Growth of *Bordetella* species in tracheobronchial washings and in other low-nutrient fluids

John Francis Porter

Presented for the Degree of Doctor of Philosophy in the Faculty of Science, University of Glasgow

Department of Microbiology, September 1991

GROWTH OF *BORDETELLA* SPECIES IN TRACHEOBRONCHIAL WASHINGS AND IN OTHER LOW-NUTRIENT FLUIDS

Dedicated to my grandparents whose courage, strength and understanding has, and always will be, an example to follow

" How could one tell that this point, rather than any other point in the whole chain of random, contingent events making up life, that *this* was the one on which history turned, that *this* was the tiny lever that formed a fulcrum and moved a world ? "

(from Michael Stewart's " Monkey Shine ", 1983)

Acknowledgements

I would like to thank Professor Alastair C. Wardlaw for his help, guidance and patience throughout this project. I am also endebted to Dr. Roger Parton for his assistance in proof-reading manuscripts and his invaluable advice.

I am grateful to Dr. D.J. Taylor of Glasgow University Veterinary School who provided a number of my clinical isolates and to Mrs E. Berry for her technical assistance. I would also like to thank Dr. N. Thompson and Dr. Hulk of the Western Infirmary who provided human bronchial lavage samples and to Professor W. MacFarlane of Glasgow Dental Hospital and School for his assistance in the chemical analysis of my TBW and water samples.

I would also like to thank the S.E.R.C. for their funding of this studentship without which the work would not have been possible.

Finally I would like to thank my mother and father for their support and understanding throughout my academic training. The understanding and patience of Miss Anne Glen during the work of the thesis puts me in her debt.

LIST OF ABBREVIATIONS

BG	Bordet-Gengou
BHIB	Brain heart infusion broth
BSA	Bovine serum albumin
CFU	Colony forming units
CL	Cyclodextrin liquid
CSM	Cyclodextrin solid medium
CW	Cohen-Wheeler
DHB	Defibrinated horse blood
DW	Distilled water
FHA	Filamentous haemagglutinin
GI	Growth index
HLT	Heat-labile toxin
ip	intraperitoneal
OD	Optical density
PBS	Phosphate buffered saline
PT	Pertussis toxin
RBC	Red blood cells
RGW	Reagent-grade water
RTF	Respiratory tract fluids
RTS	Respiratory tract secretions
SS	Stainer-Scholte
TBW	Tracheobronchial washings

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOGRAPHS

LIST OF APPENDICES

Page

SUMMARY

This study has shown that three species of *Bordetella, B. bronchiseptica, B. avium* and *B. parapertussis,* are capable of growth in vertebrate tracheobronchial washings (TBW), while *B. pertussis* is not. A total of nine samples of TBW from man, horse, mouse, rabbit, sheep, dog and chicken exhibited differences in growth-supporting abilities as judged from colony counts on Bordet-Gengou (BG) agar plates (from inocula of about 2000 CFU/ml). Single samples of TBW from horse and mouse gave higher stationary-phase counts of *B. bronchiseptica* and *B. avium,* than any of the other TBW. However additional samples would have to be tested to determine whether this is a consistent species effect or is due to between-sample variation. With human TBW, where three samples were tested, there was considerable between-sample variation.

As regards the differences between the bordetellae, *B. bronchiseptica* strain 5376 (Eldering strain) grew in all species of TBW, except that one out of three human specimens appeared to be bactericidal. The stationary-phase viable counts (colony forming units : CFU) with the other samples were all in the 12-fold range 7.9 to 9.1 (Log_{10} CFU/ml). *B. avium* strain P4091 behaved, in general, very similarly to *B. bronchiseptica* except that the average final viable counts were slightly lower. Thus although *B. avium* is exclusively an avian parasite, its growth in chicken TBW was not greater than in mammalian TBW; in fact, it grew least well in the avian fluid.

With the two human bordetellae, *B. pertussis* (strain 18334) showed no evidence of growth in any of the TBW, although there was consistent and extensive growth in the control fluid, CL medium, where counts of 10.0 log_{10} CFU/ml were reached from inocula of around 3.3 log_{10} CFU/ml. *B.*

parapertussis (strain 10520) was the organism that exhibited the greatest variation of growth in the different TBW. Thus, it grew to levels of 5.5-6.7 $\log_{10}$ CFU/ml in TBW from chicken, sheep, rabbit, mouse and two human samples, while it failed to grow in the horse and dog TBW which had supported extensive growth of *B. bronchiseptica* and *B. avium.* In Cl medium, *B. parapertussis* reached counts of 9.5 $\log_{10}$ CFU/ml i.e. slightly lower than *B. pertussis.*

With *B. pertussis,* an attempt was made to determine whether the failure to grow in TBW (rabbit was chosen) was due to lack of nutrients or to the presence of inhibitors. This was explored by comparing growth in a) TBW, b) CL medium, c) a 1:1 mixture of TBW and CL medium and d) TBW and MeßCD (methylated cyclodextrin). The observed growth in (b) and (c) but not in (a) or (d) suggested that lack of nutrients, rather than the presence of inhibitors was the likely explanation.

Since amino acids are primary nutrients of bordetellae in laboratory culture media, it was necessary to analyse TBW for their amino acid content. This was done by the ninhydrin method. All samples of TBW had readily detectable amounts of amino acids, the concentrations ranging from 0.1 to 0.81 mM, expressed as leucine equivalents. The 0.81 mM is approximately 1/200 the level of amino acids in CL medium, yet it supported growth of *B. bronchiseptica* to a level only 2-fold lower than CL medium. There was a statistically significant correlation between the amount of growth and the amino acid concentration with *B. bronchiseptica* and *B. avium* in TBW; but this was not so with *B. parapertussis.*

Nicotinic acid was likewise readily detected in TBW by microbiological assay with *Lactobacillus plantarum.* Values ranged from 0.2 to 1.06 µg/ml,

which was about 20-fold less than in conventional *Bordetella* media. As with the amino acid values, there was some correlation between the nicotinic acid content of TBW and the level of stationary-phase CFU/ml of *B. bronchiseptica* and *B. avium*, but not of *B. parapertussis*.

The possibility that nutrients in TBW had originated from blood contamination was explored by haemoglobin analysis. This showed that TBW was never contaminated by more than 0.22 % by volume of blood and that the levels of amino acids and nicotinic acid contributed by the blood were small compared with those intrinsic to the TBW.

During the growth experiments with TBW samples it was noticed that both *B. bronchiseptica* and *B. avium* showed an increase in viable count when incubated in the non-nutrient control fluid, PBS. Thus from an inoculum of 2 x 10^3 CFU/ml washed cells of *B. bronchiseptica*, there developed 2.7 x 10^6 CFU/ml after 48h at 37°C. This 1000-fold increase corresponded to about 10 cycles of cell division in the PBS. In addition, *B. bronchiseptica* also grew in reagent-grade water, and in local lake and pond waters, all without added nutrients. Within 48 to 72h at 37°C in PBS and in lake and pond waters, stationary-phase populations of around 2.7 x 10^6 CFU/ml developed from washed *B. bronchiseptica* inocula of around 2 x 10^3 CFU/ml. Much smaller increases, in the order of five- and eightfold respectively, were observed in reagent-grade water and in seawater from the same inocula.

The possibility that carry-over of nutrients was responsible for growth in PBS was discounted by showing successful serial transfer of *B. bronchiseptica* in PBS under conditions in which *Escherichia coli*, tested in parallel, rapidly died out. During long-term incubation in both PBS and lake water *B. bronchiseptica* maintained viability for at least six months at 37°C

and 10°C.

The ability of *B. bronchiseptica* strain no. 5376 to grow in these low-nutrient fluids was extended to five other strains of this species, including the type strain no. 452, a rabbit strain isolated in the 1950's and three recent clinical isolates from a pig, horse and a dog. Two strains of *Pasteurella haemolytica,* tested similarily, failed to grow in either PBS or lakewater, but gave extensive growth in the control fluid, brain heart infusion broth.

This work therefore reveals two novel observations;

1. Tracheobronchial washings from various vertebrate sources are capable of supporting the growth of *Bordetella* species, but with variability according to *Bordetella* species and the source of TBW, and

2. *B. bronchiseptica* is able to grow and survive for long periods - in the order of months - in low-nutrient fluids such as lake waters and may therefore exist in the natural enviroment.

INTRODUCTION

Part 1. The Genus *Bordetella*

The focus of this research was a study of the growth of *Bordetella* species in tracheobronchial washings from various vertebrate sources. The introduction to the thesis is designed to provide the reader with general information about the genus *Bordetella* and also to describe in some detail those aspects of direct relevance to this study. For this reason emphasis is placed on the growth and nutrition of the bordetellae and on the collection and chemical composition of respiratory tract secretions.

The opening section of the Introduction (Part 1) contains information about the historical, clinical and biochemical backgrounds of the four species of bordetellae. The main section (Part 2) deals fully with *in vivo* and *in vitro* growth of the genus and their nutritional requirements, while the final section (Part 3) concentrates on information available on the collection and chemical composition of respiratory tract secretions.

Where certain aspects have been dealt with sparingly, the reader will be referred to the appropriate review articles which contain more detailed information and primary sources.

Discovery of the bordetellae

Diseases which may , in retrospect, be attributed to members of the *Bordetella* date long before the isolation and identification of the disease-causing organism.

Bordetella pertussis , the aetiological agent of whooping cough, was first isolated and described by Bordet and Gengou in 1906. However, the first mention of a whooping cough-like disease was by Guillaume de Baillou in the Mirror of Health in 1540 (cited by Lapin, 1943). This is generally accepted as the first clear description of epidemic whooping cough:

" At the close of summer almost the same diseases prevailed as before..... Principally that common cough.... Serious are the symptoms of

this. The lung is so irritated by every attempt to expell that which is causing the trouble it neither admits air nor again easily expells it. The patient is seen to swell up and as if strangled holds his breath tightly in the middle of his throat... For they are without the troublesome coughing for the space of four or five hours at a time then this paroxysm of coughing returns, now so severe that blood is expelled with force through the nose and through the mouth. Most frequently an upset stomach follows... For we have seen so many coughing in such a manner, in whom after a vain attempt semi-putrid matter in an incredible quantity was ejected " (Linneman, Jr, 1979).

It was suggested that the disease was introduced into Europe in the Middle Ages by travellers form other parts of the world (Linneman, Jr, 1979) and was well recognized by the mid 18^{th} century. The disease was reported from diverse regions; Switzerland (1755), Island of Oeland (1763), Germany (1769), Brunswick (1770) and Milan (1815). It was Sydenham in 1769 (see Lapin, 1943) who gave the name "Pertussis" to this disease. For a more detailed historical description see Lapin (1943).

Bordetella parapertussis causes a milder form of whooping cough and went unrecognised until its isolation by Eldering and Kendrick in 1938 from cases of whooping cough. Linneman and Perry (1977) suggested that severe cases of whooping cough were mixed infections with *B. pertussis,* but now both diseases are believed to be distinct (Connor, 1986).

B. bronchiseptica causes a respiratory tract infection in a variety of domestic and wild animals (see review by Goodnow, 1980). It was originally isolated by Ferry in 1911 from the respiratory tract of dogs with a distemper-like disease. As with *B. pertussis,* previous reports of similar diseases could be attributed to this organism (review by Goodnow, 1980). A respiratory disease of swine was reported by Franque in 1830 and was the first mention of what is now known as atrophic rhinitis (see Rutter,

1985). Switzer demonstrated laboratory-induced swine atrophic rhinitis with a pure culture of *B. bronchiseptica*, thus establishing its role in the disease and this was verified by other workers (see review by Goodnow, 1980). Thompson *et al* (1974), Bemis *et al* (1977a) and McCandlish *et al* (1978) have all shown that *B. bronchiseptica* was responsible for kennel cough in dogs. Reviews on *B. bronchiseptica* and the diseases it causes include those by Thompson *et al* (1974), Bemis *et al* (1977a), Goodnow (1980), and Rutter (1985).

Bordetella avium is a pathogen of birds responsible for a disease in turkeys called turkey coryza. It was first studied and described definitively by Kersters *et al* (1984) although the disease had been reported previously (Hinz and Kunjura, 1977). Filion *et al* (1967) incorrectly blamed an upper respiratory tract infection of turkeys on *B. bronchiseptica*. In the late 1970's Hinz *et al* (1978) reported a similar disease in Germany. Simmons *et al* (1984), blamed *Alcaligenes faecalis* for turkey coryza and other reports of the disease were recorded in 1931, 1932 and 1934 (see Hinz and Kunjura, 1977).

Laboratory characteristics

The *Bordetella* are minute Gram-negative, coccobacilli, 0.2-0.5 µm in diameter and 0.5-2.0 µm in length. They are strictly aerobic, chemoorganotrophic bacteria which are parasites or pathogens of the respiratory tract of man and other animals. *B. pertussis* and *B. parapertussis* are non-motile while *B. bronchiseptica* and *B. avium* are motile. Colonies on Bordet-Gengou medium are described as "smooth, convex, pearly, glistening and nearly transparent". Those of *B. pertussis, B. parapertussis* and *B. bronchiseptica* are surrounded by a zone of haemolysis (Pittman and Wardlaw, 1981).

Table 1 outlines the main characteristics within the genus. *B. parapertussis, B. bronchiseptica* and *B. avium*, but not *B. pertussis*, utilize

Table 1 : Differentiation between *B. pertussis*, *B. parapertussis*, *B. bronchiseptica* and *B. avium*. (modified from Pittman, 1984)

Characteristics	*B. pertussis*	*B.para-pertussis*	*B. bronchi-septica*	*B. avium*
Motility.	-[a]	-	+[a]	+
Growth on Bordet-Gengou medium:				
1-2 days.	-	+	+	+
3-6 days.	+	[b]		
Growth on peptone. agar:				
Phase I.	-	+	+	+
Phase IV.	+	+	+	+
Browning.	-	+	-	-
Growth on Mac-conkey agar.	-	+	+	+
Citrate utilisation.	-	+	+	+
Nitrate reduction.	-	-	+[c]	+[d]
Urease.	-	+	+[e]	-
Oxidase.	-	-	+	+[f]
Mol% of G+C of DNA(Tm)	67-70[g]	66-70[g]	68.9[h]	61.6[i]

a Symbols : +, typically positive; - , typically negative

b Blank space indicates no information

c Regularily positive in nitrate medium supplemented with nicotinamide adenine dinucleotide (NAD) and serum. Exceptions occur in conventional nitrate-test medium

d Only in medium supplemented with NAD and serum

e Positive within 4h

f Kovac's test positive after 48-72 h incubation

g Johnson(cited by Johnson & Sneath, 1973)

h Type strain

i Strain 591/77 of Hinz

citrate as a sole carbon and energy source and are capable of growth on peptone agar and MacConkey agar (*B. pertussis* phase IV organisms also grow on peptone agar). Of the four species, only *B. parapertussis* has the ability to produce a browning from the tyrosine in the peptone agar.

Both *B. bronchiseptica* and *B. avium*, but not *B. pertussis* or *B. parapertussis* reduce nitrate and show a positive oxidase reaction. *B. bronchiseptica* and *B. parapertussis* are urease positive while the other two bordetellae show no activity. The DNA base ratios lie within the range 61-70 mole % (G+C).

The *Bordetella* also produce a wide range of toxins and other virulence factors (see reviews by Weiss and Hewlett, 1986 and Wardlaw, 1988). Table 2 shows the virulence determinants and their sharing by the four bordetellae. All four species possess heat-labile toxin (HLT), tracheal cytotoxin (TCT) and agglutinogens (AGG). The three mammalian bordetellae have filamentous haemagglutinin (FHA), adenylate cyclase toxin (ACT) and haemolysin (HLY) activities, whereas *B. avium* is deficient in all of these. *B. pertussis* is the only member of the genus to produce pertussis toxin (PT); however both *B. parapertussis* and *B. bronchiseptica* possess transcriptionally silent PT genes (Arico and Rappuoli, 1987). The virulence factors are under the control of a single genetic sequence known as the *vir* or *bvg* locus. For more detailed information about the virulence factors and their control, the reader is referred to Coote and Brownlie (1988) and other chapters in the book by Wardlaw and Parton (1988), and Coote (1991).

Taxonomy

The similarities and characteristics of the *Bordetella* and some closely related genera are shown in Table 3. All species within the *Bordetella* have, at some time, been placed in other families. *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* were finally assigned to the genus

Table 2 : Virulence factors of the genus *Bordetella.* (modified from Wardlaw, 1988)

Virulence Factor	*B. p*[a]	*B. pp*[b]	*B. br*[c]	*B. a*[d]
Heat Labile Toxin (HLT)	+[e]	+	+	+
Tracheal Cytotoxin (TCT)	+	+	+	+
Lipopolysaccharides (LPS)	+	+	+	+
Agglutinogens (AGG)	+	+	+	+
Filamentous Hemagglutinin (FHA)	+	+	+	
Adenylate Cyclase Toxin (ACT)	+	+	+	-[f]
Haemolysin (HLY)	+	+	+	
Pertussis Toxin (PT)	+	-	-	-

[a] *B. pertussis*; [b] *B. parapertussis*; [c] *B. bronchiseptica*; [d] *B. avium*;

[e] activity demonstrated; [f] no activity recorded

A blank space indicates no information available.

Table 3 : Differential characteristics of the genus *Bordetella* and other morphologically and physiologically similar genera [a]. (modified from Pittman, 1984)

Characteristic	*Bordetella*	*Alcaligenes*	*Brucella*	*Haemophilus*
Strictly parasitic.	+	–	+	–
Saprophytic.	–	+	–	–
Localise on respiratory cilia	+	–	–	–
Strictly aerobic.	+	+	+	–
Growth requirement :				
Thiamine.	–	–	+	–
Nicotinamide.	+	–	–	–
X and/or V factor.	–	–	–	+
Ferment carbohydrates.	–	–	–	+
Nitrate reduction.	D	D	+	+
Litmus milk, alkaline.	+	+	–	–
Oxidation of amino acids	+	+	+	–
Tetrazolium reduction.	+	–	b	+
Growth on 320mg potassium tellurite per litre	–	+		–
Citrate utilised.	D	+	–	–
PAGE resemblance.[c]	–		–	–
Mol% G+C of DNA.	61-70(Tm)	56-70(Tm)	55-58(Tm)	38-44(Tm)

a Symbols : +, positive reaction ; - , no reaction ; D, variable

b Blank space indicates no information

c PAGE patterns are distinct for each genus

Bordetella by Moreno-Lopez in 1952, and *B. avium* was added at a later date (Kersters *et al*, 1984). *B. pertussis* was originally a member of the *Haemophilus*, *B. parapertussis* was placed in both *Haemophilus* and *Acinetobacter*, and *B. bronchiseptica* was placed successively in *Bacillus*, *Alcaligenes*, *Brucella* and then *Haemophilus* before being allocated to *Bordetella*. Even *B. avium* was formerly assigned to *Alcaligenes*.

A comprehensive, numerical taxonomic study by Johnson and Sneath (1973) helped establish *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* as individual species within the one genus, supporting the stance taken by Moreno-Lopez in 1952. Differentiation between the *Haemophilus* and the *Bordetella* was relatively simple as the *Bordetella* species were obligate aerobes whereas the *Haemophilus* were facultative anaerobes. The guanine plus cytosine ratios also differ, the bordetellae being in the range 61 to 70% and the haemophili 38 to 44% (Table 3). It was more difficult to separate the *Bordetella* from some other genera, especially considering the close similarities between *B. bronchiseptica* and *Alcaligenes faecalis* (Johnson and Sneath, 1973, Ulrich and Needham, 1953).

The *Brucella* and *Bordetella* differed in that the former metabolized alanine and DL-asparagine, whereas the *Bordetella* prefered glutamic acid and proline. In DNA hybridization studies, Hoyer and McCullough (1968) found no detectable pairing between *Brucella* and *Bordetella* species. The essential requirement for nicotinic acid (Proom, 1955) displayed by the *Bordetella* also differentiated them from other genera. *B. avium* was classed as a member of the *Bordetella* when Berkhoff and Riddle (1984) distinguished between it and the species within *Alcaligenes*, and Kersters *et al* (1984) listed its major characteristics.

It appeared that *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*

and *B. avium* were independent species classed within a single genus based on their nutritional requirements (Proom, 1955, Berkhoff and Riddle, 1984 and Kersters *et al*, 1984), enviromental niche (respiratory tract of man and animals), DNA base ratios and the nature of the several virulence factors that they share.

More recent studies have focussed on genetic diversity to distinguish between the *Bordetella* and other closely related genera and also between species within the *Bordetella*. De Ley *et al* (1986) showed the closeness of the relationship between the bordetellae by DNA base compositions and DNA-rRNA hybridization studies. Their results indicated that *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* could not be differentiated genotypically in terms of their Tm(e) values of their DNA-rRNA hybrids i.e. the temperature at which one half of the DNA-rRNA hybrid is denatured. The calculated mean G + C contents of their genomic DNA was almost identical. These findings agreed with those of Kloos *et al* (cited by De Ley *et al*, 1986).

The Tm(e) values for the *B. avium* strains tested put it in close vicinity of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* and a study of 147 phenotypic features of *B. avium* have shown it to resemble most closely *B. parapertussis* and then *B. bronchiseptica* (90% and 82% relatedness respectively) (De Ley *et al*, 1986).

The same workers also found that some strains from the genus *Alcaligenes* were very close to the bordetellae in terms of DNA-rRNA hybridization and they proposed a new family - the *Alcaligenaceae* - to encompass both genera (De Ley *et al*, 1986).

Other studies in this area included the work of Musser *et al* (1986) involving electrophoretic mobilities of certain metabolic enzymes of the four bordetellae. These investigations indicated that *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* were very similar. In fact, these

workers claimed that recognition of the bordetellae as separate species could only be justified if based on pathogenicity considerations. However *B. avium* was found to be genetically distinct enough to warrant status as a separate species (Musser *et al*, 1986).

In 1987 Musser and coworkers, in studies similar to those performed in 1986, reported that within strains of *B. bronchiseptica* there was only limited genetic variation.

Other evidence which supported the grouping of *B. pertussis, B. parapertussis* and *B. bronchiseptica* as members of a single independent genus was the fact that both *B. parapertussis* and *B. bronchiseptica* contained transcriptionally silent copies of the pertussis toxin operon which is active in *B. pertussis* (Arico and Rappuoli, 1987). However no such genes were found in *B. avium* or in members of the genus *Alcaligenes* (Arico and Rappuoli, 1987).

More details of the taxonomy of the bordetellae can be found in the review by Johnson and Sneath (1973) which deals with the three mammalian bordetellae, and Kersters *et al* (1984) describe in detail the properties of *B. avium* . The papers of interest dealing with the genetic approach to taxonomy are those of De Ley *et al* (1986), Musser *et al* (1986, 1987) and Arico and Rappuoli, 1987).

Bordetella : The Diseases

The bordetellae cause a variety of different diseases which affect man, animals and birds. Table 4 shows some of the characteristics of these diseases.

B. pertussis is a respiratory tract pathogen of man that is responsible for whooping cough, or pertussis. A similar but less severe form of the infection is caused by *B. parapertussis*. *B. bronchiseptica* causes kennel cough in dogs (reviewed by Thompson *et al*, 1974; Bemis *et al*, 1977a, and McCandlish *et al*, 1978), atrophic rhinitis in swine (review

Table 4 : Characteristics of the diseases caused by *Bordetella* species

Characteristic	Organism			
	B. p[a]	*B. pp*[b]	*B. bron.*[c]	*B. a*[d]
Name	Whooping cough, Pertussis	Atypical whooping cough	Kennel cough, Atrophic rhinitis	Infectious coryza, Rhinotracheitis, Bordetellosis, Turkey coryza
Reservoir	Man	Man, Sheep	Dogs, pigs and various wild animals	Turkeys, chickens and other birds
Transmission	Aerosol droplets	Aerosol droplets	Aerosol droplets	Aerosol droplets and infected feed, litter and water
Symptoms	Catarrhal, paroxysmal and convalescent stages	As with B.p	Nasal atrophy and snout deformation Coughing, sneezing, Ocular and nasal discharge. Reduced weight gain	Exudative conjunctivitis. Sneezing and serous discharge Tracheal rales. Reduced feeding.
Site of infection	Cilia of respiratory epithelium	As with B.p	Nasal and respiratory epithelial cilia	Respiratory epithelial cilia
Carriers	None	None	Dogs, cats, rats, swine	Not known
Immunity	Solid after infection Short-lived with immunization	As with B.p	As with B.p	Not known
Duration	Several months	As with B.p	Up to 14 weeks	About 12 weeks
Treatment	No effective treatment	As with B.p	As with B.p	As with B.p

Table 4 (continued)

Characteristic	Organism			
	B. p	*B. pp*	*B. bron.*	*B. a*
Vaccine	Whole cell Acellular	None	Whole cell Acellular	Whole cell

[a] Information on *B. pertussis (B.p)* gathered from; Connor (1986); Friedman (1988); Pittman and Wardlaw (1981) and Wardlaw and Parton (1988)

[b] Information on *B. parapertussis (B.pp)* gathered from; Eldering and Kendrick (1938); Linneman and Perry (1977); Connor (1986); Chen *et al* (1989)

[c] Information on *B. bronchiseptica (B.bron)* gathered from; Thompson *et al* (1974); Bemis *et al* (1977a); McCandlish *et al* (1978); Goodnow (1980); Rutter (1985); Papasian *et al* (1987)

[d] Information on *B. avium (B.a)* gathered from; Hinz and Kunjara (1977); Kersters *et al* (1984); Simmons *et al.* (1978, 1984)

by Rutter, 1985) and respiratory tract infections in a variety of other domestic and wild animals (review by Goodnow, 1980). The avian pathogen *B. avium* is responsible for a disease which, in turkeys, has had a number of names: rhinotracheitis, bordetellosis, infectious coryza and turkey coryza (see Kerster's *et al*, 1984).

The reservoir of infection for the four species varies. All are thought to be obligate respiratory tract pathogens restricted to colonization of cilia on the nasal and tracheal epithelium. They are generally transmitted by aerosol via infected droplets expelled whilst speaking, coughing or sneezing and are therefore very contagious within populations. With *B. pertussis* and *B. parapertussis* it is possible that hand to mouth transmission may occur via infected objects such as handkerchiefs (Nicholl and Rudd, 1989), while with *B. avium* infected feed, litter and water are possible disease vectors (Simmons *et al*, 1984).

While *B. pertussis* appears to be strictly a human pathogen, *B. parapertussis* has recently been isolated from sheep (Chen *et al* 1989). The two animal and bird pathogens infect a variety of other mammals and birds respectively. *B. bronchiseptica* has been isolated from dogs, pigs, cats, seals, rabbits, monkeys, foxes, skunks, raccoons, koala bears and lesser bushbabies (Goodnow, 1980). It has also been isolated from man and some evidence of zoonotic transmission was recorded (Goodnow, 1980; Byrd *et al*, 1981). *B. avium* is most commonly isolated from turkeys, but chickens, geese and ducks are also susceptible (Kertsers *et al* 1984). *B. avium* did not initiate respiratory tract infection on mice, guinea pigs and hamsters under laboratory conditions (Simmons *et al*, 1984).

Different symptoms present themselves in the various disease states. In whooping cough, the symptoms vary in severity, with cases of *B. pertussis* and are generally milder with *B. parapertussis*, although this is not always the rule (Lautrop, 1971) After about 7-10 days of incubation

the catarrhal stage begins. This is the most infectious stage of the disease with symptoms similar to many upper respiratory tract infections i.e. irritation of the mucous membranes, cough and fever. This usually lasts a further 7-10 days after which the paroxysmal stage ensues with bouts of sometimes severe paroxysmal coughing followed by the characteristic whoop. The convalescent stage then follows and a return to normal activity and development without complications is completed. (Connor, 1986).

Animals infected with *B. bronchiseptica* show a variety of symptoms. In swine, atrophic rhinitis is characterized by nasal atrophy and snout deformation (Rutter, 1985) and with all *B. bronchiseptica* infections it is usual to find coughing, sneezing and discharge from the eyes and nose. A reduction in weight gain is also expected (Goodnow, 1980). Symptoms presented by birds infected by *B. avium* include reduced feed consumption, exudative conjunctivitis and sneezing with serous discharge from the nose. (Hinz *et al*, 1978; Kersters *et al* 1984 and Simmons *et al*, 1984).

A major difference between the diseases caused by the two human pathogens and those by *B. bronchiseptica* is that in the latter the presence of live organisms is required for symptoms to continue, whereas in the former cases the disease persists even if no bacteria can be recovered (Bemis *et al*, 1977a).

Only *B. bronchiseptica* appears to have a carrier state where the infected animals are asymptomatic. This has been observed in dogs, cats, rats, guinea pigs and swine (review by Goodnow, 1980). *B. bronchiseptica* has also been isolated from humans (Ghosh and Tranter, 1979 and Byrd *et al*, 1981).

With the three mammalian bordetellae immunity is usually solid after natural infection whereas it is short-lived after immunization (Connor, 1986; Rutter, 1985 and Goodnow, 1980). No evidence of immunity

in *B. avium* infections has been reported. In all of the diseases caused by the four *Bordetella* species antibiotic treatment is ineffective and once initiated the disease usually runs for the whole course. With *B. parapertussis* no vaccine has been used to date. It has also been shown that immunization against *B. pertussis* does not confer resistance to infection by *B. parapertussis* (Lautrop, 1971 and Connor, 1986). Whole cell pertussis vaccines and acellular vaccines have been used for immunization against whooping cough. The whole cell vaccines have been in general use throughout the world except in Japan, where the use of acellular vaccines has been more common place (Parton, 1991). PT, FHA and agglutinogens 2 and 3 all have protective activity in both mice and man, while ACT has protective activity in mice. More detailed information about vaccines can be found in the review by Parton (1991). Similarily with *B. bronchiseptica* both whole-cell and subcellular (cell wall material) or bacterins have been used with varying measure of success (Rutter, 1985 and Goodnow, 1980). With *B. avium* whole cell vaccines have been tested (Hofstad and Jeska, 1985) however no substantial body of work exists at present.

Part 2. Growth and nutritional requirements.

Bordetella pertussis

Growth in the vertebrate host. Ethical constraints dictate that *in vivo* growth studies of *B. pertussis* can not be done in the natural host, man, and that animal models should be sought. A notable exception was a study of whooping cough in four young children in which all were infected intranasally with *B. pertussis* (MacDonald and MacDonald, 1933). Two of the children had already been vaccinated against whooping cough and remained uninfected. The remaining two became infected , with *B. pertussis* colonizing the respiratory tract and being recovered on cough plates during the period of infectivity.

Nowadays more convenient animal models have been investigated. These have included puppies, monkeys and chimpanzees (Bradford, 1938), mice (Dolby *et al*, 1961; Sato *et al*, 1980), rabbits (Preston *et al*, 1980; Ashworth *et al*, 1982) and more recently rats (Woods *et al*, 1989). A novel method for the study of *in vivo* growth of *B. pertussis* was the intraperitoneal diffusion chamber (Coleman and Wetterlow, 1986), as used previously with staphylococci (Day *et al*, 1980).

Of these experimental animals the most utilized has been the mouse. Only two sites of infection have been used in the mouse namely the lungs and the brain (Standfast and Dolby, 1961) and both intranasal and intracerebral infections have been studied extensively (Fisher, 1955; Dolby *et al*, 1961; Dolby and Standfast, 1961; Standfast and Dolby, 1961; Dolby, 1962 and Alonso *et al*, 1987). Intranasal infection involves the instillation of a measured volume of standardised bacterial suspension into the nasal cavity of anaesthetized mice and intracerebral is simply an injection into the brain of the mouse (Standfast and Dolby, 1961).

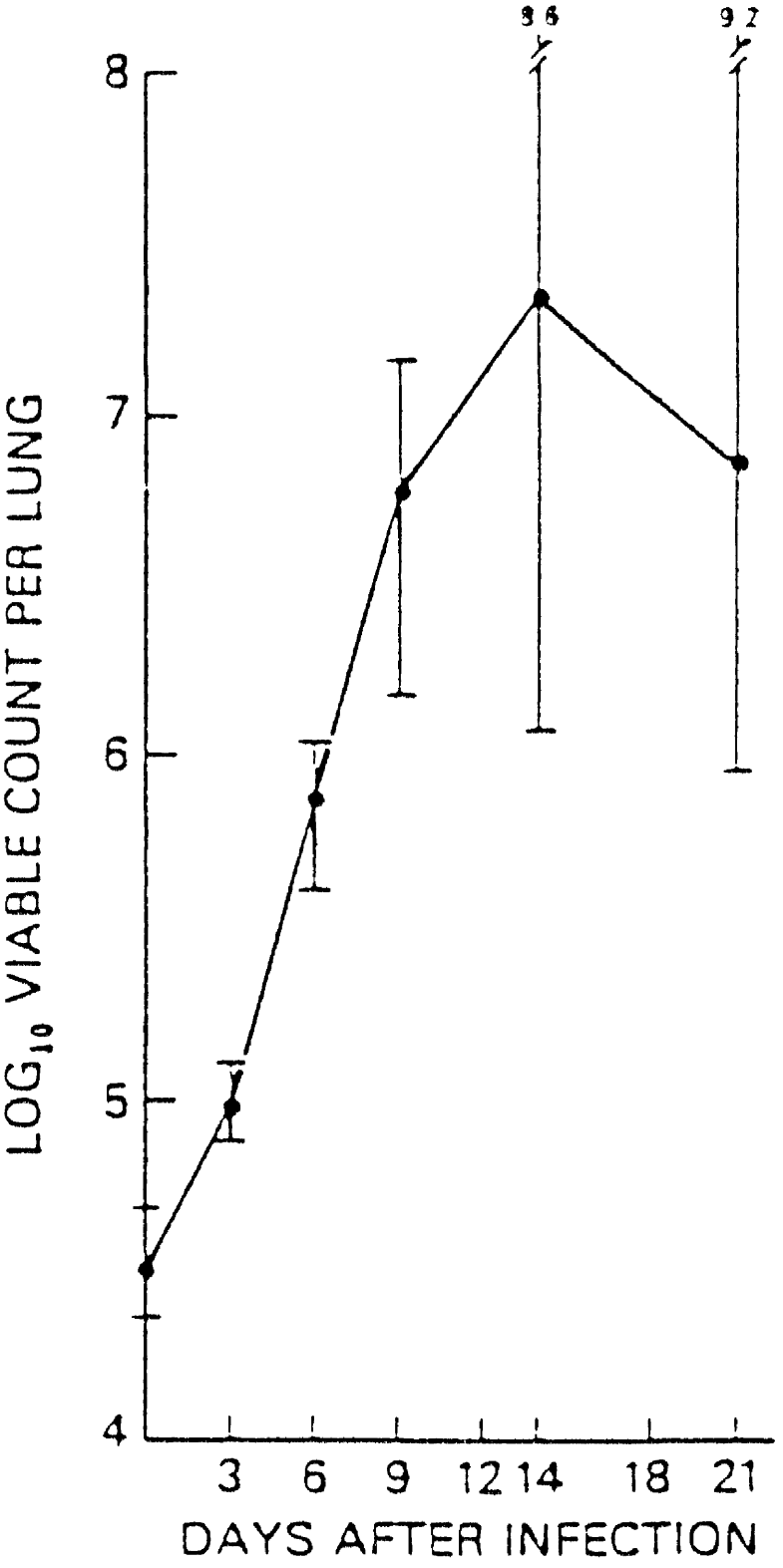
Standfast and Dolby (1961) made a comparison between the two

methods of infection drawing a number of conclusions from their study. The terminal viable count in the lungs and brain was always 10^8 CFU with death occuring on the 4^{th} or 5^{th} day after infection. In the lung sublethal infections could be observed with challenge doses of less than 10^3 CFU where numbers increased steadily to 10^4 to 10^6 until the 10^{th} to 14^{th} day after infection and then slowly declined over some weeks or even months until the lung became sterile. This was in contrast to brain infections where there was no such thing as a sublethal infection. In fact in the brain, regardless of initial inoculum, numbers consistently increased to 10^8 CFU with death ensuing. It appeared that probably a single cell, if it became lodged in the brain, could and would grow to a critical level (Dolby and Standfast, 1961). With large initial inocula there was an initial die-off followed by an increase in viable count to the critical level in both infections.

Sato *et al* (1980) claimed that aerosol infection of mice with *B. pertussis* gave a more accurate and reproducible inoculum. In this system the mice were placed on a mesh screen in a glass chamber fitted with a nebulizer. The animals were exposed to an aerosol for 30 minutes by spraying 0.4 ml of bacterial suspension per minute at a nebulizer pressure of 1.5 kg/cm^2. From a bacterial concentration of 2×10^9 CFU/ml an initial delivered inoculum of 2×10^4 CFU/lung was obtained. This reached a maximum of 10^7 CFU/lung in 14 days (Figure 1), after which the viable count decreased. Sato *et al* (1980) observed the same growth and infectivity pattern as Standfast and Dolby (1961). Again the lethal dose was found to be 10^8 CFU/lung.

A novel method of studying the growth and toxin production of *B. pertussis in vivo* was introduced by Coleman and Wetterlow (1986) following work with staphylococci (Gladstone and Glencross, 1960; Houser and Berry, 1961; Day *et al* 1980). It involved the use of a small sterile

Figure 1 : *In vivo* growth of *B. pertussis* in the lung of a mouse. (from Sato *et al.* 1980 and reproduced with permission).

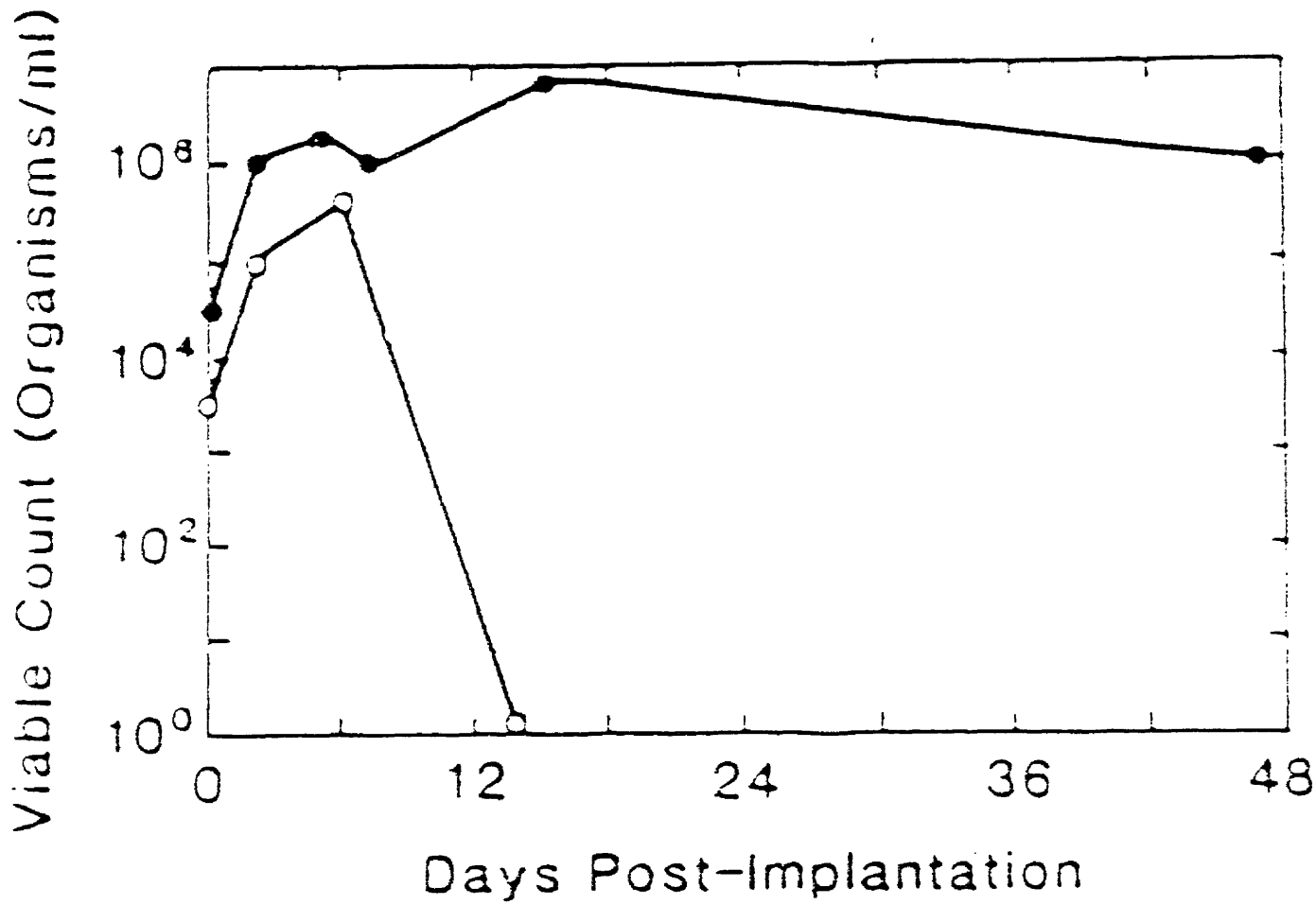

8.6
9.2
8
7
6
5
4
LOG10 VIABLE COUNT PER LUNG
3
6
9
12
14
18
21
DAYS AFTER INFECTION

chamber, bounded by 0.22 μm membrane filters, which was inoculated with *B. pertussis* , sealed and surgically implanted into the peritoneal cavity of mice. The mice were killed at intervals and viable counts performed on the contents of the chambers. A comparison was made between growth in the chambers (*in vivo*) and in Cohen and Weeler (CW) medium (*in vitro*) and is shown in Figure 2. From the Figure it can be seen that the viable counts for *B. pertussis* strain 1392 were greater in the implanted chambers reaching over 10^6 CFU/ml. Four widely different strains all showed the same growth characteristics in the chambers.

