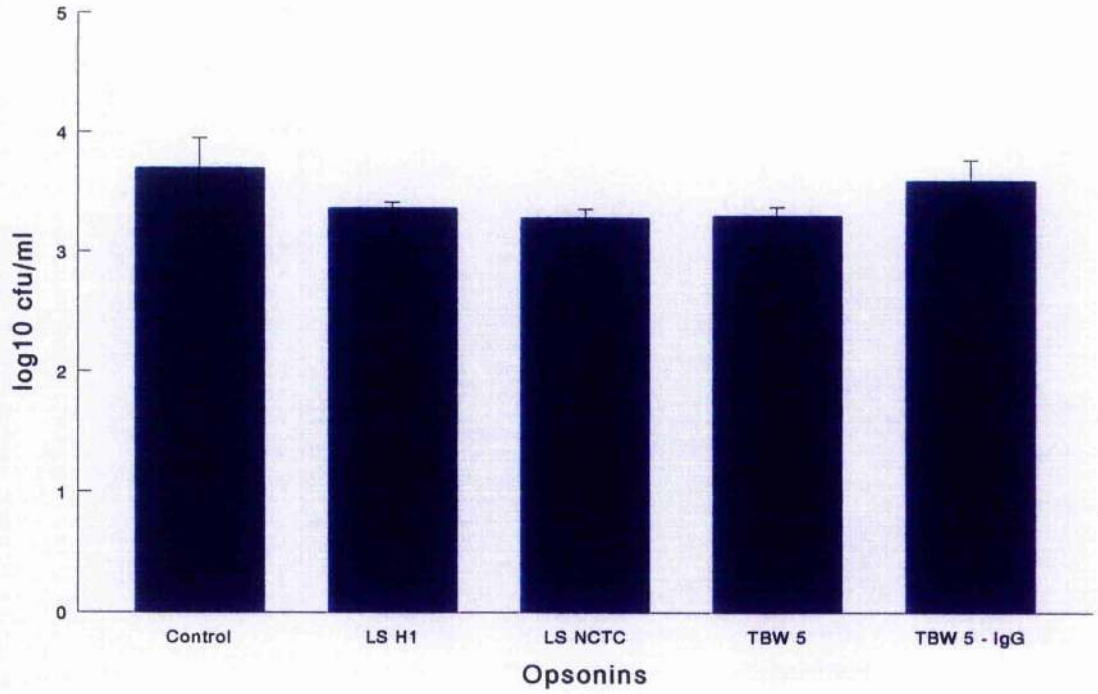
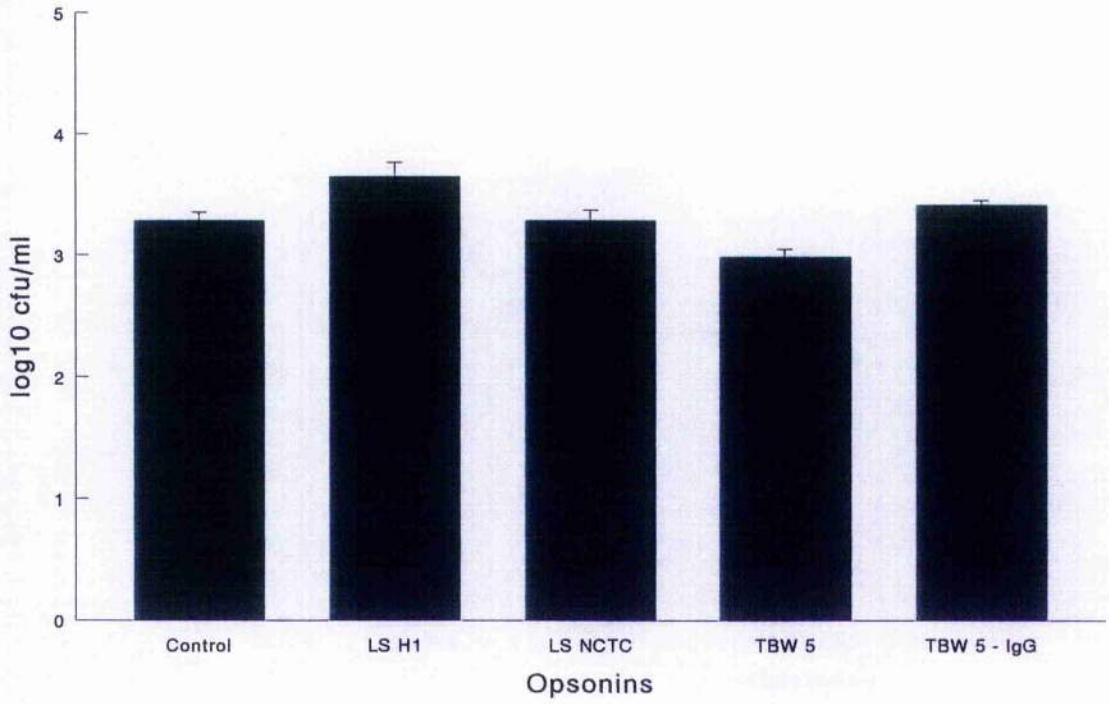
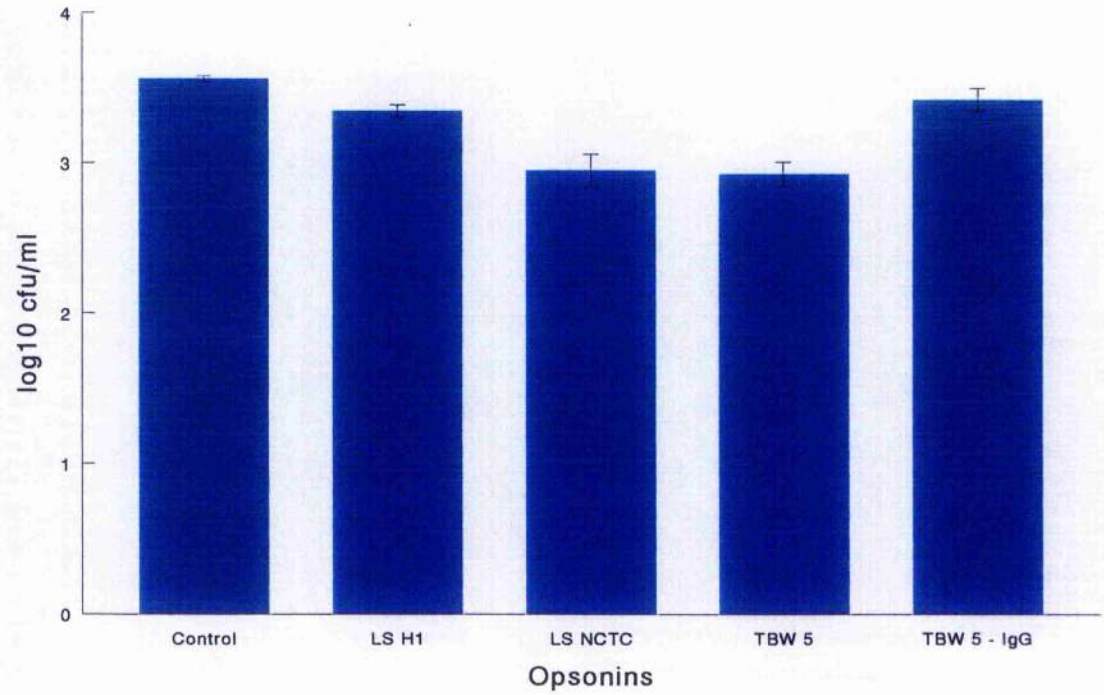

log10 cfu/ml
5
4
3
2
1
0
Control
LS H1
LS NCTC
TBW 5
TBW 5 - IgG
Opsonins

Fig. 5.10. Survival of human *B. parapertussis* B 285 within ovine alveolar macrophages following opsonisation. Opsonins used were LS H1 (lamb serum raised against H1), LS NCTC (lamb serum raised against NCTC 10520), TBW 5 and TBW 5 - IgG.

Fig 5.11. Survival of human *B. parapertussis* NCTC 10520 within ovine alveolar macrophages following opsonisation. Opsonins used were LS H1 (lamb serum raised against H1), LS NCTC (lamb serum raised against NCTC 10520), TBW 5 and TBW 5 - IgG.


log10 cfu/ml
0
1
2
3
4
5
Control
LS H1
LS NCTC
TBW 5
TBW 5 - IgG
Opsonins


log10 cfu/ml
0
1
2
3
4
Control
LS H1
LS NCTC
TBW 5
TBW 5 - IgG
Opsonins

Opsonisation of ovine and human *B. parapertussis* did not appear to significantly affect the survival of these isolates within alveolar macrophages when compared to the survival of unopsonised bacteria. The use of opsonins resulted in little difference between the survival of ovine and human isolates.

5.7. Effect of prolonged incubation on intracellular survival

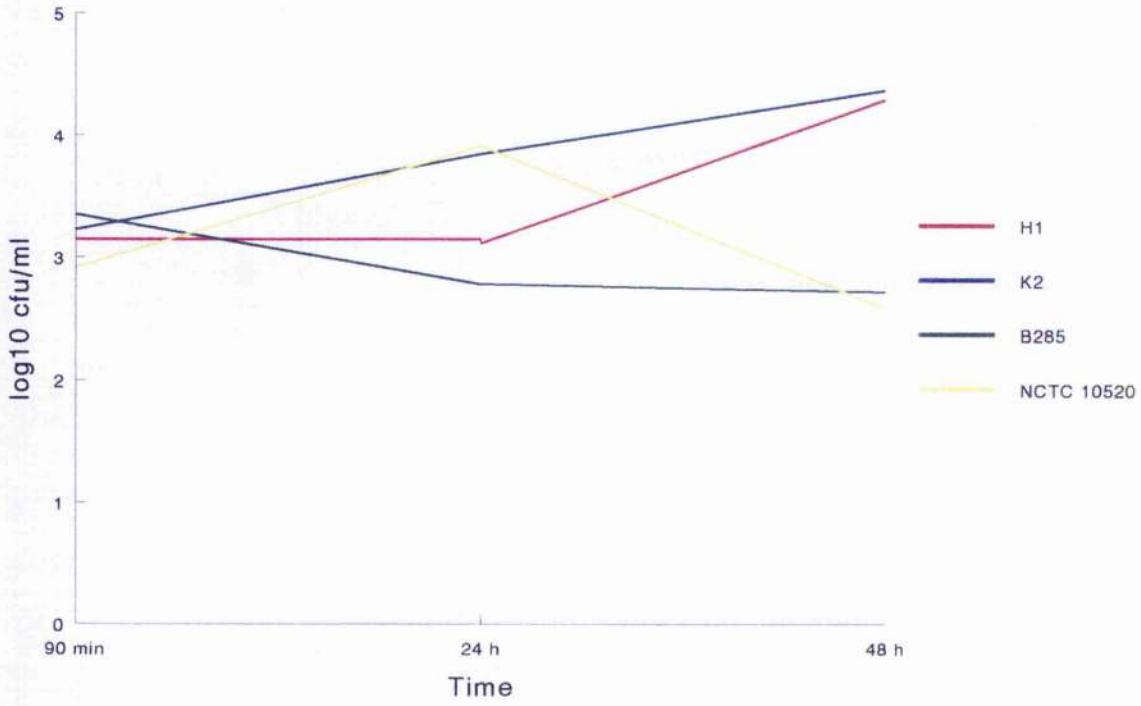
Ovine and human *B. parapertussis* survival within ovine and porcine alveolar macrophages was monitored over 48 h. Briefly, the survival was carried out as before except that following the addition of gentamicin, the cells were washed then 10 μg ml^{-1} polymyxin was added to kill any bacteria which may leave the macrophages during the 24 h or 48 h incubation. Following incubation, the cells were washed then lysed and dilutions plated onto blood agar to determine the number of viable intracellular bacteria. To ensure that the concentration of polymyxin used would kill any bacteria present, each *B. parapertussis* isolate was incubated for 24 h and 48 h in 10 μg ml^{-1} polymyxin in the absence of macrophages.