Another observation, consistent with the findings of previous workers on growth of *B. pertussis* in mice (Dolby *et al*, 1961), was that a steady-state bacterial concentration of around 10^8 CFU/ml (not presented in Figure 2) was always reached. This was also regardless of the size of the initial inoculum. If the inoculum was small the viable count increased to the maximum value in 6-7 days and if large inocula ($>10^8$) were used the viable count decreased quickly until the steady state was reached (three weeks). These workers found that it was possible to initiate an infection *in vivo* with less than 10 organisms per chamber. They also showed that virulent strains of *B. pertussis* were able to outgrow avirulent strains when implanted in the same chamber.

Another animal model which has received recent attention attention is the "coughing rat model" which closely parallels the human infection with respect to the clinical and physiological changes seen in the human infection, including the paroxysmal cough (Woods *et al*, 1989). This work was based on a previous model involving the infection of rats with *Pseudomonas aeruginosa* (Cash *et al*, 1979). With the virulent Tohama I strain a reproducible infection, similar to the human disease was produced in the rat with colonization of the lung observed. Organisms were recovered 21 days after infection.

Figure 2 : Comparison of *in vivo* growth (intraperitoneal chambers; •) and *in vitro* growth (CW medium; o) of *B. pertussis*. (from Colemen and Wetterlow, 1986 and reproduced with permission).


Viable Count (Organisms/ml)
10^8
10^4
10^2
10^0
0
12
24
36
48
Days Post-Implantation

Growth on laboratory media. Most of the literature dealing with the development of culture media for the *Bordetella* concentrated on work with *B. pertussis,* although similar media have been used for the other members of the genus. In the following sections that deal with members of the genus other than *B. pertussis* only information additional to that already provided will be presented and the reader will be referred to the appropriate sections of the text for details of other media. Since most of the media for *B. pertussis* have been used for more than one purpose e.g. Regan and Lowe (1977) medium has been used for the isolation, transport and propagation of *B. pertussis,* some repetition is unavoidable in this section.

Isolation and transport media. *B. pertussis* was first isolated in 1906 by Bordet and Gengou using a medium which consisted of glycerinated potato extract and sheep or human blood solidified with agar. Since then many workers have tried to improve upon this recipe in their attempts to create more efficient isolation media for *B. pertussis* (Table 5).

Various isolation media have been used directly or in conjunction with transport media to facilitate the recovery of *B. pertussis* from scantily populated specimens. In 1954, Lacey described a complex selective medium containing 4:4 diamidinodiphenylalanine hydrochloride, penicillin and sodium fluoride in a partially defined base which was successful in the isolation of *B. pertussis.* Most of the other recipes have involved the incorporation of selective antibiotic agents to suppress the nasopharyngeal flora present in samples. Benzyl penicillin and penicillin have been used often (Sutcliffe and Abbot, 1972; Stauffer *et al,* 1983 and Fareeduddin and Calder, 1984) . Other selective agents have included lincomycin (Hoppe and Schwaderer, 1989), methicillin (Morrill *et al,* 1988), anisomycin (Kurzynski *et al,* 1988) and cephalexin (Regan and Lowe, 1977). Of all the antibiotics tested, cephalexin at a level of 40 µg/ml proved to be the most successful.

Table 5 : Isolation media for *B. pertussis*

Medium	Main Constituents	Reference(s)
BG	glycerinated potato extract + 50% (v/v) horse blood + agar	Bordet and Gengou, 1906
Lacey's*	New Zealand agar, potato starch, glycerol, *dl*- alpha alanine, *l*-glutamic acid, sodium fluoride, sodium chloride, potassium chloride, tap water, aconitic acid, fumaric acid, malonic acid, *l* or *dl*-cysteine hydrochloride, distilled water, magnesium hydroxide, 4:4' diamidinodiphenylamine hydrochloride, penicillin and defibrinated horse blood (dhb)	Lacey, 1954
Modified charcoal blood agar*	a) Charcoal agar, 10 % (v/v) horse blood 1 % (w/v) proteose peptone, 0.25 U benzyl penicillin b) a, plus cephalexin (40 µg/ml) to replace benzyl penicillin	Sutcliffe and Abbot, 1972
Regan and Lowe*	Oxoid charcoal agar, 10 % (v/v) dhb, cephalexin (40 µg/ml)	Regan and Lowe, 1977
Jones-Kendrick (JK) charcoal agar with cephalexin/penicillin	JK charcoal agar, cephalexin (40 µg/ml) or penicillin (0.5 U/ml)	Stauffer *et al.* 1983
BG agar/ benzyl penicillin	BG agar base, 20 % (v/v) dhb, benzyl penicillin (0.25 U/ml)	Fareeduddin and Calder, 1984
Charcoal agar/ benzyl penicillin	Charcoal agar base, 10 % (v/v) dhb, benzyl penicillin (0.3 U/ml)	Fareeduddin and Calder, 1984
Cyclodextrin agar	Stainer-Scholte base, casamino acids, MeßCD, 1.8 % (w/v) agar	Aoyama *et al.* 1986 Morrill *et al.* 1988

Table 5 (continued).

Medium	Main Constituents	Reference(s)
Modified BG agar*	BG agar base, 20 % (v/v) dhb, cephalexin (40 µg/ml), anisomycin (20 µg/ml)	Kurzynski *et al.* 1988
Modified RL agar*	RL charcoal agar, cephalexin (40 µg/ml), anisomycin (20 µg/ml)	Kurzynski *et al.* 1988
BG agar/methicillin	BG agar base, 1 % (v/v) glycerol, 15 % (v/v) dhb, methicillin (2.5 µg/ml)	Morrill *et al.* 1988
SS/ MeßCD	Stainer-Scholte base, MeßCD (1 g)	Wirsing von Koenig *et al.* 1988

* also used for *B. parapertussis*

Another additive, MeßCD has also been used for the successful isolation of *B. pertussis* (Aoyama *et al*, 1986; Morrill *et al*, 1988 and Wirsing von Koenig *et al*, 1988).

Transport media (Table 6) used for *B. pertussis* had to be able to maintain the viability of the organism during its journey from the sampling point to its isolation in the laboratory. For this reason some of the transport media, like the isolation media, were enriched and contained certain selective agents (antibiotics) to improve the chances of *B. pertussis* isolation.

Amie's transport medium (Amies, 1967) was one which had been used successfully for the isolation of not only *B. pertussis* but for *B. parapertussis* and *B. bronchiseptica* It was a modification of Stuart's transport medium (Stuart, 1946) in which the glycerophosphate was replaced with an inorganic phosphate buffer and charcoal was added to the medium.

The isolation medium described by Regan and Lowe (1977) was also used as a successful transport medium. It was used directly by later workers (Kurzynski, 1988) while others made some minor modifications of the basic recipe (Morrill *et al*, 1988).

In 1988, Morrill and co-workers studied three transport media; modified Regan-Lowe, beef extract agar and charcoal yeast-extract agar. They found that *B. pertussis* survived best when the modified Regan-Lowe with cephalexin and amphotericin B was employed.

Laboratory propagation media. Both solid and liquid media have been used for the successful propagation of *B. pertussis* in the laboratory. Early work was reviewed by Rowatt (1955, 1957a and 1957b).

Table 7 shows the various solid media used for the post-isolation cultivation of *B. pertussis*.

BG agar, which was used for the first isolation of *B. pertussis*

Table 6 : Transport media for *B. pertussis*

Medium	Main Constituents	Reference
Stuart's*	Bacto agar, thioglycollic acid, sodium glycerophosphate, calcium chloride, methylene blue	Stuart, 1946
Amie's*	Charcoal pharmaceutical neutral, sodium chloride, sodium hydrogen phosphate, potassium chloride, sodium thioglycollate, calcium chloride, magnesium chloride, agar No 1	Amies, 1967
Regan and Lowe* (RL)	Charcoal agar (1/2 strength), 10% (v/v) defibrinated horse blood (DHB), cephalexin (40 µg/ml)	Regan and Lowe, 1977 Kurzynski *et al*, 1988
Modified RL	RL with amphotericin B (50 µg/ml)	Morrill *et al*, 1988
Beef-extract agar	Beef-extract, meat peptone, soluble starch charcoal, sodium chloride, nicotinic acid, agar, cephalexin (40 µg/ml) amphotericin B (50 µg/ml)	Morrill *et al*, 1988
Charcoal yeast-extract agar	Buffered charcoal yeast-extract agar, 1% (w/v) alpha-ketoglutarate, lincomycin (3 µg/ml) anisomycin (80 µg/ml)	Morrill *et al*, 1988

* also used for *B. parapertussis*

Table 7 : Solid media for the cultivation of *B. pertussis*

Medium	Main constituents	Reference
BG	glycerinated potato extract + 50% (v/v) horse blood + agar	Bordet and Gengou, 1906
Barksdale and Simpson	nutrient agar + potato extract	Barksdale and Simpson, 1934
Cruikshank and Freeman	proteose peptone + heart extract + 20% (v/v) laked horse blood with 13% (v/v) horse serum	Cruikshank and Freeman, 1937
Silverthone and Cameron	aqueous extract of beef liver + salt + agar + 33% (v/v) citrated sheep blood	Silverthorne and Cameron,1942
Modified Cohen-Wheeler(CW)	CW + agar+ charcoal	Powell *et al*, 1951
Charcoal agar	CW + agar + charcoal + catalase preparation	Mazloum and Rowley, 1955
Regan-Lowe	charcoal, cephalexin + defibrinated horse blood	Regan and Lowe, 1977

(Bordet and Gengou, 1906), has also been useful in its cultivation. Other workers modified BG medium by using heated blood (Krumwiede, *et al* 1923) or lower concentrations of blood (Debre, *et al* 1928) but they failed to notice that the organisms produced varied antigenically from Bordet and Gengou's bacillus. Leslie and Gardner (1931) explained this phenomenon when they reported that there were four phases of *B. pertussis* i.e I, II, III and IV.

Most modifications to BG medium were either by addition or removal of medium constituents or an increase or decrease in the concentration of some component. Dawson *et al* (1951) stated that the glycerol used in the medium, either added after potato extraction (Mackie and McCartney, 1953) or used in potato extraction (BG medium), was unnecessary and omitted it altogether. They also found most peptones to be inhibitory and that glutamate and aspartate improved growth, suggesting that amino acids may be responsible for the effect of the potato extract. Povitsky (1923) noticed that pH was important and that better growth could be obtained on media with a slightly acid reaction. Various other workers used lactate to improve growth (Madsen, 1925; Leslie and Gardner, 1931; Lacey, 1954).

Eldering and Kendrick (1952) made a modification to BG and this was used for routine diagnostic purposes. It contained 17% sheep blood, no peptone and penicillin was added. The medium described by Lacey (1954) and presented earlier as an effective isolation medium (Table 5) was also useful in the propagation of *B. pertussis*. However it was not a preferred medium for routine use due to the complexity of its formulation .

Blood was a possible source of many contamination problems and several attempts were made to replace it. Pollock (1947) effectively substituted charcoal, serum and serum albumin and suggested that the role of the blood was to absorb inhibitors, such as fatty acids, an effect also

shown with *M. tuberculosis* (Davis and Dubos, 1946). Powell, Culbertson and Ensminger (1951) produced a medium used for the growth of vaccine strains consisting of Cohen and Wheeler medium plus agar and charcoal suitable for growth from large but not small inocula. Others used charcoal (Mazloum and Rowley, 1955; Mishulow, Sharpe and Cohen, 1953) but again growth was only obtained from large inocula. In 1953 Ensminger *et al* in a study on the growth of *B. pertussis* on charcoal agar found that the optimum concentration of charcoal was 0.2 %, which gave a cell count of 184 billion/ml and that the optimal incubation period was 48h. They also found that Charcoal agar compared favourably with BG medium and could replace it in all of its uses.

Mazloum and Rowley (1955) combined CW medium agar, charcoal and a catalase preparation. The catalase was incorporated to counteract the inhibition by peroxidases. Mucin was also found to have a similar effect to charcoal (Miyamota *et al* 1954).

Regan and Lowe's (1977) enrichment medium, which was also used as an isolation and transport medium (Table 5 and 6), was successful in the propagation of *B. pertussis* from small populations.

Other research focussed on liquid media (Table 8) for the cultivation of *B. pertussis* Some workers believed that there could be no growth without blood (Lawson, 1939) or tissue extract (Toomey and McClelland, 1933-34). However in 1939 Hornibrook described a medium containing hydrolysed casein, soluble starch, salts, cysteine and yeast extract. In 1940, Hornibrook described another medium containing amino acids, starch and nicotinamide i.e Modified Hornibrook Medium. Other modifications to Hornibrook's recipe were made by various workers (Verwey and Sage, 1945; Wilson, 1945; Farrell and Taylor, 1945; and Cohen and Wheeler, 1946).

CW medium was recommended by the WHO for preparation of

Table 8 : Liquid media for the cultivation of *B. pertussis*

Medium	Main constituents	Reference
Hornibrook	hydrolyzed casein, soluble starch, yeast extract and salts[a]	Hornibrook, 1939
Modified Hornibrook	soluble starch, glutamic acid, L-tyrosine, glycine, L-proline, L-histidine, arginine and salt[b]	Hornibrook, 1940
Cohen-Wheeler	casamino acids, soluble starch, cysteine hydrochloride, yeast dialysate and salts[c]	Cohen and Wheeler, 1946
Stainer-Scholte	DL-alanine, L-arginine, DL-aspartic acid L-glutamine, glycine, DL-histidine, DL-serine L-proline, L-glutamic acid, L-cystine, adenine, ATP(Di Na salt),guanine, hypoxanthine, thymine, activated charcoal, tris buffer, liver co-enzymes (CoA, DPN, TPN) ascorbic acid, niacin, glutathione, 2-deoxyribose and salts[d]	Stainer and Scholte, 1971
Cyclodextrin (CL) medium	sodium L-glutamate, L-proline, tris, L-cysteine, niacin, glutathione, ascorbic acid, casamino acids, methylated ß-cyclodextrin and salts[e]	Imaizumi *et al*, 1983

a NaCl, KCl, $CaCl_2$ (anhydrous), Na_2CO_3 (anhydrous), $MgCl_2(6H_20)$, K_2HPO_4

b as in a except no Na_2CO_3 (anhydrous)

c as in d except no KCl

d as in b except add KH_2PO_4 instead of K_2HPO_4 and add $FeSO_4.7H_20$ and $CuSO_4.5H_20$

e as in d except no $MgCl_2(6H_20)$

pertussis vaccine. Verwey *et al* in 1949 compared a liquid medium they had prepared with Hornibrook's and other modified liquid media that were in use at that time. Basically, what they did was adjust the amount of the components in the medium or omitted some of them completely. Verwey and his coworkers also examined the optimal conditions for the growth of *B. pertussis*. They claimed that their medium gave better growth than the others tested and that the vaccines that were produced using this medium were comparable to those prepared from BG medium. Verwey also made an agar form of this medium and, although on its own it was unsatisfactory, when 10-20 % blood cells were added claims were made that it gave better results than the BG medium tested in parallel.

Anion exchange resins have also been shown to support the growth of virulent strains of *B. pertussis* as an alternative to starch or carbon (Kuwajima *et al*, 1957). A liquid medium containing an anion-exchange resin was described in 1961 by Sutherland and Wilkinson and they were also able to produce a solid medium that compared favourably with BG medium. The anion-exchange resin was thought to remove inhibitors from the medium and inhibitors produced during growth.

Defined media and small inocula. With defined media came a better understanding of the nutritional requirements of *B. pertussis*. The replacement of starch and casein hydrolysate by activated charcoal and an amino-acid mixture, respectively, was undertaken by Wilson in 1963 in his search to produce a defined medium. His medium contained amino acids, glutamine, glutathione, inorganic salts, niacin, ascorbic acid, ATP, coenzyme A, DPN, TPN and nucleic acid derivatives.

Some workers described chemically defined media which were more useful for studying the nutritional requirements of the organism (Ungar *et al* 1950; Jebb and Tomlinson,1955, 1957; Wilson, 1963). In 1971, Stainer and Scholte produced a simple chemically defined medium

consisting of sodium glutamate, cystine, proline, salts and growth factors, suitable for the large scale production of phase I *B. pertussis*

In most, if not all of the media described above, growth of *B. pertussis* could be obtained with large but not small inocula. In 1983, Imaizumi *et al* discovered the novel growth stimulant for *B. pertussis* heptakis (2,6-0-dimethyl)ß-cyclodextrin (MeßCD). This was added to a complete synthetic medium such as SS and the same number of individual colonies and growth rates as with BG were obtained. As well as supporting growth from small inocula, the MeßCD was found to prevent the growth of other nasopharangeal flora (Aoyama *et al* 1986).

Cyclodextrin medium was initially used as an agar, with a basal medium similar to Stainer-Scholte, to which was added a supplement (Imaizumi *et al* 1983). CSM has been compared with BG medium and it is as good, if not better for the isolation and cultivation of *B. pertussis* (Aoyama *et al* 1986). When 5µg of cephalexin was added to CSM it increased its specificity for *B. pertussis* and made it a better clinical isolation medium than BG medium. It could also be used as a liquid medium. However the major restriction is that the MeßCD used in the CSM is expensive.

B. parapertussis

Growth in the vertebrate host. *B. parapertussis* infections have been reproduced experimentally in mice (Chen *et al* 1989). Natural infections have been noted in both humans (Linneman, 1979) and sheep (cited by Chen *et al* 1989). Very few studies have dealt quantitatively with the growth of this species within the confines of the animal or human host.

One study which examined some of these points was that of Chen *et al* (1989) who induced an experimental pneumonia in mice using six strains of *B. parapertussis* which had been isolated from sheep

bronchoalveolar washings. The inoculum (2.7 x 10^7 CFU/ml) was instilled intranasally into three week old mice and sampling times over a period of 29 days were used. The number of viable bacteria colonizing the lung at each sampling time was determined by plate counts and scored semi-quantitatively i.e. large numbers (+++), small (++), very small (+) and none (-). During the study period the viable count of *B. parapertussis* increased within the first couple of days when large numbers of bacteria were recovered from the lungs. The viable count then decreased and remained constant up until day eight. The numbers subsequently decreased to zero after day 14. There are no other reports similar to that of Chen *et al* (1989).

Growth on laboratory culture media. In general, media used for the isolation and culture of *B. pertussis* have been reported as also being suitable for use with *B. parapertussis* (Wardlaw, 1990).

Isolation and transport media. Of the transport media described in Table 6 for *B. pertussis* three have also been employed for the primary isolation of *B. parapertussis.* These were Stuart's transport medium (Stuart, 1946), Amies transport medium (Amies, 1967) and the isolation and enrichment medium of Regan and Lowe (Regan and Lowe, 1977). Mishulow, Sharpe and Cohen, in 1953, described a charcoal agar slope containing penicillin for transport of *B. parapertussis* to the laboratory before isolation.

Both BG agar (Lautrop, 1960) and modified BG agar (Kendrick and Eldering, 1969) have been used for the isolation of *B. parapertussis* from human specimens. In 1974, Gopaul and Yu used two media for the isolation of *B. parapertussis* The first was BG agar containing 4 µg/ml of penicillin and the second contained the selective agent described by Lacey (1954). It consisted of a charcoal agar base with 10 % defibrinated horse blood and 2 µg/ml of M&B 938. To this was added a further 40 µg/ml of cephalexin.

Both media were used successfully for the isolation of *B. parapertussis*.

Laboratory propagation media. Those media presented in Tables 7 and 8 are also capable of supporting the growth of *B. parapertussis*. Being less fastidious than *B. pertussis*, *B. parapertussis* can be cultivated on MacConkey's agar and nutrient agar or in nutrient broth (Topley and Wilson, 1975). When *B. parapertussis* is grown on nutrient agar it causes a browning of the medium due to its ability to utilize tyrosine (see Table 1).

For references to the other media used for the cultivation of *B. parapertussis* the reader is referred to the previous section for *B. pertussis*.

B. bronchiseptica

Growth in the vertebrate host. Like *B. parapertussis*, there have been few studies of experimentally-induced infection of laboratory animals with emphasis upon quantitating the levels of bacterial growth in the animal's tissues.

In 1970 Shimizu and co-workers infected hysterectomy-derived, colostrum-deprived piglets, 5-6 days old, intranasally with a strain of *B. bronchiseptica*. The strain had been freshly isolated from the turbinate of a pig belonging to a herd with an acute rhinitis problem. The investigators attempted to recover *B. bronchiseptica* from the animals at 37 days and three months of age. No viable count was reported at time zero although reference was made to an inoculating suspension of 1 mg/ml (0.5 ml given) *B. bronchiseptica*. Upon sampling at 37 days over 50 CFU were recovered on a plate culture when a swab was taken of the mucosal surfaces of the turbinate and ethmoid, 10-49 CFU from the bronchi and 1-9 CFU from the lungs. After three months only a few bacteria were recovered from the ethmoid (10-49 CFU) and none was recovered from the turbinate, bronchi or the lung. Shimizu *et al* (1970) concluded that *B. bronchiseptica*

multiplies in the aforementioned anatomical sites, causes damage and then decreases in numbers, disappearing from the respiratory tract of pigs three months after exposure.

Koshimizu *et al* (1973) reported similar findings with intranasally infected conventional pigs aged 5-6 days of age. A volume of 0.5 ml of an initial inoculum of about 10^9 CFU/ml was instilled and samples were taken at intervals of 1-2 weeks. At three weeks of age, *B. bronchiseptica* was recovered from the nasal cavity, ethmoid, trachea and lung with greater than 100 colonies per plate from a single swab. The viable count remained constant for about 12 weeks (16 weeks in some cases) and then decreased, with no cells recovered after 22-24 weeks.

Other workers reported similar findings but no quantitation was recorded. Kemeny (1972) re-isolated *B. bronchiseptica* from young pigs after intranasal inoculation of crude nasal washings from swine with atrophic rhinitis and also with a tryptone phosphate broth culture of a *B. bronchiseptica* strain isolated from the nasal washings. A similar inoculum to that of Koshimizu *et al* (1973) was used i.e. 1.5×10^9 CFU/ml of which 4 ml was delivered with an atomizer.

Burek *et al* (1972) successfully re-isolated *B. bronchiseptica* from the trachea, nasal cavity and lung of conventional and germ-free rats after aerosol infection, while Meyer and Beamer (1973) did likewise with intranasally infected germ-free swine. In this study *B. bronchiseptica* was also isolated from the intestinal tract. Brassinne *et al* (1976) intranasally infected gnotobiotic pigs with a strain of *B. bronchiseptica* isolated from the nasal cavity of a pig with atrophic rhinitis. He witnessed sneezing and coughing and re-isolated the bacterium from the ethmoid, turbinates, trachea and pneumonic lesions.

Similar findings have been reported in dogs infected by an aerosol of *B. bronchiseptica* (Thompson *et al*, 1976). *B. bronchiseptica* was

recovered from the nasal cavity, trachea, bronchi and lung of the infected animals.

In none of these studies was reference to quantitation made.

Growth on laboratory culture media. The media that are used for the isolation of *B. bronchiseptica* can generally be employed for the further cultivation of the organism.

Isolation and transport media. A number of different isolation and transport media have been assembled in Table 9.

Blood agar and MacConkey's agar appear to be the most popular isolation media (Thompson *et al,* 1976, Bemis *et al,* 1977b and Smith and Baskerville, 1979). Other workers modified MacConkey's to give better results and less contamination by the other bacteria of the respiratory tract. Farrington and Switzer (1977) added furaltadone and nystatin to double strength MacConkey's with 2 % dextrose. Bemis *et al* (1977b, 1977c) used Levine eosin methylene blue agar for the successful isolation of *B. bronchiseptica* from both nasal and tracheal swabs from infected dogs.

In the study of Smith and Baskerville (1979) with pigs, two types of modified MacConkey's agar were used; 1) MacConkey's agar plus 1 % (v/v) glucose and 20 % MacConkey's agar plus 1 % glucose and 20 µg/ml furaltadone. During this study a number of other isolation media were employed. In addition to the modified MacConkey's medium and blood agar two other media were described for the selective isolation of *B. bronchiseptica* from pigs. Peptone agar was used which consisted of bactopeptone, glucose, lactose, sodium chloride, bromothymol blue and agar and a new medium named G 20G agar was also described. This medium was based on peptone agar with added penicillin, furaltadone and gentamicin. In these studies the anti-fungal agent fungizone was added to all of the agars described. G 20G medium proved to be the most successful

Table 9 : Isolation and transport media for *B. bronchiseptica*

Medium	Reference(s)
Blood agar	Thompson *at al*, 1976; Bemis *et al*, 1977a; Smith and Baskerville, 1979
MacConkey's agar	Thompson *et al*, 1976
Levine eosin methylene blue agar	Bemis *et al*, 1977b, 1977c
Modified MacConkey's agar	Fisk and Soave, 1973 Farrington and Switzer, 1977 Smith and Baskerville, 1979 De Jong and Borst, 1985
G 20 G agar	Smith and Baskerville, 1979
Peptone agar	Smith and Baskerville, 1979
Modified BG agar	Rutter, 1981
Pasturella multocida and *B. bronchiseptica* selective agar	De Jong and Borst, 1985

in the isolation of *B. bronchiseptica.*

Antibiotics have been used by other workers, for example, Fuzi (1973) used penicillin, streptomycin and vancomycin as selective agents. Nystatin and penicillin were used by Woode and McLeod (1967), furzolidine by Koshimizu and co-workers (1973) and furaltadone and nystatin by Farrington and Switzer (1977).

A selective BG medium containing furaltadone, penicillin, amphotericin B, streptomycin sulphate and spectinomycin was used during an investigation of atrophic rhinitis in pigs (Rutter, 1981).

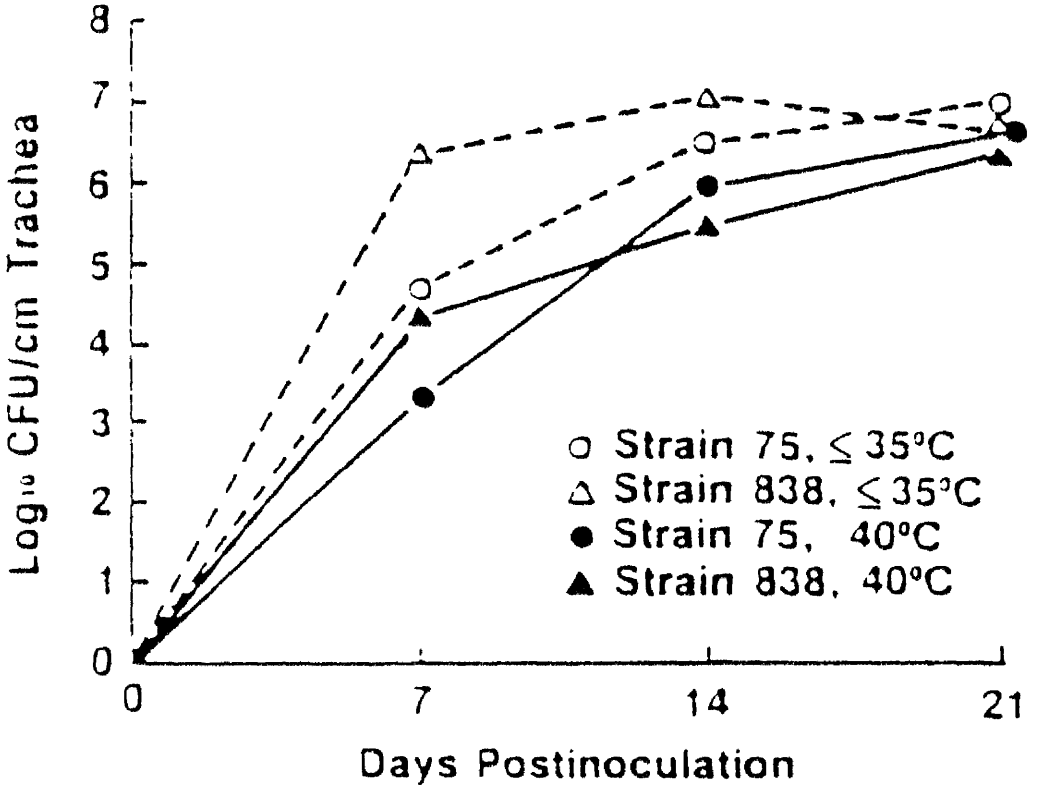
Amie's transport medium is usually the choice for *B. bronchiseptica* (Bemis *et al,* 1977b) when direct inoculation onto an isolation medium is not practicable.

Laboratory propagation media. The culture media used for the other bordetellae (Tables 7 and 8) are also useful for laboratory maintenance and cultivation of *B. bronchiseptica* Usually MacConkey's agar and blood agar are routinely employed and Brucella agar has also been used (Bemis *et al,* 1977c). The media described in Table 9 should be suitable as propagation media in addition to isolation and transport, as most are multi-purpose.

B. avium

Growth in the vertebrate host. Arp and McDonald (1985) produced a detailed study on the effect of temperature on the growth of *B. avium* in turkeys. They reported viable counts in the tracheas of turkeys which were inoculated with $10^{6.3}$ CFU/0.5ml and then either heat-stressed (40°C) or cold-stressed (35°C or less). Figure 3 was reproduced from the original paper. In all cases there was an increase in viable count of *B. avium* strains 78 and 838. The counts were expressed as $\log_{10}$ CFU/cm trachea. Seven to fourteen days after challenge, Arp and McDonald (1985) reported that colonization of cold-stressed turkeys was significantly less

Figure 3 : **Growth of *B. avium in vivo.*** Effect of temperature on growth of *B. avium* strains 75 and 838 in turkey tracheas at 35°C and 40°C. (from Arp and Mc Donald, 1985 and reproduced with permission).


Log10 CFU/cm Trachea
8
7
6
5
4
3
2
1
0
0
7
14
21
Days Postinoculation
Strain 75, ≤ 35°C
Strain 838, ≤ 35°C
Strain 75, 40°C
Strain 838, 40°C

than that in heat stressed birds i.e $\log_{10}$ CFU/cm values of 6 and 6.5 in cold-stressed birds (strains 78 and 838 respectively) compared to 5.5 and 5 for heat-stressed birds (78 and 838 respectively). However by 21 days there was no significant difference between the two types of bird. Comparing numbers of bacteria in the upper and lower trachea of the turkeys the investigators found that in cold-stressed birds there were more organisms present in the upper than the lower portion of the trachea. The counts from the upper and lower trachea of heat-stressed turkeys were not significantly different. Observations after 21 days were not reported.

No other reports similar to this one or dealing with increases in viable counts within the avian host have been uncovered despite extensive searching.

Growth on laboratory culture media. The growth and isolation media used for *B. avium* are the same as those for *B. bronchiseptica* although other media which are only used for *B. avium* are also described.

Isolation and transport media. In 1979, Simmons and co-workers isolated *B. avium* from turkey poits using chocolate agar fortified with Iso Vitale X enrichment and included chloral hydrate to prevent the swarming of motile contaminating bacteria. The same workers used MacConkey's agar with 5 % agar to serve a similar purpose as the chloral hydrate. Simmons *et al* (1984) used MacConkey's agar to isolate *B. avium* from turkey poults as did Hofstad and Jeska (1985) and Blackall and Farrah (1985). In the study of Blackall and Farrah (1985) other isolation media used included blood agar and Salmonella-Shigella agar.

Laboratory propagation media. The media used for the isolation of *B. avium* also serve as good cultivation media. For example, the isolation media used by Blackall and Farrah (1985) were employed as growth media in the same study. Kersters *et al* (1984) recommended the

use of blood agar containing Columbia agar base and 7 % defibrinated ox-blood or veal infusion agar for the cultivation of *B. avium.*

During a study of the physical, biochemical and pathological properties of *B. avium,* Simmons and coworkers (1979) used brain heart infusion broth and agar for the cultivation of the bacteria. In 1983, Rimler and Simmons grew *B. avium* on a number of different media including bovine blood agar, MacConkey's agar, Salmonella-Shigella agar and Simmons Citrate agar. Arp and McDonald (1985) used brain-heart infusion broth while Hofstad and Jeska (1985) employed dextrose-starch agar slants for cultivation.

In addition to the solid media described *B. avium* should grow in the liquid media described for the other bordetellae.

Nutritional requirements of *Bordetella*

Early work on the nutritional requirements of this group dealt with *B. pertussis, B. parapertussis* and *B. bronchiseptica* (Proom, 1955, Rowatt, 1955 and Jebb and Tomlinson, 1955, 1957). Later Kersters *et al* (1984) detailed the requirements of *B. avium.*

The *Bordetella* have relatively simple nutritional requirements with some similarities between species. All species have an absolute requirement for nicotinic acid and utilize amino acids as their primary carbon and energy source (Proom, 1955, Rowatt, 1955 and Kersters *et al,* 1984). Table 10 shows the minimal amino acid requirements of *B. pertussis, B. parapertussis* and *B. bronchiseptica* presented in a detailed study by Proom (1955). His results have been confirmed by other workers (Rowatt, 1955, Jebb and Tomlinson, 1955, 1957).

Glutamic acid appears to be the prefered amino acid and in simple amino acid mixtures is the first to be utilized by *B. pertussis, B. parapertussis* and *B. bronchiseptica* (Rowatt, 1955, Jebb and Tomlinson, 1955, 1957; Goldner *et al,* 1966). *B. bronchiseptica* has the simplest needs

Table 10 : Minimal nutritional requirements of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. (modified from Proom, 1955)

Medium	*B.p*	*B.pp*	*B.br.*
1. ABSM[a] + complex amino acid (aa) mixture + nicotinic acid (NA)	+[b]	+	+
2. Medium 1 without NA	-	-	-
3. ABSM + 7aa mixture	-[c]	+	+
4. Medium 3 without glutamic acid	-[d]	-	-
5. Medium 4 with alpha-ketoglutaric acid	+[e]	+	+
6. ABSM + NA + glutamic acid + proline + leucine	-	-[e]	+
7. Acetate citrate + NA	-	-	-
8. ABSM + NA + either alpha-ketoglutaric acid, acetate, citrate, glucose, glutamic acid or pyruvate	-	-	a. Old lab strains are + with all but glucose b. Fresh animal strains are + with lactate or citrate only

[a] Ammonia Basal Salt Medium; [b] phase I required starch; [c] with cystine; [d] with cystine; [e] with cystine and methionine

and can grow in a mixture of glutamic acid, proline and leucine. Proom (1955) suggested that *B. parapertussis* required added cysteine and methionine and *B. pertussis* required in addition alanine, asparagine and serine for good growth (Proom1955). However Stainer and Scholte (1971) grew *B. pertussis* in a mixture of glutamate, proline and cystine.

None of the *Bordetella* utilize carbohydrates (Proom, 1955; Rowatt, 1955; Jebb and Tomlinson, 1955, 1957; Kersters *et al*, 1984) although *B. bronchiseptica* can use the organic acids lactate and citrate as alternative carbon and energy sources (Proom, 1955). This also is the case with *B. avium* (Kersters *et al* 1984). Proom (1955) noticed that alpha-ketoglutaric acid could replace glutamic acid as the primary nutritional factor, even in the case of *B. pertussis*, if cystine was also added. *B. pertussis* appears to have an essential requirement for cystine, presumably as a sulphur source (Proom, 1955).

B. avium has been the subject of least investigation and to date little work on the nutritional requirements has been undertaken. Its requirements appear to be similar to those of *B. bronchiseptica* with which it shares a number of other traits (see Table 2). *B. avium* can utilize organic acids as a substitute for amino acids and in a simple amino acid mixture it used similar acids to those of *B. bronchiseptica*. For information about *B. avium* see Kersters *et al* (1984).

Part 3: Respiratory tract and its secretions

Anatomy of the respiratory tract

For purposes of this thesis, the respiratory tract may be regarded as a system of branching tubes that fuse with the main airway and whose surface supports the growth of the bordetellae.

The trachea is a thin-walled tube composed of connective tissue strengthened at intervals by incomplete, C-shaped rings of cartilage which prevent collapse. It branches into the two main bronchi, whose structure is similar to that of the trachea and which is retained through the many successive branchings within the lungs, to the alveoli. The bronchi are supplied with blood by bronchial arteries and some of the deoxygenated blood is drained by bronchial veins. The lining of the tracheobronchial system protects the lungs from dehydration and invasion by foreign particles, including microorganisms.

The lungs develop at the ends of the bronchi, each lobe being served by an internal bronchus. The functional lungs are elastic spongy organs, consisting of several lobes, that occupy the thoracic cavity. Each is covered with a membrane, the pulmonary pleura, which forms a continuous sac, enclosing the lung and continuing as the parietal pleura of the chest cavity. The potential space between the pleurae is the pleural cavity and each lung is enclosed in a separate pleural cavity. The function of the lungs is gas exchange.

Respiratory tract fluids.

Mucus and respiratory tract secretions arise from two sites, the submucosal glands and epithelial surface (goblet) cells. The collection and the composition of these secretions will be the main focus of attention for this section as it will be from the secretions that the bordetellae derive their nutrients during infection. Although there are reviews on respiratory

tract fluids (Yeager ,1971, Boat and Cheng, 1980, and Richardson 1988) none contains information on potential *Bordetella* nutrients.

Methods of Collection. Attempts to collect respiratory tract fluids date back to 1882 when Rossbank used blotting paper to dry the exposed tracheal mucous membrane of cats and dogs during his research into mucus replenishment (cited by Boyd, 1954). Table 11 summarizes early work on respiratory tract fluids.

Many of the early experiments used anesthetized animals with an indwelling tracheotomy tube, the system being brought to physiological temperature and humidity i.e. Boyd technique (Boyd, 1954). Another method, of use in short-term studies only, involved severing the trachea completely and fixing the transected ends inside a plastic cylinder, the secretions being collected from the cylinder (Hilding, 1966). Figure 4 shows a few devices used for the collection of respiratory tract fluids. The " tracheal pouch " technique was described in dogs (Wardell, *et al* 1969). With this method a 5-6 cm segment of trachea was isolated by transections above and below, the isolated segment having an intact blood and nerve supply. The two ends of the remaining airway were then anastomosed and healed satisfactorily. The proximal end of the isolated tracheal segment was then brought to the exterior and sutured to subcutaneous tissue in the neck. The distal end of the segment was ligated. The system remained sterile and the mucosa appeared normal by light microscopy. Repeated collection of secretions were made over a period of months with the dogs unanesthetized.

Most studies on human tracheobronchial secretions have been carried out on expectorated sputum from patients with chronic bronchitis, making the secretions abnormal from the beginning (see review by Yeager, 1971).

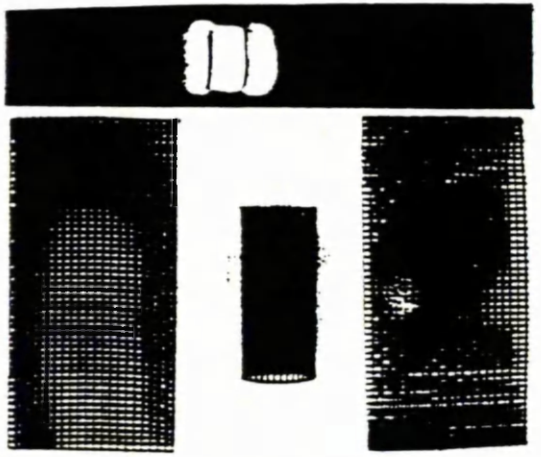
Bronchoscopy was a common method which eliminated the

Table 11 : Early research with respiratory tract fluids.

Method	Reference
Drying exposed trachea mucus membrane of dogs and cats with blotting paper.	Rossback, 1882[a] Calvert, 1896[a]
Collection in calcium chloride tubes attached to a tracheal cannula.	Henderson and Taylor, 1910
Postural drainage and bronchoscopic suction.	Jackson and Jackson, 1934
Tracheal cannula with a glass tube attatched.	Perry and Boyd, 1941

[a] cited by Boyd, 1954

Figure 4 : Collection devices for respiratory tract fluids. (Reproduced from Hilding, 1966 and Adams *et al*, 1976, with permission)



Devices used for collection of tracheobronchial secretions. A soft cotton and plastic absorbent airway is shown at the top. Below are shown collecting screens. Screen at the left is shown before placement, the middle screen is shown rolled prior to introduction, and the screen at the right shows secretions accumulated during a collection period.

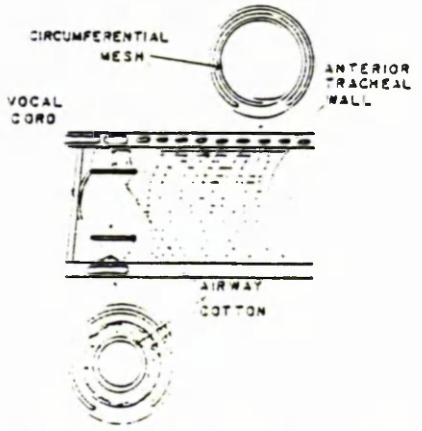
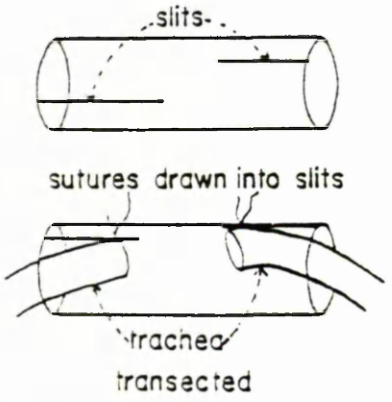
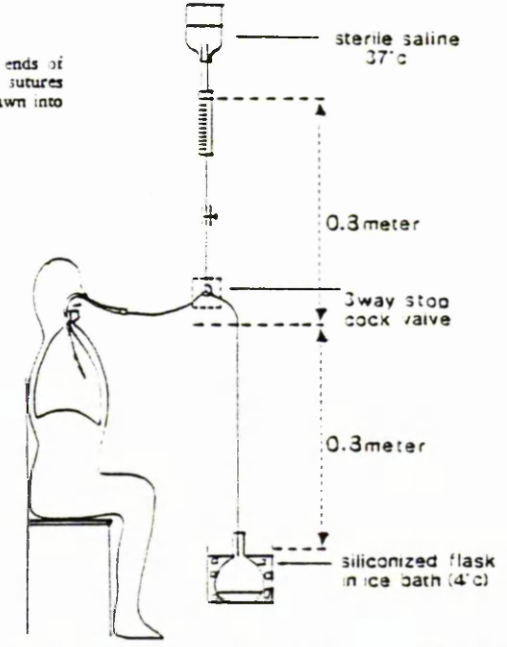


Diagram of placement of secretion collection devices in the dog trachea. Circumferential mesh of screen and absorbent cotton-covered airway are placed distal to the vocal cords. Rolled screen expands to gently contact the tracheal epithelial surface. Secretory material not collected in the interstices of the mesh is absorbed by the cotton. Upper cross-sectional diagram shows the relationship between the mesh screen and the tracheal wall. Lower cross section shows the absorbent airway consisting of a layer of cotton attached to the outside of a soft plastic tube.



Method of fixing the transected ends of the trachea within a plastic cylinder. The sutures attached to the tracheal ends are simply drawn into slits cut in the side of the cylinder.



Bronchoalveolar lavage with gravity-dependent flow. The subject may be seated or recumbent. With the fiberoptic bronchoscope wedged in a subsegment, a 100-ml aliquot of saline is run into the lung by gravity flow, then allowed to drain out into the dependent flask; the infusion is repeated three times. This is in contrast to the separate infusion and aspiration method, performed with a syringe attached to the bronchoscope.

problem of contamination with oral secretions. It was usually necessary to irrigate with a saline solution to obtain the secretions even through the bronchoscope because of the small amount of material recoverable. An ingenious suction trap device connected to a warmed, humidified tracheotomy tube has been described for the collection of human tracheobronchial secretions (Toremalm, 1960). Keimowitz (1964) described another method. Immediately after induction of anesthesia (Halothane or nitrous oxide) a catheter was passed through the endotracheal tube and approximately 10 ml of isotonic saline was instilled then suctioned into a Luken's collecting tube (about 1 ml was recovered).

During his investigations into the chemical composition of secretions from patients with cystic fibrosis, bronchiestasis and laryngectomy (Matthews *et al*, 1963), secretions were expectorated onto gauze squares and the gauze was then pressed together to absorb the saliva. The saliva-free secretions from patients with cystic fibrosis and bronchiectasis were then transferred directly into plastic vials. The "normal" secretions were aspirated directly from their tracheostomies into plastic vials. All samples were kept at 5°C during the collection period and then stored at -70°C prior to analysis.

Adams *et al* (1976) collected airway secretions from dogs on a plastic-coated glass fiber screen. This device can be seen in Figure 4. In studies on bronchoalveolar lavage fluids from smokers and non-smokers, Low *et al* (1978) employed transnasal fiberoptic bronchoscopy with pulmonary lavage. Four 60 ml boluses of sterile 0.9 % saline solution were instilled and immediately withdrawn through the channel of a 5.2 mm fiberoptic bronchoscope gently wedged into a subsegmental bronchus of the right middle lobe or lingula. Transtracheal aspiration was used in only the most urgent cases as fatalities have occured (Pratter and Irwin, 1979). Oropharyngeal contamination was eliminated with this method. It involved

insertion of a large-bore needle through the cricoid membrane extending only a few millimetres into the trachea. A catheter was then passed via the needle into the trachea and the needle removed. Specimens were obtained by suction with a 20-30 cc syringe.

Open lung biopsy has been used in compromised hosts where differential diagnosis includes infective and noninfective diseases. This method allows sampling of different anatomical sites and collection of larger specimens than less invasive biopsy proceedures (Yeager, 1971).

In most cases the processing of the secretions was similar; centrifugation was followed by decantation of supernates which were split into aliquots and stored frozen (-20°C or -70°C) if not used for immediate analysis (Yeager, 1971, Low *et al*, 1978, Boat and Cheng, 1980).

Composition.

In the text that follows a distinction will be made, where possible, between *secretions* and *washings*. Respiratory tract secretions (RTS) are taken to mean the undiluted fluid collected from the respiratory tract and tracheobronchial washings (TBW) to indicate that some kind of lavage procedure was used. Where it is not necessary to make this distinction the term respiratory tract fluid (RTF) will be employed.

Reviews on the composition of these washings and secretions include those by Ramirez, *et al* (1971), Yeager (1971) and Boat and Cheng (1980).

General Composition. The RTS from humans contained approximately 95% water and 5% solids (Matthews *et al*, 1963). Some of the water was free but much of it was either bound at one of several levels of affinity to macromolecular components or was trapped within the interstices of a gel matrix, formed by polymerization and aggregation of the mucous glycoproteins and other macromolecular components. The free water and soluble components formed an aqueous layer, the periciliary

fluid, bathing the cilia (Lucas and Douglas, 1934). The mucous gel overlay this layer. Recovery of the periciliary fluid alone for analysis was not possible, but, the aqueous fraction obtained upon centrifugation was thought to be similar in composition to the periciliary fluid (Boat and Cheng, 1980).

The solid content of human RTS included 2-3 % proteins (4 % in healthy dogs; Reasor, *et al* 1978) and glycoproteins, 1 % lipids and 1 % minerals (Matthews *et al*, 1963). The fluids obtained from the trachea were somewhat hypertonic with an osmolarity of 359 ± 56 mmole/1000 g (Boat and Cheng, 1980). The water in the RTS contained inorganic salts, proteins and glycoproteins (Richardson and Phipps, 1981).

With regard to the cellular content, degenerating macrophages were most abundantly recovered from RTF (94.8 ± 2.8%) followed by lymphocytes (3.5 ± 2%) and polymorphonuclear leukocytes (1.7 ±1.6%) (Low *et al*, 1978). These figures were taken for lavage fluids and they may therefore be greater in the whole undiluted secretions.

Inorganic Components. In 1954, Boyd reported chloride levels of 5.2 ± 2.6 mg/ml in rabbit, cat and dog RTS. He also reported a potassium level of 0.76 mg/ml in the cat RTS.

Two studies which concerned themselves with the inorganic analysis of human RTS were those by Matthews *et al* (1963) and Potter *et al* (1967). Their results can be seen in Table 12. Matthews *et al* (1963) investigated the overall chemical composition of pulmonary secretions while Potter *et al* (1967) studied the ionic enviroment of secretions from patients with cystic fibrosis, bronchiectasis and laryngectomy ("normal").

Sodium and chloride concentrations were high in " normal " secretions compared with potassium and calcium which occurred at much lower concentrations. Up to 15 % of the monovalent ions and 30 % of calcium were not removed by dialysis, suggesting that considerable ion-

Table 12 : Inorganic composition[a] of human and animal (stated) respiratory tract secretions.

Reference	Inorganic component (mean ± SD)
Boyd (1954)	Cl = 5.2 ± 2.6 (rabbit, cat and dog) K = 0.76 (cat)
Matthews *et al*, (1963)	Ca = 0.062 ± 0.02 P = 0.27 ± 0.16 Na = 1.65 ± 0.42 Cl = 1.62 ± 0.6 K = 0.132 ± 0.054
Potter *et al* (1967)	Na = 211.1 ± 33.8 mM/L K = 16.6 ± 3.4 mM/L Ca = 2.45 ± 1.11 mM/L Cl = 156.7 ± 24.6 mM/L

[a] mg/ml unless stated

-binding to macromolecular components existed (Potter *et al*, 1967).

Proteins. Several studies have reported the protein content of human and animal RTF (see Table 13). Matthews *et al* (1963) estimated that human secretions contained 10.0 ± 3 mg/ml (mean ± SD) protein. In 1978, Low and his co-workers measured the protein concentration of "normal" human TBW from healthy volunteers at 0.068 ± 0.029 mg/ml (mean ± SD), while Davis *et al*(1982) reported that human TBW contained 0.114 ± 0.005 mg/ml of protein. The differences between these workers' findings and those of Matthews *et al* (1963) could be accounted for by the dilution effect during sample collection as previously described.

In animal RTS the protein concentration is variable. Adamson *et al* (1969) reported a protein concentration of 0.3 mg/ml in the alveolar liquid from the foetal lamb. An earlier study by Boyd *et al* (1944) reported protein measurements in rabbits, cats and a cockerel of 1.04 ± 0.21 mg/ml, 1.36 ± 0.37 mg/ml and 2.6 ± 0.4 mg/ml respectively (all mean ± SEM).

The proteins in human and animal respiratory tract fluids include, as well as specific secretory products of airway cells, serum proteins which may be added by transudation from the vascular spaces of the lung. Albumin is the major serum component in the fluids, reported to be present in human TBW at a concentration of 1.42 ± 0.2 mg/ml (Reynolds and Newball, 1974) and 17.4 ± 9.0 µg/ml (Low *et al* 1978), and other serum proteins identified include the immunoglobulins, alpha-1-antitrypsin, alpha-1-antichymotrypsin, antithrombin III, alpha-2-macroglobulin, transferrin, prealbumin, alpha-1-acid glycoprotein, fibrinogen, ceruloplasmin, hemopexin, haptoglobin, several complement components and others (review by Boat and Cheng, 1980). Table 14 shows the proteins secreted by airway cells from humans, some of whom were in an immunocompromised state.

Masson and Heremans (1973) found that lysozyme and lactoferrin,

Table 13 : Protein concentrations reported for human and animal (stated) respiratory tract fluids .

Reference	Protein concentration (mg/ml) (mean ± SEM unless otherwise stated)
Boyd *et al* (1944)[a]	1.04 ± 0.21 (rabbit)
	1.36 ± 0.37 (cat)
	2.6 ± 0.4 (cockerel)
Matthews *et al* (1963)[a]	10.0 ± 3 (mean ± SD)
Adamson *et al* (1969)[a]	0.3 (foetal lamb)
Low *et al* (1978)[b]	0.068 ± 0.029 (mean ± SD)
Davis *et al* (1982)[b]	0.114 ± 0.005 (mean ± SD)

[a] refers to respiratory tract secretions

[b] refers to tracheobronchial washings

Table 14 : Proteins in respiratory tract secretions. (modified from Boat and Cheng, 1980)

Protein	Cell source	Conc. in bronchitic secretions (mg/ml)	Reference
Mucous glycoproteins	Goblet cells Mucous glands	5-10 (estimated)	Matthews *et al.* 1963.
Lysozyme	Serous glands	0.16-0.7	Brogan *et al.* 1975.
Lactoferrin	Serous glands	0.05-0.05	Brogan *et al.* 1975.
Kallikrein	Epithelium	—	Masson & Heremans 1973.
Proline-rich proteins	?	—	Bailleul *et al.* 1977
Antiproteases	Surface epithelium and glands	—	Tegner & Ohlsson, 1977.
Secretory component	Surface epithelium Mucous glands	—	Reynolds & Newball, 1974.
IgA	Plasma cells	0.4-0.2	
IgG	Plasma cells	0.05-0.21	Kaltreider, 1976.
IgM	Plasma cells	—	

which have antimicrobial properties, were secreted by submucosal glands at high concentrations. Several antiproteases appeared to be secreted by airway tissues and these inhibited trypsin, chymotrypsin and leukocyte proteases (Boat and Cheng, 1980). The majority of antiprotease activity in airways was contributed by lung-specific antiproteases while the remainder was derived by transudation of serum antiproteases. Proline-rich polypeptides have been detected in both bronchial secretions (Bailleul *et al*, 1977) and saliva (Carlson *et al*, 1970), and these may be structural components of secretory granules released during the secretory process (Boat and Cheng, 1980).

The glycoproteins, secreted by mucous cells of submucosal glands and by goblet cells of the surface epithelium, were largely responsible for the gelation of airways secretions. There has been much investigation into the glycoproteins but here it will suffice to say that they are indeed present in the secretions and have a composition similar to that of other mammalian epithelial glycoproteins, and that they give mucus its characteristic properties of viscosity and elasticity (Yeager *et al*, 1971 and Reasor *et al* 1978).

Immune system components. Specific antibodies associated with the external fluids have been identified and characterized, supporting the hypothesis that mucous membranes may have their own immune system (Yeager, 1971).

Early studies by Keimowitz (1964) and Masson *et al* (1965) detected immunoglobulins in human RTF. Keimowitz (1964) reported the presence of β_{2A}-globulin and gamma-globulin while Masson *et al* (1965) identified IgA and IgG.

Reynolds and Newball (1974) reported the presence of three immunoglobulins in human TBW (Table 15). They measured IgA concentrations of 0.91 ± 0.11 mg/ml, IgG concentrations of 0.17 ± 0.005

Table 15 : Immunoglobulins detected in human and animal (stated) respiratory tract fluids.

Reference	Immunoglobulin content (mean ± SD)
Reynolds and Newball (1974)	IgA = 0.91 ± 0.11 mg/ml
	IgG = 0.17 ± 0.05 mg/ml
	IgE = 73.2 ± 8.2 ng/ml
Reasor *et al* (1978)	IgG and IgM detected (dog)

mg/ml and and IgE concentration of 73.2 ± 8.2 ng/ml. Reasor *et al* (1978) showed the presence of IgG and IgM (not detected in human washings; Reynolds and Newball, 1974) in dog TBW and by immunodiffusion IgA was also detected. Together with albumin, Reasor *et al* (1978) found that IgG made up about 70 % of the total protein in the TBW supernates. IgG has been reported as the predominant immunoglobulin in dog pulmonary washings (Kaltreider and Chan, 1976).

Carbohydrates. Carbohydrates made up from 20-50% of the nondialyzable material in airway fluids (Yeager, 1971). In 1963 Matthews *et al* measured the concentration of carbohydrate in human RTS at a level of 9.51 ± 2.18 mg/ml (mean ± SD). Other workers confirmed the presence of carbohydrates in human washings (see Table 16). Potter *et al* (1967) reported a carbohydrate concentration of 19.3 ± 9.4 mM/ml (mean ± SD), while Low *et al* (1978) reported a concentration of 0.008 ± 0.004 mg/ml (mean ± SD). A similar concentration to that reported by Low *et al* (1978), in human TBW of 0.01 ± 0.002 mg/ml (mean ± SEM) was reported by Davis *et al*, in 1982. Again the differences between concentrations recorded by Potter *et al* (1967) and the latter two workers were probably due to the dilution effect encountered by Low, and Davis, during collection of the fluids.

In the study by Potter *et al* (1963), a description of the carbohydrate content of whole pulmonary secretions was given (Table 17). The " normal " secretions were taken from laryngectomized patients and the monosaccharides found were N-acetyl hexosamines (N-acetyl glucosamine and N-acetyl galactosamine), galactose, manose, fucose and sialic acids (Yeager, 1971). No uronic acids were detected, hence no acid mucopolysaccharides were present (Schultz and Heremans, 1966).

Lipids. Human tracheobronchial washings contain relatively large amounts of lipid, up to 40 % of the dried, insoluble material (Sahu and

Table 16 : Carbohydrate concentration[a] in human respiratory tract fluids.

Reference	Carbohydrate concentration (mg/ml unless stated)
Matthews *et al* (1963)[b]	9.51 ± 2.18
Potter *et al* (1967)[b]	19.3 ± 9.4 mM/ml
Low *et al* (1978)[c]	0.008 ± 0.004
Davis *et al* (1982)[c]	0.011 ± 0.002 (mean ± SEM)

[a] mean + SD unless otherwise stated

[b] refers to respiratory tract secretions

[c] refers to tracheobronchial washings

Table 17 : Carbohydrate content of pulmonary secretions from "normal" patients. (information from *Potter et al*, 1963).

% of total carbohydrate			
Fucose	Sialic acid	Hexosamine	Hexose
21.0 ± 3.8	20.7 ± 2.8 .	31.2 ± 2.3	25.5 ± 3.8

Lynn,1977). A number of studies have yielded different results for the concentration of lipids and phospholipids in human and animal TBW (Table 18). Again the dilution effect may explain this. In 1954, Boyd reported the lipid contents of three animal RTS. He found that while cat and rabbit RTS had similar lipid contents of 0.22 mg/ml and 0.28 mg/ml respectively the concentration of lipids in dog RTS was higher (0.85 mg/ml).

Matthews *et al* (1963), in his work with human RTS found a total lipid concentration of 8.4 ± 2.7 mg/ml (mean ± SD) with Potter *et al* (1967) reporting a concentration of 19.3 ± 9.4 mM/L (mean ± SD). Low *et al* (1978) measured the lipid concentration of human TBW in three parts. Non-polar lipid was present at a concentration of 0.0778 ± 0.0768 mg/ml, polar lipid at 0.0441 ± 0.0415 mg/ml and phospholipid at a concentration of 0.0011 ± 0.0003 mg/ml (all mean ± SD). The lipid in human washings measured by Davis *et al* in 1982 was similar to that reported by Low *et al* (1978) i.e. 0.167 ± 0.0219 mg/ml (mean ± SEM), but less than the reports for undiluted RTS.

Table 19 shows the phospholipid analysis of human TBW from healthy volunteers in Low's study (1978). Analysis of phosphatidylcholine, which is the lung surfactant responsible for surface tension in the lung to prevent collapse (Morgan, 1971), revealed large amounts of saturated fatty acids (72.5± 5.8% palmitic acid, 16:0). Other fatty acids present included stearic (18:0), oleic (18:1), linoleic (18:2), palmitoleic (16:1) and myristic (14:0) (Low *et al*, 1978). The presence of fatty acids in RTS has been reported elsewhere (Boyd, 1954) for other species i.e. cat, rabbit and dog.

Amino Acids. Only one report has been found which refered to the free amino acid concentration of human RTS. In a study by Potter *et al* (1967) the amino acid concentration of "normal" human RTS was reported to be 31.8 ± 16.6 mM/L.

Other reports refered to non-protein nitrogen concentrations (Boyd,

Table 18 : Lipid/phospholipid concentrations[a] in human and animal (stated) respiratory tract fluids.

Reference	Lipid/phospholipid concentration (mg/ml unless otherwise stated)
Boyd (1954)[b]	0.22 (cat)
	0.28 (rabbit)
	0.85 (dog)
Matthews *et al* (1963)[b]	8.4 ± 2.7
Potter *et al* (1967)[b]	19.3 ± 9.4 mM/L
Low *et al* (1978)[c]	0.0778 ± 0.00768 (non-polar lipid)
	0.0441 ± 0.0415 (polar lipid)
	0.00109 ± 0.00033 (phospholipid)
Davis *et al* (1982)[c]	0.167 ± 0.0219 (lipid; mean ± SEM)
	0.00109 ± 0.00016 (phospholipid; mean ± SEM)

[a] mean + SD unless stated

[b] refers to respiratory tract secretions

[c] refers to tracheobronchial washings

Table 19 : Phospholipid content of pulmonary washings from " normal " patients. (information from *Low et al* 1978)

Phospholipid	% of total lipid
Phosphatidylcholine	83.8 ± 7.5
Phosphatidylglycerol	12.3 ± 4.6
Diphosphatidylglycerol	1.1 ± 1.1
Phosphatidylinositol	1.2 ± 1.9
Phosphatidylethanolamine	0.3 ± 0.5
Phosphatidic acid	0.4 ± 0.5
Phosphatidylserine	0.7 ± 0.8

1954 and Matthews *et al*, 1963). Boyd's reported concentrations of non-protein nitrogen in rabbits, cats and dogs of 0.63 ± 0.25 mg/ml, 0.35 ± 0.35 mg/ml and 0.3 ± 0.3 mg/ml respectively (all mean ± SD). Matthews *et al* (1963) reported non-protein nitrogen levels of about 0.5 mg/ml in "normal" human RTS.

An interesting point of note was the change in the composition of the secretions with the disease status of the host. In studies with patients with cystic fibrosis, bronchiectasis and laryngectomy it was found that as the severity of the disease increased so did the content of organic constituents, such as DNA, protein, lipid, and carbohydrate, with a corresponding decrease in the inorganic content i.e sodium, calcium, potassium, etc, (Potter *et al*, 1967). It has also been reported that the amino acid content of washings increases with the severity of infection (Potter *et al* 1967). Table 20 shows the relationship of solids, amino acids Na, Cl, and Ca in tracheobronchial secretions of various diseases. (Potter *et al*, 1967 and Chernick and Barbero, 1963).

Table 20 : Relationship of solids, Na, Cl, Ca and amino acids in respiratory tract fluids in various diseases.

(modified from Chernick & Barbero, 1963)

	% solids	% Na and Cl	% Ca	% Amino acids
Respiratory infections (e.g. common cold)	↓	↑	↑	↓
Tuberculosis				
Bronchiectasis				
Cystic Fibrosis				

The arrows indicate increasing (downwards) or decreasing (upwards) concentrations of constituents in respiratory-tract fluids in different disease states.

OBJECT OF RESEARCH

Previous studies on the *in vitro* growth of the bordetellae have focused almost exclusively on the isolation and cultivation of the bacteria in complex or defined media. Hitherto there has been no study of the possible growth of the organisms in the fluids of the respiratory tract of host species.

The objects of this investigation were therefore :

a) to determine whether each of the four species of *Bordetella* is capable of *in vitro* growth in tracheobronchial washings (TBW) from a variety of host and non-host species.

b) Whether there is any evidence of host/parasite growth specificity which might account for the host preferences of the bordetellae.

During this work there emerged the unexpected observation that *Bordetella bronchiseptica* could grow in non-nutrient control fluids such as phosphate buffered saline and distilled water. This lead to a study of the growth and survival of this species in lakewater and seawater.

MATERIALS AND METHODS

Part 1. Bacterial cultivation, preparation and standardization.

Organisms, strains and storage

A total of nine strains of bordetella was used in this investigation, comprising one strain each of *B. pertussis*, *B. parapertussis* and *B. avium* and six strains of *B. bronchiseptica* (Table 21). Some of the strains such as *B. bronchiseptica* no. 5376 had been in long-term laboratory culture while others (*B. bronchiseptica* strain no. 13325) were recent isolates.

Other organisms used included *Escherichia coli* strain no. JM 83, *Lactobacillus plantarum*, both from the departmental culture collection and *Pasturella haemolytica* strains S/C 82/1 and W/D 83/4, provided by Mr. Qurban Ali.

For preservation of cultures, a turbid bacterial suspension was prepared with growth from a Bordet-Gengou (BG) agar plate in 2 ml, 1 % (w/v) casamino acids (Gibco), and 300 µl of this suspension was added to a solution of 2.1 ml casamino acids and 0.6ml glycerol and stored at -70°C in 0.3 ml portions. *E. coli* JM 83 was grown on nutrient agar (Oxoid) and frozen suspensions were prepared in a similar manner. The two strains of *P. haemolytica* were stored at -70°C in a 50 %(v/v) glycerol/ brain heart infusion broth (BHIB) mixture, while *L. plantarum* was stored in a refrigerator (4°C) on stab cultures of Micro Assay Culture Agar (Difco).

Reagent-grade water (RGW)

RGW was produced from tap water by serial passage through 1) a pre-filter (Sparkler Filters GB/LTD); 2) a Milli-RO^{TM} 4/15 Laboratory Grade Water System, comprising a Rogard prefilter cartridge followed by a reverse osmosis cartridge; and 3) a Milli-Q^{TM} Reagent-Grade Water System (Millipore), consisting of a carbon cartridge and two ion-exchange cartridges. The final conductivity was not more than 0.2 µS/cm.

Table 21 : Organisms and strains used in this research.

Species	Strain	Obtained from	Comments
B. pertussis	18334	Connaught Lab. Ltd. Toronto	A vaccine strain
B. parapertussis	10520	Dr. R. Parton[a]	NCTC strain
B. bronchiseptica	5376	Dr. R. Parton	As used by Eldering (1962)
	452	NCTC	Type strain
	10541	NCTC	As used by Lacey (1952)
	6353	Dr. D. J. Taylor[b]	Isolated from a dog (1985) with severe respiratory disease
	13325	Dr. D. J. Taylor	Isolated from a horse (1990) with chronic respiratory disease
B. bronchiseptica	11	Dr. D. J. Taylor	Isolated from a pig (1986) used in pathogenicity studies
B. avium	P4091	Dr. R. Parton	Dr Rimler, USA

Table 21 (continued)

Escherichia coli	JM 83	Dr. R. Parton	no information
Lactobacillus plantarum		Dr. R. Parton	Standard strain for analysis of nicotinic acid.
Pasturella haemolytica	S/C 82/1	Glasgow University Veterinary School	From bovine lungs field case of pneumonic Pasturellosis.
Pasturella haemolytica	W/D 83/4	Glasgow University Veterinary School	Healthy animal Bovine Nasopharyngeal swab.

[a] Glasgow University. Microbiology Department Culture Collection.

[b] Glasgow University Veterinary School

Single-distilled water (DW)

DW of conductivity not more than 1.0 µS/cm was collected from a Fistreem^{TM} Cyclon (Fisons) distillation apparatus.

Fresh lake and pond water

Samples were obtained from a garden pond, a reservoir and various lakes in the Glasgow area (Ordnance Survey map coordinates; pH): garden pond (OS 64, 514715; pH 7.6), Milngavie Reservoir (OS 64, 556765; pH 7.1), Loch Achray (OS 57, 513064; pH 7.0), Loch Ard (OS 57, 475020; pH 7.1), Loch Katrine (OS 57, 495074; pH 7.2), Loch Lomond (OS 59; pH 7.1) and Lake of Menteith (OS 57, 588005; pH 6.9). In addition, sea water was obtained from the pier of the University Marine Biological Station Millport, Isle of Cumbrae (OS 63, 176545; pH 8.4). All samples were sterilized by filtration through a 0.45 µm membrane filter (Gelman Sciences), the initial portion of filtrate being discarded, and stored frozen at -20°C.

Phosphate-buffered saline (PBS)

PBS was prepared with analytical grade chemicals, by dissolving 7.99 g NaCl, 1.196 g Na_2HPO_4, 0.212 g KH_2PO_4 (all BDH Ltd) and 0.199 g KCl (Koch-Light Laboratories Ltd), in DW or RGW to a final volume of 1.0 litre. By removing small portions of the PBS and testing the pH after the addition of alkali or acid the pH was adjusted to 7.2. The solution was sterilized in a pressure cooker (121°C for 15 min) containing DW or RGW as the source of steam.

Bordet-Gengou (BG) medium.

BG medium consisted of 40 g BG agar base (Gibco Europe) with 1 % (v/v) glycerol, dissolved at 100°C in DW to a final volume of 1 litre. It was adjusted to pH 7.6 and sterilized at 121°C for 15 min. After cooling the base to 50°C, defibrinated horse blood (Becton Dickinson) was added to give 15-20 % (v/v) and 15 ml portions poured into 50 mm deep-vented petri

dishes (BDH Ltd.). The plates were stored at +4°C in sealed plastic bags and discarded after three weeks if not used.

Cyclodextrin liquid (CL) medium.

CL medium was prepared as described by Imaizumi *et al* (1983) by dissolving 10.7 g sodium l-glutamate, 2.5 g NaCl, 0.5 g KH_2PO_4, 0.1 g $MgCl_2.6H_2O$, 0.02 g $CaCl_2$ (all from BDH Ltd), 0.2 g KCl (Hopkin and Williams), 6.25 g Tris (Boehringer Mannheim GmbH), 10 g Casamino Acids (Gibco), 0.24 g L-proline (Sigma) and 1.00 g cyclodextrin (a gift from J. Shimizu, Teijin Ltd., Tokyo, Japan) in DW to a final volume of 1 litre at pH 7.4. It was sterilized in a pressure cooker at 121°C for 15 min. A supplement consisting of 0.01 g $FeSO_4$ (BDH Ltd), 0.04 g L-cysteine, 0.004 g niacin, 0.15 g glutathione and 0.4 g ascorbic acid (all from Sigma) in 10 ml DW, was sterilized by passage through a 0.45 µm membrane filter (Gelman Sciences) and added to the autoclaved components, immediately before use, in volumes of 0.1 ml per 10 ml of medium. The supplement was stored at -20°C and discarded after two months.

Preparation and standardization of inoculum

Deep-frozen suspensions (-70°C) of bacteria were plated onto BG medium and incubated for 24h at 37°C. The purity of the grown cultures was checked by Gram staining, after which the growth was scraped from the BG medium, suspended in 3 ml PBS and the optical density at 540 nm adjusted with PBS to 0.45 in a 1 cm cuvette. The cell suspension (1 ml) was then washed three times in PBS by centrifuging at 10,500 rpm for 15 min in a Haemicrofuge (Wifug Laboratory Centrifuges) at room temperature, the supernate removed and the pellet resuspended in 1.0 ml of PBS. During the centrifugations the temperature in the fluid typically rose to 36°C. The thrice-washed suspension was diluted in PBS to give an inoculating

suspension of 1900 ± 850 (SEM) CFU/ml. Figure 5 shows the dilution scheme for each of the four bordetellae.

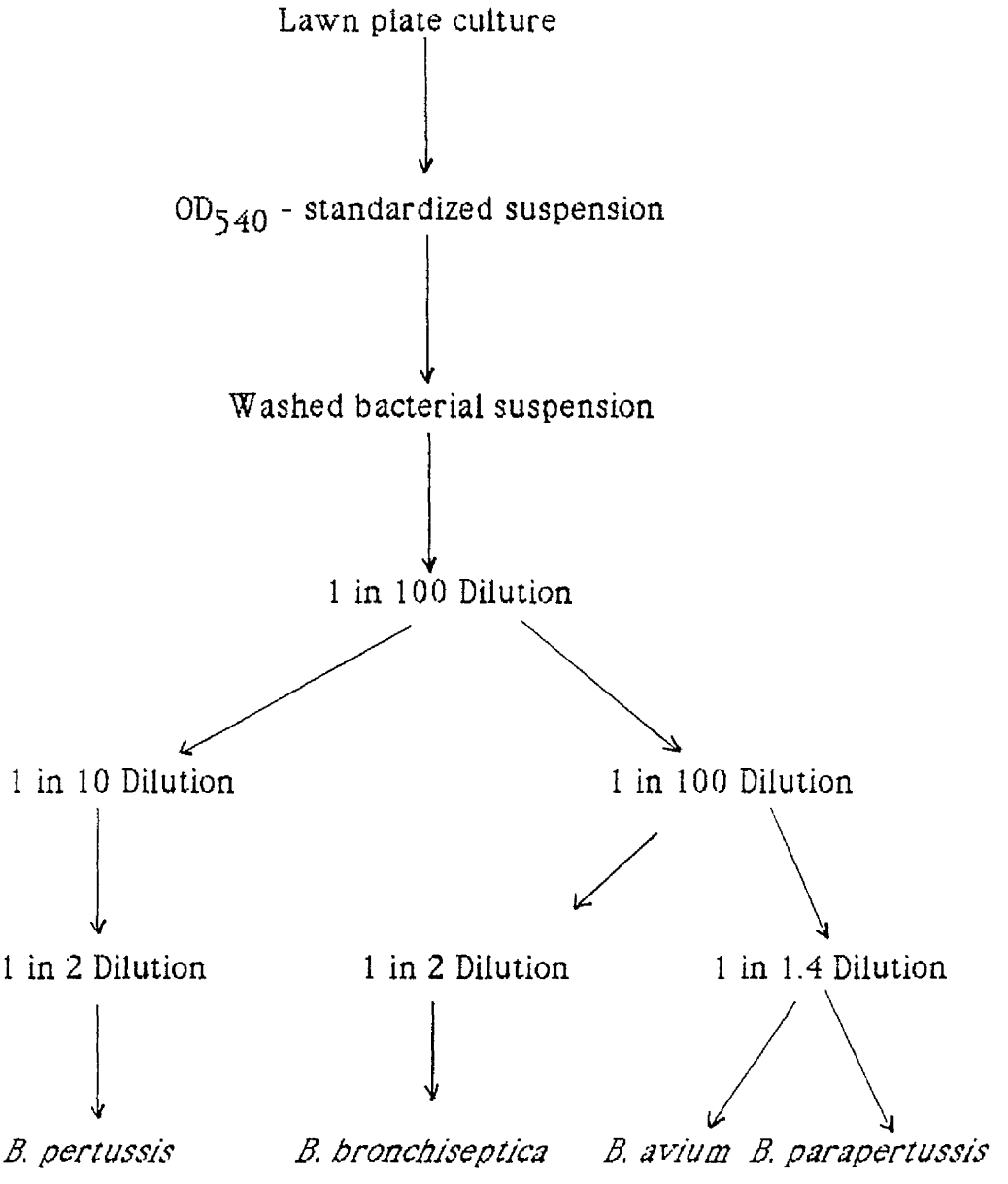
Special precautions

Glassware in some experiments was cleaned by overnight immersion in concentrated nitric acid, after which it was rinsed five times with DW and soaked for a further 24h in DW. A final rinse in DW was given and the complete removal of acid was verified by showing that the rinsings had the same pH as DW straight from the still. Plastic screw caps for the bottles were boiled for 15 min in DW, placed in a covered beaker and allowed to dry overnight at 37°C. Sterile plastic 30 ml universal containers and 7 ml bijou bottles were obtained from BDH Ltd. Plastic pipette tips were boiled for 5 min in RGW and sterilized in a pressure cooker (121°C for 15 min) containing RGW. Triflex vinyl examination gloves (Baxter) were worn when handling the tips before autoclaving.

Growth model for experimentation

Fluids (975 µl) to be tested for growth-supporting ability were placed in sterile 7 ml plastic bijou bottles (Sterilin) and inoculated with 25 µl of washed bacterial suspension containing approximately 1900 ± 850 (SEM) CFU/ml. A sample of 20 µl was taken immediately after inoculation and plated on BG medium to give the zero-time viable count. The bottles were then incubated at 37°C on a Mk IIIB shaker (LH Engineering Co. Ltd) rotating at 110 rpm. To monitor the death, survival and/or growth of *Bordetella* species at 37°C, samples were removed from the inoculated fluids at 0, 24 and 48h, and at later times up to 2-3 wk in some experiments, and plated (20 µl) on to BG medium. Based on preliminary experiments, the samples were plated either undiluted or after a number of 10-fold dilutions in CL medium. The BG plates were incubated at 37°C for 48h, or longer with *B. pertussis*, when colonies were counted.

Figure 5 : Dilution scheme for the inoculum preparation of the four species of *Bordetella.*


Lawn plate culture
OD_{540} - standardized suspension
Washed bacterial suspension
1 in 100 Dilution
1 in 10 Dilution
1 in 100 Dilution
1 in 2 Dilution
1 in 2 Dilution
1 in 1.4 Dilution
B. pertussis
B. bronchiseptica
B. avium
B. parapertussis

Dry weight determination of *B. bronchiseptica* strain no. 5376.

The confluent growth from four small (50 mm) BG plates was scraped into 10 ml of PBS to form a thick bacterial suspension of *B. bronchiseptica* strain no. 5376. A 1 ml sample of the suspension was diluted to an OD_{540} of 0.45 with PBS (as described above) and the OD of the undiluted suspension was thus calculated. Two 1 ml aliquots were placed in tared microcentrifuge tubes and cell pellets obtained after centrifugation at 10, 500 rpm for 15 min in a Haemicrofuge (Wifug Laboratory Centrifuges) at room temperature and decantation of the supernate. Two empty microcentrifuge tubes were weighed as zero-sample reference controls.