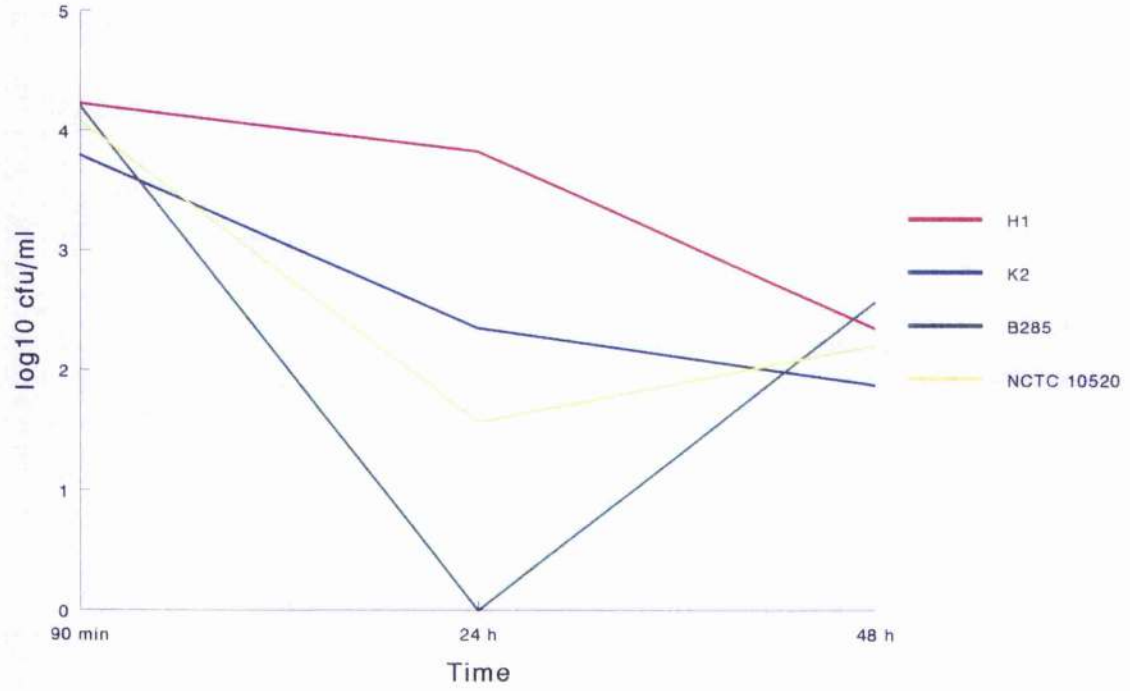
The survival rates represented in Fig. 5.12 show that by 24 h, both the ovine isolate K2 and human isolate NCTC 10520 had increased in numbers while both H1 and B285 had decreased. However, after incubation for 48 h the survival of the human isolates had decreased whereas the survival of the ovine isolates had increased.

Fig. 5.13 represents the survival of *B. parapertussis* within porcine alveolar macrophages over 48 h and shows that the number of viable intracellular bacteria

Fig. 5.12. Survival of ovine (H1 and K2) and human (B 285 and NCTC 10520) *B. parapertussis* within ovine alveolar macrophages over 48 h.

Fig. 5.13. Survival of ovine (H1 and K2) and human (B 285 and NCTC 10520) *B. parapertussis* within porcine alveolar macrophages over 48 h.


5
4
3
2
1
0
log10 cfu/ml
90 min
24 h
48 h
Time
H1
K2
B285
NCTC 10520


5
4
3
2
1
0
log10 cfu/ml
90 min
24 h
48 h
Time
H1
K2
B285
NCTC 10520

for both ovine and human isolates decreased over the first 24 h. However, after 48 h, the decline in survival of ovine isolates continued whereas the numbers of human isolates had begun to recover.

5.8. Discussion

Until recently the bordetellae were thought to be obligate extracellular pathogens with no known environmental reservoirs of infection. However, research into the intracellular capabilities of both *B. pertussis* and *B. bronchiseptica* has revealed these species can invade, and survive within, epithelial cells (Lee *et al.*, 1990; Schipper *et al.*, 1994), polymorphonuclear leucocytes (Steed *et al.*, 1992; Register *et al.*, 1994) and macrophages (Saukkonen *et al.*, 1991; Friedman *et al.*, 1992; Forde *et al.*, 1998).

The interaction of *B. parapertussis* with macrophages has been little studied which is perhaps surprising considering that its two closest relatives in the *Bordetella* genus, *B. pertussis* and *B. bronchiseptica*, have been shown to use different mechanisms for the entry into and survival within these cells. *B. pertussis* uses *bvg*-activated factors such as FHA, PT and ACT for phagocytosis and survival within macrophages (Friedman *et al.*, 1992), whereas, the expression of *bvg*-activated factors is not necessary for the uptake and intracellular survival of *B. bronchiseptica* (Banemann and Gross, 1997).

The adherence of *B. pertussis* to macrophages is dependent upon the production of PT which interacts with carbohydrate receptors on the cell surface, and on FHA which interacts with both carbohydrate receptors and the macrophage integrin CR3

(Relman *et al.*, 1990; Saukkonen *et al.*, 1991). Both these factors can independently mediate the adherence of *B. pertussis* to macrophages (Relman *et al.*, 1990) yet the presence of PT enhances the binding of FHA to CR3 (van't Wout *et al.*, 1992). The mechanism of *B. parapertussis* adherence to macrophages has not been determined. However, this species does not produce PT therefore it is likely that FHA is the major adhesin. The results presented in this study (Fig 5.1) show that ovine isolate H1 was more adherent to ovine alveolar macrophages than the other isolates tested. This isolate produced more FHA than the other isolates (Chapter 4) and suggests that FHA plays an important role in the adherence of *B. parapertussis* to macrophages. The ovine isolate K2 was found to produce less FHA than B285 and NCTC 10520 yet this isolate adhered better than the human isolates which suggests either that there is some host specificity of the isolates or that K2 produces more of another adhesin, such as PRN. Friedman *et al.* (1992) suggested a role for PRN in the attachment of *B. pertussis* to macrophages and this may also be the case for *B. parapertussis*.

Investigation into intracellular entry of *B. parapertussis* into ovine alveolar macrophages (Table 5.1) showed that ovine isolate H1 had a greater level of uptake compared with ovine isolate K2 and the human isolates. Friedman *et al.* (1992) showed that FHA is important for the internalisation of *B. pertussis* into macrophages following interaction of the RGD sequence on FHA with the macrophage integrin CR3 (Relman *et al.*, 1990; Saukkonen *et al.*, 1991). H1 produced more FHA than the other *B. parapertussis* therefore it is possible that the higher macrophage uptake of this isolate is due to the presence of FHA. This suggests a possible uptake mechanism of *B. parapertussis* similar to that of *B.*

pertussis. B285 and NCTC 10520 were found to produce similar concentrations of FHA yet a higher concentration of NCTC 10520 was internalised. PRN also contains an RGD sequence which may interact with CR3 thereby enabling macrophage uptake of the bacteria. The PRN of *B. pertussis* plays a role in the invasion of Hep-2 (Roberts *et al.*, 1991) and HeLa (Leininger *et al.*, 1992) cells and a possible role for PRN in macrophage uptake of *B. pertussis* has been suggested by Friedman *et al.* (1992).

Intracellular uptake is dependent upon the initial adherence of bacteria to cells. Since the ovine isolates adhered to ovine alveolar macrophages better than the human isolates, ovine *B. parapertussis*, as expected, invaded the macrophages better than human *B. parapertussis*. It would be interesting to screen more strains of ovine and human *B. parapertussis* to determine if the interaction between *B. parapertussis* and macrophages is host specific.

The uptake of ovine and human *B. parapertussis* in this study was approximately 0.1-14.5%. Such large variation of uptake is possibly due to the production of different concentrations of the necessary virulence factors by the four isolates tested and may also be due to some host-specificity of the isolates. This value is similar to those from studies of *B. pertussis* and *B. bronchiseptica* in which the bacterial uptake was 1-12.4% (Friedman *et al.*, 1992; Banemann and Gross, 1997; Forde *et al.*, 1998).

The adherence of all the isolates except K2 increased following opsonisation with lamb serum. The use of TBW 5 as an opsonin either decreased or had no effect on the adherence of the isolates whereas, TBW 5 - IgG had little effect on the adherence of both ovine and human *B. parapertussis*. However, this did not

completely correlate with the number of intracellular bacteria. Opsonisation with lamb serum increased both the adherence and intracellular count of H1 and B285 but using lamb serum raised against H1 increased the adherence of NCTC 10520 but decreased the number of intracellular bacteria. Also, opsonisation with lamb serum reduced the adherence of K2 but increased the intracellular uptake of this isolate. Opsonisation with TBW 5 decreased the adherence and the number of intracellular bacteria for all the isolates. Whereas, opsonisation with TBW 5 - IgG had little effect on the adherence of ovine and human isolates but this increased the intracellular count of all isolates except H1.

Opsonisation with lamb serum raised against the human isolate NCTC 10520 increased the intracellular uptake of all the isolates. Yet, opsonisation with lamb serum raised against ovine isolate H1 decreased the uptake of NCTC 10520 and the uptake of B285 was not as high as with the other serum. It is possible that the lamb serum raised against H1 did not interact with the human isolates as effectively as the other serum and this resulted in a reduced uptake when compared with opsonisation using lamb serum raised against NCTC 10520. It may be that the two sera react to different antigens present on the bacterial cell surface and that on the surface of human *B. parapertussis* these antigens may be masked, resulting in less interaction with the bacteria and ultimately, less macrophage uptake.

Human bronchoalveolar lavage fluid has been shown to stimulate phagocytosis due to the presence of natural opsonins such as surfactant protein A, which can act as a stimulator of phagocytosis (Tino and Wright, 1996). In this study, TBW 5 only slightly increased the intracellular count of one of the isolates, B285. In the absence of IgG this TBW increased the macrophage uptake of ovine isolate K2 and

both the human isolates. This suggests that the IgG in TBW 5 may somehow prevent the uptake of bacteria when they are pre-incubated in this fluid. However, in chapter 3, neither TBW 5 nor any purified IgG from this fluid reacted with ovine and human *B. parapertussis*. It is possible that the purification procedure also removed some other antimicrobial component(s) of the fluid. The low bacterial uptake following incubation with TBW 5 may be due to antimicrobial factors present in TBW such as peptides, free fatty acids and lysozymes, which have been found to be bactericidal (Coonrod, 1986). The low intracellular count of the ovine isolates opsonised with TBW 5 - IgG in comparison with the other isolates suggest that some of the substances present in this fluid may act specifically against ovine *B. parapertussis* thereby reducing macrophage uptake.

The increased bacterial uptake into macrophages following opsonisation may be due to invasion of the cells via an alternative route. Hazenbos *et al.* (1994) suggested that in the absence of opsonins, *B. pertussis* is internalised in monocytes via the RGD-CR3 interaction whereas in the presence of opsonins, the bacteria enter monocytes via Fc receptors. This may also be the case for the macrophage uptake of *B. parapertussis*.

Uptake via the CR3 receptor does not initiate the oxidative burst within macrophages (Wright and Silverstein, 1983). As a result, the bacteria will not be exposed to reactive oxygen intermediates which are bactericidal. Therefore, this route may promote intracellular survival of the bacteria. On the other hand, entry into the macrophages via Fc receptors activates the respiratory burst which can then kill the bacteria.