All four tubes were placed in a dessiccator containing P_2O_5 (BDH) and dried to constant weight at room temperature, over a period of one week. The dry weight of the cell pellet was taken as the average weight in the two tubes, after subtracting the weight of the empty tubes and accounting for any weight change in those tubes. The number of bacteria present in a cell suspension of OD_{540} = 0.45 was calculated from the initial viable counts of previous growth experiments and this was used to give the viable count for the original thick bacterial suspension. From this figure the weight of one CFU of *B. bronchiseptica* was calculated by dividing the weight of the cell pellet by the number of bacteria present in the pellet.

Long-term survival of *B. bronchiseptica* in PBS and fresh water

B. bronchiseptica strains NCTC 452 (type strain), 6353 (from dog) and No. 11 (from pig) were incubated and sampled over a period of six months at different temperatures in an attempt to assess the longevity of the bacteria in PBS and fresh water. The preparation of the inoculum and experimental system were as previously described. Each strain was inoculated into 18 bottles of PBS and 18 bottles of membrane-filtered fresh

water collected from Loch Lomond. Incubation was at 37°C shaken (nine bottles) and 10°C static (nine bottles) and the caps of the bottles were screwed tight. Some of the bottles were weighed before inoculation and all were weighed after inoculation in order to monitor, and correct for, evaporation over the incubation period. Two empty bottles (blanks) were also weighed and incubated (one at each temperature) to detect weight changes in the bottles themselves (e.g. due to variable atmospheric pressure). Two uninoculated controls of each fluid were included, one incubated at 37°C and the other at 10°C. With strain No. 11 only 10 bottles of each fluid were set up (five at each temperature) although all other conditions were as described for the other two strains.

Weighings were taken throughout the incubation period. Bottles 7, 8 and 9 (strain Nos. 452 and 6353), 4 and 5 (strain No. 11) and the blanks were weighed after 1 week and thereafter at the end of each month. Any weight loss as a result of evaporation was corrected by the addition of sterile distilled water.

Samples from one of the other bottles were taken at 1 week, 1 month, three months and six months and plated onto BG agar and incubated at 37°C until growth appeared. Undiluted samples and serial 10-fold dilutions were taken to obtain a viable count at each interval.

After approximately three months, one bottle (number1) from each set of conditions was sacrificed for an investigation of wall growth. The contents were discarded into a disposal jar, the remaining fluid being removed with a pipette. A fresh 975 µl portion of sterile PBS was added to the bottles and, after vigorous vortexing, an undiluted 20 µl sample was plated onto BG agar and incubated for 24-48h at 37°C until growth appeared. If no growth had appeared after 4-5 days incubation, the plates were discarded.

Part 2. Collection of tracheobronchial washings (TBW)

Mouse TBW

Adult mice were used to collect the TBW. Each mouse was killed in a glass jar containing CO_2 and pinned out on its back on a board. The fur and skin were removed from the throat using a scalpel and folded back. An incision was made to expose the trachea and a hole pierced in the trachea with dissection scissors. A canula, of diameter approximately 1 mm, with a 2 ml syringe attatched to the end was inserted into the trachea and tied in with fine thread and 1.5 ml of PBS passed into the lungs. After 10-15 seconds the fluid was sucked out and transferred to a 30 ml plastic universal. The TBW collected from 20-30 mice on the same day was pooled, except that any with visible blood contamination (pinkish tinge) was discarded.

The washings were spun in a laboratory bench centrifuge (MSE minor 'S') at 10,000rpm for 15 min. The supernate was then filter-sterilized through a 0.45 µm filter (Gelman Science) into 1.2 ml aliquots dispensed into microcentrifuge tubes. The tubes were stored at -20°C as were the pellets from the centrifugations.

Horse TBW

Approximately 93 ml of TBW was obtained by Professor A.C. Wardlaw at Glasgow University Veterinary School from a 16-year old Arab stallion with a spinal weakness which had had to be put down. The fluid was centrifuged at 10,000 rpm for 15 min. Some of the supernate was stored in bulk before sterilization. The remainder was filter-sterilized (as with mouse TBW) into 3 x 20 ml portions, in 30 ml sterile plastic universals, and in 1.2 ml aliquots in microcentrifuge tubes and stored at -20°C.

Rabbit TBW

The collection of rabbit TBW was similar to that for mouse TBW. The only differences were that the rabbits were killed by an overdose of Sagittal (1-1.5 ml). The canula used was larger (diameter of 2-3 mm) and 40 ml of PBS was used to irrigate the lungs. Centrifugation, sterilization and storage were as before.

Sheep TBW

TBW was provided by Glasgow University Veterinary School. The lungs from freshly slaughtered normal sheep were removed and rinsed with approximately 100 ml of PBS, the first rinsings being separated from rinses 2 and 3. The PBS was poured into the sheep trachea and removed after 10-15 seconds. Centrifugation, sterilization and storage of the TBW and cell pellets were as before.

Chicken TBW

Chickens were provided by The Royal Hospital for Sick Children (Glasgow). Two year old black-spotted chickens used for blood harvest were used for lung washing. The procedure was similar to that used in the collection of mouse TBW and approximately 20-30 ml of fluid was obtained. Centrifugation, sterilization and storage of the TBW and cell pellets were as before.

Dog TBW

TBW was obtained from a 14 kg mongrel bitch at the Glasgow University Veterinary School. The dog was premedicated with acetylpromazine. Anaesthesia was induced with 5 %(w/v) sodium thiopentone and maintained with halothane/nitrous oxide/oxygen during a 4h operation. The dog was killed with an injection of barbiturate. The trachea had previously been intubated with a leak-proof cannula and approximately 200 ml of sterile PBS was poured in within 1-2 minutes of

death. This was then recovered by tilting the operating table and letting the fluid run out under gravity. Processing of the TBW was as previously described.

Human bronchial lavage specimens

A total of six human specimens were provided by Dr. Neil Thompson and Dr. Hulk of Glasgow Western Infirmary. These samples were taken from elderly male and female patients (by bronchial lavage with sterile saline) who had some form of non-infectious lung or bronchial disorder. Only three of the six samples were used in this study

The pH of all the TBW was measured with Johnson's test paper and found, in all cases to be approximately 7.

Part 3. Biochemical methods

Blood contamination

During the collection of the TBW samples it was normal for some blood contamination to occur. Since this could lead to additional nutrients being supplied by the blood, it was necessary to measure the amount of blood in the sample.

The assay employed was a modification of the Sigma kit, procedure number 525, used for the quantitative, colourimetric determination of whole blood at 530-550 nm. The reaction involves the conversion of all of the haemoglobin present in the sample to cyanmethaemoglobin by addition of a mixture of potassiun ferricyanide and potassium cyanide (Drabkin reagent). This compound is red in colour and can be measured spectrophotometrically at 540 nm.

Drabkin solution was prepared by reconstituting Drabkin reagent (Sigma Catalogue No. 525-2) with 1000 ml distilled water. To this was added 0.5 ml of 30 % Brij-35 solution (Sigma Catalogue No. 430AG-6) and the resultant solution was mixed well and stored in an amber bottle at room temperature. The haemoglobin standards were dilutions of whole mouse blood . The fresh blood was added, immediately upon collection, into PBS to give a 2% (v/v) solution and stored frozen until used.

The test was performed as follows. Two or more test tubes were labelled BLANK, TEST 1, 2, 3, etc and 2.0 ml of Drabkin solution was added to each. The TBW samples (0.5 ml) were added to the tubes labelled TEST, mixed and allowed to stand for at least 15 min at room temperature (18-26°C). The BLANK contained 2.0 ml of Drabkin solution plus 0.5 ml of PBS. The absorbance at 540 nm of TEST vs BLANK was recorded for each TBW and the concentration of haemoglobin in the sample calculated from

the calibration curve. The standards for the calibration curve were dilutions of whole mouse blood at 1 %, 0.5 %, 0.25 % and 0.125 % (v/v). These were treated in the same way as the samples. The haemoglobin contents of the unknown samples were interpolated on to the standard curve and expressed as mean ± SEM % (v/v) mouse blood.

Amino acids

The amino acid content of TBW was measured by a modification of the ninhydrin method of Moore and Stein (1948) applied after the precipitation of the proteins of TBW with sulphosalicylic acid.

The precipitation of proteins from the TBW was performed as follows; 500 µl of 4 %(w/v) 5-sulphosalicylic acid (AnalaR) was added to 500 µl of the TBW in a microcentrifuge tube, and left at 4°C for at least 4h. The mixture was then centrifuged in a Hemicrofuge (WIFUG Laboratory Centrifuges) at 10,500 rpm for 10 min after which the supernate was decanted and then used for analysis.

A citrate solution was prepared with 4.3 g citric acid (M&B) plus 8.7 g sodium citrate (BDH) in 250 ml of distilled water and adjusted to pH 5.0 with HCl or NaOH. To this, 400 mg of stannous chloride (BDH) was added immediately before use. Gentle heating in a water bath at 40°C was required to dissolve fully the stannous chloride. A 50 % (v/v) iso-propanol solution (Koch-Light Laboratories Ltd) was prepared (500 ml). A ninhydrin solution, consisting of 1 g ninhydrin (Sigma) dissolved in 25 ml methoxyethanol (BDH), was prepared immediately before use and added to the stannous chloride solution in a 1:1 ratio (colour development reagent). Boiling tubes (14 x 150 mm) were used for the reaction mixtures which consisted of 100 µl of sample or standard made up to 0.5 ml with distilled water plus 1.5 ml of the colour development reagent. The mixture was boiled for 5 min in a water bath, the tubes removed and allowed to cool,

before 8 ml of the propanol solution was added to each tube. The contents were mixed on a vortex and left at room temperature. The absorbance was read at 570 nm after 30 min on a SP6 550 UV/Visible spectrophotometer (Pye Unicam). A calibration curve was constructed using L-leucine (Sigma) as the standard amino acid dissolved in PBS. The concentration of the standards were 0.25 mM, 0.5 mM, 1 mM and 2 mM leucine. A water blank was treated in the same way as the standards and samples as a comparison. The amino acid content of the unknown samples was calculated by interpolation from the curve. The mean and the SEM were calculated from a number of observations on the same TBW sample.

Nicotinic acid (NA)

A microbiological assay was used to determine the concentration of nicotinic acid in the various TBW samples tested. The test organism was *Lactobacillus plantarum* which, like the bordetellae, has an essential requirement for NA. The extent of growth of the bacterium in the culture medium was in direct correlation with the concentration of NA present. During growth, *L. plantarum* produced lactic acid which was titrated with NaOH, the volume of which required for neutralisation was directly proportional to the growth of the bacterium and hence to the NA concentration added to the medium.

An initial inoculum of *L. plantarum* was made from a stab stock culture on Micro Assay Culture Agar (Difco). After activation at 30°C for 24h a loopful was transferred to 10 ml of Micro Inoculum Broth (Difco) and incubated at 30°C for 24h or until a silky turbidity developed. The cell suspension was then pelleted at 9,000 rpm in a MSE minor 'S' bench centrifuge and washed twice in 0.85 % (w/v) sterile saline to avoid the possibility of nutrient carryover from the broth. Niacin Assay Medium

(Difco) was prepared (200 ml) and 5 ml of this was transferred to 15 ml test tubes. A stock NA solution (100 µg/ml) was prepared with 0.05 mg of NA (niacin, Sigma) dissolved in 500 ml of distilled water. This was diluted to 0.1µg/ml by adding 0.1 ml stock solution to 99.9 ml distilled water, and added to the test tubes to give 0, 0.05, 0.1, 0.2, and 0.4 µg/ml NA (i.e. 0, 0.5, 1, 2 and 4 ml respectively). TBW samples (0.5 ml) were added to the sample tubes and all tubes were made up to a final volume of 10 ml with distilled water.

The assay medium was sterilized at 121°C for 10 min and cooled. Each test tube had 50 µl of *L. plantarum* inoculum added and was incubated at 30°C for around 24h. Once turbidity had developed in the media the tubes were removed. The contents of each tube was transferred to a 100 ml conical flask and 50 ml of distilled water and 30 µl of bromothymol blue indicator added. Sodium hydroxide (0.1 M) was used to titrate the acid produced by the *L. plantarum* in the medium. The volume of NaOH required for neutralization was equivalent to the acid produced, which in turn, was directly related to the concentration of NA in the medium.

Total protein determination

The method used was the Lowry protein estimation (Lowry *et al*, 1951). A series of bovine serum albumin (BSA, Sigma) standards were prepared in boiling tubes in concentrations ranging from 50 µg/ml to 50 µg/ml. The samples tested in duplicate, were prepared undiluted and at a 1 in10 dilution in distilled water. To 0.5 ml of sample and the standard BSA solutions and 0.5 ml water (blanks), 0.5 ml of 1N NaOH was added and the tubes were then placed in a boiling water bath for 5 min.

The experimental protocol was as follows; 50 ml of 5 % (w/v) Na_2CO_3 plus 2 ml of 0.5 % (w/v) $CuSO_4$ $5H_2O$ in 1 % (w/v) sodium potassium tartarate (all BDH) were prepared immediately before use. When the tubes

from the water bath had cooled, 2.5 ml of this reagent was added, mixed and the tubes were allowed to stand for 10 min. To each of the tubes 0.5 ml of 1N Folin reagent (Folin-Ciocalteaus phenol reagent diluted 1:1 in distilled water) was added and mixed. After 30 min at room temperature the absorbance at 750 nm in a cuvette of path length 1 cm was read in a Shimadzu UV 240 spectrophotometer. The protein concentrations in the unknown samples were calculated and presented in the read-out from the spectrophotometer.

Part 4. Characterization of *B. bronchiseptica* strains

Cultural characteristics

A 25 µl sample from each of six *B. bronchiseptica* strains, which had been stored as previously described, was spread onto two BG agar plates to give single colonies after incubation at 37°C for 24-48h. Purity of the resultant plate culture was determined by colony morphology and Gram stain.

A single colony from each plate was suspended in about 0.3 ml of PBS and mixed vigorously. A 10 µl sample was taken for Gram stain by the method described in Mackie and McCartney (1989). A further 10 µl sample was taken for motility testing by the "hanging drop" method (Mackie and McCartney, 1989).

All six strains of *B. bronchiseptica* tested Gram negative and were actively motile.

API tests

The six strains of *B. bronchiseptica* used in this research were tested for their reactions on an API 20NE identification strip (API Laboratory Products Ltd).

A saline suspension was prepared from the 24-48h growth, on BG agar, of each *B. bronchiseptica* strain : A single colony from each BG plate was suspended in an ampoule of NaCl (0.85 %) and mixed vigorously.

An API strip for each strain was placed in an incubation box, and about 5 ml of distilled water was added to the box, to create a humid enviroment. The tubes, but not the cupules, of the tests NO_3 to PNPG (see Appendix 1a) were inoculated with the saline suspension of the corresponding *B. bronchiseptica* strain. Sterile mineral oil was then added to the cupules of the tests GLU, ADH and URE.

To an ampoule of AUX medium, 200 µl of the remaining saline suspensions was added and mixed, avoiding excess bubble formation. The tubes and cupules of tests GLU through to PAC were filled with the corresponding AUX suspension. The incubation boxes containing the API strips were closed and incubated for 24h at 30°C.

After incubation the results were recorded as either positive or negative, based upon the test interpretation table (Appendix 1a). Each strain was then allocated an identification number which was cross-checked with the identification table in Appendix 1b (this table listed results for 66 different non-fermentative, Gram negative bacteria).

Filamentous haemagglutinin (FHA)

The six strains (20-30 µl of a thawed deep-freeze culture) of *B. bronchiseptica* were grown on BG plates for 24-48h at 37°C, and the resultant growth was scraped into 3 ml of PBS. The optical density at 450 nm was adjusted to 1.9-2.0 with PBS.

A 5 ml volume of horse red blood cells (Becton-Gibson) was washed three times in PBS by centrifugation in a bench centrifuge (MSE minor 'S') at 3,500 rpm for 10 min. A 2 % (v/v) solution of the cells, in PBS, was prepared by adding 1 ml of washed cells to 49 ml of PBS.

A 50 µl sample of bacterial suspension was added to the first well of a round-bottomed microtitre plate (Gibco). To the successive wells was added 50 µl of PBS. Serial 2-fold dilutions of the bacterial suspension were made in the PBS and then 50 µl of the washed erythrocytes was added to all wells. A semi-purified preparation of FHA from *B. pertussis* (provided by Miss Farhat Mirza), and PBS, were each mixed (50 µl) with the red blood cells and used as positive and negative controls respectively.

The microtitre plate was incubated at 37°C for 1-2h after which the results were recorded. A positive agglutination was indicated by the

non-formation of a red button of erythrocytes at the bottom of the well.

Heat Labile Toxin (HLT)

The six strains of *B. bronchiseptica* were assayed (by Professor A.C. Wardalw) for heat labile toxin (HLT). Each strain was plated onto four BG plates and incubated for 24h at 37°C. The confluent growth was scraped into sterile plastic universals (Sterilin) and stored frozen overnight. After thawing, the bacterial pellets were resuspended in PBS to give 30 mg/ml and kept on ice (see Table 22).

The resuspended pellets were sonicated on ice in 5 x 1 min bursts with 1 min. cooling in between. For testing, the preparations were diluted to 10 mg/ml. Dilutions of the adjusted bacterial suspensions (4-fold) were made over the range 1/4 - 1/64 and a control heated at 56°C for 30 min to inactivate any HLT activity was also included. As a positive control a crude preparation of HLT from *B. pertussis* strain no. 77/18319 (provided by Professor A.C. Wardlaw) was diluted to give 10 mg/ml and then treated in the same manner as the test samples.

Two mice were tested at each dose point in a mouse toxicity test and the animals were monitored for adverse reactions over a period of five days.

Table 22 : Preparation of *B. bronchiseptica* suspensions for use in HLT analysis.

Strain number	Moist weight (mg)	PBS for 30 mg/ml (ml)
5376	338	11.3
452	383	12.6
10541	292	9.7
11	283	9.4
13325	287	9.6
6353	368	12.3

RESULTS

Part 1 : Characterization of stock *B. bronchiseptica* strains.

It is convenient to introduce the results of these investigations by describing the six strains of *B. bronchiseptica* with which much of the work was done. In fact, this characterization was performed mid-way through the study, after the surprizing observation had been made that *B. bronchiseptica* strain 5376 could grow in PBS without nutrient supplementation.

All six strains were small Gram-negative coccobacilli. Hanging drop preparations of colonies from BG plates suspended in PBS showed convincing motility with each of them. Biochemical tests with API 20 NE strips gave the results summarized in Table 23 (see also Appendix 1a), which include the API identification numbers. It will be noted that all six strains were positive for urease and oxidase and for assimilation of adipate, malate, citrate and phenyl-acetate. All six strains were negative for indole production, acidification of glucose, arginine dehydrolase, ß-glucosidase, protease, ß-galactosidase and assimilation of glucose, arabinose, mannose, mannitol, N-acetylglucosamine, maltose and gluconate. Differences between strains were observed with nitrate reduction and caprate assimilation.

According to the API scheme, the interpretation code (last line within Table 23) for typical *B. bronchiseptica* is 11 000 23. This was exhibited by strain nos. 5376, 6353, 11 and 10541. Strain No. 13325, a recent isolate from a horse with respiratory disease, differed in one test (caprate assimilation) and had the code 11 000 33. The type strain 452, which had been isolated in 1920, differed in two tests, caprate assimilation and nitrate reduction and had the code 01 000 33. All six strains fell within the limits of variability for typical and atypical *B.bronchiseptica* as defined by API tests (Appendix 1b).

Additional confirmation of identity was sought by assaying the

Table 23 : API identification of *B. bronchiseptica* strains

Test	Strain no. (API 20 NE code)					
	5376	6353	11	10541	13325	452
NO_3 TRP GLU	+ - -	+ - -	+ - -	+ - -	+ - -	- - -
ADH URE ESC	- + -	- + -	- + -	- + -	- + -	- + -
GEL PNPG GLU	- - -	- - -	- - -	- - -	- - -	- - -
ARA MNE MAN	- - -	- - -	- - -	- - -	- - -	- - -
NAG MAL GNT	- - -	- - -	- - -	- - -	- - -	- - -
CAP ADI MLT	- + +	- + +	- + +	- + +	+ + +	+ + +
CIT PAC OXI	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
Code	(11 000 23)	(11 000 23)	(11 000 23)	(11 000 23)	(11 000 33)	(01 000 33)

For description of tests see API 20 NE interpretation table (Appendix 1a)

11 000 23 is the API code for *B. bronchiseptica* (Appendix 1b)

11 000 33 and 01 000 33, from the API identification table, are atypical of *B. bronchiseptica* although they fall within the range of variability

strains for ability to produce filamentous haemagglutinin (FHA) and heat-labile toxin (HLT), both of which virulence factors are regarded as characteristic of this bordetella (Pittman and Wardlaw, 1981). As shown in Table 24 (see also Appendices 2 and 3), the six strains varied considerably in ability to produce these two factors. Four of the strains produced FHA in readily detectable amounts, whereas two strains, one of which was the type strain and the other a recent isolate, did not. However all six strains produced HLT although the amount varied over a 250-fold range.

The overall conclusion from these biochemical and other tests was that all six strains were confirmed as as *B. bronchiseptica*

Table 24 : Characteristics of *B. bronchiseptica* strains.

Strain	Investigator,or Source, and date	API 20 NE number	FHA activity[a]	HLT activity[b]
5376	Eldering, 1962	11 000 23[c]	1.6	6.2
6353	Dog, 1985	11 000 23	12.5	100
11	Pig, 1986	11 000 23	3.1	100
13325	Horse, 1990	11 000 33	< 0.1	0.4
10541	Lacey, 1952	11 000 23	0.8	12.5
452	Ferry, 1920	01 000 33	< 0.1	1.6

[a] expressed as a percentage of HA activity of a semi-purified preparation of FHA from *B. pertussis*

[b] expressed as a percentage of the activity of a semi-purified preparation of HLT from *B. pertussis*

[c] 11 000 23 is the number which is assigned to *B. bronchiseptica* strains.

See Appendices 2 and 3 for FHA and HLT data

Part 2 : Growth of *Bordetella* species in TBW

Development of the test method

The main initial object of this research was to determine whether the tracheobronchial washings (TBW) from various vertebrate species could support the growth of the four species of *Bordetella,* and if so, whether evidence to explain host-parasite species specificity might emerge.

Initial experiments involved establishing a suitable protocol for the growth experiments with the four species. A different dilution scheme had to be developed for each organism (Figure 5) in order to obtain the desired count of approximately 50 colonies from a 20 µl sample of inoculated TBW at time zero. Once established, the protocol was used, with minor variations, throughout the research.

The first fluid tested was mouse TBW, with cyclodextrin liquid (CL) medium and PBS as the positive and negative control fluids respectively. Initial results of the growth-supporting properties of these three fluids are presented in Table 25. These indicated that mouse TBW supported growth of *B. parapertussis, B. bronchiseptica* and *B. avium*, as shown by the confluent growth given by undiluted 20 µl samples taken after incubation for 48h at 37°C and plated onto BG medium. *B. pertussis* failed to give any colonies when tested in parallel, indicating greater than 98 % loss of viability in TBW. All four species grew in the CL medium used as a positive control. Surprisingly, *B. bronchiseptica* and *B. avium* grew also in PBS. However, the two human-pathogenic bordetellae failed to survive in this fluid.

Growth of the bordetellae was then studied with better quantitation and with various sampling times over one week. In addition, the samples were serially diluted before plating so as to yield countable number of colonies on at least some of the BG plates. Results of a typical

Table 25 : Qualitative observation of growth of *Bordetella* species during 48h at 37°C in CL medium, mouse TBW and PBS.

Fluid	Qualitative observation of growth of :			
	B. pertussis	*B. parapertussis*	*B. bronchiseptica*	*B. avium*
CL medium	++	++	++	++
Mouse TBW	-	++	++	++
PBS	-	-	++	++

++ and - refer respectively to confluent growth and no growth when a 20 µl sample was spread on a BG plate after the period of incubation.

With each organism, the inoculum contained an initial viable count such that 30-50 colonies developed from 20 µl of the inoculated fluids spread on BG plates.

experiment with *B. bronchiseptica* strain 5376, with sampling times of 48h and 1 wk are given in Table 26.

The Table shows that from an average inoculum of about 40 CFU/20 µl (equivalent to 2 x 10^3 CFU/ml) the viable counts increased to 3.6 x 10^9 CFU/ml in CL medium, to 5.7 x 10^6 in PBS, and to values between 8 x 10^7 and 1 x 10^9 CFU/ml in TBW from three mammalian species. It was therefore apparent that this strain of *B. bronchiseptica* was capable of extensive growth in TBW samples from horse, sheep and rabbit.

The sections that follow deal with the detailed examination of growth of the four species of bordetella on each of seven species of TBW. Figures 6 to 9 summarize the results which are recorded in detail in Appendices 4a to 7c.

B. bronchiseptica

Figure 6 shows the viable counts recorded over 1 week when *B. bronchiseptica* strain no. 5376 was incubated at 37°C in PBS, in CL medium and in TBW samples from man, horse, sheep, dog, mouse, rabbit and chicken. The viable counts were presented as mean $\log_{10}$ CFU/ml ± SEM, calculated from viable counts similar to those in Table 26.

The Figure shows that in most of the fluids, the growth of *B. bronchiseptica* reached its maximum value within two days, after which the viable count was generally stable for at least a further five days. In PBS, a 2500-fold increase in viable count was recorded at seven days, i.e. the initial $\log_{10}$ viable count of 3.3 ± 0.05 CFU/ml (N = 15) increased progressively and reached 6.7 ± 0.1 (N = 5) at the end of 1 week. This 2500-fold increase corresponded to about 11 cycles of cell division.

In CL medium, from the same initial $\log_{10}$ CFU/ml of 3.3 ± 0.04 (N = 21), the *B. bronchiseptica* reached a maximum $\log_{10}$ viable count of 9.4 ± 0.1 (N = 14) after 48h incubation at 37°C. This level was maintained for a

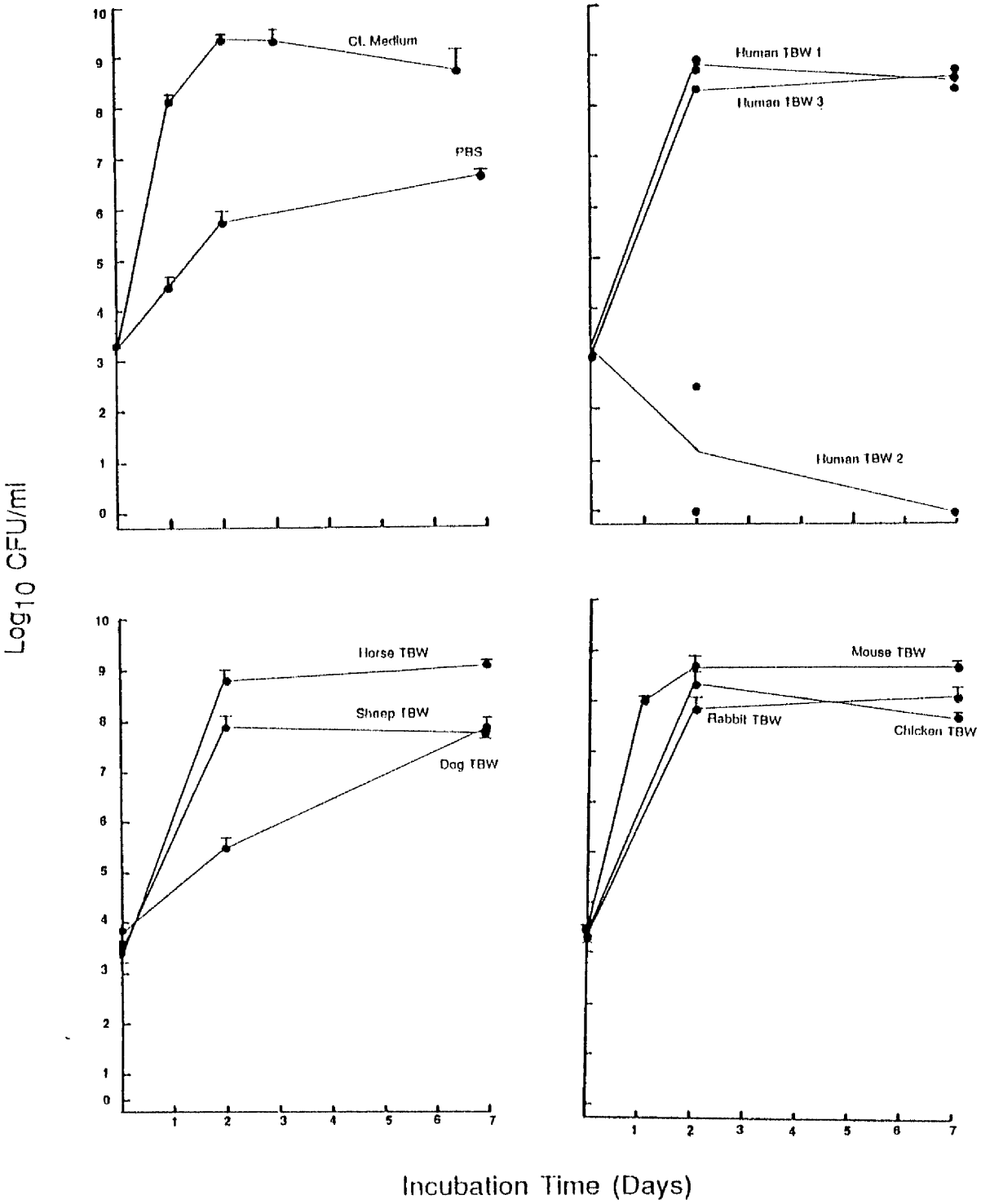
Table 26 : Typical experiment (Expt III 1) on the growth of *B. bronchiseptica* strain no. 5376 at 37°C in three species of TBW as compared to CL medium and PBS. The data are presented as actual colony counts at the different sample dilutions.

Fluid	No. of colonies from 20 µl at						CFU/ml	
	Zero time	48h (dilution)		7d (dilution)			48h	7d
	Undiluted	10^5	10^6	10^4	10^5	10^6		
CL medium	39[a]	±	44	+	±	73	2.2×10^9	3.6×10^9
		10^2	10^3	10^2	10^3			
PBS	43[a]	±	26	+	115		1.3×10^6	5.7×10^6
		10^4	10^5	10^2	10^3	10^4 10^5		
Horse TBW	39[a]	±	32	++	++	± 209	1.6×10^8	1×10^9
		10^4	10^5	10^2	10^3	10^4		
Sheep TBW	..	±	23	++	+	209	1.1×10^8	1.1×10^8
		10^4	10^5	10^2	10^3	10^4		
Rabbit TBW	..	±	12	++	+	159	6×10^7	8.0×10^7

[a] The average of these three values gives a zero time average count of 2×10^3 CFU/ml in the samples

.. signifies not tested; ++ indicates confluent growth; + indicates almost confluent growth and ± indicates too many colonies to count.

Figure 6 : ***B. bronchiseptica* growth in TBW : Tracheobronchial washings (TBW) from various vertebrate species were compared with CL medium and PBS.** *B. bronchiseptica* strain no. 5376 was incubated at 37° C and the error bars represent SEM. With the human TBW, three separate specimens were tested.


Log10 CFU/ml
Ct. Medium
PBS
Human TBW 1
Human TBW 3
Human TBW 2
Horse TBW
Sheep TBW
Dog TBW
Mouse TBW
Rabbit TBW
Chicken TBW
Incubation Time (Days)

further 24h, after which the count decreased slightly to 8.8 ± 0.4 (N = 5) at 1 week. The maximum increase in viable count corresponded to about 20 cycles of cell division.

Each of the species of TBW tested supported the growth of *B. bronchiseptica* over the 1 week incubation and gave final $\log_{10}$ viable counts within the 16-fold range of 7.8 ± 0.1 CFU/ml (sheep and chicken TBW) to 9.1 ± 0.1 CFU/ml (horse TBW). The counts with the other species of TBW were intermediate. With horse, mouse and two of the human TBW, these maximum counts were close to those recorded for CL medium and corresponded to about 18 cycles of cell division. Although dog TBW gave the same 7-day viable count as sheep and chicken TBW, the rate of growth was significantly less.

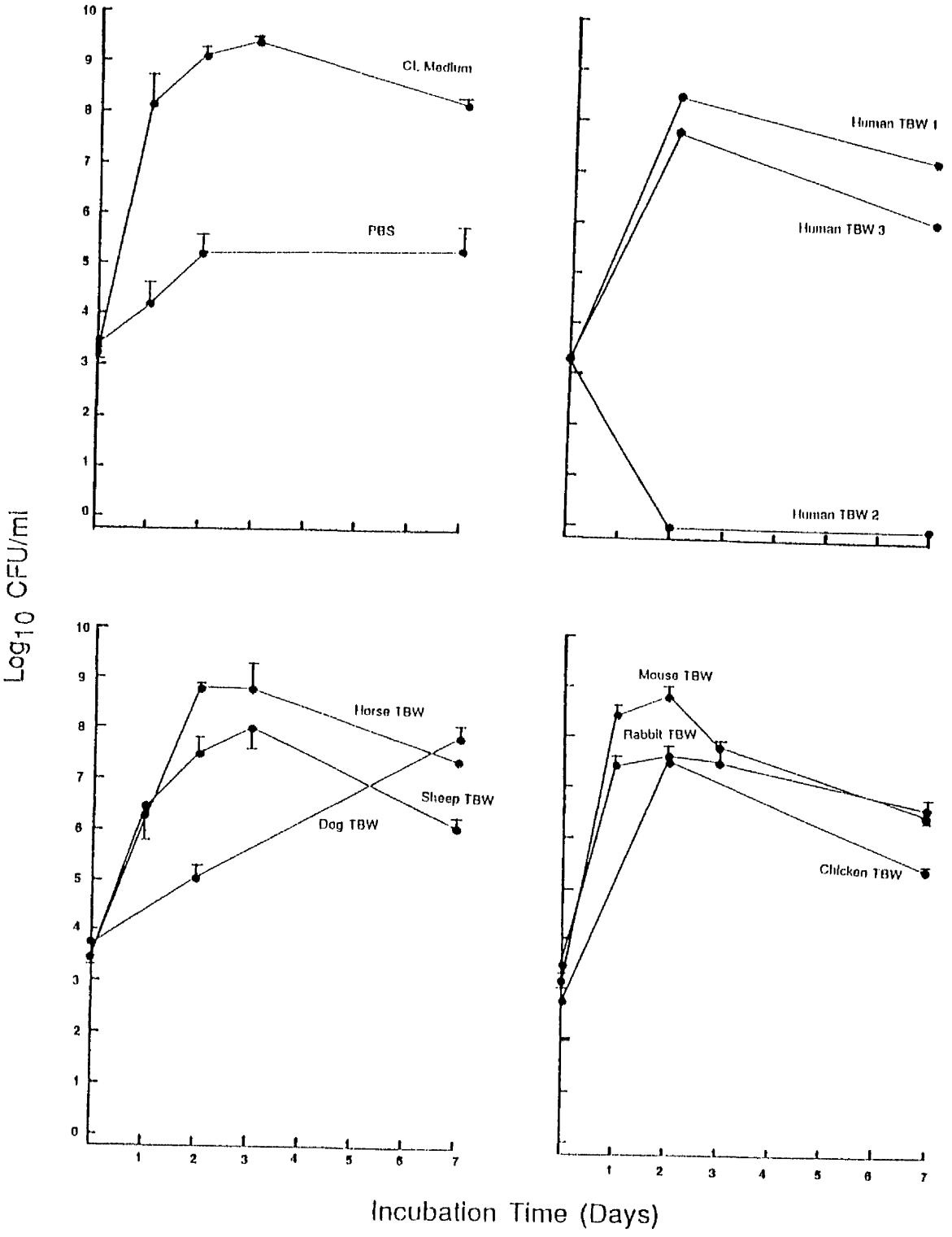
The majority of the TBW samples gave very similar growth patterns, with one exception. This was one of the human samples (TBW No. 2) which not only failed to support growth, but caused a decrease in viable count such that after incubation for 48h the culture appeared to be sterile.

B. avium.

B. avium strain P4091 gave results qualitatively similar to those obtained with *B. bronchiseptica* strain 5376 but with definite quantitative differences (Figure 7). Thus in PBS, there was only an 80-fold increase in viable count (N = 4) compared with the 2500-fold seen with *B. bronchiseptica.* In CL medium, the maximum $\log_{10}$ viable count was 9.4 ± 0.1 CFU/ml (N = 6) which was indistinguishable from that obtained with *B. bronchiseptica.*

In the various species of TBW the maximum viable counts attained by *B. avium* were quite similar to those of *B. bronchiseptica* except that there was no plateau between day 2 and day 7. Typically, the *B. avium* counts showed an average 33-fold decline over this interval. As with *B.*

Figure 7 : *B. avium* growth in TBW : Tracheobronchial washings (TBW) from various vertebrate species were compared with CL medium and PBS. *B. avium* strain no. P4091 was incubated at 37^{o} C and the error bars represent SEM. With the human TBW, three separate specimens were tested.


Log10 CFU/ml
Cl. Medium
PBS
Human TBW 1
Human TBW 3
Human TBW 2
Horse TBW
Sheep TBW
Dog TBW
Mouse TBW
Rabbit TBW
Chicken TBW
Incubation Time (Days)

bronchiseptica the dog TBW supported slower growth of *B. avium* than did the other species, although the final viable count was just as high, and human TBW no. 2 also failed to support the growth of the bacterium. With the exception of the human TBW each of which was only tested once, all of the other samples were examined with between three to seven observations. The detailed viable counts with the various fluids are recorded in Appendices 5a to 5e.

As with *B. bronchiseptica* the maximum viable counts in TBW from horse, mouse and human no. 1 were close to (within a factor of five) those obtained in CL medium. Sheep and chicken TBW gave least growth with both of the *Bordetella* species.

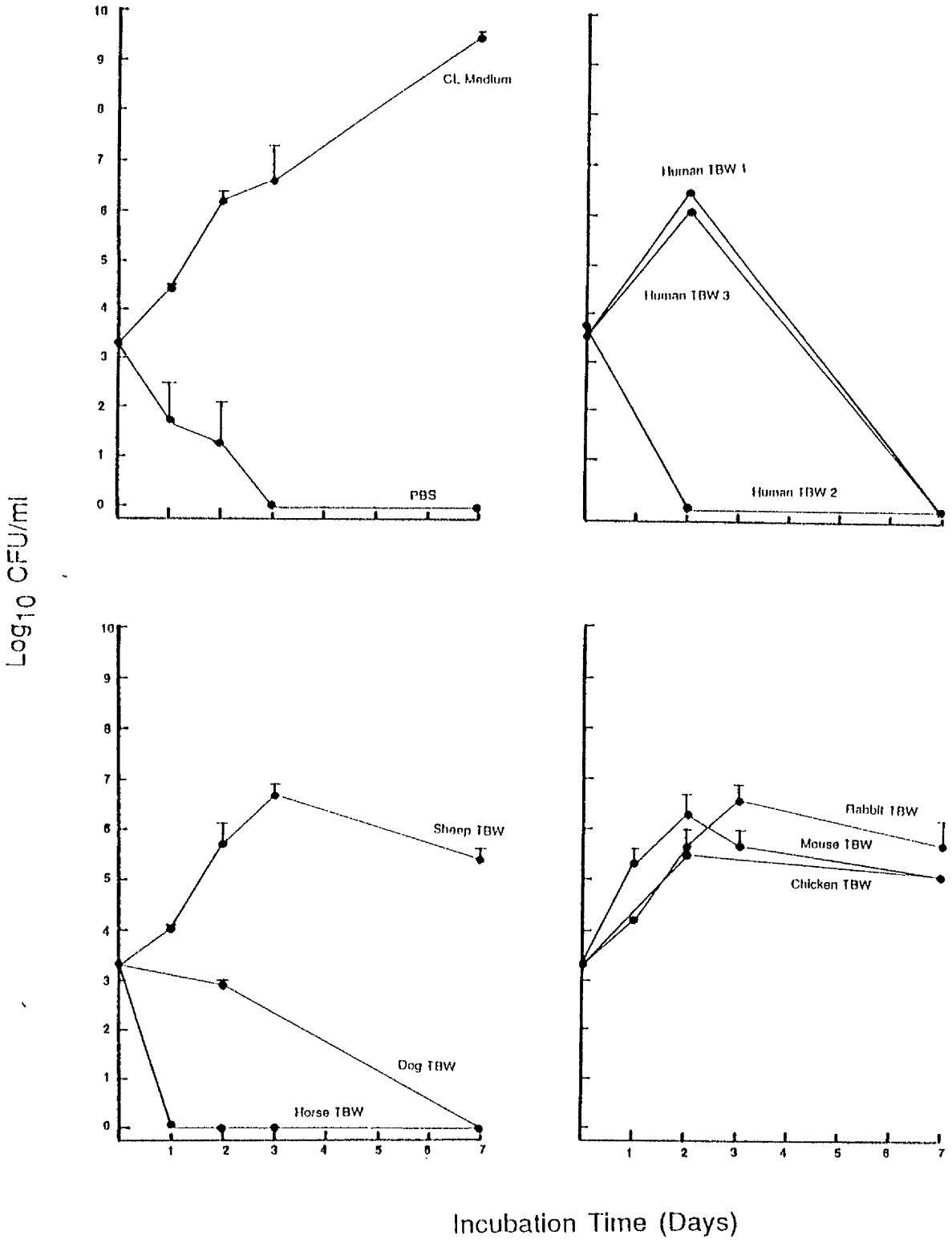
B. parapertussis

Figure 8 shows that the results with *B. parapertussis* strain no. 10520 were markedly different from those with the two species of *Bordetella* described above. In CL medium, growth was much slower, although at 1 week there was the same $\log_{10}$ viable count (9.5 ± 0.1; N = 3). With PBS in contrast, *B. parapertussis* showed no evidence of growth; the viable count had declined by 97 % at 24h and by 99 % at 48h (N = 3 and 4 respectively). By three days there were no viable cells detectable.

B. parapertussis showed four patterns of response in the various TBW samples but in each case the maximum viable count was substantially lower than with *B. bronchiseptica* or *B. avium.* The most common pattern with *B. parapertussis* was growth that reached a peak of $\log_{10}$ viable count between 5.5 and 6.7 around days 2-3 and then declined slightly. This was shown by TBW from sheep, rabbit, mouse and chicken. With the human samples 1 and 3, there was a similar peak at day 2 but thereafter the viable count decreased to undetectable numbers by day 7.

The dog and horse TBW which had supported good growth of *B.*

Figure 8 : ***B. parapertussis* growth in TBW : Tracheobronchial washings (TBW) from various vertebrate species were compared with CL medium and PBS.** *B. parapertussis* strain no. 10520 was incubated at 37° C and the error bars represent SEM. With the human TBW, three separate specimens were tested.


Log10 CFU/ml
CL Medium
PBS
Human TBW 1
Human TBW 3
Human TBW 2
Sheep TBW
Dog TBW
Horse TBW
Rabbit TBW
Mouse TBW
Chicken TBW
Incubation Time (Days)

bronchiseptica and *B. avium* were not growth-supporting for *B. parapertussis*; the viable count declined slowly but steadily in the dog TBW and very rapidly in that from the horse. The human TBW sample 2 which had been unable to support the growth of the other two species of *Bordetella* was similarily lethal for *B. parapertussis*. The detailed viable counts with the various fluids are recorded in Appendices 6a to 6e.

B. pertussis

B. pertussis strain no. 18334 failed to grow in any of the seven TBW tested (Figure 9 and Appendices 7a to 7c). PBS was also unable to support growth of this species. However in CL medium the viable count increased to 5.4 ± 0.2 (N = 6) in 48h from an initial $\log_{10}$ CFU/ml of 3.5 ± 0.2 (N = 5) and reached 10.0 ± 0.2 (N = 5) after 1 week at 37°C.

Although none of the TBW supported growth, there were differences in the time taken for the viable count to reach zero. In horse, dog, mouse, chicken and the three human TBW no culturable bacteria were recovered from a 48h sample, whereas with sheep and rabbit TBW and PBS the $\log_{10}$ CFU/ml values at 48h were 2.5 ± 0.2 (N = 2), 1.5 ± 1.5 (N = 2) and 0.7 ± 0.7 (N = 4) from initial values of 3.4 ± 0.5 (N = 2), 3.4 ± 0.5 (N = 2) and 3.5 ± 0.3 (N = 3) respectively. Thereafter no culturable bacteria were recovered from the 1 week sample.

Supplementation of rabbit TBW for the growth of *B. pertussis*

An experiment was done to investigate the effect on the growth of *B. pertussis* of supplementing rabbit TBW with the nutrients in CL medium and with MeßCD. The latter was added to the rabbit TBW to give 1 mg/ml, the same concentration as in CL medium. The results in Table 27 show that adding this ingredient to TBW did not permit growth, although it retarded slightly the loss of viability which *B. pertussis* underwent in rabbit TBW. A 1:1 mixture of CL medium and rabbit TBW supported more growth than CL

Figure 9 : *B. pertussis* growth in TBW : Tracheobronchial washings (TBW) from various vertebrate species were compared with CL medium and PBS. *B. pertussis* strain no. 18334 was incubated at 37^{o} C and the error bars represent SEM. With the human TBW, three separate specimens were tested.

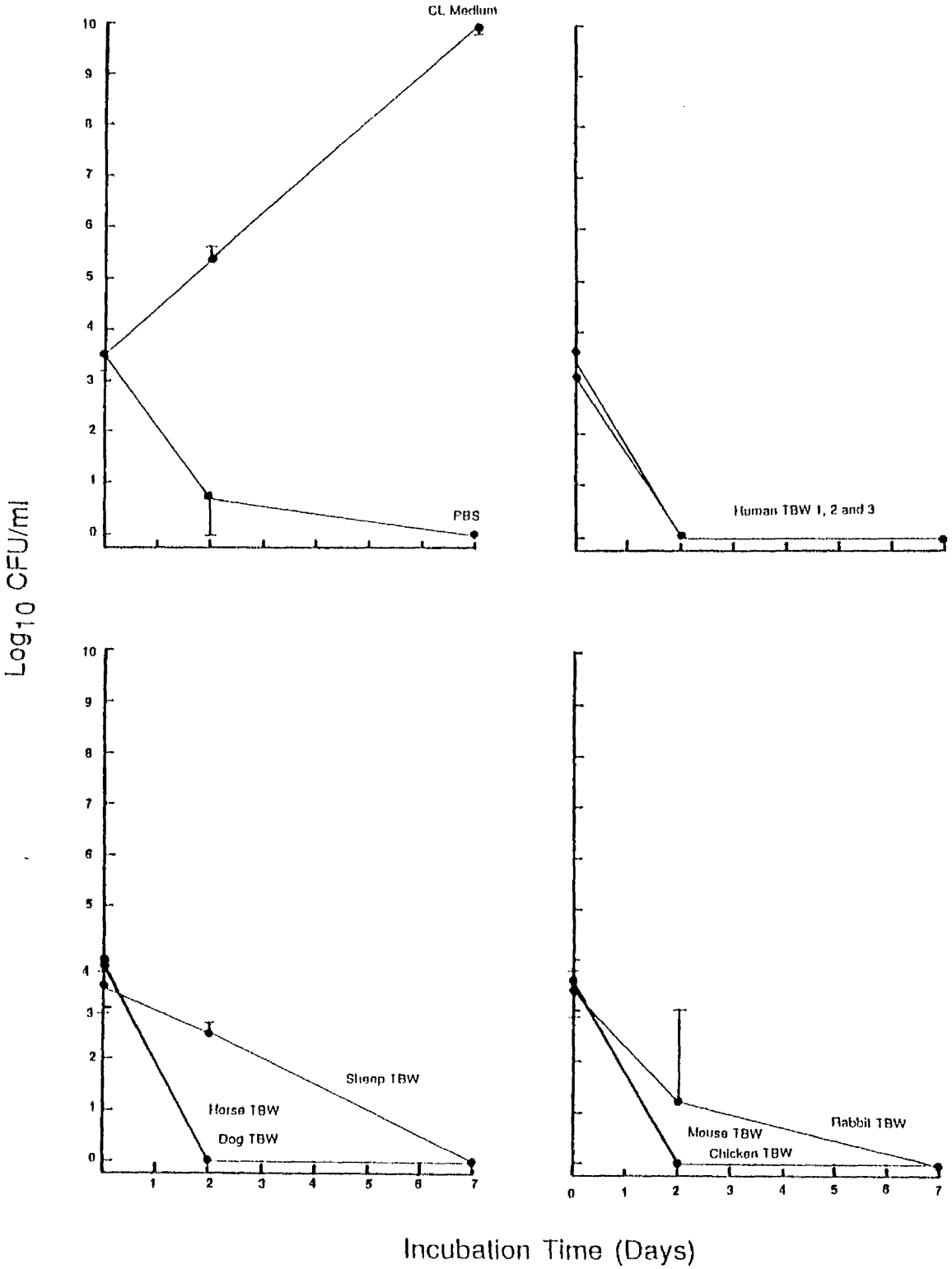

CL Medium
PBS
Human TBW 1, 2 and 3
Sheep TBW
Horse TBW
Dog TBW
Rabbit TBW
Mouse TBW
Chicken TBW
Log10 CFU/ml
Incubation Time (Days)

Table 27 : Effect of supplementing rabbit TBW with the nutrients of CL medium (1:1 ratio) and with MeßCD (1 mg/ml) on the growth of *B. pertussis* strain no. 18334 at 37°C.

Fluid	CFU/ml after incubation (h)[a]		
	0	48	168
CL medium	800	4×10^5	7×10^9
Rabbit TBW + CL medium	1450	14.4×10^5	16.5×10^9
Rabbit TBW + MeßCD	950	50	< 50
Rabbit TBW	..[b]	< 50	< 50
PBS	..	< 50	< 50

[a] average zero time count was $1.1 \times 10^3 \pm 196$ (N=3) CFU/ml

[b] not tested

medium alone, suggesting that the rabbit fluid was contributing nutrients which compensated for dilution of the CL medium and did not have intrinsic bactericidal or other activities that might reduce the apparent viable count.

B. parapertussis gave qualitatively similar results, when incubated for 48h at 37°C, in a 1:1 mixture of horse TBW and CL medium, again showing that the loss of viable count with this organism and horse TBW was not due to bactericidal or agglutinating effects.

Part 3. Chemical Analysis of TBW

The TBW samples tested above for ability to support the growth of the four species of *Bordetella* were analyzed for known *Bordetella* nutrients, viz amino acids, fatty acids and nicotinic acid. In addition, the samples were assayed for haemoglobin, as an index of blood contamination and also for total protein.

Blood contamination.

The majority of the TBW samples at the time of collection were colourless turbid liquids and only a few of them showed red cells in the pellet obtained by centrifugation. However, the possibility that small quantities of blood with or without haemolysis may have contaminated the TBW during collection could not be discounted. Therefore a sensitive assay for haemoglobin with the Drabkin reagent was done on the pellets resuspended to original TBW volume and also on the membrane-filtered supernates from the centrifugation.

Figure 10 (see also Appendix 8) is the standard curve from the Drabkin test for absorbance at 540 nm against haemoglobin concentration expressed as the % (v/v) equivalent of mouse blood. This assay had a detection limit down to about 0.001 % (v/v) of blood in the diluent. Table 28 summarizes the results on 13 samples of TBW from the seven vertebrate species (Appendices 9 to 11), each sample being analyzed for haemoglobin in both the pellet and the supernate.

The Table shows that all samples of TBW contained some measurable haemoglobin, but even the highest level of contamination was below 1 % (v/v) equivalent of blood. The TBW sample with the most blood in it was human No. 1 with 0.86 % (v/v) blood equivalent. On the other hand, the least contaminated sample in the series was one of the other human specimens with 0.02 %(v/v) blood equivalent. In the non-human

Figure 10 : Estimation of blood contamination in TBW. Standard curve for the Drabkin assay of haemoglobin calibrated in terms of the percent (v/v) whole mouse blood.

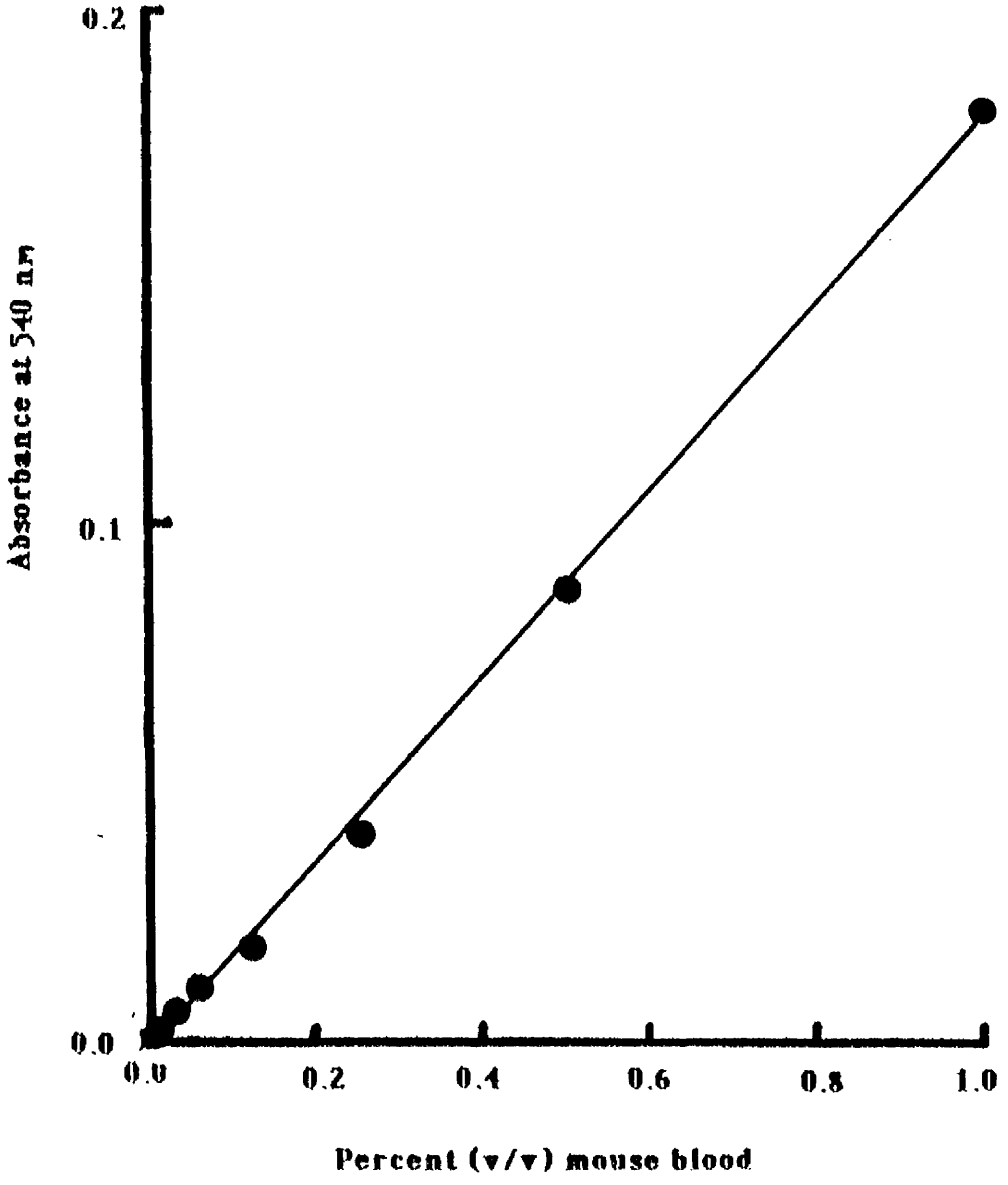

0.2
0.1
0.0
Absorbance at 540 nm
0.0
0.2
0.4
0.6
0.8
1.0
Percent (v/v) mouse blood

Table 28 : Blood contamination[a] of TBW (based on a minimum of three observations). Each TBW was centrifuged to deposit unhaemolysed erythrocytes (and other cells). This "TBW pellet" was resuspended in PBS to the original volume of the TBW and analysed for haemoglobin. The " TBW supernate " was membrane-filtered before testing.

	Blood contamination (expressed as % v/v whole mouse blood)		
TBW	TBW Pellet	TBW Supernate	Total
Horse	0.12 ± 0.01	0.12 ± 0.01	0.24 ± 0.02
Rabbit 1	0.13 ± 0.01	0.08 ± 0.01	0.21 ± 0.02
Rabbit 2/3	0.05 ± 0.01	0.07 ± 0.004	0.12 ± 0.014
Sheep 1	0.13 ± 0.01	0.03 ± 0.01	0.16 ± 0.02
Sheep 2	0.05 ± 0.01	0.007 ± 0.002	0.057 ± 0.012
Mouse (I57)	0.26 ± 0.03	0.22 ± 0.01	0.48 ± 0.04
Mouse (I58)	0.16 ± 0.004	0.13 ± 0.01	0.29 ± 0.014
Mouse (I65)	0.31 ± 0.04	0.15 ± 0.01	0.46 ± 0.05
Chicken	0.03 ± 0.01	0.01 ± 0.005	0.04 ± 0.015
Dog	0.12 ± 0.01	0.01 ± 0.002	0.13 ± 0.012
Human 1	0.86 ± 0.02	< 0.001	0.86 ± 0.02
Human 2	0.50 ± 0.03	0.16	0.66 ± 0.03
Human 3	0.02 ± 0.003	< 0.001	0.02 ± 0.003

[a] Expressed as percent (v/v) whole mouse blood ± SEM

For full details see Appendices 9, 10 and 11

TBW samples, the blood contamination extended over a 10-fold range from 0.04 %(v/v), in chicken TBW, to 0.48 %(v/v) in mouse TBW 157. The haemoglobin levels in the other animal TBW samples were intermediate.

In most cases, the majority of the haemoglobin measured was located in the resuspended cell pellet, particularily with the human TBW samples, while in the membrane-filtered supernates the values recorded ranged between 0, in human TBW Nos. 1 and 3, to 0.22 % in mouse TBW 157. These data suggested that although some blood had leaked into the TBW during collection, little haemolysis had occurred.

Variations were observed in different batches of TBW from the same species; an 8-fold range was seen in the three human TBW, a 3-fold range in the two sheep TBW and a 2-fold range in both the mouse TBW and rabbit TBW samples.

Amino acids.

The average dose-response curve for amino acid determination by the ninhydrin method, with leucine as standard, is shown in Figure 11 which summarizes the results of seven calibration curves (Appendix 12). It shows that there is a linear relationship between concentration of leucine and absorbancy at 570 nm, at least up to 2 mM of the amino acid. The lower limit of detection was at approximately 0.017 mM (equivalent to where1 SEM below the standard curve intersects zero absorbance).

All 13 assayed samples of TBW from seven species had measurable levels of amino acids, within the 8-fold range from 0.10 to 0.81 mM (Table 29 and Appendices 13 to 15). With four of the species - rabbit, sheep, mouse and human - there was more than one sample available for assay which permitted investigation of within-species and between-species variation. The Table shows that there was little convincing evidence of between-species variation after the within-species between-sample differences had been taken into account. For example, there was a 3.5-fold

Figure 11 : Standard curve of amino acid concentration by the ninhydrin method. Results are expressed as mM leucine equivalents (LE).

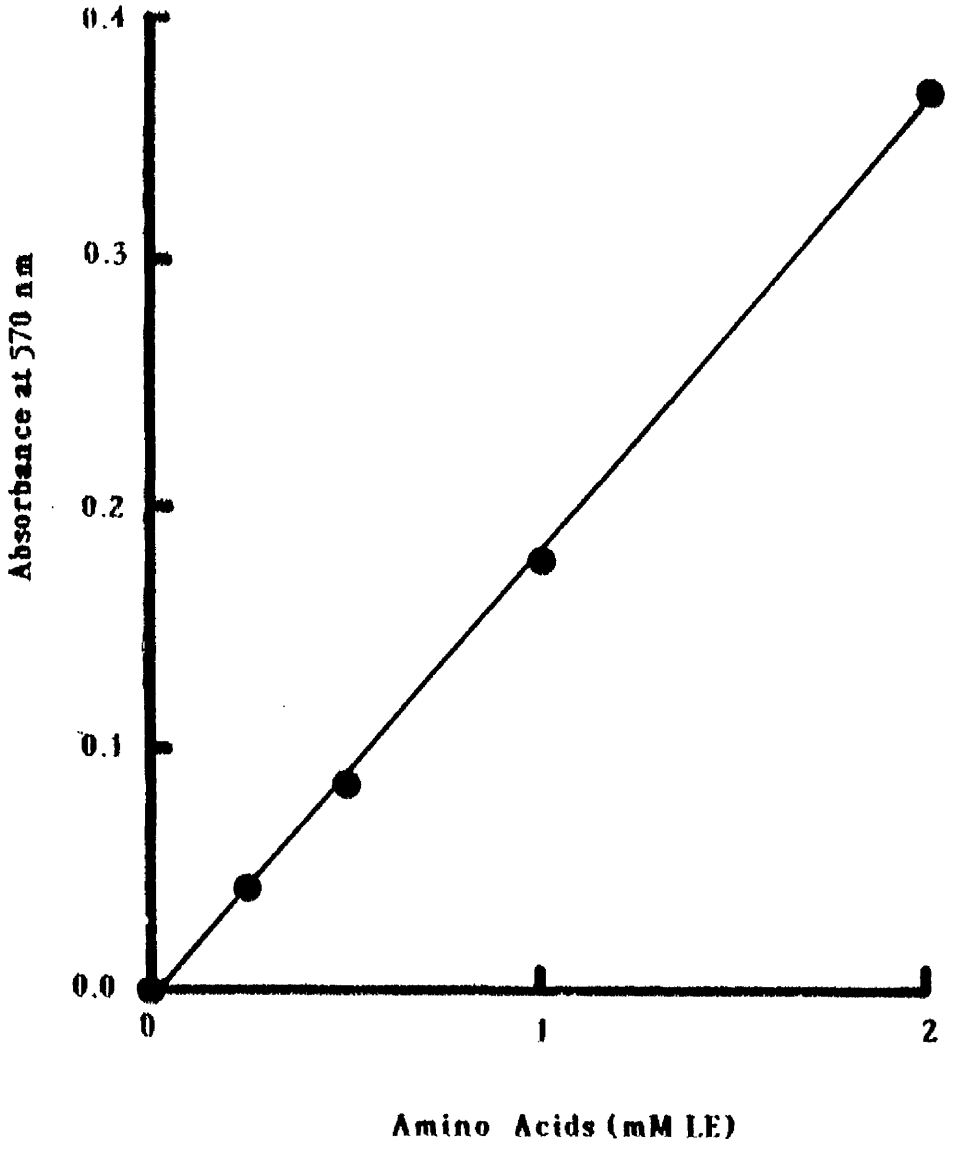

0.4
0.3
0.2
0.1
0.0
Absorbance at 570 nm
0
1
2
Amino Acids (mM LE)

Table 29 : Amino acid content[a] of TBW (based on at least three observations). The TBW had been centrifuged and membrane-filtered and deproteinized before analysis.

TBW	Amino acid content in leucine equivalents (mM ± SEM)
Horse	0.74 ± 0.08
Rabbit 1	0.20 ± 0.03
Rabbit 2/3	0.12 ± 0.0
Sheep 1	0.15 ± 0.02
Sheep 2	0.09 ± 0.01
Mouse I57	0.81 ± 0.09
Mouse I58	0.47 ± 0.01
Mouse I65	0.39 ± 0.09
Chicken	0.10 ± 0.03
Dog	0.23 ± 0.02
Human 2	0.73 ± 0.04
Human 3	0.21 ± 0.01

[a] expressed as mM leucine equivalents (LE) + SEM

For full details see Appendices 13, 14 and 15

variation between two of the human samples and differences of the order of 2-fold between the two rabbit specimens, the two sheep samples and the three TBW pools from mice. It was concluded that the observed differences had arisen mainly through unintended variations in the process of collection.

Nicotinic Acid (NA).

The mean standard curve, based on three tests (Appendix 16), for the assay of nicotinic acid (NA) by the promotion of growth of *Lactobacillus plantarum* is presented in Figure 12. The graph was linear up to 0.16 µg NA/ml and the limit of detection was approximately 0.001 µg NA/ml.

Application of this microbiological method to the assay of NA in TBW yielded the results presented in Table 30 (see also Appendices 17 to 18) . All samples of TBW had readily detectable amounts of NA within the 5-fold range from 0.2 to 1.06 µg/ml. The human samples which were from different patients gave closely similar values at around 0.65 µg NA/ml.

Contribution of blood amino acids to TBW amino acids

Having found that the TBW supernates which were used for the bacterial growth experiments had between 0.001 %(v/v) and 0.22 %(v/v) equivalent of blood, it was of interest to determine the quantity of amino acids which might be contributed by these measured levels of blood contamination. To provide a calibration scale, the ninhydrin assay was therefore applied to mouse blood which had been diluted in PBS to give 0.25, 0.5 and 1.0 %(v/v) mixtures and the protein precipitated with sulphosalicylic acid. The results in Table 31 show that the deproteinized supernates of mouse blood that had been diluted to 1 %(v/v) in PBS contained amino acids equivalent to 0.24 mM leucine.

Based on this value for the amino acid content of mouse blood, and assuming it would be the same for other species, the proportion of the amino acids which had been contributed to the various TBW samples by

Figure 12 : Standard curve of nicotinic acid by the *Lactobacillus* method. The concentration of nicotinic acid (µg/ml) is plotted against the volume of NaOH (ml) required for acid neutralization.

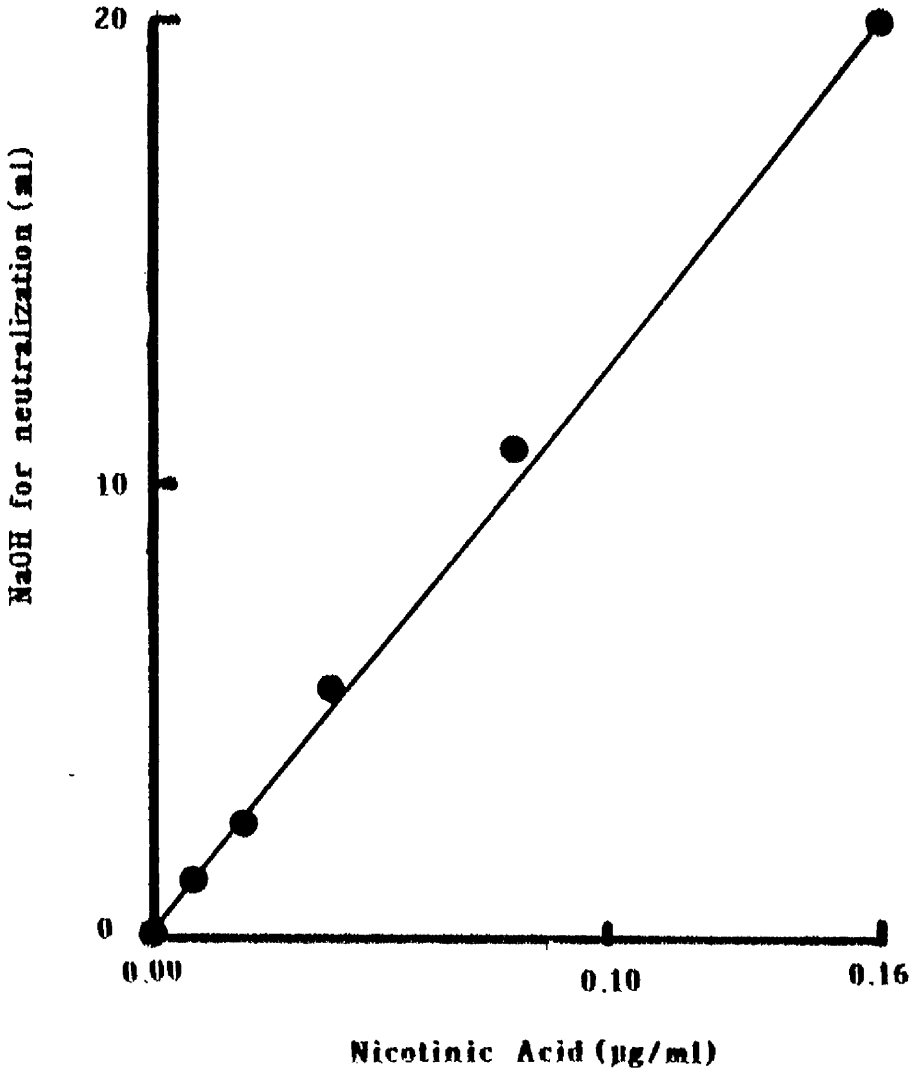

20
10
0
NaOH for neutralization (ml)
0.00
0.10
0.16
Nicotinic Acid (μg/ml)

Table 30 : Nicotinic acid (NA) concentration[a] of membrane-filtered TBW.

TBW	NA concentration : µg/ml ± SEM (N)
Horse	1.06 ± 0.05 (4)
Rabbit 2/3	0.23 ± 0.03 (4)
Sheep 1	0.33 ± 0.08 (4)
Mouse (I57)	0.84 ± 0.08 (4)
Chicken	0.20 ± 0.00 (2)
Dog	0.54 ± 0.00 (3)
Human 2	0.63 ± 0.09 (3)
Human 3	0.67 ± 0.08 (3)

[a] measured in µg/ml

For full details see Appendices17 and 18

Table 31 : Amino acid concentration[a] of whole mouse blood at different dilutions in PBS, expressed as mM leucine equivalents (based on two observations).

Mouse blood % (v/v)	Amino acid concentration (mM LE) individual values (mean)
1	0.25, 0.23 (0.24)
0.5	0.20, 0.16 (0.18)
0.25	0.11, 0.10 (0.10)

[a] (mean + SEM)

blood contamination was then calculated. For this calculation, the data on blood contamination were taken from Table 28 and on amino acids from Table 29 with the results presented in Table 32 . Of the eight samples examined in this way, the majority had less than 10 % of the total amino acids contributed by blood contamination, while two samples showed 16.7 and 20 %. It therefore appeared that although blood contamination contributed some amino acids to TBW it was not a major source relative to that which could not be accounted for by blood contamination (i.e. the amino acids endogenous to the TBW).

Total Protein.

The protein concentrations of the various TBW samples (Table 33 and Appendices 19 to 20), as determined by the method of Lowry *et al* 1951, lay within the range 88 µg/ml (chicken) to 2172 µg/ml (human No. 1). The protein concentrations in the other TBW tested were intermediate.

Table 32 : Estimation of the concentration of amino acids[a] in membrane-filtered TBW contributed by blood contamination. The data in columns 2 and 3 are from Table 29 and 28 respectively.

TBW	Total amino acids (leucine equivalents mM)	Blood contamination %(v/v) mouse blood equivalents	% of total amino acids contributed by blood contamination (%)
Horse	0.74	0.12	3.9
Mouse I57	0.81	0.22	7.1
Rabbit 2/3	0.12	0.07	16.7
Sheep 1	0.15	0.03	6.7
Chicken	0.10	0.01	20.0
Dog	0.23	0.01	1.0
Human 2	0.73	0.16	5.3
Human 3	0.21	> 0.001	> 0.1

[a] expressed as the percent (v/v) of the total amino acids present

Table 33 : Protein concentration[a] of membrane-filtered TBW

TBW	Protein concentration (µg/ml ± SEM)
Horse	1294 ± 119
Rabbit 2/3	318 ± 23
Sheep 1	769 ± 72
Mouse (I57)	1010 ± 33
Chicken	88 ± 16
Dog	599 ± 163
Human 1	2172 ± 98
Human 2	1865 ± 77

[a] measured in µg/ml

For full details see Appendices 19 and 20

Part 4. Growth of *B. bronchiseptica* in PBS and natural waters

The observation that *B. bronchiseptica* strain no. 5376 could survive and grow in PBS without any other added nutrients was the basis for the studies described in this section. Various batches of PBS and samples of fresh waters from lakes in proximity to the laboratory were tested for growth-supporting properties towards *B. bronchiseptica* strain 5376. In all, a total of six strains of *B. bronchiseptica* were tested in both PBS and lake water, and rough estimations of the amount of nutrient in these fluids were made.

Dry weight determination of *B. bronchiseptica* strain no. 5376

In an attempt to estimate the amount of nutrient required for the observed growth of *B. bronchiseptica* strain 5376 in PBS and the lake waters, the dry weight of a single cell of *B. bronchiseptica* was calculated (Figure 13)

Assuming that one CFU corresponded to a single viable cell of *B. bronchiseptica*, it was estimated that the dry weight of one bacterial cell of *B. bronchiseptica* was equivalent to 105 fg.

Growth of *B. bronchiseptica*, strain no. 5376, in PBS and RGW

PBS prepared from analytical grade salts dissolved in either DW or RGW supported the growth of *B. bronchiseptica* (Table 34). In PBS made with DW (expt 1) the initial viable count of 1.6×10^3 CFU/ml increased to 8.4×10^5 CFU/ml in 24h and to 3.5×10^6 CFU/ml at 48h. Thus, over 48h, there was an increase in viable count of approximately 2200-fold, coresponding to 11 cycles of cell division. Essentially the same results were obtained with PBS made in RGW (expt 2), where the viable count increased by a factor of 2700. In neither case was there development of visible turbidity in the PBS.

In CL medium which was inoculated in parallel as a positive control

Figure 13 : Dry weight determination of *B. bronchiseptica* strain no. 5376.

Confluent growth from four BG plates was scraped into 10 ml distilled water to give a thick suspension. This was diluted 60X with PBS to reach an OD_{540} = 0.45. Thus the original suspension had an OD= 27

Tube	No.	Weight of tubes (g)		Increase	Average Increase
		Initial	Final	(g)	(mg)
Blank	1	0.8893	0.8901	0.0008	
					0.5
	2	0.8863	0.8865	0.0002	
Sample	1	0.9030	0.9147	0.0117	
					11.5
	2	0.9415	0.9528	0.0113	

Increase in weight of tubes due to the bacteria = 11 mg

Therefore an OD_{540} equal t0 0.45 contains 11/60 x 1000 µg/ml of *B. bronchiseptica* i.e. 183 µg/ml

From previous viable count data the CFU/ml at an OD_{540} of 0.45 = 1.7356 x 10^9 which is equivalent to 183 µg/ml (from above)

Therefore if ; 1.7356 x 10^9 CFU/ml =183µg/ml

1.7356×10^9 CFU = 183×10^{-6} g

and 1 CFU = $183 \times 10^{-6} / 1.7356 \times 10^9$

= 105 fg

TABLE 34 : Typical results showing growth of *B. bronchiseptica* strain no. 5376 in PBS and in CL medium during incubation for 48h at 37°C.

Expt no.	Fluid	Incubation time (h)	CFU/20 µl from dilution							CFU/ml
			10^0	10^1	10^2	10^3	10^4	10^5	10^6	
1	PBS	0	32, 33[a]	..[b]	..	..	..	..	..	1.6×10^3
	in DW	24	..	ac[c]	168	15	..	..	..	8.4×10^5
		48	..	..	ac	70	..	..	..	3.5×10^6
	CL	0	30, 38	..	..	..	..	..	..	1.7×10^3
	medium	24	..	..	..	..	ac	65	..	3.2×10^8
		48	..	..	..	..	..	ac	31	1.5×10^9
		0	15, 16	..	..	..	..	..	..	775
2	PBS	24	c[d]	ac	ac	13	..	..	..	6.5×10^5
	in RGW	48	c	ac	424	..	2	..	..	2.1×10^6
	CL	0	18, 19	..	..	..	..	..	..	925
	medium	24	c	..	..	..	16	..	1	8×10^6
		48	..	..	..	..	c	..	48	2.4×10^9

[a] Duplicate samples; [b] not tested; [c] almost confluent growth; [d] confluent growth.

, the same initial inocula produced respectively, viable counts of 1.5×10^9 and 2.4×10^9 CFU/ml at 48h for experiments 1 and 2 i.e. about 430 and 1100 times higher than the respective counts in PBS. Here, however, visible turbidity developed in the medium.

In repeat experiments (n=42, see appendix 14), the initial viable count on different days had a standard deviation of 850 around the mean value of 1900 CFU/ml. To facilitate comparisons between experiments, it was therefore convenient to express the extent of growth on a common basis as the logarithmic (base 10) increase from the initial viable count. For this purpose, the Growth Index (GI) defined as $\log_{10}$ (count at time t/count at time zero) was used. The several repeat experiments with PBS in DW and RGW presented in this fashion (Figure 14 and Appendix 21) show average GIs of around 3.0, corresponding to a 1000-fold increase in viable count during 48h.

In an attempt to remove the presumed traces of nutrient from PBS, 50 ml of the buffer made with RGW was passed slowly through a 1 x 10cm column packed with coarse-grade charcoal (NORIT) and then sterilized by membrane filtration. Figure 14 shows that the growth-promoting property of PBS for *B. bronchiseptica* was not impaired by the charcoal treatment; if anything, it was slightly enhanced.