The intracellular count of *B. parapertussis* presented in Table 5.3. was much lower

than those in Table 5.1. This may be because the macrophages used in these experiments were isolated from different sheep, resulting in different intracellular counts and survival rates.

Comparison of the survival of ovine and human *B. parapertussis* within ovine alveolar macrophages revealed that H1 was more capable of intracellular survival compared with K2, B285 and NCTC 10520 (Fig 5.3) . It is possible that the higher survival rate of H1 is a result of better adherence and uptake into the macrophage compared with the other isolates. Alternatively, it may be that H1 possesses higher concentrations of the virulence factors of *B. parapertussis* and is therefore better equipped to withstand the antimicrobial mechanisms within the macrophage. *B. pertussis* uses AC to increase intracellular cAMP thereby inhibiting the fusion of the *B. pertussis*-containing phagosome and the lysosome and preventing killing of the bacteria (Masure, 1993). It is possible that H1 produces more AC than the other isolates and utilises the same mechanism as *B. pertussis* for intracellular survival.

In contrast with *B. pertussis*, *B. bronchiseptica* survives within macrophages by a process which does not require the production of *bvg*-activated virulence factors. *B. bronchiseptica*-containing phagosomes fuse with the lysosome thereby exposing *B. bronchiseptica* to attack by antimicrobial products such as reactive oxygen metabolites and lysozyme. However, *B. bronchiseptica* survives the antimicrobial attack which suggests that this organism may produce virulence factors which are negatively-regulated by the BvgAS system in order to survive intracellularly (Banemann and Gross, 1997). The actual mechanism of *B. parapertussis* intracellular survival is unknown. As *B. pertussis* and *B. bronchiseptica* both use

different methods of survival within the macrophage it would be useful to determine if *B. parapertussis* uses the same mechanism as one of the other bordetellae or if it has a unique survival strategy.

Opsonisation with a variety of fluids did not significantly affect the survival of *B. parapertussis* (Fig. 5.8-5.11). Fig. 5.9 shows that a proportion of the unopsonised ovine isolate K2 appears to survive intracellularly yet counts of intracellular bacteria carried out microscopically showed that, during this experiment, unopsonised K2 was not intracellular. It is possible that K2 is closely associated with the macrophage cell surface but not internalised. This may protect the bacteria from killing when antibiotics are added to the macrophage layer in order to kill extracellular bacteria and therefore these bacteria are included in the intracellular survival count. This type of protection has been observed for the interaction of *B. bronchiseptica* with macrophage monolayers (Forde *et al.*, 1998). Following 48 h incubation within ovine alveolar macrophages, the number of viable intracellular ovine *B. parapertussis* was beginning to increase whereas the concentration of viable intracellular human *B. parapertussis* was in decline (Fig. 5.12). The difference in the ability of human and ovine *B. parapertussis* to survive intracellularly may be due to host specificity of the isolates. Unfortunately, the survival of ovine and human isolates within fresh human alveolar macrophages could not be studied to determine if the intracellular survival of *B. parapertussis* is host specific. However, porcine alveolar macrophages were used to determine if *B. parapertussis* survival within alveolar macrophages was host specific. If this was the case then you would expect the number of viable ovine and human *B. parapertussis* to decrease. Over the first 24 h, the viability of both ovine and

human *B. parapertussis* decreased (Fig. 5.13). This was followed by a continual decrease in viable ovine *B. parapertussis* for a further 24 h whereas the numbers of viable human *B. parapertussis* increased. It is possible that the increase of human *B. parapertussis* is the result of a release of nutrients from dead bacteria therefore enabling growth of the remaining viable bacteria. Alternatively, the recovery in viable numbers of human *B. parapertussis* could represent the selection of a small percentage of bacteria which are then able to grow because of the lack of competition from other bacteria within the macrophage.

Using an infection ratio of approximately 30 bacteria per cell, ovine *B. parapertussis* could survive and increase in numbers within ovine macrophages for 48 h. Friedman *et al.* (1992) demonstrated that *B. pertussis* could survive intracellularly for 3 days at a infection rate of 100 bacteria per cell but required a ratio of 400 bacteria per cell for multiplication to occur. It has also been shown that the persistence of *B. bronchiseptica* within cultured murine macrophages was dependent upon the infectivity ratio. Forde *et al.* (1998) showed that *B. bronchiseptica* could survive within macrophages for 4 days, with little reduction in number, when an infectivity ratio of ≥500:1 (bacteria:macrophages) was used. The 48 h incubation period in this study only permitted a study of short-term survival of *B. parapertussis* within macrophages. Investigation over a longer period of time might reveal the extent of *B. parapertussis* intracellular survival and indicate the importance of this in the disease process.

For all of the phagocytic survival experiments, there were large margins of error. It is likely that this was due to the fact that for each experiment, macrophages were isolated from a group of different sheep. Similar differences in bacterial survival

were also noted by Wasswnaar *et al.* (1997) who stated that the viability of phagocytosed *Campylobacter jejuni* was dependent upon the individual donating the macrophage.

The experiments presented in this chapter suggest that there is a relationship between the adherence of *B. parapertussis* to macrophages and any subsequent invasion of these macrophages. However, analysis of the survival of *B. parapertussis* within macrophages revealed that despite the ovine isolates adhering to and invading ovine alveolar macrophages better than human isolates, neither of the ovine isolates survived intracellularly better than the human isolates.

It is clear that the interaction of *B. parapertussis* with host macrophages and the intracellular survival of these bacteria requires further study to determine if the mechanisms of these interactions are similar to those of *B. pertussis* or *B. bronchiseptica* or if *B. parapertussis* uses a different strategy. Further investigation, possibly using *B. parapertussis* mutants defective in the virulence factors implicated in macrophage interaction, would determine the importance of intracellular survival in *B. parapertussis* disease.

CHAPTER 6

COMPARISON OF OVINE AND HUMAN *BORDETELLA PARAPERTUSSIS* LIPOPOLYSACCHARIDE

6.1. Introduction

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria and one of the main virulence factors produced by these bacteria. Studies have revealed that the LPS of human *B. parapertussis* is smooth and consists of O-antigen, which produces a ladder arrangement of high molecular mass in addition to a region of low molecular mass on PAGE (Amano *et al.*, 1990; Di Fabio *et al.*, 1992). In comparison, the LPS of ovine *B. parapertussis* is reported to rough, consisting of only the core region which produces an area of low molecular mass on PAGE (van den Akker, 1998). However, little is known about the role of LPS in infections caused by *Bordetella* species, although the LPS of *B. parapertussis* has been reported to be less toxic than the LPS of *B. pertussis* and *B. bronchiseptica* (Watanabe *et al.*, 1990).

This study was carried out to compare the PAGE-generated LPS profiles of ovine and human *B. parapertussis* and determine whether the LPS of ovine and human were immunologically distinct.

6.2. Materials and methods

The isolates used in this chapter are listed below in Table 6.1.

Table 6.1. Strains of *B. parapertussis* used in LPS analysis

Ovine	Human
C	B285
H1	NCTC 10520
K2	NCTC 5952
J1	B268
NZ 928	B271

The LPS of these bacteria were analysed using PAGE and Western blots. Ovine *B. parapertussis* H1 and K2 and human *B. parapertussis* B285 and NCTC 10520 were also used in bactericidal assays comparing the bactericidal activity of ovine and rabbit sera raised against ovine and human *B. parapertussis*. Further details of the materials and methods used can be found in sections 2.6 and 2.7.

6.3. LPS PAGE

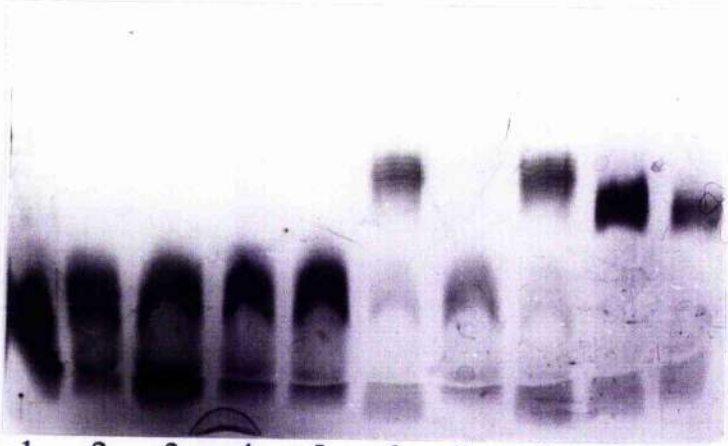
LPS was extracted from nutrient broth and cyclodextrin liquid cultures of different *B. parapertussis* isolates. These were then compared using PAGE and visualised using a silver stain. The results are shown in Fig. 6.1 – 6.4.

The LPS of *B. parapertussis* grown in cyclodextrin liquid (Fig. 6.1 and 6.2) was different from the LPS of *B. parapertussis* grown in nutrient broth (Fig. 6.3 and 6.4) when observed using PAGE.

The 12 % acrylamide gel of cyclodextrin liquid cultures (Fig 6.1) shows that the

Fig. 6.1. 12 % acrylamide PAGE of LPS of ovine and human *B. parapertussis* extracted from cyclodextrin liquid cultures visualised using silver staining. Ovine isolates lanes 1-5 and human isolates lanes 6-10. Lane order: (1) H1, (2) K2, (3) C, (4) J1, (5) NZ 928, (6) B 285, (7) NCTC 5952, (8) NCTC 10520, (9) B268, (10) B271.

Fig. 6.2. 14 % acrylamide PAGE of LPS of ovine and human *B. parapertussis* extracted from cyclodextrin liquid cultures visualised using silver staining. Ovine isolates lanes 1-5 and human isolates lanes 6-10. Lane order: (1) H1, (2) K2, (3) C, (4) J1, (5) NZ 928, (6) B 285, (7) NCTC 10520, (8) NCTC 5952, (9) B268, (10) B271.


1 2 3 4 5 6 7 8 9 10

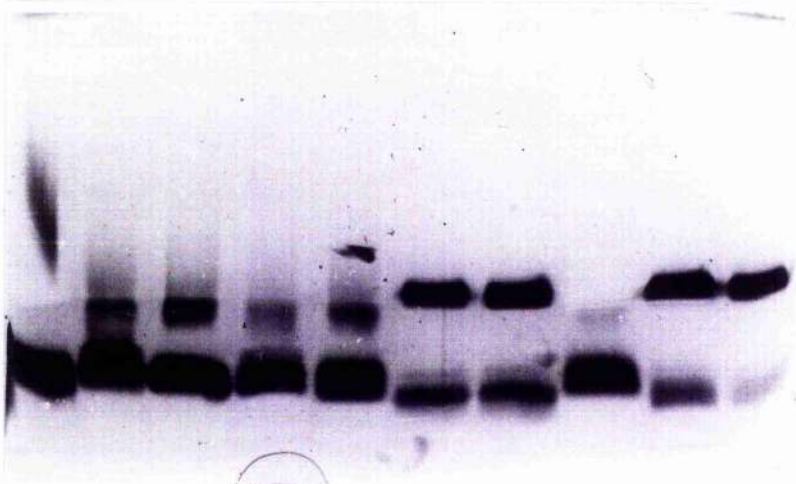
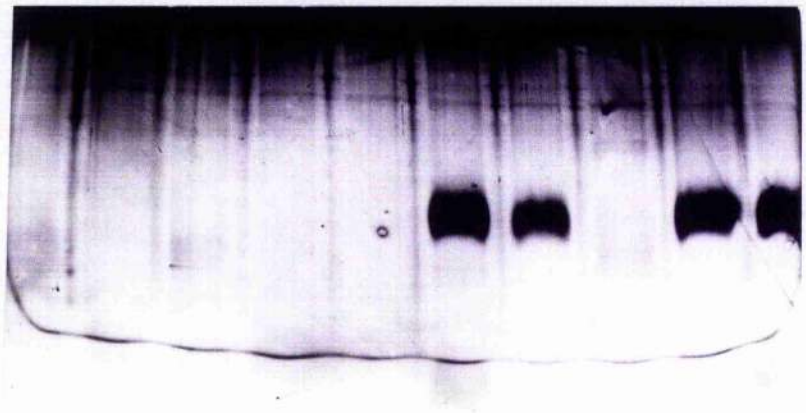
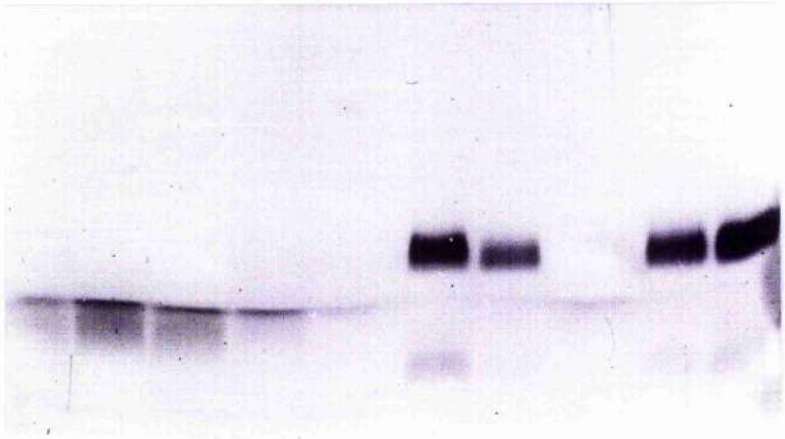