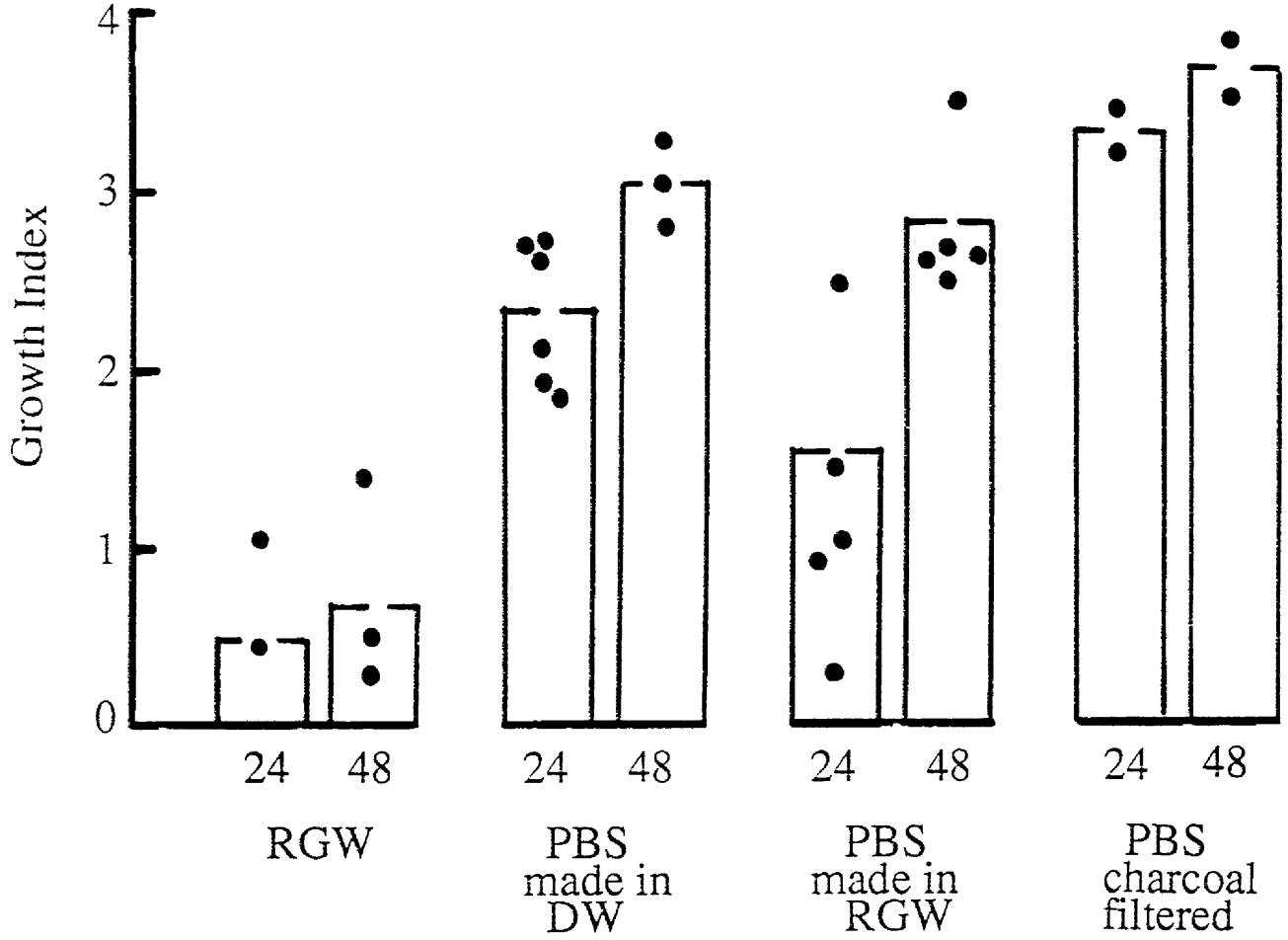
RGW without added salts was also tested for ability to support growth of the bacteria by the same method. The mean GI at 48h was 0.7 (Figure 14), corresponding to a 5-fold increase in viable count i.e. 2.25 cell divisions. It therefore appeared from these experiments that the main source of nutrients in PBS was one or more of the analytical grade salts used to prepare the buffer, rather than the water itself or the atmosphere of the laboratory.

Serial transfer in PBS (made in DW)

Serial transfer experiments, with a 40-fold dilution at each step,

Figure 14 : Growth of *B. bronchiseptica* strain no. 5376 during 24h and 48h incubation in water and PBS at 37°C.

The dots represent individual values and the bars the SEM.


Growth Index
4
3
2
1
0
24
48
RGW
PBS made in DW
PBS made in RGW
PBS charcoal filtered

were made to investigate the possibility that the previously observed growth of *B. bronchiseptica* strain no. 5376 in PBS might have been due to nutrients carried over from the original BG medium. A thrice-washed inoculum (25 µl) of the bacteria was therefore added to 975 µl of PBS, and also to 975 µl of CL medium as a nutrient-positive control. Samples for viability counting were taken from both media at zero time and after incubation for 24h at 37°C. The first of the serial transfers consisted in inoculating fresh 975 µl aliquots of PBS or CL medium with 25 µl from the corresponding 24h cultures. These in turn were incubated for 24h at 37°C and then subjected to a second round of serial transfer, with counting at zero time and 24h. Figure 15 shows that *B. bronchiseptica* grew up to the same viable count of around 10^6 CFU/ml after the third serial transfer as it did from the initial inoculum (see also Appendix 22). This indicated that the source of nutrients was not the initial washed inoculum. In CL medium, the 24h viable count in each serial transfer was about 10^8 CFU/ml (Figure 15).

When *E. coli* JM 83 was subjected to the same serial transfers, it failed to grow in the same batch of PBS which gave positive results with *B. bronchiseptica*, and did not survive to the second or third transfers (Figure 16 and Appendix 23). Viable counts of *E. coli* in CL medium reached approximately 10^8 CFU/ml in each of the three transfers (Figure 16). Thus the *B. bronchiseptica* strain was able to survive and grow during serial transfer in PBS under conditions in which the *E.coli* strain failed to persist.

Dilutions of CL medium

To compare roughly the level of nutrients in PBS (made in DW) with those in CL medium, the growth of *B. bronchiseptica* was measured in a series of ten-fold dilutions of CL medium in PBS. In two independent experiments (Figure 17 and Appendix 24), a 10^4 dilution of CL medium

Figure 15 : **Changes in $\log_{10}$ viable count during three serial transfers of *B. bronchiseptica* strain no. 5376 in a) PBS and b) CL medium. Each serial transfer involved a 1 in 40 dilution.** The dots represent individual values and the bars the SEM.

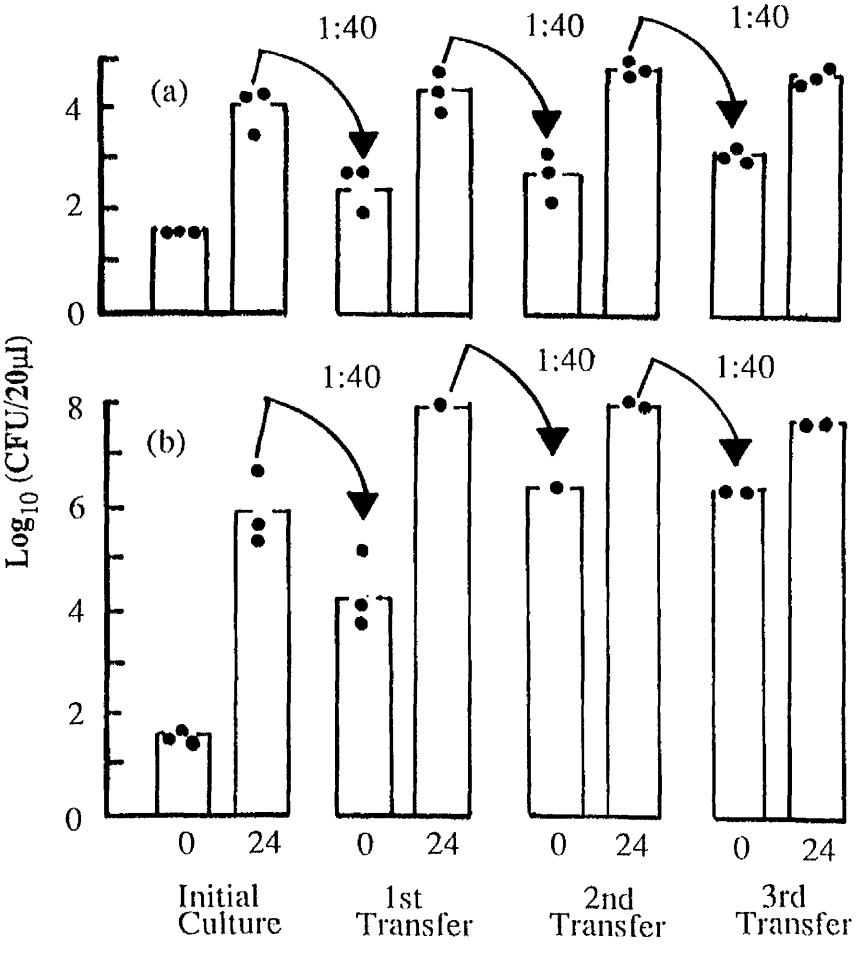

1:40
1:40
1:40
(a)
4
2
0
1:40
1:40
1:40
(b)
8
6
4
2
0
Log10 (CFU/20µl)
0
24
0
24
0
24
0
24
Initial Culture
1st Transfer
2nd Transfer
3rd Transfer

Figure 16 : Changes in $\log_{10}$ viable count during three serial transfers of *E. coli* strain no. JM 83 in a) PBS and b) CL medium. Each serial transfer involved a 1 in 40 dilution. The dots represent individual values and the bars the SEM.

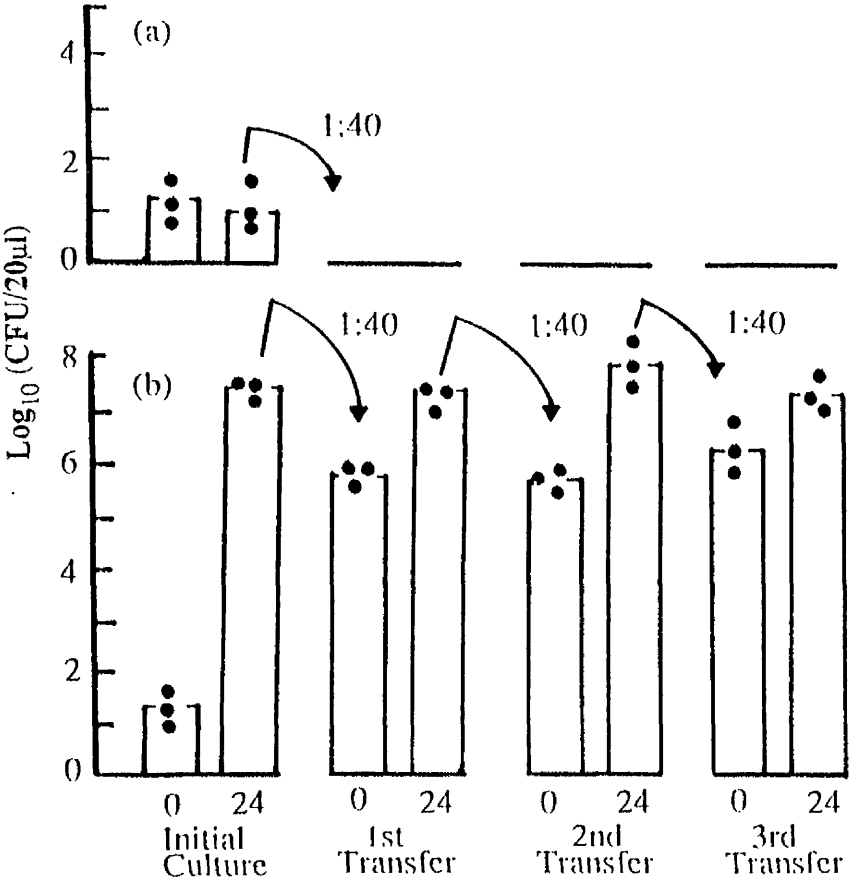
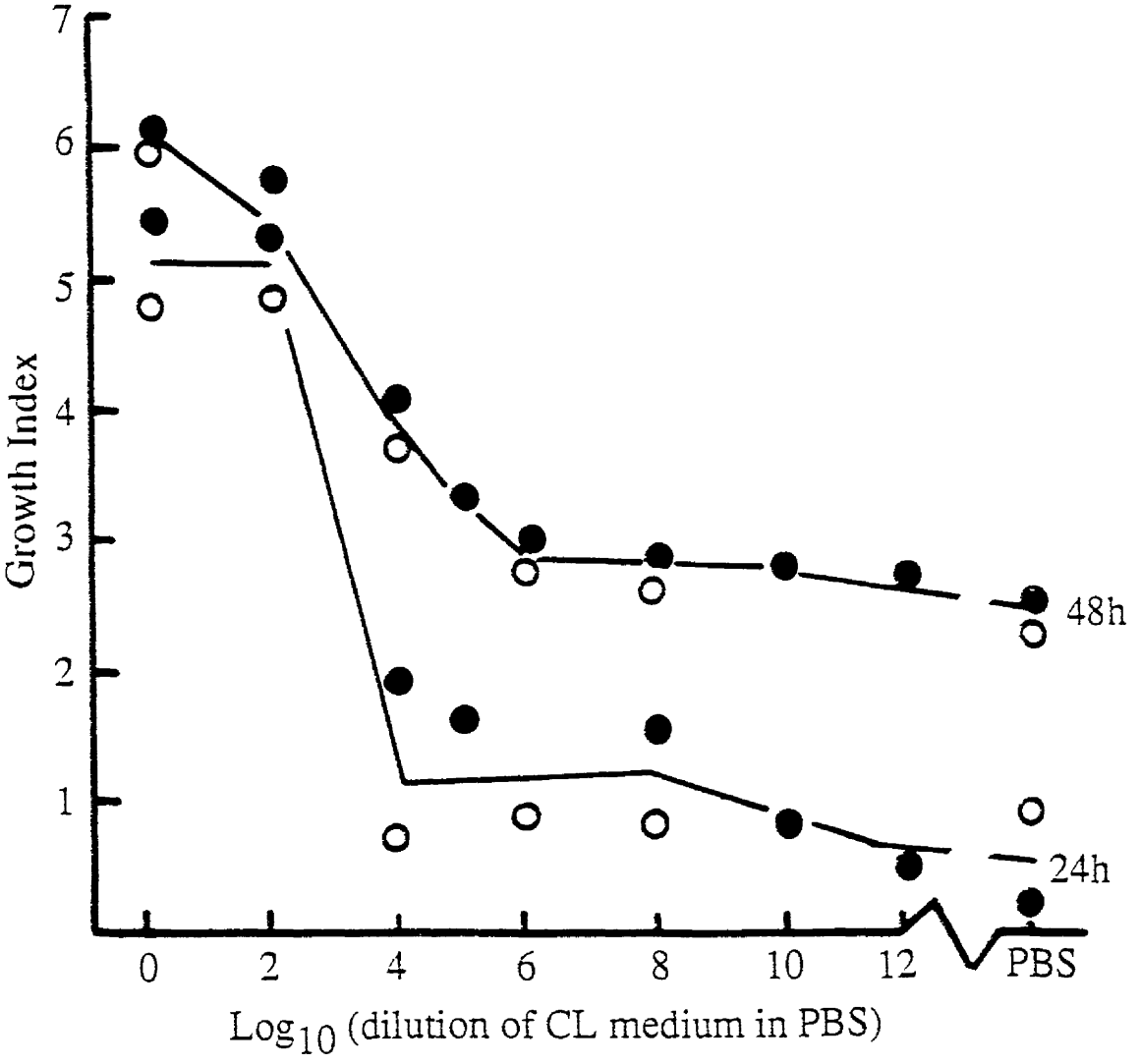

(a)
4
2
0
1:40
(b)
8
6
4
2
0
1:40
1:40
1:40
Log_{10} (CFU/20µl)
0
24
Initial Culture
1st Transfer
2nd Transfer
3rd Transfer

Figure 17 : Estimation of the concentration of nutrients in PBS by determining the dilution of CL medium which failed to stimulate growth of *B. bronchiseptica* strain no. 5376 above the level achieved in PBS. The dots represent individual values.


7
6
5
4
3
2
1
Growth Index
48h
24h
0
2
4
6
8
10
12
PBS
Log10 (dilution of CL medium in PBS)

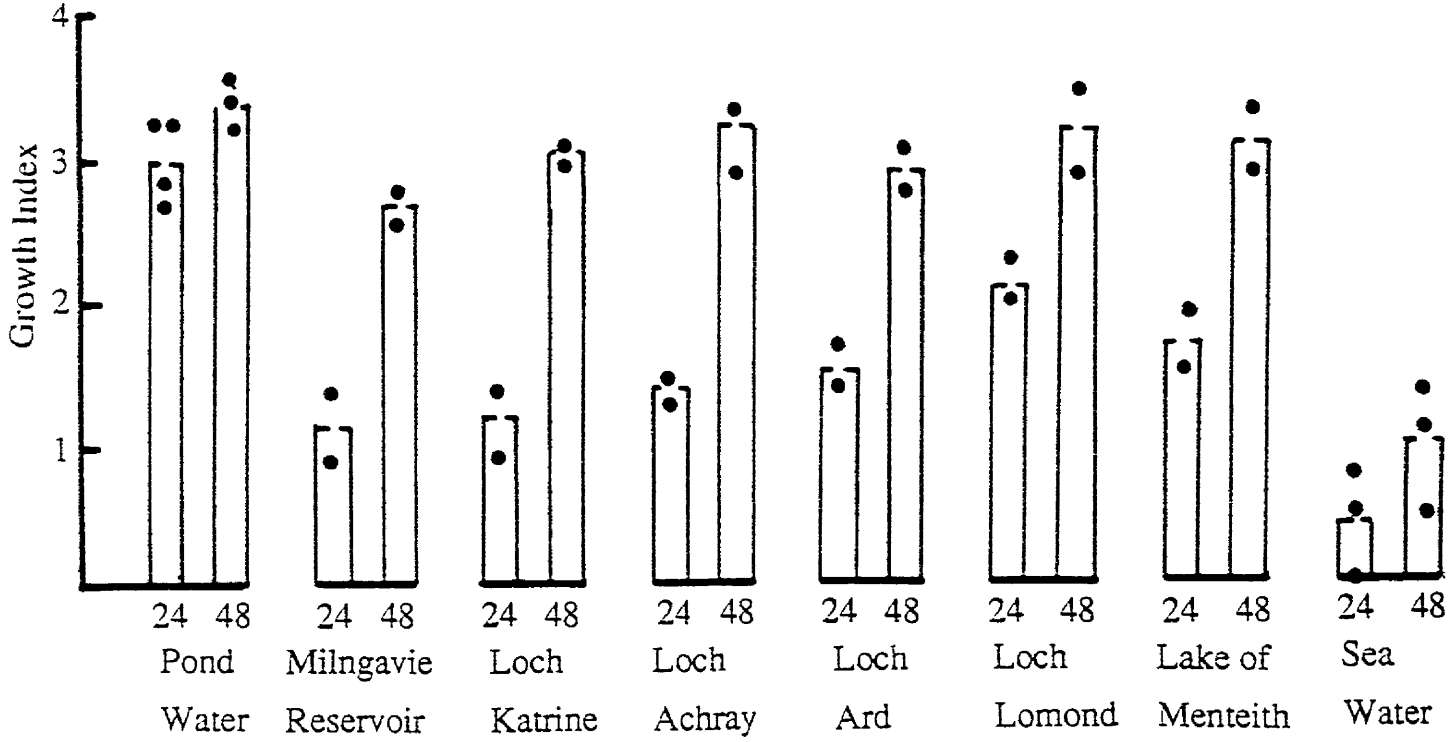
gave a slightly higher GI at 48h than PBS alone, and in one experiment a small increase in growth was detected with a 10^5 dilution. Since undiluted CL medium contains amino acids at about 22 g/l, the growth in PBS alone could be explained by nutrient impurities in the range 0.2-2 mg/l.

Growth in natural waters

In an attempt to discover if *B. bronchiseptica* strain 5376 could survive in waters from the natural enviroment various samples of fresh water and sea water were tested for their growth-supporting properties.

Figure 18 shows that *B. bronchiseptica* grew and survived in natural fresh water and sea water at 37°C (see also Appendices 25 to 26). In the various fresh water samples from a garden pond, a reservoir and five lakes in the vicinity of Glasgow, the 48h GI values all lay within a narrow range from 2.7 to 3.2, corresponding to between 8 and 10 cycles of cell division from the inocula of approximately 1900 CFU/ml. At 24h however, the GI values varied between 1.2 and 3.0, indicating larger differences in initial rate of growth in the various fluids. These latter differences appeared to be correlated with the amount of particulates in the water samples before membrane filtration. Thus the highest 24h GI of 3.0 was obtained with the garden pond water, which was the most turbid sample prior to membrane filtration. This was followed by two similarily turbid waters, from Loch Lomond and the Lake of Menteith, which yielded 24h GIs of 2.1 and 1.7 respectively. In contrast, the clearest waters produced the lowest GIs after 24h. In this category were Loch Katrine and Milngavie Reservoir, the source and storage lake respectively for Glasgow's drinking water. With further incubation of both waters to 48h respective GI values of 2.7 and 3.1 were recorded. Continued incubation at 37°C beyond 48h, showed that all of the fresh water samples supported approximately three additional generations within 1 wk, after which the viable counts changed little for at least 3 wk.

Figure 18 : Growth of *B. bronchiseptica* strain no. 5376 in a variety of natural lake and pond waters during 24h and 48h incubation at 37°C. The dots represent individual values and the bars the SEM.


Growth Index
4
3
2
1
24 48
Pond Water
24 48
Milngavie Reservoir
24 48
Loch Katrine
24 48
Loch Achray
24 48
Loch Ard
24 48
Loch Lomond
24 48
Lake of Menteith
24 48
Sea Water

In sea water, although the growth of *B. bronchiseptica* was much less than in the fresh water samples, the average GI of 0.9 corresponded to an 8-fold increase in viable count during 48h at 37°C (Figure 18 and Appendix 25).

Other strains of *B. bronchiseptica*

To investigate the possibility that the ability of *B. bronchiseptica* to grow in low-nutrient fluids was specific for strain 5376, five additional strains of this species, including the type strain 452 and a number of recent isolates from a pig, horse and a dog, were tested for their ability to grow in PBS (made in RGW) and fresh water from Loch Lomond. Figure 19 shows that all five strains resembled strain 5376 in their viable count increases in the two test fluids (see also Appendices 27 to 28).

Incubation of *Pasteurella haemolytica* in PBS and Loch Lomond water

Another bacterium which is an obligate pathogen of ruminant species and, like the *Bordetella* usually in the respiratory tract is *Pasteurella haemolytica*. This organism was also tested for the ability to grow in PBS and fresh water from Loch Lomond.

It was found that when a growth experiment of the type described above was set up using two *P. haemolytica* strains (S/C 82/1 and W/D 83/4) inoculated into PBS, Loch Lomond water and BHIB only the latter supported the growth of both organisms (Table 35). From an initial viable count of around 200 CFU/ml, both strains of *P. hemolytica* showed visible turbidity in BHIB after 48h incubation at 37°C. The viable count in PBS and the fresh water was zero after the same time period.

Long-term survival of *B. bronchiseptica* in low-nutrient fluids

Having established that the *B. bronchiseptica* strains tested above all posessed the ability to grow in PBS and fresh water samples, a study was done to establish how long *B. bronchiseptica* would survive under the

Figure 19 : **Comparison of six strains of *B. bronchiseptica* for their ability to grow in PBS (A) and Loch Lomond water (B).** The dots represent individual values and the bars the SEM.

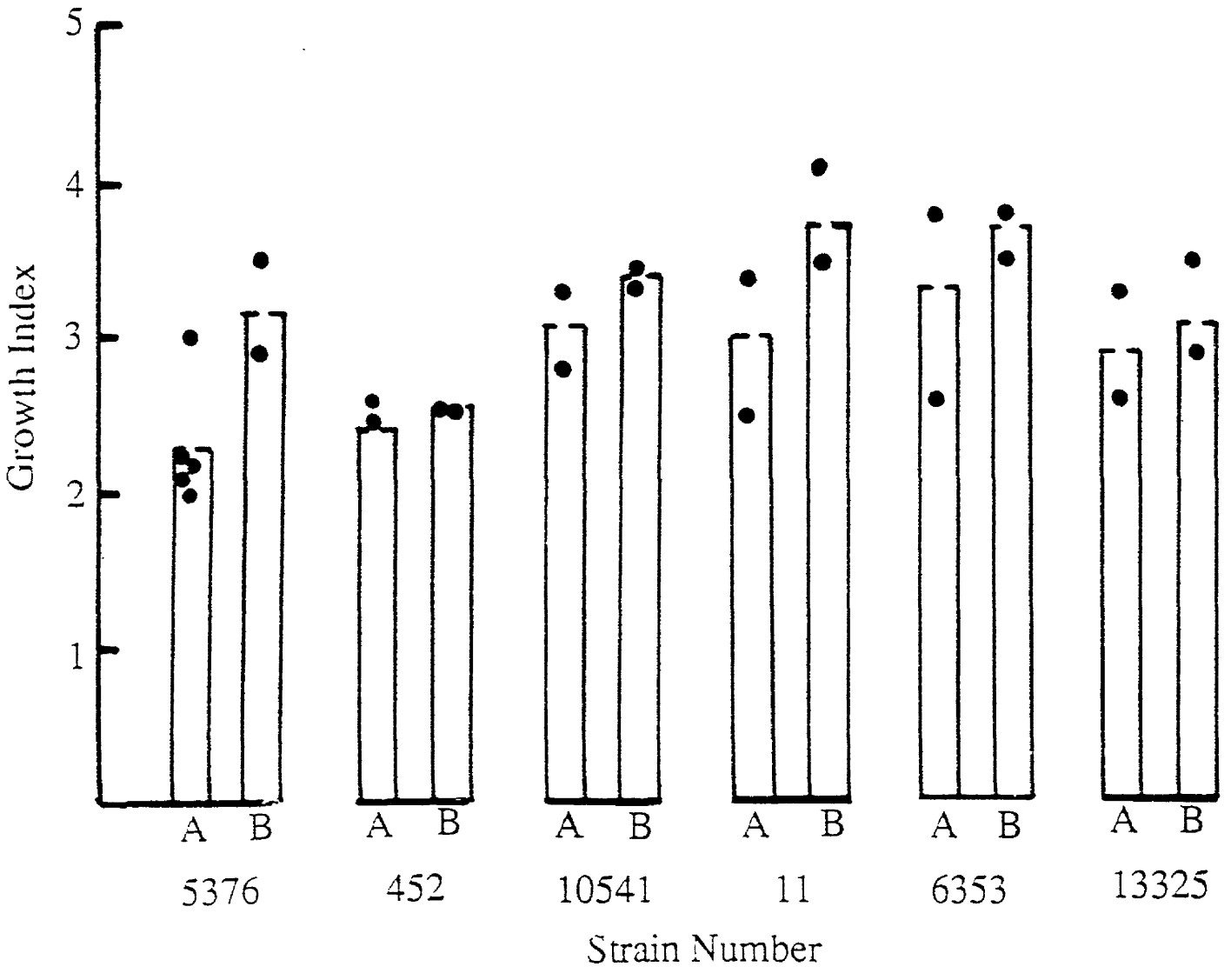

Growth Index
5
4
3
2
1
A B
A B
A B
A B
A B
A B
5376
452
10541
11
6353
13325
Strain Number

Table 35 : Colony count changes with *Pasteurella haemolytica* in PBS and Loch Lomond water, as compared to those in Brain Heart Infusion Broth (BHIB).

Fluid	CFU/20 µl after incubation (h)			
	P. haemolytica S/C 82/1		*P. haemolytica* W/D 83/4	
	0h	48h	0h	48h
BHIB	4	++	4	++
PBS	4	0	3	0
Loch Lomond	3	0	8	0

++ : confluent growth

low-nutrient conditions.

Three strains of *B. bronchiseptica* (452, 6353 and 11) were incubated in closed, screw-cap bottles containing PBS and Loch Lomond water and incubated at temperatures of 37°C and 10°C. Samples (20 µl) were taken at zero time and after 1 week, 1 month, three months and six months at each incubation temperature. These samples were plated either undiluted or after a number of 10-fold dilutions onto BG agar. The viable counts are summarized in Table 36 with the actual counts presented in Appendix 29 .

All three strains of *B. bronchiseptica* remained viable for at least three months at both 37°C and 10°C. With strain 11, no viable count was recorded in the fluids incubated at 37°C after six months. In the other fluids incubated at 10°C, although slight decreases were recorded after six months, the viable count remained considerably higher than the zero time count.

Growth in the fluids incubated at 10°C was slower than at 37°C with all three species. However the stationary phase counts were similar. No obvious differences were observed between growth and survival in PBS as compared to that in Loch Lomond water with any of the strains tested.

In most cases, viability was maintained even after incubation for six months, maximum counts were reached at between 1-3 months and the viable count remained steady or decreased slightly thereafter. An exception was with strain 452 incubated in PBS at both 37°C and 10°C where the viable count continued to rise slightly after six months.

Colony morphology. An interesting observation was made with all strains over the six month incubation period. It was observed that as the incubation period proceeded the colony size on BG agar decreased, with a mixture of small and large colonies observed on the plates. Photograph 1 shows four plates containing *B. bronchiseptica* strain 6353. Plates A and B

Table 36 : Long-term survival of *B. bronchiseptica* strain nos. 11, 6353 and 452 in PBS and Loch Lomond water when incubated at 37°C (shaken) and 10°C (static) over six months.

Strain	Fluid	CFU/ml after incubation (wk)				
		0	1	4	12	24
	PBS A[a]	300	9.5×10^5	5.5×10^5	25.5×10^5	0
	PBS B[b]	300	2.15×10^5	63×10^5	36.5×10^5	16.5×10^4
11	LLA[c]	350	30×10^5	9.5×10^5	14.7×10^6	0
	LLB[d]	400	8.5×10^4	36.5×10^5	28×10^5	83.5×10^5
	PBS A	300	62×10^5	19×10^5	9.5×10^5	6.5×10^5
	PBS B	..	2.05×10^5	14×10^5	50×10^5	2.05×10^6
6353	LLA	450	27.5×10^5	1.75×10^5	12×10^6	12.5×10^5
	LLB	..	8×10^5	17.5×10^5	36.5×10^5	35.5×10^5
	PBS A	2.3×10^3	2.7×10^5	11.5×10^5	5.5×10^5	6×10^5
	PBS B	..	4.1×10^3	4.5×10^4	6.5×10^5	12×10^5
452	LLA	2.9×10^3	15×10^5	2.6×10^5	5.5×10^5	5.5×10^4
	LLB	..	4×10^4	3.45×10^5	7×10^5	9.5×10^4

[a] phosphate-buffered saline, incubated at 37°C

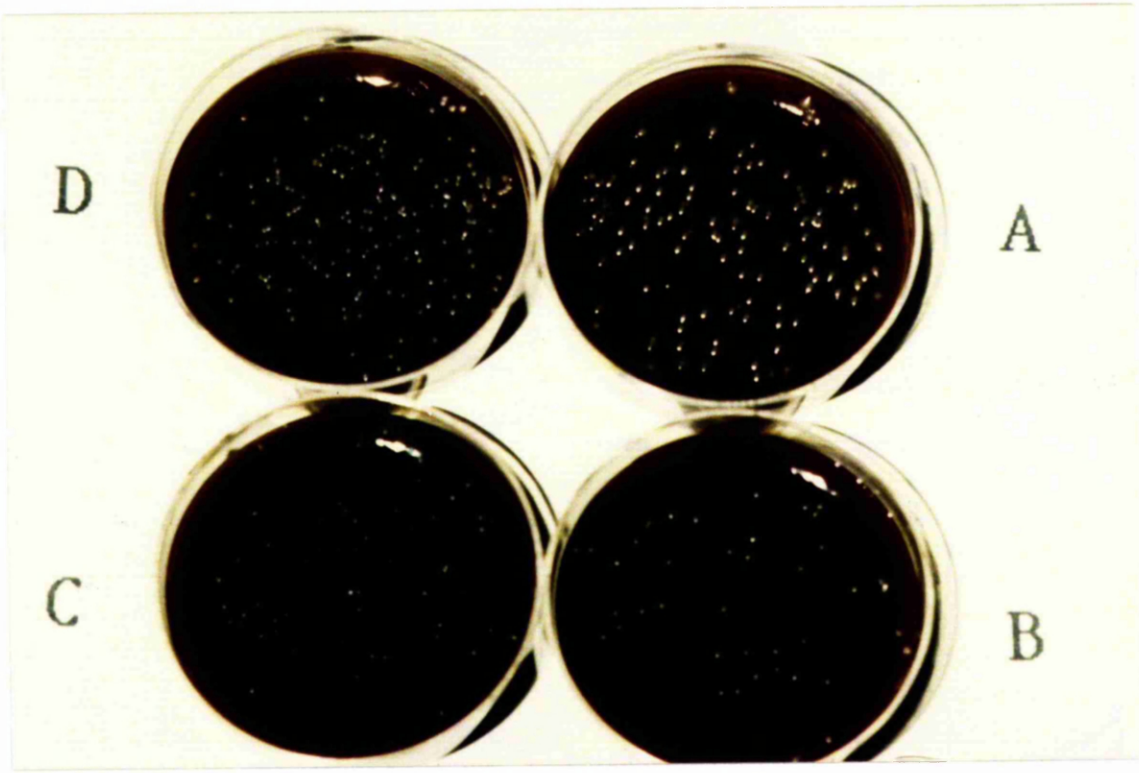
[b] phosphate-buffered saline, incubated at 10°C

[c] Loch Lomond water, incubated at 37°C

[d] Loch Lomond water, incubated at 10°C

.. not tested

Photograph 1 : Variation in the colony morphology of *B. bronchiseptica* strain no. 6353 on BG agar, when a 20 µl sample was taken from PBS and Loch Lomond water after incubation at 37°C and 10°C for 1 month.
(see last line of page 134 for reference to A, B, C and D)


D
A
C
B

are 1 month samples from PBS at 37°C and 10°C respectively and plates C and D are those from the respective Loch Lomond samples at 37°C and 10°C. The differences in the colonial sizes can be clearly seen and both types of colonies are evident on plate D.

Photograph 2 is a close-up of a plate containing *B. bronchiseptica* strain 6353 incubated for 1 month in Loch Lomond water at 10°C. It shows two very distinctive colony sizes. As time passed the predominant phenotype was the small-form. Both types of colony were tested on the API 20 NE strip and both gave the identical code, characteristic of a *B. bronchiseptica* strain i.e. 11 000 23.

Wall growth In the study described above the possibility was considered that during the incubation of the *B. bronchiseptica* strains, attatchment to the sides of the culture vessel might have occurred. A qualitative estimation of this possible wall growth from each of the three *B. bronchiseptica* strains used in the long-term survival study was therefore made. The fluid from one culture vessel (PBS and Loch Lomond water) at 10°C and 37°C was discarded and replaced with a fresh 1 ml of PBS. The contents were then mixed vigorously and a 20 µl sample was plated onto a BG plate.

All three strains showed an apparent degree of wall growth or adherence in PBS at both 37°C and 10°C (Table 37). In the Loch Lomond water at 10°C, both strains 452 and 6353 showed considerable evidence of wall growth while that seen with strain 11 was much less. In the same fluids at 37°C there appeared to be less adherence than at 10°C with all three species.

Photograph 2 : Detail of colony morphology of *B. bronchiseptica* strain no. 6353 on BG agar, when a 20 µl sample was taken from Loch Lomond water incubated at 37°C for 1 month.

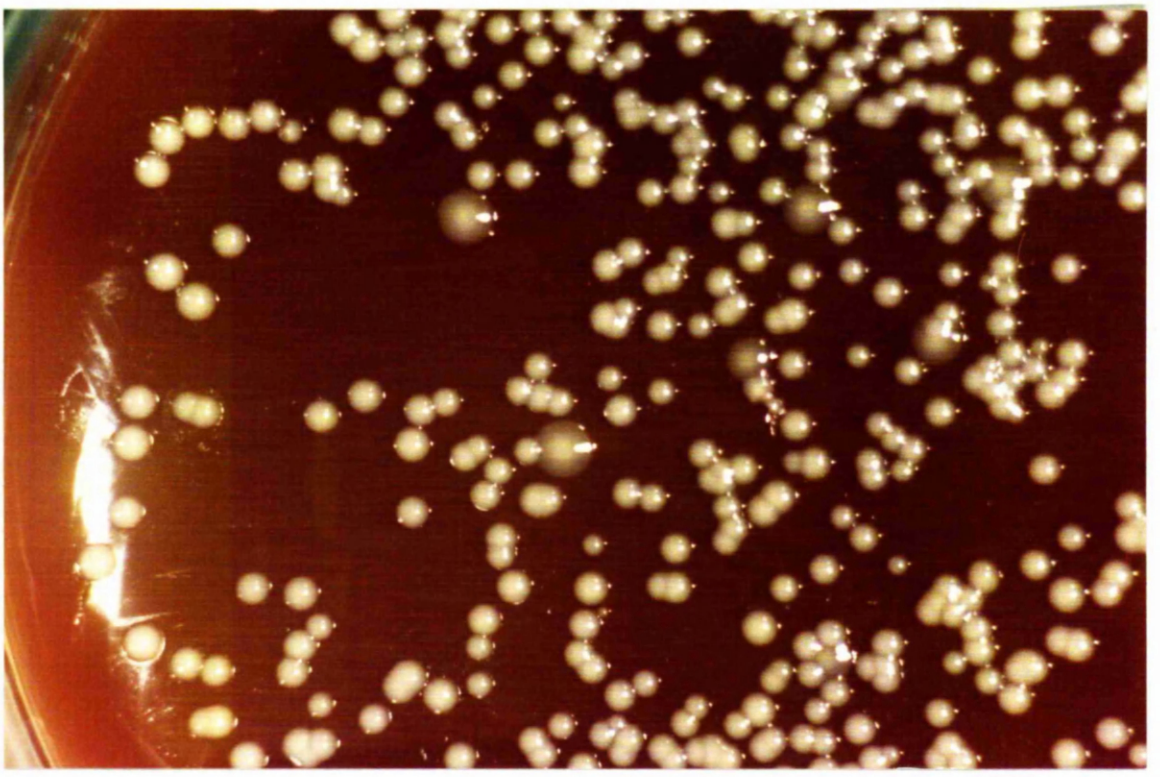


Table 37 : Qualitative assessment of wall growth of *B. bronchiseptica* strain nos. 11, 6353 and 452 in PBS and Loch Lomond water after incubation for three months at 37°C and 10°C.

Strain no.	CFU/20 µl from 1 ml rinsing after three months at :			
	37° C in :		10°C in :	
	PBS	Loch Lomond water	PBS	Loch Lomond water
11	++	42	++	+
6353	++	0	++	++
452	+	+	++	++

+ and ++ indicate almost confluent and confluent growth on a BG plate from a 20 µl sample

DISCUSSION

Part 1. Growth of *Bordetella* species in TBW

Despite the early recognition that *in vivo* growth of the bordetellae was confined to the respiratory tract of animals and birds, there appear to have been no studies prior to the present work on the growth of these organisms in TBW *in vitro*. Yet the harvesting of fluids from the respiratory tract for a variety of experimental and diagnostic purposes goes back at least 40-50 years. For example, in 1944 Boyd and co-workers reported on the lipid, sodium, chloride and nitrogen content of the respiratory tract fluid of normal animals. Moreover lack of usage of TBW for growth of respiratory pathogens is not restricted to the *Bordetella*. In fact, extensive searches of the literature have failed to uncover any recorded use of TBW or bronchial lavage fluids for *in vitro* growth of any respiratory-tract pathogens e.g. *Haemophilus, Pneumococcus, Legionella,* or *Candida*. In contrast, there is a substantial body of literature dealing with the growth of bacteria in other body fluids, such as saliva, breast milk and urine (Table 38). Experience gained in the present work suggests that such investigations might be fruitful in the context of defining growth in such media which arguably are more natural than conventional culture media.

One of the initial hopes in this investigation was that the four species of *Bordetella* might show growth patterns in TBW from different vertebrate species which would be in accordance with their known patterns of host specificity. In fact, the results did not work out this way. *B. pertussis* failed to grow in any of seven species of TBW (including human); *B. parapertussis* grew in five of the seven, while *B. bronchiseptica* and *B. avium* grew in all of the TBW species tested (except human TBW no. 2). Rather than revealing any host specificity within the bordetellae for TBW, the results indicated more the degree of fastidiousness of each of the four organisms, a point which has long been known.

Table 38 : Growth of microorganisms in body fluids as reported in the literature.

Fluid	Microorganism	Reference
Saliva	*Actinomyces viscous, Aerobacter cloacae, Bacillus cereus, Bacillus subtilus, Candida albicans, Corynebacterium diptheriae, Lactobacillus, Staphylococcus aureus, Streptococcus faecalis, Streptococcus pyogenes*	Williams and Powlen, 1959 Cowman *et al*, 1977 De Jong *et al*, 1984
Serum	*Streptococcus* spp.	Sukroongreung *et al*, 1988
Breast-milk	*Staphylococcus, Streptococcus, E. coli,*	Roberts and Severn, 1978 Nwankwo *et al*, 1988
Urine	*E. coli, Proteus, Klebsiella, Enterococci, Staphylococcus epidermidis, Aerobacter aerogenes, Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus albus*	Kaye, 1968 Stamey and Kaufman, 1975 Greenwood and O' Grady, 1978 Stamey and Mihara, 1980

Table 39 shows the maximal $\log_{10}$ CFU/ml recorded for each of the four bordetellae in the TBW samples and compares them with those for CL medium. With all four organisms, the viable count was always highest in CL medium (as was expected) due to the nutritious nature of this medium. However, the counts recorded for *B. bronchiseptica* and *B. avium* in horse and mouse TBW were similar to those for CL medium. In most cases, *B. bronchiseptica* gave the most growth in the TBW samples, although *B. avium* was similar. This was as expected considering the fact that these two organisms are the least fastidious of the bordetellae. With *B. parapertussis* the maximum $\log_{10}$ CFU/ml in all six TBW samples was similar suggesting that a nutritional deficiency prevented *B. parapertussis* from emulating the viable counts of *B. bronchiseptica* and *B. avium*. Although *B. pertussis* failed to grow in any of the TBW samples without supplementation, the viable counts recorded in the CL medium were the highest of the four species. A surprizing observation was that rabbit TBW supplemented with CL medium gave an approximately 2-fold greater viable count with *B. pertussis* than did CL medium alone. This suggested that the rabbit TBW may contain a stimulant for *B. pertussis* which is lacking in the CL medium. The reasons for the quantitative differences in the viable counts recorded for the different bordetellae in the TBW was thought to reflect the nutritional content of the TBW and the nutrient-scavenging abilitiy of the organisms. The ability of *B. bronchiseptica* and *B. avium* to use alternative carbon and energy sources would also help explain the larger viable counts recorded with these two bacteria.

The fact that members of the *Bordetella* can even grow in TBW from different animal and bird species presents a number of future areas of research. A study of the infectivity and pathogenicity of the bacteria

Table 39 : Viable counts[a] of the four species of *Bordetella* in various samples of vertebrate TBW after two and seven days incubation at 37°C.

Fluid	Day	Log_{10} CFU/ml (N) of			
		B. bronchiseptica	*B. avium*	*B. parapertussis*	*B. pertussis*
PBS	2	5.9 ± 0.1 (12)	5.2 ± 0.4 (5)	1.3 ± 0.8 (5)	0.7 ± 0.7 (4)
	7	6.7 ± 0.1 (5)	5.3 ± 0.5 (4)	0 (6)	0 (4)
CL	2	9.4 ± 0.1 (14)	9.1 ± 0.2 (10)	6.2 ± 0.2 (11)	5.4 ± 0.2 (6)
medium	7	8.8 ± 0.4 (5)	8.2 ± 0.1 (4)	9.5 ± 0.1 (4)	10.0 ± 0.2 (5)
TBW					
Horse	2	8.8 ± 0.2 (4)	8.8 ± 0.1 (5)	0 (3)	0 (2)
	7	9.1 ± 0.1 (3)	7.4 ± 0.03 (3)	0 (3)	0 (2)
Sheep	2	7.9 ± 0.2 (5)	7.5 ± 0.3 (5)	5.7 ± 0.4 (3)	2.5 ± 0.2(2)
	7	7.8 ± 0.1 (4)	6.1 ± 0.2 (3)	5.4 ± 0.2 (3)	0 (2)
Dog	2	5.5 ± 0.2 (3)	5.1 ± 0.2 (3)	2.9 ± 0.1 (6)	0 (3)
	7	7.9 ± 0.2 (3)	7.9 ± 0.2 (3)	0 (6)	0 (3)
Mouse	2	8.7 ± 0.2 (4)	8.8 ± 0.2 (3)	6.3 ± 0.5 (5)	0 (3)
	7	8.8 ± 0.1 (3)	6.5 ± 0.1 (3)	5.1 (1)	0 (3)
Rabbit	2	7.9 ± 0.2 (4)	7.6 ± 0.2 (5)	5.6 ± 0.4 (3)	1.5 ± 1.5 (2)
	7	8.2 ± 0.2 (3)	6.6 ± 0.1 (4)	5.7 ± 0.5 (3)	0 (2)
Chicken	2	8.4 ± 0.2 (3)	7.5 ± 0.03 (3)	5.5 ± 0.2 (3)	0 (3)
	7	7.8 ± 0.1 (3)	5.4 ± 0.1 (3)	5.1 ± 0.2 (3)	0 (3)
Human 1	2	8.9 (1)	8.5 (1)	6.5 (1)	0 (1)
	7	8.7 (1)	7.3 (1)	3.3 (1)	0 (1)
Human 2	2	1.2 ± 1.2 (2)	0 (1)	0 (2)	0 (2)
	7	0 (2)	0 (1)	0 (2)	0 (2)
Human 3	2	8.3 (1)	7.8 (1)	6.1 (1)	0 (1)
	7	8.7 (1)	6.1 (1)	3.2 (1)	0 (1)

[a] expressed as log10 CFU/ml

grown in TBW as compared to the conventionally-grown bacteria would perhaps reveal more information on phenotypic modulation and provide us with an organism for study,which is more like the one involved in the natural infection. A model system using tissue culture with TBW as the bathing fluid would provide a model similar, if not better than the current animal models. Another area of research could concentrate on supplementation studies with *B. pertussis* and *B. parapertussis* to investigate the question of which essential nutrients are missing from the TBW. This could lead to the development of a new artificial medium, solid or liquid, based upon the different TBW and tested as an isolation and cultivation medium for each of the four bordetellae. Further studies to reveal which alternative energy sources are being exploited by *B. bronchiseptica* and *B. avium* would also be a potential area of research. Thinking laterally, this work could be extended to other respiratory tract pathogens such as *Pasteurella* and *Haemophilus* species.

Part 2. Chemical analysis of TBW

It is known that the *Bordetella* require amino acids and nicotinic acid for growth and that since three of the four species grew in the TBW then these nutrients must be present. The fact that there were differences in the extent of growth in the different fluids and between species of *Bordetella* suggested that nutritional differences, in terms of amino acids and nicotinic acid, within the TBW existed. To verify these assumptions a series of chemical analysis were employed to quantify the amino acid and nicotinic acid contents of the different TBW samples. Some of the nutrient in the TBW could have come from blood contamination and the haemoglobin content of the TBW was measured as an indicator of this contamination. Despite extensive searching, no relevant literature dealing with analysis of TBW for these particular nutrients has been reported. There is vast literature on analysis of human and animal TBW for proteins, lipids and immunoglobulins as was presented in the Introduction. Therefore this section will focus only on the amino acids and nicotinic acid present in the TBW.

Amino acids were found in all of the TBW tested although the concentrations were much lower than those in CL medium (Table 40). In terms of amino acids, and with respect to CL medium, the TBW could be regarded, on average, as a 1 in 500 dilution of the laboratory medium. The nicotinic acid content of the TBW also varied but was again less than that of CL medium (on average, a 1 in 7 dilution). Table 41 shows a comparison between the maximal viable counts recorded for each of the four *Bordetella* and the amino acid contents of the culture fluids. There was a significant relationship between nutrient concentration (both amino acids and nicotinic acid) and growth of *B. bronchiseptica* and *B. avium*. Comparing amino acid concentrations of the various TBW samples with the growth

Table 40 : *Bordetella* nutritional factors in CL medium and TBW

TBW/Media	Average amino acid concentration (mg/ml)	Average nicotinic acid concentration (μg/ml)
CL medium	20.94	4
Horse TBW	0.10	1.06
Mouse TBW	0.07	0.84
Rabbit TBW 2/3	0.02	0.23
Sheep TBW 1	0.02	0.33
Chicken TBW	0.01	0.20
Dog TBW	0.03	0.54
Human TBW	0.06	0.65

Table 41 : Comparison of the amino acid concentrations of TBW with the maximum viable counts[a] recorded for *B. bronchiseptica, B. avium* and *B. parapertussis*

		Maximum $\log_{10}$ CFU/ml of :		
Fluid	Amino acid (mg/ml)	*B. bronchiseptica*	*B. avium*	*B. parapertussis*
CL medium	20.94	9.4	9.4	9.5
TBW				
Horse	0.10	9.1	8.8	< 1.7
Mouse	0.07	8.8	8.8	6.3
Human[b]	0.05	8.8	8.2	6.3
Dog	0.03	7.9	7.9	< 1.7
Rabbit	0.02	8.2	7.6	6.6
Sheep	0.02	7.9	8.0	6.7
Chicken	0.01	8.4	7.5	5.5

[a] expressed as log10 CFU/ml

[b] average of two different samples

achieved by *B. bronchiseptica* in each fluid a Kendall correlation statistic of 0.667 was recorded. For *B. avium* the correlation statistic was 0.950, indicating a closer relationship. A similar pattern was seen when the growth of each organism was compared to the amount of nicotinic acid present. Kendall correlation statistics of 0.551 and 0.927 were recorded for *B. bronchiseptica* and *B. avium* respectively.

B. parapertussis did not show any relationship between the amino acid or nicotinic acid content of TBW and the viable count. The counts recorded for this bacterium, in all of the TBW, were similar and no visible turbidity was observed. This suggested that certain essential amino acids may not have been present at the correct concentrations to give as good growth as in the CL medium. Also, *B. pertussis* failed to grow in any of the TBW, thereby strengthening the argument made for *B. parapertussis*.

Therefore there appears to be a definite relationship between the amino acid content of the TBW and ability to support the growth of *B. bronchiseptica* and *B. avium* but not for *B. parapertussis* or *B. pertussis*. The possibility that the two animal and bird pathogens are growing on both amino acids and organic acids cannot be discounted.

Preliminary experiments using a technique of Isotachophoresis (Shan *et al*, 1985 and Andrews, 1988) revealed the presence of compounds in some of the TBW which could be fatty acids. However no detailed study was made and it would be unwise to assume that these results were valid without replication.

It was concluded that the level of blood contamination in the TBW samples was insufficient to affect its overall nutritional content (Table 32). In the literature there was some information on the amino acid and nicotinic acid content of whole blood. In the Documenta Geigy (1970), blood is regarded as having an amino acid content of 60 mg/l and a nicotinic acid content of 6.3 mg/l. The amino acid values are similar to those

recorded for the TBW samples tested in these studies, while the nicotinic acid values are about 6-fold greater in blood. Also documented is the fact that whole human adult blood contains fatty acids at a concentration of 80 mg/l. This would suggest that the TBW may also contain levels of fatty acids similar to those in blood providing more encouragement for further investigations with the Isotachophoresis. Both amino acids and fatty acids have been reported in other body fluids such as breast-milk, saliva and urine (see Documenta Geigy, 1970). There also appears to be species differences in the nutritional content of the TBW samples. However in order to verify this, extensive sampling would have to be completed and factors such as the age of animals, enviroment, health and diet considered.

An important factor to consider is that the TBW being dealt with in this study are a dilute form of the natural fluid of the respiratory tract. Table 42 shows estimated dilution factors for dog, human and chicken TBW in terms of protein concentrations. The protein concentration of each TBW species calculated in this study, was compared to those reported for undiluted secretions in the literature. The TBW used in this study could be regarded as a 1 in 3 (chicken), 1 in 5 (human) and a 1 in 68 (dog) dilution of their respective whole respiratory tract secretions. Therefore it is possible that the undiluted form of TBW could support the growth of the *Bordetella* species to a greater extent than is reported. It may also be the case that *B. pertussis* will grow in these secretions without the need for supplementation. However, the concentration of amino acids reported by Potter *et al* (1967) of 31.8 $\pm$ 166 mM/L for whole human secretions, was less than that recorded for one of the human samples used in this research (i.e. human TBW no. 2 contained 0.73 mM LE). This suggests that within-species variation may occur, with regards to the nutritional content of secretions, although extensive sampling would be necessary before any definite conclusions could be made.

Table 42 : Estimation of dilution factors for TBW samples based upon reports, in the literature, for undiluted respiratory-tract secretions (RTS).

Species	Reference	Protein concentration (µg/ml) of :		
		RTS[a]	TBW[b]	Dilution factor
Dog	Reasor *et al*, 1978	41, 000	599	1 in 68
Chicken	Boyd *et al*, 1944	260	88	1 in 3
Human	Matthews *et al*, 1963	10, 000	2018	1 in 5

[a] from the literature

[b] from this research

This work has shown that a range of TBW species contained sufficient nutrients to support the growth of three of the four *Bordetella* species. Supplementation of these fluids improved growth and, in the case of *B. pertussis,* resulted in a greater viable count than in CL medium alone. Future elucidation of the amino acids present in the TBW and of the fatty acid content are necessary to establish which amino acids are missing or not present in sufficient concentrations to support the growth of all four *Bordetella* species, and whether *B. bronchiseptica* and *B. avium* are utilizing an alternative energy source as is suggested.

Part 3. The growth of *B. bronchiseptica* in PBS and natural waters

This study has shown that *B. bronchiseptica*, despite its generally-accepted role as an obligate respiratory-tract parasite, is able to increase in viable count and survive in a variety of natural waters, in RGW and in PBS. Although most of our observations were made with strain, no. 5376, which had been subjected to long maintenance in the laboratory, similar growth in lake water and in PBS was seen with fresh isolates from domestic animals and with the type strain NCTC 452. It seems, therefore, that the ability of *B. bronchiseptica* to grow in these several types of low-nutrient fluids is a species characteristic and is not restricted to particular selected strains.

The extent of increase in viable count in the various fluids to which no nutrients had been added could be separated into two categories, viz in the region of 5- to 8-fold, as observed in RGW and sea water, and around 1000-fold, as recorded with PBS and all the samples of lake and pond water. It seems likely that this distinction reflects order-of-magnitude differences in the nutrient concentrations in the respective fluids. Such quantitative differences may also be accompanied by qualitative differences in the actual nutrients present.

With other species of bacteria there is an extensive literature on increases in viable count under starvation conditions (Postgate and Hunter; 1962 Jones and Rhodes-Roberts, 1981; Morita, 1982; Harder and Dijkhuizen, 1983). For example, Morita and Novitsky (Morita, 1982; Novitsky and Morita, 1977, 1978) reported 4-fold increases, over 1 week, in the CFU/ml of a marine vibrio in a starvation menstruum. Various authors have used terms such as starvation-survival (Morita, 1982), cryptic growth (Postgate and Hunter, 1962), re-growth (Strange *et al*,

1961) and cannibalism (Harrison, 1961), to describe the observed increases in viable count of bacteria in environments with very low concentrations of nutrient. It is possible that a variety of mechanisms may be involved, such as extremely efficient scavenging of trace nutrients, including those such as ammonia which may be absorbed from the atmosphere (Jones and Rhodes-Roberts, 1981); reductive division, i.e. cell division with a reduction in cell size (Morita, 1982); endogenous respiration (Pine, 1980); and death of part of a population, with reutilization of leaked nutrients by the survivors (Postgate and Hunter, 1962). Any or all of these mechanisms may have been involved in the 5-fold and 8-fold average increases in viable count of *B. bronchiseptica* observed in RGW and sea water, respectively. With regard to the biological significance of growth and survival of these organisms in the sea water, it may be noted that *B. bronchiseptica* has recently been isolated from seals (Tewes, 1989)

The greater increases in viable count, in the order of 1,000-fold, observed in PBS and lake and pond water presumably reflect the much greater level of nutrients in these fluids than in the fluids just considered. However, even these higher levels are probably only in the region of 1/10,000 to 1/100,000 of those present in a normal *Bordetella* culture medium, such as CL medium. With PBS, it seems that one or more of the analytical-grade salts, rather than the water supplies or the laboratory atmosphere, was the main source of nutrients.

Over 30 years ago, Garvie (1954) reported the growth of *E. coli* and *Aerobacter aerogenes* in PBS made from purified ingredients. Other bacteria either failed to grow or died out. In our studies, the nature of the nutrients in PBS or lake water has not been determined; they may be different from those in conventional media for *Bordetella* species. In this connection it may be noted that *B. bronchiseptica* can grow on ammonia plus lactate or citrate (Proom, 1955), although conventional media for this

species usually supply amino acids as the carbon, nitrogen and, energy source.

The stationary-phase population of *B. bronchiseptica* in PBS and lake or pond water was never greater than about 7 x 10^6 CFU/ml, a concentration insufficient to give visible turbidity. By drying and weighing pelleted cells of *B. bronchiseptica* of known CFU from growth on BG medium for 24h, the mass of an individual bacterium was established as about 0.1 pg. This is assuming that 1 CFU represents a single viable cell of *B. bronchiseptica*. On this basis, the dry weight of 10^6 CFU would be 0.1 µg, and the amount of major nutrients needed to yield this number of cells would be about 0.3 µg. While we have not attempted to analyse or measure the nutrients in the lake or pond water samples, it may be noted that 0.3 µg/ml is 0.3 mg/L and would represent the very low biological oxygen demand expected for the unpolluted waters sampled. Also, the 0.3 mg/L is nutritionally equivalent to a 1/73,000 dilution of the amino acids of CL medium, and is within the range estimated (0.2-2 mg/L) by the experiment in which CL medium was diluted in PBS to the point where it gave no more growth than PBS alone.

The ability of *B. bronchiseptica* strains to survive in the low-nutrient fluids tested was maintained over a period of six months at both 37°C and 10°C. This raises the question of whether it is feasible to assume that *B. bronchiseptica,* and even *B. avium* which shares similar properties, exist in the natural enviroment and may cause infection when ingested by the host species. The ability of other bacteria, particularly in the marine enviroment, to maintain viability over long-term starvation has been well documented although this is the first report of a similar action of *B. bronchiseptica*. In 1970 Ensign reported that *Arthrobacter crystallopoietes* maintained 100 % viability under starvation conditions for 30 days and after 60 days remained 65 % viable. In another study

involving 16 marine isolates Amy and Morita (1983) studied their starvation survival patterns and reported survival times of at least eight months. Also in relation to the marine enviroment, work with the marine vibrio Ant 300 revealed survival times of 70 weeks (Novitsky and Morita, 1978 and Morita 1982) and over 1 year (Novitsky and Morita, 1977). Jones and Rhodes-Roberts (1981) reported that a marine *Pseudomonas* species remained viable for 40 days in a starvation menstruum. Other non-marine organisms include *Campylobacter fetus* subsp. *jejuni* which showed the ability to survive in a number of different biological milieu (Blaser *et al,* 1980) such as bile (two months), faeces (three weeks), urine (five weeks) and water (four weeks). Survival of *B. bronchiseptica* in soil may be for as long as 45 days (Mitscherlich and Marth, 1984), while *B. pertussis* in the same report, survived for shorter periods on various materials; baby food (four days), air (20 h), paper (two days) and plastic (3-5 days).

A more recent investigation generated by the initial findings of this study was undertaken by McCaig (1991). The work involved an investigation into the effect of supplementation of PBS and lake and sea water (LW and SW) on the growth of *B bronchiseptica.* Initially the findings of this thesis were verified, although the viable counts recorded for growth of *B. bronchiseprica* in sea water were greater than those reported originally. This could be explained by the fact that the two samples in question were taken from different locations resulting in possible different organic contents. Supplementation of LW and SW with nicotinic acid, lactate and ammonium chloride was investigated. Of the three potential nutrients nicotinic acid was found to be most limiting and in the presence of all three the final viable count recorded with *B. bronchiseptica* was 40-fold greater in LW alone and 20-fold greater than in SW alone. Even with this supplementation and increased viable count visible turbidity was never

achieved. McCaig (1991) also showed, by means of the BOD test, that LW contained at least 0.8 µg/ml dissolved organic carbon per ml; more than enough to support the reported growth of *B. bronchiseptica.*

During the long-term incubation of *B. bronchiseptica* in PBS and fresh water, the 20 µl platings on BG exhibited two distinct colony forms (Photographs 1 and 2). As the time of incubation in the dilute-nutrient fluids progressed, the large-form colonies of *B. bronchiseptica* on BG agar tended to switch to the smaller form. This could be a response to the lack of nutrients in the PBS or lake water and may represent a method of energy conservation by the bacterium. Further study would be needed to see if the smaller colonies cotained smaller cells e.g in line with the phenomenon of miniaturization of cells which has been reported extensively elsewhere (Amy and Morita, 1983; Morita, 1982; Baker *et al,* 1983 and Marden *et al,* 1985). It may also be the case that a form of antigenic modulation or phenotypic variation is in effect, where *B. bronchiseptica* may switch off expression of its virulence factors and adopt a more economical morphological form in response to the low-nutrient conditions. This method of energy conservation under stressed conditions, such as starvation, has been observed in other bacteria. For example, Morita (1985) reported this phenomenon in marine bacteria in response to a lack of energy source, and Marden and co-workers (1985) showed similar response with three marine isolates.

The effect of temperature on the survival of *B. bronchiseptica* could be important. Towards the end of the observation period the viable counts for *B. bronchiseptica* in the fluids incubated at 37°C decreased and in some cases no viability was recorded. This was in contrast to those incubated at 10°C where the viable count, generally, remained constant over the final three months. Initially, as was to be expected, *B. bronchiseptica* grew more slowly in the fluids incubated at the lower

temperature although the maximum counts, when reached, were similar to those at 37°C. McCaig (1991) presented similar findings when she incubated the bacterium at 18°C. During these long-term starvation studies *B. bronchiseptica* could be recovered from the walls of the culture vessels. This suggests that the viable counts recorded in the experiments reported in this study, and perhaps in other investigations with *B. bronchiseptica*, may be lower than the true value if wall growth or adherence is taken into account.

Part 4. Conclusions and future areas of research

This study, as far as is known, is the first to report on the growth of *Bordetella* species in the natural host fluid from the site of infection. It has been noted that the two less fastidious species, *B. bronchiseptica* and *B. avium*, grew in all TBW samples tested while the two human pathogens either did not grow (*B. pertussis*) or showed a variable response (*B. parapertussis*). No species specificity was demonstrated . Chemical analysis of the TBW showed that at least some of the appropriate nutrients (amino acids and nicotinic acid) were present, although these may not be in the most suitable concentrations for growth of *Bordetella.*

From this work a number of possibilities for future research have emerged. The work with the TBW could be extended to other respiratory tract pathogens such as *Haemophilus* and *Legionella* species. A study of the *Bordetella* cell properties i.e. virulence factors, when grown in TBW compared to those in CL medium would reveal whether or not antigenic modulation occurred in the TBW samples. Preliminary studies of gel profiles and of FHA activity of *B. bronchiseptica* strain no. 11 grown in dog TBW (results not presented), suggested that the bacterium does possess toxin activity, while a number of differences between cells from TBW and those from CL medium were observed in the gel profiles. Supplementation of the TBW would reveal which nutrients were missing and whether when added, the TBW would then be growth-supporting for *B. pertussis*. Also there could be an investigation of possible alternative energy sources being utilized by *B. bronchiseptica* and *B. avium.* Perhaps a new medium could be developed, based upon TBW composition and could be tested in both liquid and solid form for isolation and cultivation purposes.

The other area in which novel discoveries have been made is perhaps more challenging : The ability of *B. bronchiseptica* to grow and

survive over long periods in fresh water. We concluded from these observations that it should be possible for *B. bronchiseptica* to survive in ponds and lakes and hence create a reservoir of infection. From this could emerge an investigation to isolate *B. bronchiseptica* from enviroments in which it is prevalent. For example, in a dog kennel where there is a naturally occurring infection. If *B. bronchiseptica* could be isolated from, say, infected drinking water and this water could be used to initiate infection in other animals, then the evidence for a new mode of transmission would be very strong. Another study could focus on the question of antigenic modulation under the low-nutrient conditions. We found that during long-term incubation of *B. bronchiseptica* in fresh water a smaller morphological form developed. The investigation could focus on a comparison of the two morhological cell types, looking at virulence factors such as HLT and FHA. If it was found that *B. bronchiseptica* is avirulent in these low-nutrient fluids it might help explain why it has never been isolated from other enviromental sources. Another area of research stimulated by this work could be an investigation into the starvation-survival strategies employed by *B. bronchiseptica.* It could be that the efficient scavenging ability of the organism is due to the presence of amino acid binding proteins, similar to those used in iron acquisition, or to dormancy, miniaturization of cells and/or cannibalism. Also, nothing is known about the pathogenicity of this organism when it is grown in these natural fluids. For example, is it possible that *B. bronchiseptica* grown in lake water can initiate an infection when introduced into an appropriate host species and, if so, is this infection similar to that caused by organisms grown in conventional culture media ? A major difficulty encountered here would be the problem of scaling-up to obtain sufficient numbers of cells for the study, particularily for electron microscopy studies.

A further study which has already been described earlier in the

previous section (McCaig, 1991), is the supplementation of the low-nutrient fluids and their chemical examination and could also include *B. avium* which has been shown to possess this self-same ability as *B. bronchiseptica*. It is interesting to note that both *B. bronchiseptica* and *B. avium* were formerly assigned (Pittman and Wardlaw, 1981) to the *Alcaligenes*, a genus of soil and fresh water bacteria with which they share many properties. In fact, the main differential characters additional to habitats are the virulence factors possessed by these bordetellae. This also suggests a new look at the taxonomy of the *Bordetella* and *Alcaligenes* and supports the opinion of De Ley *et al* (1986) who proposed a new family, *Alcaligeneaceae*, encompassing both genera.

REFERENCES

ADAMS, G.K., AHARONSON, E.F. REASOR, M.J. and PROCTOR, D.F. (1976). Collection of normal canine tracheobronchial secretions. *J. Appl. Physiol.* **40** : 247- 249.

ADAMSON, T.M., BOYD, R.D.H., PLATT, H.S. and STRANG.M L.B. (1969). Composition of alveolar liquid in the foetal lamb. *J. Physiol.* **204** : 159-168.

ALONSO, J.M., RAVISSE, P. and BREZIN, C. (1987). *Bordetella pertussis* respiratory infection in C57B1/6 and Balb/c mice : pathophysiology and immune responses. *Fems Microbiology Letters.* **40** : 273-277

AMIES, C.R. (1967). A modified formula for the preparation of Stuart's transport medium. *Can. J. Pub. Hlth.* **58** : 296-300.

AMY, P.S. and MORITA, R.Y. (1983). Starvation-survival patterns of sixteen freshly isolated open-ocean bacteria. *Appl. Env. Micro.* **45** : 1109-1115.

ANDREWS, A.T. (1988). Isotachophoresis (Displacement electrophoresis). In, *Electrophoresis, theory, techniques and biochemical and clinical applications.* pp. 289-302. O.V.P. Oxford.

AOYAMA, T. MURASE, Y. IWATA, T. IMAIZUMI, A. SUZUKI, Y. and SATO, Y. (1986) Comparison of blood-free medium (cylodextrin solid medium) with Bordet-Gengou medium for clinical isolation of *Bordetella pertussis*. *J. Clin. Micro.* **23** : 1046-1048.

ARICO, B and RAPPUOLI, R. (1987). *Bordetella parapertussis* and *Bordetella bronchiseptica* contain transcriptionally silent pertussis toxin genes. *J. Bact.* **169** : 2847-2853.

ARP, L.H. and McDONALD, S.M. (1985). Influence of temperature on the growth of *Bordetella avium* in turkeys and *in vitro*. *Avian Diseases.* **29** : 1066-1077.

ASHWORTH, L.A.E., FITZGEORGE, R.B., IRONS, L.I., MORGAN, C.P. and ROBINSON, A. (1982). Rabbit nasopharyngeal colonization by *Bordetella pertussis* : the effects of immunization on clearance and on serum and nasal antibody levels. *J. Hyg. Camb.* **88** : 475-486.

BAILLEUL, V., RICHERT, C., HAYEM, H. and DEGAND, P. (1977). Properties rheologiques des secretions bronchiques : mise en evidence et roles des polypeptides riches

en proline (PRP). *Clin. Chim. Acta.* **74** : 115-123.

BAKER, R.M., SINGLETON, F.L. and HOOD, M.A. (1983). Effects of nutrient deprivation on *Vibrio cholerae*. *Appl. Env. Micro.* **46** : 930-940.

BARKSDALE, I.S. and SIMPSON, F.P. (1934). A simplified cough-plate method for the early diagnosis of whooping cough. *15th Med. J. Nashville.* **27** : 943.

BEMIS, D.A. and GREISEN, H.A. (1977a). Pathogenesis of canine Bordetellosis. *J. Inf. Dis.* **135** : 753-762.

BEMIS, D.A., GREISEN, H.A. and APPEL, M.J.G. (1977b). Bacteriological variation among *Bordetella bronchiseptica* isolates from dogs and other species. *J. Clin. Micro.* **5** : 471-480.

BEMIS, D.A., CARMICHAEL, L.E. and APPEL, J.G. (1977c). Naturally occuring respiratory disease in a kennel caused by *Bordetella bronchiseptica*. *Cornell Vet.* **67** : 282-293.

BERKHOFF, H.A. and RIDDLE, G.D. (1984). Differentiation of *Alcaligenes*-like bacteria of avian origin and comparison with *Alcaligenes* spp. reference strains. *J. Clin. Micro.* **19** : 477-481.

BLACKALL, P.J. and FARRAH, J.G. (1985). Isolation of *Bordetella avium* from poultry. *Australian Veterinary Journal.* **62** : 370-372.

BLASSER, M.J., HARDESTY, H.L., POWERS, B. and WANG, W.L. (1980). Survival of *Campylobacter fetus* subsp. *jejuni* in bilological milieus. *J. Clin. MIcro.* **11** : 309-313

BOAT, T.F. and CHENG, P.W. (1980). Biochemistry of airway mucus secretions. *Fed. Proc.* **39** : 3067-3074.

BORDET, J. and GENGOU, O. (1906). Le microbe de la Coqueluche. *Ann. Inst Pasteur.* **20** : 731-741.

BOYD, E.M. (1954). Expectorants and respiratory tract fluid. *Pharmacol. Rev* **6** : 521-542.

BOYD, E.M., JACKSON, S., MacLACHLAN, M., PALMER, B., STEVENS, M. and WHITTAKER, J. (1944). The lipid, sodium, chloride, and nitrogen content of the respiratory tract fluid of normal animals. *J. Biol. Chem.* **153** : 435-438.

BRADFORD, W. L. (1938). Experimental infection in the mouse produced by intratracheal inoculation with *Hemophilus pertussis*. *Am. J. Pathol.* **14** : 377-385.

BRASSINNE, M., DEWAELE, A. and GOUFFAUX, M. (1976). Intranasal infection with *Bordetella bronchiseptica* in gnotobiotic piglets. *Res. Vet. Sci.* **20** : 162-166.

BROGAN, T.D., RYLEY, H.C., NOALE, L. and YASSA, J. (1975). Soluble proteins of bronchopulmonary secretions from patients with cystic fibrosis, asthma and bronchitis. *Thorax.* **36**: 72-79.

BUREK, J.D., JERSEY, G.C., WHITEHAIR, C.K. and CARTER, G.R. (1972). The pathology and pathogenesis of *Bordetella bronchiseptica* and *Pasteurella pneumotropica* infection in conventional and germ-free rats. *Lab. Anim. Sci.* **22** : 844-849.

BYRD, L.H., ANAMA, L., GUTKIN, M. and CHMEL, H. (1981). *Bordetella bronchiseptica* peritonitis associated with continuous ambulatory peritoneal dialysis. *J. Clin. Microbiol.* **14** : 232-233.

CARLSON, D.M., IYER, R.N. and MAYO, J. (1970). Aminoff, D., ed. *Blood and tissue antigens,* pp. 229-247. New York: Academic Press.

CASH, H.A., WOODS, D.E., McCULLOUGH, B. JOHANSON, W.G. JR. and BASS, J.A. (1979). A rat model of chronic respiratory infection with *Pseudomonas aeruginosa*. *Am. Rev. Respir. Dis.* **119** : 453-459.

CHEN, W., ALLEY, M.R. and MANKTELOW, B.W. (1989). Experimental pneumonia in mice with *Bordetella parapertussis* isolated from sheep. *J. Comp. Path.* **100** : 77-89.

CHERNICK, W.S. and BARBERO, G.J. (1963). Studies on human tracheobronchial and submaxillary secretions in normal and pathophysiological conditions. *Ann. NY. Acad. Sci.* **106** : 698-708.

COHEN, S.M. and WHEELER, M.W. (1946). Pertussis vaccine prepared with phase-I cultures grown in fluid medium. *Amer. J. Publ. Hlth.* **36** : 371-376.

COLEMAN, K.D. and WETTERLOW, L.H. (1986). Use of implantable intraperitoneal diffusion chambers to study *Bordetella pertussis* pathogenisis : growth and toxin production in vivo. *J. Inf. Dis.* **154** : 33-39.

CONNOR, J.D. (1986). Pertussis. *Infectious diseases and medical microbiology* (2nd. ed.), pp. 787-792. Braude. W.B. Saunders.

COOTE, J.G. (1991). Antigenic switching and pathogenicity : enviromental effects on virulence gene expression in *Bordetella pertussis*. *J. Gen. Micro.* (in press)

COOTE, J.G. and BROWNLIE, R.M. (1988). Genetics of virulence of *Bordetella pertussis*. In, Wardlaw, A.C. and Parton, R. (eds.), *Pathogenesis and Immunity in Pertussis*, pp. 39-67. John Wiley and Sons Ltd.

COWMAN,R.A., FITZGERALD, R.J., PERRELLA, M.M. and CORNELL, A.H. (1977). Human saliva as a nitrogen source for oral streptococci. *Caries Res.* **11** : 1-8.

CRUIKSHANK, J.C. and FREEMAN, G.G. (1937). Immunising fractions isolated from *Hemophilus pertussis*. *Lancet.* **ii** : 567.

DAVIS, B.D. and DUBOS, R.J. (1946). Interaction of serum albumin, free and esterified oleic acid and lipase in relation to cultivation of the tubercle bacillus. *Arch. Bioch.* **11** : 201-207.

DAVIS, G.S., GIANCOLA, M.S. COSTANZA, M.C. and LOW, R.B. (1982). Analyses of sequential bronchoalveolar lavage samples from healthy human volunteers. *Am Rev. Res. Dis.* **126** : 611-616.

DAWSON, B., FARNWORTH, E.H., McLEOD, J.W. and NICHOLSON, D.E. (1951). Observations on the value of the Bordet-Gengou medium for the cultivation of *Hemophilus pertussis*. *J. Gen Microbiol.* **5** : P408.

DAY, S.E.J., VASLI, K.K., RUSSELL, R.J. and ARBUTHNOTT, J.P (1980). A simple method for the study *in vivo* growth of bacterial growth and accompanying host response. *J. Infect.* **2** : 39-51.

DEBRE, R. , MARIE, J. and PRETET, H. (1928). Les races de bacilles de Bordet-Gengou isolees a Paris. *C.R. Soc. Biol. Paris.* **98** : 761.

DE JONG, M.F. and BORST, G.H.A. (1985). Selective medium for the isolation of *P. multocida* and *B. bronchiseptica*. *Vet. Rec.* **116** : 167.

DE JONG, M.H., Van Der HOEVEN, J.S., Van OS, J.H. and OLIJVE, J.H. (1984). Growth of oral *Streptococcus* species and *Actinomyces viscous* in human saliva. *Appl. Env. Micro.* **47** (5) : 901-904.

DE LEY, J., SEGERS, P., KERSTERS, K. MANNHEIM, W. and LIEVENS, A. (1986). Intra- and intergeneric similarities of the *Bordetella* ribosomal ribonucleic acid cistrons : proposal for a new family *Alcaligenaceae*. *Int. J. Syst. Bact.* **36** : 405-414.

DOCUMENTA GEIGY. (1970). Scientific Tables, pp. 572-578.

DOLBY, J.M. (1962). Passive protection of mice against intracerebral infections with *Bordetella pertussis*. *J. Hyg. Camb.* **70** : 707-718.

DOLBY, J.M. and STANDFAST, A.F.B. (1961). The intracerebral infection of mice with *Bordetella pertussis*. *J. Hyg. Camb.* **59** : 205-216.

DOLBY, J.M., THOW, D.C.W. and STANDFAST, A.F.B. (1961). The intranasal infection of mice with *Bordetella pertussis*. *J. Hyg. Camb.* **59** : 191.

ELDERING, G. and KENDRICK, P.L. (1938). *Bacillus parapertussis* : A species resembling both *B. pertussis* and *B. bronchiseptica* but identical with neither. *J. Bact.* **35** : 561-572.

ELDERING, G. and KENDRICK, P.L. (1952). Incidence of parapertussis in the Grand Rapids area as indicated by 16 years experience with diagnostic cultures. *Am. J. Pub. Hlth.* **42** : 27-31.

ENSIGN, J.C. (1970). Long-term starvation survival of rod and spherical cells of *Arthrobacter crystallopoietes*. *J. Bact.* **103** : 569-577.

ENSMINGER, P. W., CULBERTSON, C. G. & POWELL, H. M. (1953). Antigenic *Hemophilus pertussis* vaccines grown on charcoal agar. *J. infect. Dis.* **93**, 266.

FAREEDUDDIN, A. and CALDER, M.A. (1984). Isolation of *Bordetella pertussis* : benefits of using both Bordet-Gengou and charcoal agar media. *J. Clin. Pathol.* **37** : 1071-1072.

FARRELL, L. and TAYLOR, E.M. (1945). Notes on the production of phase I pertussis vaccine on fluid medium. *Canad. J. Publ. Hlth.* **36** : 326-327.

FARRINGTON, D.O. and SWITZER, W.P. (1977). Evaluation of nasal culturing proceedures for the control of atrophic rhinitis caused by *Bordetella bronchiseptica* in swine. *J. A. V. M. A.* **170** : 34-36.

FERRY,N.S. (1911). Etiology of canine distemper. *J. Infect. Dis.* **8** : 399-420.

FILION, R., CLAUTIER, S., VRANCKEN, E.R. and BERNIER, G. (1967). Infection respiratoire du dindonneau causee par un microbe apparente au *Bordetella bronchiseptica*. *Can. J. Comp. Med. Vet. Sci.* **31** : 129-134.

FISHER, S. (1955). Multiplication of *H. pertussis* in the mouse lung following intranasal infection. *Aust. J. Exp. Biol.* **33** : 609-628.

FISK, S.K. and SOAVE, O.A. (1973). *Bordetella bronchiseptica* in laboratory cats from central California. *Lab. Anim. Sci.* **23** : 33-35.

FRIEDMAN, R.L. (1988). Pertussis : the disease and new diagnostic methods. *Clin. Micro. Rev.* **1** : 365-376.

FUZI, M. (1973). Selective isolation of *Bordetella bronchiseptica*. *Zbl. Bakt. Hyg. I. Abt. Orig. A.* **224** : 270-272.

GARVIE, E.I. (1954). The growth of *Escheichia coli* in buffer substrate and distilled water. *J. Bact.* **69** : 393-398.

GHOSH, H.K. and TRANTER, J. (1979). *Bordetella bronchicanis* (*bronchiseptica*) infection in man : review and a case report. *J. Clin. Pathol.* **32** : 546-548.

GLADSTONE, G.P. and GLENCROSS, E.J.G. (1960). Growth and toxin production of staphylococci in cellophane sacs *in vivo*. *Br. J. Exp. Pathol.* **41** : 313-333.

GOLDNER, M. JAKUS, C.M. RHODES, H.K., and WILSON, R.J. (1966). The amino acid utilisation by phase 1 *Bordetella pertussis* in a chemically defined medium. *J. Gen. Micro.* **44** : 439-444.

GOODNOW, R.A. (1980). Biology of *Bordetella bronchiseptica*. *Microbiol. Rev.* **44** (4) : 722-738.

GOPAUL, D.L. and YU, C. (1974). Notes on the isolation of *Bordetella pertussis* and

Bordetella parapertussis. *Can. J. Med. Tech.* **36** : 135-138.