1 2 3 4 5 6 7 8 9 10

Fig. 6.3. 12 % acrylamide PAGE of LPS of ovine and human *B. parapertussis* extracted from nutrient broth cultures visualised using silver staining. Ovine isolates lanes 1-5 and human isolates lanes 6-10. Lane order: (1) H1, (2) K2, (3) C, (4) J1, (5) NZ 928, (6) B 285, (7) NCTC 5952, (8) NCTC 10520, (9) B268, (10) B271.

Fig. 6.4. 14 % acrylamide PAGE of LPS of ovine and human *B. parapertussis* extracted from nutrient broth cultures visualised using silver staining. Ovine isolates lanes 1-5 and human isolates lanes 6-10. Lane order: (1) H1, (2) K2, (3) C, (4) J1, (5) NZ 928, (6) B 285, (7) NCTC 5952, (8) NCTC 10520, (9) B268, (10) B271.



1 2 3 4 5 6 7 8 9 10



1 2 3 4 5 6 7 8 9 10

ovine isolates contained only a low molecular weight area, presumably the core region of the LPS whereas 4 out of 5 of the human isolates also contained higher molecular mass structures. Two of the human isolates (B285 and NCTC 10520) clearly show a ladder arrangement of O-antigens. Another two of the human isolates (B268 and B271) displayed a band in the same region of the O-antigens of B285 and NCTC 10520 but no ladder arrangement was discernible. It is interesting to note that the human isolate NCTC 5952 has an LPS pattern similar to those of the ovine isolates.

The 14 % acrylamide gel of cyclodextrin liquid cultures (Fig. 6.2) revealed that the core region of the LPS extracted from ovine isolates consisted of at least two distinct low molecular weight bands compared with the one distinct low molecular weight band found in the LPS of most of the human isolates, again with the exception of NCTC 5952 which had an profile similar to those of the ovine isolates.

Analysis of LPS extracted from nutrient broth cultures in 12% and 14% acrylamide gels (Fig. 6.3 and 6.4) showed that the ovine isolates consisted of a single low molecular weight band whereas the human isolates contained a single low molecular weight band and a single band of a higher molecular weight. No ladder arrangement could be discerned within the higher molecular weight bands. On the 12% gel the ovine isolate C and human isolate NCTC 5952 both produced faint bands at a high molecular weight, but lower than those found in the other human *B. parapertussis* LPS, as well as a low molecular weight band. In comparison, the LPS of both of these isolates consisted of a single low molecular weight band on the 14% gel.

6.4. Western blots of LPS

The LPS extracted from cyclodextrin cultures of both ovine and human *B. parapertussis* were run on a 12% acrylamide gel then transferred onto nitrocellulose membrane and probed with a range of different sera. The sera used are listed below.

Table 6.2. Sera raised against *B. parapertussis*.

Rabbit sera	Sheep sera
Anti-*B. parapertussis* isolate C (No. 355)	Anti-*B. parapertussis* isolate H1 (No. 39)
Anti-*B. parapertussis* NCTC 10520 (No.38)	Anti-*B. parapertussis* NCTC 10520 (No.227)

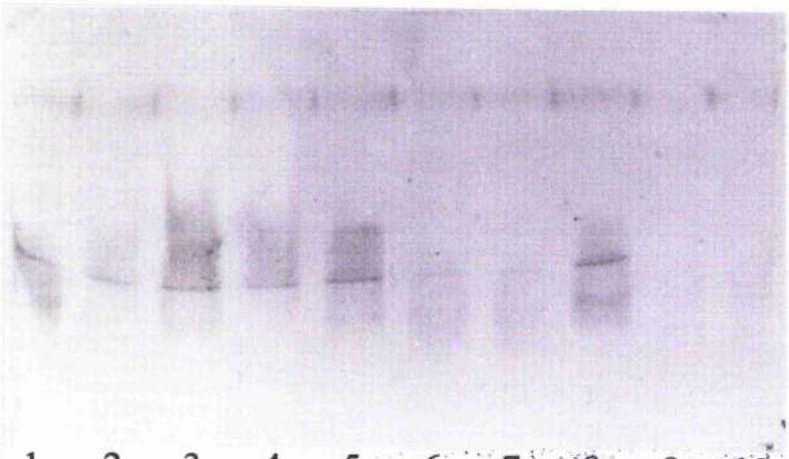
The resulting blots using rabbit sera are pictured in Fig. 6.5 and 6.6. Nothing was observed on the blots probed with sheep sera.

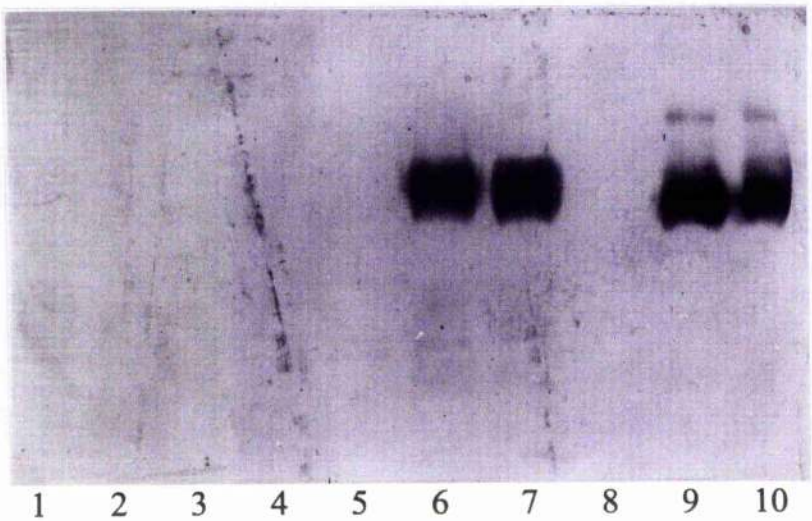
Rabbit serum raised against ovine isolate C recognised weakly all the ovine and human isolates on a Western blot (Fig. 6.5), although it did not recognise the O-antigens of the human isolates B285 and NCTC 10520.

In comparison, the rabbit serum raised against the human isolate NCTC 10520 recognised only the human *B. parapertussis* isolates. However, this serum recognised neither the human isolate NCTC 5952 nor the ovine isolates. This serum reacted with only the higher molecular weight bands that could be seen on a silver stain of the human isolates. The ladder arrangement of B 285 and NCTC 10520 can be seen on the Western blot.

Fig. 6.5. Western blot of ovine and human *Bordetella parapertussis* LPS probed with rabbit sera raised against ovine isolate C. Ovine isolates lanes 1-5 and human isolates lanes 6-10. Lane order: (1) H1, (2) K2, (3) C, (4) J1, (5) NZ 928, (6) B285, (7) NCTC 10520, (8) NCTC 5952, (9) B268, (10) B271.

Fig. 6.6. Western blot of ovine and human *Bordetella parapertussis* LPS probed with rabbits sera raised against human isolate NCTC 10520. Ovine isolates lanes 1-5 and human isolates lanes 6-10. Lane order: (1) H1, (2) K2, (3) C, (4) J1, (5) NZ 928, (6) B285, (7) NCTC 10520, (8) NCTC 5952, (9) B268, (10) B271.


1 2 3 4 5 6 7 8 9 10


1 2 3 4 5 6 7 8 9 10

6.5. Bactericidal Assay

Bactericidal assays were carried out to determine if the sera used for probing the LPS of *B. parapertussis* on Western blots were bactericidal to the ovine and human isolates of *B. parapertussis* tested. Test sera were heat-inactivated at 56°C for 30 min, added to a bacterial suspension followed by the addition of an exogenous source of complement (specific-pathogen-free lamb serum). This mixture was incubated for 45 min at 37°C then samples were taken and plated onto blood agar to determine the number of viable bacteria remaining. The results are tabulated in Table 6.3.

From this table it is clear that the complement source alone (PBS + complement) kills 100% of K2 and a proportion of the other bacteria. To compensate for this difference, the percentage of bacteria killed by the complement alone was subtracted from the percentage killed to give a more accurate reflection of the bactericidal effects of the sera tested. These results are displayed in Table 6.4.

These results show that, as with the Western blots of LPS using these sera, the sera raised against ovine *B. parapertussis* killed approximately the same amount of both ovine and human *B. parapertussis*. In comparison, sera raised against human *B. parapertussis* killed a much higher percentage of human isolates compared to ovine isolates.

Table 6.3. Percentage of ovine (H1 and K2) and human (B285 and NCTC 10520) *B. parapertussis* killed by various sera.

Sera	Percentage killing of isolate: H1 Mean ± SEM	K2 Mean ± SEM	B285 Mean ± SEM	NCTC 10520 Mean ± SEM
Sheep α H1	96.47 ± 1.97	100 ± 0	96.43 ± 1.82	92.5 ± 5.12
Sheep α NCTC 10520	58.9 ± 10.1	100 ± 0	100 ± 0	100 ± 0
Rabbit α C	68.6 ± 10.5	100 ± 0	25.3 ± 11.4	15.2 ± 7.81
Rabbit α NCTC 10520	61.77 ± 2.89	100 ± 0	100 ± 0	100 ± 0
Controls				
PBS + complement	53.4 ± 12.3	100 ± 0	36.9 ± 12.9	35.1 ± 13.2
Heat-inactivated complement + sheep αH1	1.37 ± 1.37	12.6 ± 7.08	15.6 ± 10.8	11.7 ± 7.31
Heat-inactivated complement + sheep α NCTC 10520	0 ± 0	8.9 ± 6.86	19.1 ± 12.2	9.07 ± 9.07
Heat-inactivated complement + rabbit α C	9.53 ± 7.89	6.2 ± 0.86	31.97 ± 9.37	28.4 ± 15
Heat-inactivated complement + rabbit α NCTC 10520	3.73 ± 1.87	11.3 ± 6.28	30.07 ± 9.32	28.6 ± 15.5

Table 6.4. Percentage of ovine (H1 and K2) and human (B285 and NCTC 10520) *B. parapertussis* killed by various sera following the subtraction of the percent killed by the complement source alone.

Sera	Percentage killing of isolate: H1 Mean ± SEM	K2 Mean ± SEM	B 285 Mean ± SEM	NCTC 10520 Mean ± SEM
Sheep α H1	43.1 ± 10.8	0 ± 0	65.1 ± 17.9	57.4 ± 16
Sheep α NCTC 10520	6.27 ± 5.02	0 ± 0	68.7 ± 16.3	66.9 ± 13.2
Rabbit α C	15.23 ± 1.93	0 ± 0	0 ± 0	5.03 ± 5.03
Rabbit α NCTC 10520	12.2 ± 6.10	0 ± 0	68.7 ± 16.3	66.9 ± 13.2

6.6 Discussion

PAGE of the LPS of *B. parapertussis* revealed differences in LPS produced in cyclodextrin liquid when compared with the LPS produced in nutrient broth. The LPS of four out of five human *B. parapertussis* produced a high molecular weight

band when extracted from both cyclodextrin liquid and nutrient broth cultures. However, O-antigens could only be visualised on two of the isolates (B285 and NCTC 10520) when they were grown in cyclodextrin liquid (Fig. 6.1). The LPS of ovine isolates grown in cyclodextrin liquid produced three bands, two of which formed the core region and another that was of a higher molecular weight (Fig. 6.2). In comparison, the LPS of ovine isolates grown in nutrient broth consist of a single low molecular weight band (Figure 6.4). This implies that the structure of the LPS when visualised on PAGE gel is dependent on the medium used to grow *B. parapertussis*. A study of *Bacteroides* species (Allan and Poxton, 1994) also revealed that the growth medium used affects the LPS pattern that is produced on PAGE.

The LPS of human *B. parapertussis* has been previously described as consisting of low molecular mass bands together with a ladder arrangement of high molecular mass bands (Amano *et al.*, 1990; Di Fabio *et al.*, 1992). Only two out of the five human isolates tested (B285 and NCTC 10520) displayed this LPS pattern (Fig. 6.1). However, a further two human isolates (B268 and B271) appeared to contain a high molecular weight band in the same region as the ladder arrangement of B285 and NCTC 10520. It is possible that better resolution of this band would reveal a ladder arrangement similar to those seen in the other isolates. van den Akker (1998) reported that growth temperature affected the LPS profile of *B. parapertussis* isolates. A reduction in temperature from 37°C to 25°C resulted in the absence of a ladder of O-antigens on the LPS of human *B. parapertussis* but this was replaced by a faster-migrating O-antigen containing a single band. This is similar to the LPS profile of B268 and B271 and it may be that growth of these

isolates at a different temperature will result in a ladder arrangement of O-antigens. In contrast, the LPS of all the ovine isolates and the human isolate NCTC 5952 appeared to be of the rough type i.e. no O-antigens were seen. The presence of O-antigens in the LPS of most of the human *B. parapertussis* and their absence in the LPS of ovine *B. parapertussis* reflects the findings of van den Akker (1998) who also determined that the LPS type of *B. parapertussis* was host specific. Yet, in this study, the LPS of the human isolate NCTC 5952 had a similar profile to the ovine isolates therefore questioning the suggestion that the LPS of *B. parapertussis* is host-specific. This is supported by the analysis of *B. parapertussis* LPS using anti-LPS monoclonal antibodies which resulted in NCTC 5952 being grouped with ovine isolates rather than the other human isolate tested (Porter *et al.*, 1995c). It would be useful to screen a larger sample of human and ovine *B. parapertussis* to determine the prevalence of rough-type LPS.

Different LPS types from the same host species have also been described for *B. bronchiseptica* (Le Blay *et al.*, 1997; van den Akker, 1998). Le Blay *et al.* (1997) suggested that the variability of LPS structures of *B. bronchiseptica* from the same host species could occur to evade the immune response and that the smooth LPS may correspond to a primary infection whereas the rough LPS may correspond to a recurrent or chronic infection. This could also be the case for *B. parapertussis* as ovine *B. parapertussis* isolated from apparently healthy sheep (Cullinane *et al.*, 1987; Porter *et al.*, 1994) was reported to cause a subclinical infection in mice (Chen *et al.*, 1989) and sheep (Porter *et al.*,1995a). This could explain the presence of only rough LPS on *B. parapertussis* isolated from sheep.

The Western blots of LPS from *B. parapertussis* revealed different specificities of

the sera used. The rabbit sera raised against the ovine *B. parapertussis* isolate C recognised the core region of all the isolates tested (Fig. 6.5) whereas the rabbit sera raised against the human isolate NCTC 10520 recognised only the O-antigens of the human *B. parapertussis* tested (Fig. 6.6). The sera raised against human *B. parapertussis* did not recognise the core region of any of the isolates and as such did not recognise the human isolate NCTC 5952. The lack of recognition of the core region of *B. parapertussis* LPS with this sera suggests that when the host comes into contact with smooth LPS of *B. parapertussis* the O-antigens mask the core region and therefore only an immune response against the O-antigens is raised. The absence of O-antigens on ovine LPS could explain why rabbit sera raised against the ovine isolates recognises both ovine and human LPS as all the isolates tested presented a core region on a silver stain of the LPS gel. This could also explain why the O-antigens on the human isolates are not recognised by sera raised against ovine isolates.

Ovine sera raised against ovine and human *B. parapertussis* did not recognise any of the *B. parapertussis* LPS on a Western blot. This may be because to raise ovine sera against these isolates, live bacteria were inoculated into the sheep. This could result in the bacteria masking the LPS or it could be that LPS is non-immunogenic in sheep. In comparison, the rabbits were inoculated with formalin-killed bacteria plus an adjuvant. In this state the LPS of the bacteria may be exposed and the presence of an adjuvant would enhance any immune response to the virulence factors present.

The use of a bactericidal assay to determine if the sera used to probe Western blots for LPS were bactericidal to ovine and human *B. parapertussis* revealed that the

isolates tested were sensitive to the complement source (Table 6.3). *B. parapertussis* has been reported to have a serum resistance locus (*brk*) (Fernandez and Weiss, 1994) although it is thought to be transcriptionally silent. Therefore this is unlikely to affect the complement killing of the *B. parapertussis* isolates tested here. The presence of O-antigens on the LPS of *Bordetella* species has been suggested to protect the bacteria from antibody and complement-mediated killing (Byrd *et al.*, 1991). The absence of O-antigens on the LPS of a *B. bronchiseptica* was determined to be the reason why this strain was sensitive to unheated pre-colostrum sera and hyperimmune porcine sera. However, despite the presence of O-antigens on the LPS of the human isolates B285 and NCTC 10520 approximately 35% of these bacteria were killed by complement alone. A higher percentage of the ovine strains (53% and 100%) were killed by the complement source and as these bacteria had rough LPS it is possible that the LPS does play a role in protecting *B. parapertussis* from complement killing. However, other factors must also be involved. Colostrum-deprived SPF lamb sera was used as the complement source and as such, no antibodies should be present. Therefore, it is possible that the bacteria activate the alternative complement pathway resulting in the serum complement killing the bacteria.

The percentage killing of bacteria by the various sera (Table 6.4) revealed that sera raised against the human isolate NCTC 10520 were more bactericidal for the human isolates than the ovine isolates. In comparison, there was little difference in the bactericidal effects of sera raised against ovine *B. parapertussis* on both ovine and human isolates. This reflects the reaction of rabbit sera with Western blots of LPS from these bacteria (Fig. 6.5 and 6.6) where the sera raised against an ovine

isolate reacted with all the isolates yet sera raised against a human isolate only reacted with the human isolates. These findings suggest a possible role for LPS as a target for the bactericidal activity of sera.

CHAPTER 7

GENERAL DISCUSSION

This study was designed to compare ovine and human *B. parapertussis* with particular reference to the possibility of host specificity of these isolates. In the context of this thesis, *B. pertussis* and *B. bronchiseptica* were discussed as comparators for *B. parapertussis*. These species have been shown to be so closely related (De Ley *et al.*, 1986; van der Zee *et al.*, 1997) and have been extensively researched that comparison with these was of more immediate relevance than with other unrelated bacterial species.

Ovine tracheobronchial washings (TBW) were used as growth media for ovine and human isolates to determine any host preference in the survival of *B. parapertussis*. The work presented here showed that there was possible host specificity of ovine and human *B. parapertussis* in their ability to grow in ovine TBW. Moreover, the results suggested that the potential for growth of *B. parapertussis* in these TBW was dependent upon the sheep that the TBW were taken from. The different growth rates of ovine and human *B. parapertussis* in the 5 TBW and the differing intracellular count and survival rates of these bacteria within ovine alveolar macrophages do suggest that each sheep reacts differently to the presence of these bacteria and this may depend upon prior exposure to *B. parapertussis*. It would be interesting to screen more sheep for the presence of *B. parapertussis* and monitor how exposure to *B. parapertussis* affects both the growth of this bacterium in TBW and interaction with macrophages taken from the sheep that the *B. parapertussis*

strain was isolated from.

The use of nutrient broth (NB) as a positive control for the growth of ovine and human *B. parapertussis* revealed that human isolates of *B. parapertussis* were slower growing than ovine *B. parapertussis*. This suggests that ovine and human *B. parapertussis* have different nutritional requirements and it may be that human *B. parapertussis* are more fastidious than the ovine isolates.

Further work is needed to fully ascertain the reason for the differing growth rates of ovine and human *B. parapertussis* in the variety of fluids tested. Perhaps, these isolates have different requirements for nicotinic acid and essential amino acids. Assaying the presence of these factors in TBW and NB and monitoring the growth of ovine and human *B. parapertussis* in varying concentrations of these factors might help to resolve this question. It may also be that ovine and human *B. parapertussis* have different susceptibilities to antibacterial factors present in TBW and monitoring the growth of these isolates in varying concentrations of factors such as lung surfactant, lysozyme, fatty acids and lactoferrin may reveal the reason for the different growth rates observed. It is also possible that, because these factors may be present in ovine TBW, the ovine isolates may possess mechanisms which could overcome these antibacterial proteins whereas the human isolates may be susceptible.

The suggestion of host specificity of ovine and human *B. parapertussis* could be further studied by monitoring the growth of these isolates in human TBW. This work could also benefit from the use of a wider range of ovine and human *B. parapertussis* strains which would give a better indication of any host specificity.

Adherence to the host respiratory tract is an important step in the establishment of

B. parapertussis infection. Previous studies have shown that the adherence of human *B. parapertussis* to ciliated epithelial cells demonstrates some host specificity (Tuomanen *et al.*, 1983). The results of the present study suggested that the adherence of ovine *B. parapertussis* to ovine tracheal organ culture was host specific. However, the adherence to continuous cell culture lines did not reveal host specificity of the ovine and human *B. parapertussis*. These cells are non-ciliated whereas *B. parapertussis* normally adheres to cilia of the host tracheal epithelium (Tuomanen *et al.*, 1983). Therefore, the ovine tracheal organ culture assay used in this project more closely resembles an *in vivo* situation. An interesting comparison would be to study the adherence of a greater number of ovine and human *B. parapertussis* strains to both ovine and human tracheal organ culture to determine if the adherence of ovine and human *B. parapertussis* to trachea is indeed host specific.

Investigation into the invasive potential of ovine and human *B. parapertussis* suggested that the invasion of cells by *B. parapertussis* was dependent upon adherence to these cells. Ovine isolate H1 was more adherent and invasive than the other isolates. H1 also appeared to produce more FHA therefore reinforcing the suggestion that this protein is an important virulence factor of *B. parapertussis*. FHA is not the only potential adhesin produced by ovine and human *B. parapertussis*. The adherence assays revealed differences in the ability of the two human isolates (B285 and NCTC 10520) to adhere to both cell lines and ovine tracheal organ culture despite both these isolates producing similar levels of haemagglutinin, presumably FHA. This raised the question of a possible role for other adhesins, such as PRN and fimbriae, in the adherence of human *B.*

parapertussis. It is also likely that these virulence factors are produced to enhance the adherence capabilities of ovine *B. parapertussis*. The development of specific assays to measure for PRN, fimbriae and FHA as well as the use of monoclonal antibodies raised against these factors would establish how essential these proteins are for the adherence of *B. parapertussis* to different target cells. The production of mutants defective in these virulence factors, by using site-directed mutagenesis, would be useful in the study of their role, if any, in the adherence of *B. parapertussis*.

In the present study, incubation of *P. haemolytica* with *B. parapertussis* culture fluid did not increase the adherence of *P. haemolytica*. It may be that, unlike *B. pertussis*, *B. parapertussis* does not contribute to the adherence of other bacteria to the respiratory tract. The results presented in this study suggest that *B. parapertussis* and *P. haemolytica* adhesins compete for the same receptors. However, it is possible that the production of other *B. parapertussis* virulence factors may enhance *P. haemolytica* infection.

Human *B. parapertussis* strains produce TCT and HLT which are capable of damaging the ciliated epithelial cells of the respiratory tract. This could predispose the host to a secondary infection by another respiratory pathogen. It may be that ovine *B. parapertussis* also produce these toxins and the action of these enable *P. haemolytica* to cause disease. If these factors are produced by ovine *B. parapertussis* then the establishment of mutants defective in these toxins followed by their use in organ culture or sheep which are then infected with *P. haemolytica* may determine their role in the predisposition of sheep to *P. haemolytica* infection.

Porter *et al.* (1995a) demonstrated that *B. parapertussis* predisposed specific

pathogen-free (SPF) lambs to *P. haemolytica* infection but that *B. parapertussis* alone did not result in any signs of clinical disease. However, Porter *et al.* (1995a) used ovine *B. parapertussis* isolate C which has subsequently been shown to be less adherent to cell lines than both the ovine isolates, H1 and K2, used in this current study (F. McCallum, personal communication). It is possible that the use of an apparently more virulent strain, such as H1, would result in greater predisposition of SPF lambs to *P. haemolytica* which, in turn, may cause a more severe infection than observed by Porter *et al.* (1995a). It is also possible that infection of SPF lambs with ovine *B. parapertussis* isolate II1 alone may result in some signs of clinical disease.

Analysis of the LPS produced by ovine and human *B. parapertussis* revealed that all the ovine isolates tested had rough-type LPS whereas the majority of the human isolates had smooth-type LPS. The one exception was NCTC 5952 which is the type strain of *B. parapertussis* and was isolated from a case of whooping cough. These results conflicted with an earlier report (van den Akker, 1998) which stated that ovine and human *B. parapertussis* have different LPS structures. The interaction of *B. parapertussis* LPS with rabbit sera raised against ovine and human *B. parapertussis* did suggest that LPS may be immunogenic and possibly a target of bactericidal activity. A bactericidal assay using sera absorbed with LPS would clarify whether anti-LPS antibodies are an important part of the host immune defence against *B. parapertussis* infection.

An interesting observation was that the pattern of LPS visualised on a silver-stained PAGE gel varied depending on the growth medium used. A study of the LPS produced by ovine and human *B. parapertussis* grown in a variety of different

fluids, such as ovine and human TBW, lamb serum and human serum, may reveal further variation of *B. parapertussis* LPS and the use of *in vivo* fluids may determine the LPS pattern produced during infection.

Analysis of interaction between alveolar macrophages and *B. parapertussis* showed that the isolates which were more adherent to macrophages were also more easily taken up by macrophages. This also reflects the findings in chapter 4 where the isolates which were more adherent to MRC-5 cells were also more invasive. In this study, both the ovine isolates adhered to and invaded ovine alveolar macrophages better than human isolates thereby suggesting some host specificity. However, the survival of any intracellular bacteria did not follow the same pattern. Instead, the survival of *B. parapertussis* within macrophages was possibly dependent upon the production of virulence factors. Ovine isolate H1 produced more haemagglutinin, presumably FHA, than the other isolates and as most of the virulence factors of *B. parapertussis* are regulated by the *bvg*-operon, it is likely that H1 will also produce more of the other virulence factors. The production of these virulence factors and other proteins may result in the better survival of H1 within alveolar macrophages compared to the other isolates tested.

This study highlighted the importance of the production of virulence factors by *B. parapertussis*. Throughout this project, ovine *B. parapertussis* H1 stood out as the isolate most capable of growth in *in vivo* fluids, adherence to and invasion of cells and survival within macrophages. It is known that this isolate produces more haemagglutinin, presumably FHA, than the other isolates and it is possible that H1 also produces more of the known *B. parapertussis* virulence factors. Measurement of these other virulence factors, production of antibodies against them and

development of mutants defective in their production may reveal their role in the pathogenicity of ovine *B. parapertussis* and their possible role in the predisposition of sheep to *P. haemolytica* infection.

The growth studies, adherence to tracheal organ culture and adherence to and invasion of alveolar macrophages have all suggested possible host specificity of ovine and human *B. parapertussis*. This project was limited in the study of host specificity because only ovine tissues and fluids were accessible. For an accurate comparison of potential host specificity of ovine and human *B. parapertussis* to be carried out, the interaction of both these types of *B. parapertussis* with both human and ovine tissues and fluids must be studied. It has been suggested by van der Zee *et al.* (1996) that ovine and human *B. parapertussis* evolved separately from *B. bronchiseptica*. This may explain the possible host specificity of the isolates tested in this project.

Any further comparison of ovine and human *B. parapertussis* should comprise of more strains and include the human isolate NCTC 5952. In this study, NCTC 5952 produced the same LPS profile as ovine *B. parapertussis*. Previous work has also shown NCTC 5952 to have antibiotic sensitivities similar to ovine *B. parapertussis* (Porter *et al.*, 1995) and a PFGE banding pattern identical to ovine *B. parapertussis* (Porter *et al.*, 1996). Further study of this isolate may reveal more about the evolution of ovine and human *B. parapertussis* and the potential of any cross-infection caused by these isolates.

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APPENDIX 1

Bordet-Gengou agar

15 g	Bordet-Gengou agar base
5 g	Glycerol
75 ml	Sheep blood
500 ml	Distilled water

Cyclodextrin liquid (as Imaizumi *et al.*, 1983)

Basic medium

10.7 g	Sodium 1-glutamate
2.5 g	NaCl
0.5 g	KH_2PO_4
0.1 g	$MgCl_2.6H_20$
0.02 g	$CaCl_2$
0.2 g	KCl
6.1 g	Tris
0.5 g	Casamino acids
0.24 g	L-proline
1 g	Heptakis(2,6-di-O-methyl)-β-cyclodextrin
1 l	Distilled water

Adjust to pH 7.4. Sterilise at 121°C for 15 min. Before use, add 100µl of supplement to 10ml of cyclodextrin liquid.

Supplement

0.01 g	$FeSO_4$
0.04 g	L-cysteine
0.004g	Niacin
0.15 g	Glutathione
0.02 g	Ascorbic acid
10 ml	Distilled water

Filter sterilise using a 0.45µm filter. Store at -20°C for up to 3 months.

SDS-PAGE for protein detection

4 x 0.75mm 10% gels (BIO-RAD Mini-PROTEAN II electrophoresis system)

Resolving gel

7.85 ml	Distilled water
5 ml	Buffer (1.91 g Tris, 0.4 g SDS, distilled water to 100ml, pH 8.8)
6.35 ml	Acrylamide (30%)
1.11 g	Sucrose
7.5 µl	TEMED
7.5 µl	Ammonium persulphate (10%)

Stacking gel

4 ml	Distilled water
5 ml	Buffer (3.03 g Tris, 1 g SDS, water to 100 ml, pH 6.8
1 ml	Acrylamide (30%)
10 µl	TEMED
75 µl	Ammonium persulphate (10%)

Electrode buffer

14.4 g	Glycine
3.03 g	Tris
1 g	SDS

Make up to 1 l with distilled water

Coomassie blue

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

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

Destain for Coomassie blue

70 ml	Glacial acetic acid
230 ml	Methanol
400 ml	Distilled water

Western blotting of SDS-PAGE mini gels

(BIO-RAD Mini trans-blot electrophoretic transfer system)

Electrode buffer

3 g	Tris
14.42 g	Glycine
250 ml	Methanol

Make up to 1 l with distilled water

Blot wash buffer

5 ml	Tween
0.37 g	EDTA
20.45 g	NaCl
100 ml	10 x PBS (400 g NaCl, 10 g KCl, 57.5 g Na_2HPO_4, 10 g KH_2PO_4, distilled water to 5 L)

Make to 1 l with distilled water

199S medium

40 ml	10x 199 medium
400 ml	Sterile distilled water
60 ml	Tryptose phosphate broth
2.6 ml	8% Sodium bicarbonate

Before use, add 10% foetal bovine serum (FBS) and 1% glutamine

Eagles minimum essential medium (EMEM) for MRC-5 cells

500 ml	EMEM
5 ml	Basic amino acid solution

Before use, add 10% FBS.

EMEM for tracheal organ culture

500 ml	EMEM
50 ml	FBS
5 ml	HEPES (2.5 M)

Cell lysis solution

17.6 ml	PBS
2 ml	Saponin (10%)
400 µl	FBS

Sterilise using a 0.45µm filter before use.

RPMI 1640

164 ml	RPMI 1640
20 ml	FBS
4 ml	Glutamine (14.6 g l^{-1})
2 ml	Pen/Strep (10000 U ml^{-1}/ 10000 µg ml^{-1})
3 ml	Nystatin (10000 U ml^{-1})
1 ml	Mercaptoethanol (10 µl in 10 ml PBS)
5 ml	Sodium bicarbonate (8%)
2.4 ml	HEPES (2.5M)

PAGE for detection of LPS

4 x 0.75 mm gels (BIO-RAD Mini-PROTEAN II electrophoresis system)

Electrode buffer same as for SDS-PAGE (1.1)

Resolving gel (12%)

2.6 ml	Distilled water
8.75 ml	Double strength buffer (90.855 g Tris, 1 L distilled water, pH 8.8)
5.25 ml	Acrylamide (40%)
25 µl	TEMED
0.875 ml	Ammonium persulphate (1.5%)

Resolving gel (14%)

1.725 ml	Distilled water
8.75 ml	Double strength buffer (90.855g Tris, 1 L distilled water, pH 8.8)
6.125 ml	Acrylamide (40%)
25 µl	TEMED
0.875 ml	Ammonium persulphate (1.5%)

Stacking gel (4%)

3.5 ml	Distilled water
5 ml	Double strength buffer (3.03 g Tris, 100 ml distilled water, pH 6.8)
1 ml	Acrylamide (40%)
20 µl	TEMED
0.5 ml	Ammonium persulphate (1.5%)

Western blotting of LPS PAGE gels

(BIO-RAD Mini trans-blot electrophoretic transfer system)

Electrode buffer same as for western blotting of SDS-PAGE gels (1.2)

Tris-buffered saline (TBS)

4.84 g	Tris
58.48 g	NaCl
2 l	Distilled water

Dissolve Tris and NaCl in approximately 1.5 l of water. Adjust pH to 7.5 and make up to 2 l.

Tween Tris-buffered saline (TTBS)

To 2 l of TBS add 0.5 ml of Tween 20.

.APPENDIX II

Chapter 3

All viable counts are the mean of duplicate counts from 4 samples.

TBW 1

	Mean ± SEM (cfu ml^{-1})			
Isolate	**0 h**	**24 h**	**48 h**	**72 h**
H1	$8.38 \times 10^2 \pm 9.62 \times 10^1$	$4.00 \times 10^2 \pm 3.27 \times 10^1$	$2.38 \times 10^2 \pm 5.32 \times 10^1$	$2.19 \times 10^3 \pm 5.08 \times 10^2$
K2	$1.34 \times 10^3 \pm 1.89 \times 10^2$	$9.88 \times 10^2 \pm 9.72 \times 10^1$	$5.00 \times 10^2 \pm 1.21 \times 10^2$	$1.33 \times 10^3 \pm 2.43 \times 10^2$
B285	$3.89 \times 10^3 \pm 4.69 \times 10^2$	$3.38 \times 10^2 \pm 1.02 \times 10^2$	$3.75 \times 10^1 \pm 1.83 \times 10^1$	$2.50 \times 10^1 \pm 1.64 \times 10^1$
NCTC 10520	$2.70 \times 10^3 \pm 3.71 \times 10^2$	$3.80 \times 10^1 \pm 1.83 \times 10^1$	$1.67 \times 10^1 \pm 1.67 \times 10^1$	$8.33 \times 10^1 \pm 3.07 \times 10^1$

TBW 2

	Mean ± SEM (cfu ml^{-1})			
Isolate	**0 h**	**24 h**	**48 h**	**72 h**
H1	$8.63 \times 10^2 \pm 2.82 \times 10^2$	$4.63 \times 10^2 \pm 5.96 \times 10^1$	$1.89 \times 10^3 \pm 3.97 \times 10^2$	$8.64 \times 10^3 \pm 8.73 \times 10^2$
K2	$1.58 \times 10^3 \pm 1.16 \times 10^2$	$1.06 \times 10^3 \pm 1.31 \times 10^2$	$2.61 \times 10^3 \pm 2.26 \times 10^2$	$7.68 \times 10^3 \pm 9.67 \times 10^2$
B285	$2.65 \times 10^3 \pm 4.16 \times 10^2$	$1.28 \times 10^3 \pm 1.13 \times 10^2$	$8.33 \times 10^1 \pm 3.07 \times 10^1$	$2.37 \times 10^3 \pm 3.25 \times 10^2$
NCTC 10520	$2.21 \times 10^3 \pm 3.2 \times 10^2$	$8.38 \times 10^2 \pm 1.03 \times 10^2$	$2.63 \times 10^2 \pm 9.44 \times 10^1$	$2.64 \times 10^3 \pm 6.14 \times 10^2$

TBW 3

	Mean ± SEM (cfu ml^{-1})			
Isolate	**0 h**	**24 h**	**48 h**	**72 h**
H1	$1.56 \times 10^3 \pm 1.05 \times 10^2$	$4.03 \times 10^3 \pm 5.37 \times 10^2$	$2.74 \times 10^4 \pm 3.20 \times 10^3$	$1.69 \times 10^5 \pm 3.46 \times 10^4$
K2	$1.35 \times 10^3 \pm 1.53 \times 10^2$	$1.74 \times 10^3 \pm 1.84 \times 10^2$	$6.20 \times 10^3 \pm 4.49 \times 10^2$	$5.21 \times 10^4 \pm 3.90 \times 10^3$
B285	$1.28 \times 10^3 \pm 1.44 \times 10^2$	$1.25 \times 10^1 \pm 1.25 \times 10^1$	$1.63 \times 10^2 \pm 6.25 \times 10^1$	$1.13 \times 10^3 \pm 3.75 \times 10^2$
NCTC 10520	$1.19 \times 10^3 \pm 7.66 \times 10^2$	0 ± 0	$2.51 \times 10^1 \pm 1.64 \times 10^1$	$3.50 \times 10^2 + 6.45 \times 10^1$

TBW 4

	Mean ± SEM (cfu ml^{-1})			
Isolate	**0 h**	**24 h**	**48 h**	**72 h**
H1	$2.09 \times 10^3 \pm 2.12 \times 10^2$	$1.13 \times 10^3 \pm 7.01 \times 10^2$	$3.46 \times 10^3 \pm 5.49 \times 10^2$	$1.25 \times 10^4 \pm 9.82 \times 10^2$
K2	$1.80 \times 10^3 \pm 1.82 \times 10^2$	$1.23 \times 10^3 \pm 1.13 \times 10^2$	$1.06 \times 10^3 \pm 1.75 \times 10^2$	$4.29 \times 10^3 \pm 3.44 \times 10^2$
B285	$1.75 \times 10^3 \pm 1.15 \times 10^2$	0 ± 0	0 ± 0	0 ± 0
NCTC 10520	$1.26 \times 10^3 \pm 1.45 \times 10^2$	0 ± 0	$2.50 \times 10^1 \pm 1.64 \times 10^1$	$8.75 \times 10^1 \pm 4.80 \times 10^1$

TBW 5

	Mean ± SEM (cfu ml^{-1})			
Isolate	**0 h**	**24 h**	**48 h**	**72 h**
H1	$1.71 \times 10^3 \pm 3.81 \times 10^2$	$1.66 \times 10^3 \pm 2.20 \times 10^3$	$2.26 \times 10^5 \pm 1.71 \times 10^4$	$7.00 \times 10^6 \pm 8.23 \times 10^5$
K2	$1.64 \times 10^3 \pm 1.77 \times 10^2$	$1.36 \times 10^3 \pm 1.97 \times 10^2$	$4.89 \times 10^4 \pm 4.93 \times 10^3$	$1.08 \times 10^6 \pm 3.59 \times 10^4$
B285	$1.80 \times 10^3 \pm 1.67 \times 10^2$	$1.25 \times 10^2 \pm 4.91 \times 10^1$	0 ± 0	0 ± 0
NCTC 10520	$1.06 \times 10^3 \pm 1.13 \times 10^2$	0 ± 0	0 ± 0	0 ± 0

The viable counts below are the mean of duplicate counts taken from 12 samples.

NB

	Mean ± SEM (cfu ml^{-1})			
Isolate	**0 h**	**24 h**	**48 h**	**72 h**
H1	$1.51 \times 10^3 + 1.74 \times 10^2$	$3.59 \times 10^4 \pm 6.55 \times 10^3$	$1.42 \times 10^7 \pm 2.75 \times 10^6$	$4.29 \times 10^8 \pm 5.81 \times 10^7$
K2	$1.42 \times 10^3 \pm 1.25 \times 10^2$	$3.52 \times 10^4 \pm 7.59 \times 10^3$	$1.96 \times 10^7 \pm 5.12 \times 10^6$	$3.16 \times 10^8 \pm 6.33 \times 10^7$
B285	$2.80 \times 10^3 \pm 2.49 \times 10^2$	$4.73 \times 10^3 \pm 8.64 \times 10^2$	$1.72 \times 10^5 \pm 5.44 \times 10^4$	$2.08 \times 10^7 \pm 5.31 \times 10^6$
NCTC 10520	$1.20 \times 10^3 \pm 1.66 \times 10^2$	$1.71 \times 10^3 \pm 2.56 \times 10^2$	$9.78 \times 10^4 \pm 1.73 \times 10^4$	$1.23 \times 10^7 \pm 2.79 \times 10^6$

PBS

	Mean ± SEM (cfu ml^{-1})			
Isolate	**0 h**	**24 h**	**48 h**	**72 h**
H1	$3.46 \times 10^3 \pm 4.92 \times 10^2$	$3.48 \times 10^2 \pm 5.55 \times 10^1$	$1.00 \times 10^2 \pm 2.27 \times 10^1$	$1.04 \times 10^2 \pm 2.70 \times 10^1$
K2	$4.05 \times 10^3 \pm 5.19 \times 10^2$	$4.46 \times 10^2 \pm 4.34 \times 10^1$	$1.54 \times 10^2 \pm 3.66 \times 10^1$	$1.08 \times 10^2 \pm 2.75 \times 10^1$
B285	$4.94 \times 10^3 + 5.95 \times 10^2$	$2.17 \times 10^2 \pm 5.47 \times 10^1$	$2.08 \times 10^1 \pm 8.47$	$8.33 \times 10^1 \pm 3.33 \times 10^1$
NCTC 10520	$3.62 \times 10^3 \pm 4.69 \times 10^2$	$2.17 \times 10^2 \pm 5.83 \times 10^1$	$2.08 \times 10^1 \pm 1.34 \times 10^1$	$5.00 \times 10^1 \pm 2.18 \times 10^1$

Chapter 4

Raw data for Fig. 4.1.

		Cell line		
		FLT	**FLL**	**MRC-5**
Isolate	**No. bacteria added to 1 x 10^5 cells**	**No. bacteria adhering to 100 cells**	**No. bacteria adhering to 100 cells**	**No. bacteria adhering to 100 cells**
H1	5.30 x 10^6	1546		
	5.17 x 10^6	2026		
	4.87 x 10^6	986		
	2.20 x 10^6	241		
	3.50 x 10^5		244	
	1.25 x 10^6	471		
	1.75 x 10^6			625
	8.50 x 10^5	320	345	57
	4.45 x 10^6	240		110
	2.60 x 10^6	337	266	
K2	5.97 x 10^6	260		
	6.80 x 10^6	308		
	3.85 x 10^6	59		
	1.65 x 10^6	9		
	8.65 x 10^5	66	54	
	2.65 x 10^6	51		
	2.50 x 10^6			335

		FLT	FLL	MRC-5
Isolate	**No. bacteria added to 1 x 10^5 cells**	**No. bacteria adhering to 100 cells**	**No. bacteria adhering to 100 cells**	**No. bacteria adhering to 100 cells**
K2	1.70×10^6	143	47	1
	6.10×10^6	145		47
	6.90×10^6	1565		
	3.35×10^6	67	66	
B285	3.90×10^6	1008		
	4.05×10^6	288	134	
	2.85×10^6	79	55	4
	1.19×10^7	152		216
	8.30×10^6	74	49	
NCTC 10520	6.45×10^6	1676		
	4.87×10^6	146		
	6.90×10^6	275		
	3.75×10^6	54		
	2.10×10^6	42		
	4.90×10^6	529	167	
	4.15×10^6	207		
	3.40×10^6			1684
	1.45×10^6	41	36	19
	1.10×10^7	216		307
	3.25×10^6	49	40	

Raw data for Fig. 4.2.

		Cell line		
		FLT	**FLL**	**MRC-5**
Isolate	**No. bacteria added to 1 x 10^5 cells (cfu ml^{-1})**	**No. viable bacteria recovered from cells (cfu ml^{-1})**	**No. viable bacteria recovered from cells (cfu ml^{-1})**	**No. viable bacteria recovered from cells (cfu ml^{-1})**
H1	1.25×10^6	4.75×10^3		
	1.75×10^6			1.05×10^4
	8.50×10^5	2.70×10^5	2.05×10^3	8.05×10^4
	4.45×10^6	2.15×10^3		5.10×10^4
	2.60×10^6	3.35×10^3	1.00×10^2	
K2	2.65×10^3	1.15×10^3		
	2.50×10^6			1.30×10^4
	1.70×10^6	5.50×10^2	5.00×10^2	6.05×10^4
	6.10×10^6	1.85×10^3		2.95×10^4
	3.35×10^6	5.00×10^2	1.15×10^3	
B 285	2.85×10^6	2.30×10^3	5.70×10^3	1.12×10^5
	1.19×10^7	4.50×10^3		2.95×10^4
	8.25×10^6	4.35×10^3	7.00×10^2	
NCTC 10520	4.15×10^6	7.00×10^3		
	3.40×10^6			4.25×10^4
	1.45×10^6	3.80×10^3	2.20×10^3	7.60×10^4

		Cell line		
		FLT	FLL	MRC-5
Isolate	No. bacteria added to 1 x 10^5 cells (cfu ml^{-1})	No. viable bacteria recovered from cells (cfu ml^{-1})	No. viable bacteria recovered from cells (cfu ml^{-1})	No. viable bacteria recovered from cells (cfu ml^{-1})
NCTC 10520	1.10 x 10^7	5.90 x 10^3		3.55 x 10^5
	3.25 x 10^6	1.75 x 10^3	7.00 x 10^2	

Raw data for Fig. 4.4. Viable counts are the mean of duplicate counts.

Isolate	Inoculum (cfu ml^{-1})	No. cfu ml^{-1} from tracheal organ culture		
H1	1.15 x 10^6	2.40 x 10^4	7.05 x 10^4	3.95 x 10^4
K2	1.25 x 10^6	3.05 x 10^4	6.30 x 10^3	4.90 x 10^3
B285	2.00 x 10^6	2.05 x 10^4	2.20 x 10^4	4.25 x 10^4
NCTC 10520	2.10 x 10^6	1.70 x 10^4	3.90 x 10^4	1.35 x 10^4
P. haemolytica A2	2.95 x 10^6	1.20 x 10^5	2.05 x 10^5	1.80 x 10^5

Isolate	Inoculum (cfu ml^{-1})	No. cfu ml^{-1} from tracheal organ culture		
H1	7.75 x 10^5	7.55 x 10^4	1.35 x 10^4	1.25 x 10^4
K2	6.10 x 10^5	4.15 x 10^4	1.80 x 10^4	2.15 x 10^4
B285	1.11 x 10^6	1.55 x 10^4	2.15 x 10^4	1.85 x 10^4
NCTC 10520	1.23 x 10^6	1.45 x 10^4	7.00 x 10^3	9.00 x 10^3
P. haemolytica A2	3.95 x 10^5	3.20 x 10^3	4.30 x 10^5	3.20 x 10^3

Raw data for Fig. 4.5 Viable counts are the mean of duplicate counts.

Treatment	Inoculum (cfu ml^{-1})	No. cfu ml^{-1} from tracheal organ culture		
A2 control	1.95×10^6	1.20×10^5	2.05×10^5	1.80×10^5
H + A/1	1.95×10^6	7.10×10^4	9.50×10^4	3.90×10^5
H + A/2	1.60×10^5	2.70×10^3	2.30×10^3	4.30×10^3
H + A/3	8.40×10^5	2.75×10^4	2.10×10^4	2.75×10^4
H + A/4	4.75×10^5	3.80×10^3	3.30×10^3	5.80×10^3

Raw data for Fig. 4.6 Viable counts are the mean of duplicate counts.

Treatment	Inoculum (cfu ml^{-1})	No. cfu ml^{-1} from tracheal organ culture		
A2 control	6.25×10^7	1.10×10^6	1.70×10^6	1.25×10^6
A/1	6.25×10^7	6.50×10^5	2.70×10^6	2.45×10^6
A/2	6.25×10^7	2.05×10^6	1.90×10^6	2.05×10^6
A/3	3.90×10^6	2.40×10^4	1.25×10^2	9.50×10^3
A/4	2.95×10^6	1.30×10^4	7.00×10^3	2.90×10^4

Chapter 5

Raw data for Fig. 5.1

Isolate	No. bacteria added to 1×10^5 cells	No. bacteria adhering to 100 cells	
H1	1.25×10^6	219	64
K2	7.35×10^6	41	37
B285	5.10×10^6	75	52
NCTC 10520	5.75×10^6	27	36
P. haemolytica A2	1.75×10^5	5	15
C. bovis	1.60×10	2	24

Isolate	No. bacteria added to 1×10^5 cells	No. bacteria adhering to 100 cells	
H1	5.45×10^5	116	14
K2	8.00×10^5	21	14
B285	3.25×10^6	28	3
NCTC 10520	4.30×10^6	4	5
P. haemolytica A2	3.10×10^5	15	4
C. bovis	9.50×10^4	35	3

Raw data for Table 5.1

Isolate	**No. bacteria added to 1 x 10^5 cells**	**No. bacteria intracellular within 100 cells**	
H1	1.25 x 10^6	335	65
K2	7.35 x 10^6	0	0
B285	5.10 x 10^6	0	0
NCTC 10520	5.75 x 10^6	0	18
P. haemolytica A2	1.75 x 10^6	0	0
C. bovis	1.60 x 10	0	0

Isolate	**No. bacteria added to 1 x 10^5 cells**	**No. bacteria intracellular within 100 cells**	
H1	5.45 x 10^5	60	2
K2	8.00 x 10^5	19	0
B285	3.25 x 10^6	17	0
NCTC 10520	4.30 x 10^6	5	0
P. haemolytica A2	3.10 x 10^5	0	0
C. bovis	9.50 x 10^4	0	1

Raw data for Fig. 5.3

Isolate	**log_{10} Inoculum**			
H1	5.90	5.23	5.69	5.78
K2	5.89	6.85	5.88	5.93
B285	6.74	6.67	6.52	6.51
NCTC 10520	6.82	6.69	6.67	6.59
P. haemolytica A2	6.32	6.15	5.51	5.48
C. bovis	5.15	5.26	5.04	4.90

Isolate				
H1	3.76	3.45	3.16	2.70
K2	2.93	0.00	2.74	1.70
B285	3.56	3.18	2.93	2.88
NCTC 10520	3.38	3.24	3.04	2.81
P. haemolytica A2	0	0	0	0
C. bovis	0	0	0	0

Raw data for Fig. 5.4-5.7

Isolate	Opsonin	No. bacteria added to 1 x 10^5 cells	No. bacteria adhering to 100 cells		
H1	Control	4.15 x 10^6	138	26	25
	LS H1		55	745	1072
	LS NCTC		375	431	580
	TBW 5		129	41	26
	TBW 5 - IgG		26	72	100
K2	Control	2.90 x 10^6	193	222	139
	LS H1		78	162	141
	LS NCTC		218	117	143
	TBW 5		56	137	62
	TBW 5 - IgG		311	90	148
B285	Control	2.00 x 10^6	41	46	8
	LS H1		110	156	79
	LS NCTC		45	127	150
	TBW 5		10	25	0
	TBW 5 - IgG		14	53	16
NCTC 10520	Control	2.40 x 10^6	29	44	30
	LS H1		41	471	25
	LS NCTC		81	75	24
	TBW 5		8	9	3

Isolate	Opsonin	No. bacteria added to 1 x 10^5 cells	No. bacteria adhering to 100 cells		
NCTC 10520	TBW 5 - IgG		175	4	3
P. haemolytica	Control	6.70 x 10^6	19	25	29
C. bovis	Control	4.95 x 10^6	193	138	228

Raw data for Table 5.3

Isolate	Opsonin	No. bacteria added to 1 x 10^5 cells	No. bacteria intracellular within 100 cells		
H1	Control	4.15 x 10^6	0	24	30
	LS H1		234	0	0
	LS NCTC		233	53	0
	TBW 5		0	1	19
	TBW 5 - IgG		42	0	0
K2	Control	2.90 x 10^6	0	0	0
	LS H1		0	0	210
	LS NCTC		307	19	0
	TBW 5		0	0	0
	TBW 5 - IgG		38	7	0
B285	Control	2.00 x 10^6	0	0	6
	LS H1		5	43	0

Isolate	Opsonin	No. bacteria added to 1×10^5 cells	No. bacteria intracellular within 100 cells		
B285	LS NCTC		163	0	7
	TBW 5		1	0	7
	TBW 5 - IgG		71	0	40
NCTC 10520	Control	2.40×10^6	0	0	19
	LS H1		2	0	0
	LS NCTC		147	120	0
	TBW 5		1	2	2
	TBW 5 - IgG		0	1	45
P. haemolytica	Control	6.70×10^6	30	0	0
C. bovis	Control	4.95×10^6	0	0	0

Raw data for Fig. 5.8-5.11

Isolate	Opsonin	$\log_{10}$ No. viable bacteria recovered (cfu ml^{-1})		
H1	Control	4.27	2.81	3.47
	LS H1	3.53	3.08	3.16
	LS NCTC	3.74	3.57	3.74
	TBW 5	3.02	2.85	3.49
	TBW 5 - IgG	3.69	3.43	3.53
K2	Control	4.19	3.38	3.53
	LS H1	3.45	3.35	3.30
	LS NCTC	3.30	3.15	3.40
	TBW 5	3.38	3.16	3.37
	TBW 5 - IgG	3.89	3.30	3.60
B285	Control	3.31	3.39	3.15
	LS H1	3.76	3.41	3.77
	LS NCTC	3.38	3.11	3.36
	TBW 5	3.10	2.88	2.98
	TBW 5 - IgG	3.36	3.48	3.40
NCTC 10520	Control	3.59	3.52	3.56
	LS H1	3.29	3.31	3.42
	LS NCTC	3.16	2.90	2.78
	TBW 5	3.06	2.93	2.78
	TBW 5 - IgG	3.27	3.44	3.54

Isolate	Opsonin	$\log_{10}$ No. viable bacteria recovered (cfu ml^{-1})		
P. haemolytica	Control	2.69	2.48	2.69
C. bovis	Control	3.00	2.30	2.69

Raw data for Fig. 5.12

Isolate	No. viable bacteria recovered (cfu ml^{-1})											
	90 min				24 h				48 h			
H1	1250	250	1150	550	0	0	1150	0	0	3250	0	27000
	2000	1650	3100		4500	2400	1250		11500	21000	74000	
K2	150	3300	0	0	0	0	0	0	0	0	0	0
	2250	2900	3300		2250	41500	5750		2100	85500	76500	
B285	50	150	150	0	0	0	0	0	0	250	400	0
	1750	2050	12050		1700	650	1900		1000	1850	200	
NCTC 10520	300	250	350	300	0	1150	0	0	0	0	0	0
	450	1200	3400		54000	2700	50		1250	1450	0	
P. haemolytica	0	0	0	0	0	0	0	0	0	0	0	0
	0	95000	0		1650	0	0		0	0	950	
C. bovis	0	0	100	150	50	150	0	250	150	400	1400	1000
	350	1100	1250		4000	700	250		0	100	1400	

Raw data for Fig. 5.13

Isolate	**No. viable bacteria recovered (cfu ml^{-1})**											
	90 min				**24 h**				**48 h**			
H1	6550	6000	28000	27500	800	600	14500	11000	300	100	500	0
K2	2350	1150	11000	10500	0	0	850	50	0	0	300	0
B285	28000	13000	14000	10500	0	0	0	0	0	1500	0	0
NCTC 10520	11500	11500	17000	9500	0	0	100	50	650	0	0	0
P. haemolytica	0	0	0	0	0	0	0	0	0	0	0	0
C. bovis	1650	2000	19000	4000	0	0	250	0	0	1800	0	0

Chapter 6

Raw data for Table 6.3

Treatment	% killing of isolate:											
	H1			K2			B285			NCTC 10520		
Sheep α H1	93.2	96.2	100	100	100	100	94.0	100	95.3	82.7	100	94.8
Sheep αNCTC 10520	60.8	40.4	75.4	100	100	100	100	100	100	100	100	100
Rabbit α C	62.2	54.5	89.1	100	100	100	5.3	0	44.8	0	25.9	19.7
Rabbit α NCTC 10520	62.6	56.4	66.3	100	100	100	100	100	100	100	100	100
PBS + complement	44.6	37.8	77.7	100	100	100	39.2	0	54.7	38.5	10.8	56.1
Cx + sheep α H1	4.1	0	0	13.3	0	24.5	2.6	0	37.0	1.9	7.2	26.0
Cx + sheep α NCTC 10520	0	0	0	22.4	0	4.3	15.5	0	41.7	0	0	27.2
Cx + rabbit α H1	25.2	0	3.4	5.6	5.1	7.9	14.7	11.7	46.9	0	34.3	50.9
Cx + rabbit α NCTC 10520	5.4	5.8	0	21.7	0	12.2	12.8	0	44.8	0	32.6	53.2

Where, Cx = heat-inactivated complement.