GREENWOOD, D. and O' GRADY, F. (1978). An *in vitro* model of the urinary bladder. *J. Antimic. Chem.* **4** : 113-120.

HARDER, W. and DIJKHUIZEN, L. (1983). Physiological responses to nutrient limitation. *Ann. Rev. Micro.* **37** : 1-23.

HARRISON, A.P., Jr. (1961). The response of *Bacterium lactis aerogenes* when held at growth temperature in the absence of nutriment: an analysis of survival curves. *Proc. Roy. Soc.* **152** : 418-428.

HENDERSON, V.E. and TAYLOR, A.H. (1910). Expectorants. *J. Pharmacol. and Exper. Therap.* **2** : 153-164.

HILDING, A.C. (1966). A method for collecting near-normal secretions from the tracheobronchial tree in experimental animals. *Arch. Environ. Health.* **12** : 793-794.

HINZ, K.H. and KUNJARA, C. (1977). *Haemophilus avium* , a new species from chickens. *Int. J. Sys. Bact.* **27** (4) : 324-329.

HINZ, K.H., GLUNDER, G. and LUDERS, H. (1978). Acute respiratory disease in turkey poults caused by *Bordetella bronchiseptica* - like bacteria. *Vet. Rec.* **103** : 262-263.

HOFSTAD, M.S. and JESKA, E.L. (1985). Immune response of poults following intranasal inoculation with Artvax™ vaccine and a formalin-inactivated *Bordetella avium* bacterin. *Avian Diseases.* **29** : 746-754.

HOPPE, J.E. and SCHWADERER, J. (1989). Comparison of four charcoal media for the isolation of *Bordetella pertussis*. *J. Clin. Micro.* **27** : 1097-1098.

HORNIBROOK, J.W. (1939). Cultivation of phase I *H. pertussis* in a synthetic liquid medium. *Pub. Hlth Rep.* **54** : 1847-1851.

HORNIBROOK, J.W. (1940). Nicotinic acid as a growth factor for *H. pertussis*. *Proc. Soc. Exp. Biol.* N.Y. **45** : 598.

HOUSER, E.D. and BERRY, L.J. (1961). The pathogenesis of staphylococcus infections. I. The use of diffusion chambers in establishing the role of staphylococcus toxins. *Br. J. Exper. Path.* **109** : 24-30.

HOYER, B.H. and McCULLOUGH, N.B. (1968). Homologies of deoxyribonucleic acids from *Brucella ovis* canine abortion organisms, and other *Brucella* species. *J. Bact.* **96** : 1783-1790.

IMAIZUMI, A. SUZUKI, Y. ONO, S., SATO, H. and SATO, Y. (1983). Effect of Hepatikis(2,6-0-Dimethyl)ß-Cyclodextrin on the production of Pertussis Toxin by *Bordetella pertussis*. *Inf.Imm.* **41** (3) : 1138-1143.

JACKSON, C. and JACKSON, C.L. (1934). Bronchoscopy, esophagoscopy and gastroscopy. W.B. saunders company, Philadelphia.

JEBB. W.H.H. and TOMLINSON, A.H. (1955). The nutritional requirements of *Haemophilus pertussis*. *J. Gen. Micro.* **13** : 1-8.

JEBB. W.H.H. and TOMLINSON, A.H. (1957). The minimal amino acid requirements of *Haemophilus pertussis*. *J. Gen. Micro.* **17** : 59-63.

JOHNSON, R. and SNEATH, P.H.A. (1973). Taxonomy of *Bordetella* and related organisms of the families *Achromobacteraceae*, *Brucellaceae*, and *Neisseriaceae*. *Int. J. Syst. Bact.* **23** : 381-404.

JONES, K.L. and RHODES-ROBERTS, M.E. (1981). The survival of marine bacteria under starvation conditions. *J. Appl. Bact.* **50** : 247-258.

KALTREIDER, H.B. (1976). State of the art: Expression of the immune mechanisms in the lung. *Am. Rev. Respir. Dis.* **113** : 347-479.

KALTREIDER, H.B. and CHAN, M.K.L. (1976). The class specific immunoglobulin composition of fluids obtained from various levels of the canine respiratory tract. *J. Immun.* **116** : 423-429.

KAYE, D. (1968). Antibacterial activity of Human Urine. *J. Clin. Invest.* **47** : 2374-2390.

KEIMOWITZ, R.I. (1964). Immunoglobulins in normal human tracheobronchial washings. *J. Lab. Clin. Med.* **63** (1) : 54-59.

KEMENY, L.J. (1972). Experimental atrophic rhinitis produced by *Bordetella bronchiseptica* culture in young pigs. *Cornell. Vet.* **62** : 477-485.

KENDRICK, P.L. and ELDERING, G. (1969). Microbiology of whooping cough. pp. 259-282. In : Ocklitz, H.W. (ed.), Der Keuchhusten, vol. 8. Infectionskrankheiten und ihre Erreger. Jena : Veb Gustav Fischer.

KERSTERS, K. HINZ,K.H. HERTLE, A. SEGERS, P. LIEVENS, A. SIEGMAN, O. and DE LEY, J. (1984). *Bordetella avium* sp. nov. , Isolated from the respiratory tracts of turkeys and other birds. *Int. J. Sys. Bact.* **34** (1) : 56-70.

KOSHIMIZU, K., KODAMA, Y. and OGATA, M. (1973). Studies on the etiology of infectious atrophic rhinitis of swine V. Experimental *Bordetella bronchiseptica* infection in conventional piglets. *Jap. J. Vet. Sci.* **35** : 223-229.

KRUMWIEDE, C., MISHULOW, L. and OLDENBUSCH, C. (1923). The existance of more than one immunologic type of *B. pertussis*. *J. Infect. Dis.* **32** : 22P

KURZYNSKI, T.A., BOEHM, D.M., ROTT-PETRI, J.A., SCHELL, R.F. and ALLISON, P.E. (1988). Comparison of modified Bordet-Gengou and modified Regan-Lowe media for the isolation of *Bordetella pertussis* and *Bordetella parapertussis*. *J. Clin. Micro.* **26** : 2661-2663.

KUWAJIMA, Y. MATSUI, T. and KISHIGAMI, M. (1957). The growth-supporting effects of some anion exchange resins for phase 1 *Haemophilus pertussis*. *Japan. J. Microb.* **1** (4) : 375-381.

LACEY, B.W. (1954). A new selective medium for *Hemophilus pertussis* containing a diamidine, sodium fluoride and penicillin. *J. Hyg. Camb.* **52** :273-304.

LAPIN, J. H. (1943). Historical. In. *Whooping Cough*, pp.3-7. Thomas, C.C. (ed.).

LAUTROP, H. (1960). Laboratory diagnosis of whooping-cough or *Bordetella* infections. *Bulletin of the World Health Organization*. **23** : 15-35.

LAUTROP, H. (1971). Epidemics of Parapertussis. 20 years' observations in Denmark. *Lancet.* **1** : 1195-1198.

LAWSON, G. McL. (1939). Immunity studies in pertussis. *Am. J. Hyg.* (B) **29** : 119-131.

LESLIE, P.H., and GARDNER, A.D. (1931). The phases of *Haemophilus pertussis*. *J. Hyg.* **31** : 423-434.

LINNEMANN, Jr. C.C. (1979). Host-Parasite interactions in Pertussis. In, *Third International Symposium on Pertussis.* pp.3-18.

LINNEMANN, Jr. C.C. and PERRY, E.B. (1977). *Bordetella parapertussis*: Recent experience and a review of the literature. *Am. J. Dis. Child.* **131** : 560-563.

LOW, R.B., DAVIS, G.S. and GIANCOLA, M.S. (1978). Biochemical analysis of bronchoalveolar lavage fluids of healthy human volunteer smokers and nonsmokers. *Am. Rev. Res. Dis.* **118** : 863-875.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951). Protein measurement with the folin phenol regent. *J. Biol. Chem.* **193** : 265-275.

LUCAS, A.M. and DOUGLAS, L.C. (1934). Principles underlying ciliary activity in the respiratory tract.II. Mucous clearance in man, monkey and other mammals. *Arch. Otolaryngol.* **20** : 518-541.

Mc CAIG, A. (1991). Growth and survival of *Bordetella bronchiseptica* in lakewater. *Senior Honours project.* Department of Microbiology, University of Glasgow.

Mc CANDLISH, I.A.P., THOMPSON, P.H., CORNELL, H.J.C. and WRIGHT, N.G. (1978). A study of dogs with kennel cough. *Vet. Rec.* **102** : 298-301.

MACDONALD, H. and MACDONALD, E.J. (1933). Experimental pertussis. J. Inf. Dis. **53** : 328-330.

MACKIE, T.J. and McCARTNEY, J.E. (1953). Handbook of practical bacteriology, 9th edn. pp. 189. Edinburgh: E. and S. Livingstone Ltd.

MACKIE, T.J. and McCARTNEY, J.E. (1989). Practical medical microbiology, 13th edn. pp. 21 and 41. Churchill Livingstone.

MADSEN, T. (1925). Whooping cough. Its bacteriology, diagnosis, prevention and treatment. *Boston Med. Surg. J.* **192** : 50.

MARDEN, P., TUNLID, A., MALMCRONA-FRIBERG, K., ODHAM, G. and KJELLEBERG, S.

(1985). Physiological and morphological changes during short term starvation of marine bacterial isolates. *Arch. Micro.* **142** :326-332.

MASSON, P.L. and HEREMANS, J.F. (1973). Sputum proteins. In, Dulfano, M.J., ed. *Sputum: fundamentals and clinical pathology.* pp. 412-475. Springfield, IL: Charles C Thomas.

MASSON, P.L. HEREMANS, J.F. and PRIGNOT, J. (1965). Studies on he proteins of human bronchial secretions. *Biochim. Biophys. Acta.* **III** : 466-478.

MATTHEWS, L.W., SPECTOR, S., LEMM, J. and POTTER, J.L. (1963). Studies on pulmonary secretions. I. The overall chemical composition of pulmonary secretions from patients with cystic fibrosis, bronchiectisis and laryngectomy. *Am. Rev. Res. Dis.* **88** : 199-204.

MAZLOUM, H.A. and ROWLEY, D. (1955). The growth requirements of *Hemophilus pertussis* on solid media. *J. Path. Bact.* **70** : 439-447.

MEYER, R.C. and BEAMER, P.D. (1973). *Bordetella bronchiseptica* infections in germ-free swine : an experimental pneumonia. *Vet. Path.* **10** : 550-556.

MISHULOW, L., SHARPE, L.S. and COHEN, L.L. (1953). Beef-heart charcoal agar for the preparation of pertussis vaccines. *Amer. J. Pub. Hlth.* **43** : 1466-1472.

MITSCHERLICH, E. and MARTH, E.H. (1984). Bacteria and Rickettsiae important in human and animal health. In *Microbial survival in the enviroment.* pp. 45-47. Springer-Verlag, Berlin Heidelberg, New York, Tokyo.

MIYAMOTA, H., KAMEZAWA, K., YUKINO,S. and AKAMA, K. (1954). On mucin-added liquid media for *Hemophilus pertussis. Gumma J. Med. Sci.* **3** : 97-103.

MOORE, S. and STEIN, W.H. (1948). Photometric ninhydrin method for use in the chromatography of amino acids. *J. Biol. Chem.* **176** :367-388.

MORENO-LOPEZ, M. (1952). El Genero *Bordetella*. *Microbiologia Espanola.* **5** : 177-181.

MORGAN, T.E. (1971). Pulmonary surfactant. *New. Eng. J. Med.* **284** : 1185-1193.

MORITA, R.Y. (1982). Starvation-survival of heterotrophs in the marine enviroment.

pp. 171-198. In K.C. Marshall (ed.), Advances in microbial ecology, vol. 6. Plenum Publishing Corp., New York.

MORITA, R.Y. (1985). Starvation and miniturization of heterotrophs, with special emphasis on maintenance of the starved viable state. In, Fletcher,M., and Floodgate, D. (eds.), *Bacteria in their natural enviroments*, pp. 111-130 Academic Press.

MORRILL, W.E., BARBAREE, J.M., FIELDS, B.S., SANDEN, G.N. and MARTIN, W.T. (1988). Effects of transport temperature and medium on recovery of *Bordetella pertussis* fron nasopharyngeal swabs. *J. Clin. Micro.* **26** : 1814-1817.

MUSSER, J.M., HEWLETT, E.L., PEPPLER, M.S. and SELANDER, R.K. (1986). Genetic diversity and relationships in populations of *Bordetella* spp. *J. Bact.* **166** : 230-237.

MUSSER, J.M., Bemis, D.A., ISHIKAWA,H. and SELANDER, R.K. (1987). Clonal diversity and host distribution in *Bordetella bronchiseptica*. *J. Bact.* **169** : 2793-2803.

NICOLL, A. and RUDD, P (1989). Whooping cough. In *British Paediatric Association Manual on Infections and Immunization in Children*, pp. 148-151. Oxford Medical Publications.

NOVITSKY, J.A. and MORITA, R.Y. (1977). Survival of psychrophillic marine vibrio under long-term nutrient starvation. *Appl. Envir. Micro.* **33** : 635-641.

NOVITSKY, J.A. and MORITA, R.Y. (1978). Possible strategy for the survival of marine bacteria under starvation conditions. *Mar. Biol.* **48** : 289-295.

NWANKWO, M.U., OFFOR, E., OKOLO, A.A. and OMENE, J.A. (1988). Bacterial growth in expressed breast milk. *Annals. Trop. Ped.* **8** : 92-95.

PAPASIAN, C.J., DOWNS, N.J., TALLEY, R.L., ROMBERGER, D.J. and HODGES, G.R. (1987). *Bordetella bronchiseptica* bronchitis. *J. Clin. Micro.* **25** : 575-577.

PARTON, R. (1991). Changing perspectives on pertussis and pertussis vaccination. *Rev. Med. Micro.* **2** : 121-128.

PERRY, W.F. and BOYD, E.M. (1941). A method for studying expectorant action in

animals by direct measurement of the output of respiratory tract fluids. *J. Pharmacol. Ther.* **73** : 65-77.

PINE, M.J. (1980). Turnover of microbial cell proteins and their peptide residues, pp. 459-490. *In* J.W. Payne (ed.), Microorganisms and nitrogen sources. John Wiley and Sons, Ltd., New York.

PITTMAN, M. (1984). Genus *Bordetella* Moreno-Lopez, 1952. *Bergey's Manual of Systematic Bacteriology.* **2** : 388-393.

PITTMAN, M. and WARDLAW, A.C. (1981). The genus *Bordetella.* In, Starr, M.P., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H.G.(eds). *The prokaryotes. A handbook on habits, isolation and identification of bacteria,* pp. 1075-1085. Springer-Verlag Publications.

POLLOCK, M.R. (1947). The growth of *H. pertussis* on media without blood. *Br. J. Exp. Path.* **28**: 295.

POSTGATE, J.R. and HUNTER, J.R. (1962). The survival of starved bacteria. *J. Gen. Micro.* **29** : 233-263.

POTTER, J.L., MATTHEWS, L.W., LEMM, J. and SPECTOR, S. (1963). Human pulmonary secretions in health and disease. *Ann. NY. Acad. Sci.* **106** : 692-697.

POTTER, J.L., MATTHEWS, L.W., SPECTOR, S. and LEMM, J. (1967). Studies on pulmonary secretions. II. Osmolarity and the ionic enviroment of pulmonary secretions from patients with cystic fibrosis, bronchiectasis and laryngectomy. *Am. Rev. Res. Dis.* **88** : 83-87.

POVITSKY, O.R. (1923). Improved methods for the isolation and later cultivation of B. pertussis. *J. Infect. Dis.* **32** : 8-14.

POWELL, H.M., CULBERTSON, C.G. and ENSMINGER, P.W. (1951). Charcoal agar culture medium for preparing *Haemophilus pertussis* vaccine. *Publ. Hlth. Rep. Wash.* **66** : 346-348.

PRATTER, M.R. and IRWIN, R.S. (1979). Transtracheal aspiration: guidelines for safety. *Chest* **76** : 518-520.

PRESTON, N.W., TIMEWELL, R.M. and CARTER, E.J. (1980). Experimental pertussis infection in the rabbit: similarities with infections in primates. *J. Infect.* **2** : 227-235.

PROOM, H. (1955). The minimal nutritional requirements of organisms of the genus *Bordetella* Lopez. *J. Gen. Microbiol.* **12** : 63-75.

RAMIREZ, J.R., SCHWARTZ, B., DOWELL, A.R. and LEE, S.D. (1971). Biochemical composition of human pulmonary washings. *Arch. Intern. Med.* **127** : 395-400.

REASOR, M.J., ADAMS III, G.K., PROCTOR, D.F. and RUBIN, R.J. (1978). Tracheobronchial secretions collected from intact dogs : I. Protein and mucous glycoprotein composition. *J. Appl. Physiol.* **45** : 182-189.

REGAN, J. and LOWE, F. (1977). Enrichment medium for the isolation of *Bordetella*. *J. Clin. Micro.* **6** (3) : 303-309.

REYNOLDS, H.Y. and NEWBALL, H.H. (1974). Analysis of proteins and respiratory cells obtained from human lungs by bronchial lavage. *J. Lab. Clin. Med.* **84** : 559-573.

RICHARDSON, P.S. and PHIPPS, R.J. (1981). The anatomy, physiology, pharmacology and pathology of tracheobronchial mucus secretion and the use of expectorant drugs in human disease. In Widdicombe, J.G. (ed.), *Respiratory Pharmacology*, pp. 437-476. Section 104 of the *International Encyclopedia of Pharmacology and Therapeutics*. Pergamon Press, Oxford.

RICHARDSON, P.S. (1988). The function of the airway mucosa. In, W. Donachie, E. Griffiths and J. Stephen (eds.). *Bacterial Infections of Respiratory and Gastrointestinal Mucosae*, pp. 1-8. IRL Press, Oxford.

RIMLER, R.B. and SIMMONS, D.G. (1983). Differentiation among bacteria isolated from turkeys with coryza (rhinotracheitis). *Avian Diseases* **27** : 491-500.

ROBERTS, S.A. and SEVERN, M. (1978). Bacterial growth in raw and pasteurised human milk. *Br. Med. J.* **1** : 1196.

ROWATT, E. (1955). Amino acid metabolism in the genus *Bordetella*. *J. Gen. Micro.* **13** : 552-560.

ROWATT, E. (1957a). The growth of *Bordetella pertussis* : a review. *J. Gen. Micro.* **17** (2) : 297-326.

ROWATT, E. (1957b). Some factors affecting the growth of *Bordetella pertussis*. *J. Gen. Micro.* **17** (2) : 279-296.

RUTTER, J.M. (1981). Quantitative observations on *Bordetella bronchiseptica* infection in atrophic rhinitis of pigs. *Vet. Rec.* **108** : 451-454.

RUTTER, J.M. (1985). Atrophic rhinitis in swine. *Adv. Vet Sci. Comp. Med.* **29** : 239-279.

SAHU, S. and LYNN, W.S. (1977). Lipid composition of airway secretions from patients with asthma and patients with cystic fibrosis. *Am. Rev. Respir. Dis.* **115** : 233-239.

SATO, Y., IZUMIYA, K., SATO, H., COWELL, J.L. and MANCLARK,C.R. (1980). Aerosol infection of mice with *Bordetella pertussis*. *Inf. immun.* **29** : 261-266.

SCHULTZ, H.E. and HEREMANS, J.F. (1966). Molecular biology of human proteins, vol 1, p. 816. New York, Elsevier Publishing Co.

SHAH, H.N., NASH, R.A., and HARDIE, J.M. (1985). Detection of acidic end products of metabolism of anaerobic gram-negative bacteria. In, Goodfellow, M. and Minnitain, D.E. (eds.), *Chemical methods in bacterial systematics*. Soceity for Applied Bacteriology, Technical series no. 20.

SHIMIZU, T., NAKAGAWA, M., SHIBATA, S. and SUZUKI, K. (1970). Atrophic rhinitis produced by intranasal inoculation of *Bordetella bronchiseptica* in hysterectomy produced colostrum-deprived pigs. *Cornell. Vet.* **61** : 696-705.

SILVERTHORNE, N. and CAMERON, C. (1942). Whooping cough. IV. Beef liver extract blood agar media for liquid growth of *H. pertussis*. *J. Pediat.* **20** : 16-25.

SIMMONS, D.G., GRAY, J.G., ROSE, L.P., DILLMAN, R.C. and MILLER, S.E. (1978). Isolation of an etiologic agent of acute respiratory disease (rhinotracheitis) of turkey poults. *Avian Diseases*. **23** : 194-203.

SIMMONS, D.G., ROSE, L.P. and GRAY, J.G. (1979). Some physical, biochemic and pathologic properties of *Alcaligenes faecalis*, the bacterium causing rhinotracheitis (coryza) in turkeys. *Avian Diseases*. **24** : 82-90.

SIMMONS, D.G., GRAY, J.G., ROSE, L.P., Dillman, R.C. and MILLER, S.E. (1984). Turkey coryza. In, M.S. Hofstad *et al.* (eds.), *Diseases of Poultry*, (8^{th} ed), pp. 251-256. Iowa State Univ. Press.

SMITH, I.M. and BASKERVILLE, A.J. (1979). A selective medium facilitating the isolation and recognition of *Boredetella bronchiseptica* in pigs. *Res. Vet. Sci.* **27** : 187-192.

STAINER, D.W. and SCHOLTE, M.J. (1971). A simple chemically defined medium for the production of phase I *Bordetella pertussis*. *J. Gen. Micro.* **63** : 211-220.

STAMEY, T.A. and KAUFMAN, M.F. (1975). Studies of introital colonization in women with recurrent urinary tract infections. II. A comparison of growth in normal vaginal fluid of common versus uncommon serogroups of *Escherichia coli*. *J. Urol.* **114** : 264-267.

STAMEY, T.A. and MIHARA, G. (1980). Observations on the growth of urethral and vaginal bacteria in sterile urine. *J. Urol.* **124** : 461-463.

STANDFAST, A.F.B. and DOLBY, J.M. (1961). A comparison between the intranasal and intracerebral infection of mice with *Bordetella pertussis*. *J. Hyg. Camb.* **59** : 217-229.

STAUFFER, L.R., BROWN, D.R. and SANDSTROM, R.E. (1983). Cephalexin-supplemented Jones-Kendrick charcoal agar for selective isolation of *Bordetella pertussis* : Comparison with previously described media. *J. Clin. Micro.* **17** : 60-62.

STRANGE, R.E., DARK, F.A. and NESS, A.G. (1961). The survival of stationary phase cells of *Aerobacter aerogenes* stored in aqueous suspension. *J. Gen. Micro.* **25** : 61-76.

STUART, R.D. (1946). The diagnosis and control of gonorrhoea by bacteriological cultures : With a preliminary report on a new method for transporting clinical material. *Glasgow, Med. J.* **27** : 131-142.

SUKROONGREUNG, S. and WASI, P. (1988). Serum of thalassemic patients promotes

growth of streptococci. *Birth. Def.* **23**: 543-546.

SUTHERLAND, I.W. and WILKINSON, J.F. (1961). A new growth medium for virulent *Bordetella pertussis*. *J. Path. Bact.* **82** (2) : 431-438.

SUTCLIFFE, E.M. and ABBOT, J.D. (1972). Selective medium for the isolation of *Bordetella pertussis* and *parapertussis*. *J. Clin. Pathol.* **25** :732-733.

TEGNER, H. and OHLSSON, K. (1977). Localization of a low molecular weight protease inhibitor to tracheal and maxillary sinus mucosa. *Hoppe-Seyler's Z. Physiol. Chem.* **358** : 425-429.

TEWES, S. (1989). Bacteriological examination of seals. pp. 24. Presented at the International Workshop on Current Research in Seal Diseases, Hanover Veterinary School. Hanover. Germany.

THOMPSON, H., McCANDLISH, I.A.P. and WRIGHT, N.G. (1976). Experimental respiratory disease in dogs due to *Bordetella bronchiseptica*. *Res. Vet. Sci.* **20** : 16-23.

TOOMEY, J.A. and McCLELLAND, J.E. (1933-34). Pertussis toxin or antigen. *Proc. Soc. Exp. Biol.* **31** : 34-35.

TOPLEY and WILSON (1975). *Bordetella*. Principles of bacteriology, virology and immunology, pp. 1036-1050. Vol 1.

TOREMALM, N.G. (1960). The daily amount of tracheo-bronchial secretions in man. *Acta oto-laryng. Suppl.* **158** : 44-53.

ULRICH, J.A. and NEEDHAM, G.M. (1953). Differentiation of *Alcaligenes faecalis* from *Brucella bronchiseptica* by biochemical and nutritional methods. *J. Bact.* **65** : 210-215.

UNGAR, J., JAMES, A.M., MUGGLETON, P.W., PEGLER, H.F. and TOMICH, E.G. (1950). The cultivation of *Haemophilus pertussis* in partially defined liquid media. *J. Gen. Micro.* **4** : 345-359.

VERWEY, W.F. and SAGE, D.N. (1945). An improved liquid culture medium for the growth of *Hemophilus pertussis*. *J. Bact.* **49** : 520-521.

VERWEY, W.F., THIELE, E.H., SAGE, D.N. and SCHUCHARDT, L.F. (1949). A simplified liquid culture medium for the growth of *Hemophilus pertussis*. **58** : 127-134.

WARDELL (Jr), J.R. CHAKRIN, L.W. , PAYNE, B.J. and MARSHALL, R. (1969). The canine " tracheal pouch"; A model for use in respiratory mucus research. *Fed. Proc.* **28** : 800.

WARDLAW, A.C. (1988). Virulence factors and species specificity in *Bordetella*. In, Donachie, W., Griffiths, E. and Stephen, J. (eds.), *Bacterial Infections of Respiratory Tract and Gastrointestinal Mucosae*. Special Publications of the Society for General Microbiology. **24** : 41-56.

WARDLAW, A.C. (1990). *Bordetella*. In, *Topley and Wilson, Principles of bacteriology, virology and immunology*, (8th ed.) pp. 321-338. Vol 2. Systematic bacteriology.

WARDLAW, A.C. and PARTON, R. (1988). Pathogenesis and immunity in pertussis. John Wiley and Sons Ltd..

WEISS, A.A. and HEWLETT, E.L. (1986). Virulence factors of *Bordetella pertussis*. *Ann. Rev. Micro.* **40** : 661-686.

WILLIAMS, N.B. and POWLEN, D.O. (1959). Human paratoid saliva as a sole source of nutrient for microorganisms. *Arch. Oral. Biol.* **1** : 48-61.

WILSON, R.J. (1945). The production of phase I pertussis vaccine in casein hydrolosate broth. *Canad. J. Publ. Hlth.* **36** : 321-326.

WILSON, R.J. (1963). Cultivation of *Bordetella pertussis* (Phase 1) in a chemically defined medium. *Canad. J. Pub. Hlth.* **54** : 518-523.

WIRSING VON KOENIG, C.H., TACKEN, A. and FINGER, H. (1988). Use of supplemented Stainer-Scholte broth for the isolation of *Bordetella pertussis* from clinical material. *J. Clin. Micro.* **26** : 2558-2560.

WOODE, G.N. and McLEOD, N. (1967). Control of acute *Bordetella bronchiseptica* pneumonia in a guinea-pig colony. *Lab. Anim.* **1** : 91-94

WOODS, D.E., FRANKLIN, R. CRYZ, S.J. JR., GANNS, M. PEPPLER, M. and EWANOWICH, C. (1989). Development of a rat model for respiratory infection with *Bordetella*

pertussis. *Inf. Immun.* **57** : 1018-1024.

YEAGER, H.(Jr). (1971). Tracheobronchial secretions. *Am. J. Med.* **50** : 493-509.

APPENDICES

Appendix 1a : API interpretation table.

TESTS	SUBSTRATES	REACTIONS/ENZYMES	RESULTS	
			NEGATIVE	POSITIVE
NO_3	potassium nitrate	reduction of nitrates to nitrites	NIT 1 + NIT 2 / 5 min	
			colourless	pink-red
		reduction of nitrates to nitrogen	Zn / 5 min	
			pink	colourless
TRP	tryptophan	indole production	JAMES immediate	
			pale green-yellow	Pink
GLU	glucose	acidification	blue or green	yellow
ADH	arginine	arginine dihydrolase	yellow	orange/ pink/ red
URE	urea	urease	yellow	orange/ pink/ red
ESC	aesculin	hydrolysis (ß-glucosidase)	yellow	grey/ brown/ black
GEL	gelatin	hydrolysis (protease)	no pigment diffusion	diffusion of black pigment
PNPG	p-nitrophenyl-ßD-galactopyranoside	ß-galactosidase	colourless	yellow
GLU	glucose	assimilation	transparent	opaque
ARA	arabinose	assimilation	transparent	opaque
MNE	mannose	assimilation	transparent	opaque
MAN	mannitol	assimilation	transparent	opaque
NAG	N-acetyl-glucosamine	assimilation	transparent	opaque
MAL	maltose	assimilation	transparent	opaque
GNT	gluconate	assimilation	transparent	opaque
CAP	caprate	assimilation	transparent	opaque
ADI	adipate	assimilation	transparent	opaque
MLT	malate	assimilation	transparent	opaque
CIT	citrate	assimilation	transparent	opaque
PAC	phenyl-acetate	assimilation	transparent	opaque
OX	tetramethyl-p-phenylene diamine	cytochrome oxidase	colourless	violet

Appendix 1b : API identification codes.

	NO3	TRP	GLU	ADH	URE	ESC	GEL	PNPG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX
Pseudomonas aeruginosa	98	0	0	80	20	1	90	1	99	2	4	84	72	6	97	98	75	99	98	1	98
Pseudomonas aureofaciens	90	0	0	60	0	0	90	30	100	99	100	99	90	0	100	90	30	100	100	90	70
Pseudomonas cepacia	39	0	30	1	2	50	70	72	100	72	98	35	97	8	97	99	93	100	99	98	98
Pseudomonas chlororaphis	7	0	0	7	0	27	7	0	100	80	93	80	7	0	93	73	0	100	100	7	40
Pseudomonas diminuta	6	0	0	0	0	0	33	0	1	1	1	1	0	1	0	35	12	24	1	0	94
Pseudomonas fluorescens	31	0	0	55	1	1	39	4	99	70	90	85	81	7	98	99	6	99	99	16	99
Pseudomonas mallei	100	0	0	50	33	0	0	0	100	0	67	7	100	0	100	0	99	0	0	0	100
Pseudomonas mendocina	100	0	0	94	1	0	0	0	100	0	0	0	0	0	94	100	1	100	100	0	100
Pseudomonas mesophilica	24	0	0	0	76	0	0	0	29	43	0	0	0	0	29	0	12	80	19	0	99
Pseudomonas paucimobilis	10	0	0	0	1	99	2	90	95	78	75	15	59	95	46	18	8	61	46	1	73
Pseudomonas pickettii	95	0	0	3	3	0	6	0	95	70	1	4	6	0	100	87	30	100	94	3	100
Pseudomonas pseudomallei	100	0	0	96	0	12	100	0	100	0	98	100	100	0	100	100	98	100	97	89	100
Pseudomonas putida	3	0	1	70	1	0	1	1	98	56	57	5	2	0	92	96	1	100	99	30	98
Pseudomonas putrefaciens	92	0	4	0	1	71	95	0	6	11	0	0	95	9	1	71	1	90	2	0	100
Pseudomonas stutzeri	94	0	0	5	1	0	1	0	95	4	10	87	0	89	81	86	2	95	84	2	100
Pseudomonas vesicularis	23	0	0	1	0	98	15	40	72	0	0	3	10	72	1	6	1	40	0	0	99
Comamonas testosteroni/ Ps.alcaligenes	80	0	0	6	2	0	4	1	8	3	3	4	1	2	42	55	38	90	32	2	98
Comamonas acidovorans	97	0	0	0	0	0	3	0	1	1	0	75	0	0	99	53	91	98	53	81	100
Xanthomonas maltophilia	37	0	0	0	1	99	99	87	84	3	93	1	95	98	1	1	1	98	98	0	4
Chryseomonas luteola	57	0	13	71	1	100	30	95	99	99	99	90	12	65	85	62	1	90	94	3	2
Flavimonas oryzihabitans	0	0	0	0	1	0	11	11	100	99	100	100	0	80	99	88	2	98	99	0	0
Chromobacterium violaceum	95	11	99	99	0	0	99	1	99	0	86	22	88	1	99	86	1	100	44	1	95
Bordetella bronchiseptica	72	0	0	0	87	0	0	0	0	0	0	0	0	0	0	7	82	53	76	77	100
Bordetella avium	0	0	0	0	0	0	0	0	13	0	0	0	0	0	0	0	100	100	100	100	75
Oligella ureolytica	71	0	0	0	99	0	0	0	0	0	0	0	0	0	0	0	1	95	95	28	90
Oligella urethralis	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	98	30	0	100
CDC gr IV C-2	1	0	0	0	28	0	1	0	0	0	0	0	0	1	50	60	74	99	75	12	96
Alcaligenes faecalis	20	0	0	2	0	0	1	2	2	0	0	0	1	0	12	88	12	99	92	96	95
Alcaligenes xylosoxidans ssp denitrificans	98	0	0	0	2	0	0	0	1	0	0	0	0	1	80	20	90	99	96	93	100
Alcaligenes xylosoxidans ssp xylosoxidans	88	0	0	0	0	0	3	0	99	0	29	3	3	0	100	79	88	100	100	97	99
Achromobacter CDC gr Vd	80	0	0	0	84	0	0	3	84	79	60	25	83	80	39	35	4	99	50	1	99
Agrobacterium radiobacter	99	0	0	0	50	99	1	98	100	99	100	100	99	90	90	2	1	100	1	8	50
Flavobacterium breve	2	5	0	0	1	5	93	0	10	1	1	0	0	10	0	0	1	0	0	10	100
Flavobacterium indologenes	20	85	2	0	44	98	99	31	65	27	82	3	0	62	3	0	3	6	17	17	98
Flavobacterium meningosepticum	1	83	2	0	5	96	94	33	77	7	75	76	70	61	0	0	1	0	25	0	99
Flavobacterium odoratum	1	10	0	0	35	2	97	1	1	0	1	0	5	1	0	0	1	5	0	9	39
Sphingobacterium multivorum	1	0	16	0	95	100	1	100	99	91	98	0	98	98	0	0	1	0	0	0	99
Sphingobacterium spiritivorum	0	0	0	0	1	100	0	100	100	1	100	19	100	100	0	0	0	0	0	0	100
Weeksella virosa	4	5	0	0	0	1	99	0	0	1	0	0	0	0	2	0	0	0	0	1	99
Weeksella zoohelcum	0	0	0	0	99	0	87	0	0	0	2	0	0	0	0	0	1	0	0	0	99
Acinetobacter baumannii	1	0	20	0	2	0	1	0	52	79	0	0	0	0	30	99	90	100	99	98	0
Acinetobacter calcoaceticus	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	1	0	100	100	0
Acinetobacter haemolyticus	2	0	24	0	0	0	99	0	0	0	0	0	0	0	0	98	2	98	78	1	0
Acinetobacter johnsonii	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	100	33	0	100	0	0
Acinetobacter junii	2	0	8	0	3	0	0	0	19	24	1	1	0	0	3	83	38	100	68	0	0
Acinetobacter lwoffii	1	0	1	0	3	0	0	1	3	4	1	1	1	0	1	70	28	48	0	36	0
Moraxella lacunata	90	0	0	0	0	0	99	0	0	0	0	0	0	0	0	0	0	9	7	0	100
Moraxella phenylpyruvica	60	0	0	0	95	0	0	0	0	0	0	0	0	0	0	0	1	3	1	0	100
Moraxella spp	45	0	0	0	2	0	5	0	1	0	0	0	0	0	0	4	2	5	1	0	100
Pasteurella aerogenes	100	0	97	0	99	0	0	99	95	75	95	3	80	95	95	0	1	95	1	0	77
Pasteurella haemolytica	95	0	2	0	0	2	0	62	0	0	0	0	0	0	0	0	1	0	0	0	90
Pasteurella multocida	89	98	5	0	0	0	0	10	3	0	5	5	5	2	2	0	1	3	0	0	79
Pasteurella pneumotropica	100	60	28	0	85	0	0	75	3	3	6	0	6	3	6	0	8	6	0	0	34
Pasteurella spp	96	1	1	16	0	0	5	4	50	6	2	5	1	1	2	0	1	50	1	0	37
Aeromonas hydrophila/ caviae	98	86	98	75	0	94	97	99	99	81	79	100	98	100	93	89	0	99	37	0	99
Aeromonas sobria	100	86	96	80	0	1	99	97	100	29	99	97	99	100	100	90	0	99	75	0	100
Aeromonas salmonicida salmonicida	100	1	57	39	0	99	96	18	75	11	1	32	82	99	99	0	4	89	4	0	100
Aeromonas salmonicida masoucida/ achromogenes	100	21	17	8	0	27	42	8	33	4	24	25	4	19	8	0	1	6	1	0	100
Plesiomonas shigelloides	100	100	97	96	0	0	0	86	88	0	15	0	42	42	68	68	0	73	0	0	99
Vibrio alginolyticus	97	93	93	0	0	85	91	10	74	2	38	75	57	74	72	2	0	93	10	0	97
Vibrio cholerae	96	100	100	0	0	7	94	99	98	1	30	79	75	95	99	5	1	99	95	2	100
Vibrio damsela	94	0	94	94	94	13	0	13	13	0	13	0	6	13	0	0	0	83	0	0	100
Vibrio hollisae	100	100	35	0	0	0	0	5	10	59	53	0	24	6	41	0	0	94	0	0	100
Vibrio metschnikovii	0	50	1	0	0	50	100	0	100	0	50	100	100	100	100	50	0	100	0	0	0
Vibrio parahaemolyticus	97	98	100	4	8	2	97	94	79	71	82	94	51	93	90	1	2	99	21	0	99
Vibrio vulnificus	100	95	95	0	0	95	99	95	9	0	10	9	1	8	28	0	0	95	91	0	100

(% of positive reactions after 24-48 h at 30°C)

Appendix 2 : Results of FHA tests on the six strains of *B. bronchiseptica*

Fluid or Strain no.	HA at sample dilution									
	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
PBS	-	-	-	-	-	-	-	-	-	-
FHA	+	+	+	+	+	+	+	+	+	+
5376	+	+	+	+	+	-	-	-	-	-
6353	+	+	+	+	+	+	+	+	-	-
11	+	+	+	+	+	+	-	-	-	-
10541	+	+	+	-	-	-	-	-	-	-
452	-	-	-	-	-	-	-	-	-	-
13325	-	-	-	-	-	-	-	-	-	-

+ and - indicate haemagglutination and no haemagglutination respectively

Appendix 3 : HLT toxicity results in mice with the six strains of *B. bronchiseptica*

Source of HLT	48h (dead and very sick mice)/no. infected : after with 0.5ml i.p. of 10mg/ml crude HLT diluted :				
	Undiluted (heated at 56°C)	Undiluted	1/4	1/6	1/64
B. bronchiseptica					
5376	0/2	2/2	1/2*	0/2	0/2
452	0/2	2/2 *	0/2	0/2	0/2
10541	1/2	2/2	2/2	1/2*	0/2
11	0/2	2/2	2/2	2/2	2/2 *
13325	0/2 *	0/2	0/2	0/2	0/2
6353	0/1	2/2	1/1	1/1	2/2 *
B. pertussis					
crude HLT	0/2 *	2/2	2/2	2/2	2/2

* indicates region of LD_{50} endpoint

Appendix 4a : Growth of *B. bronchiseptica* strain 5376 in PBS : Accumulated colony count data.

Expt no.	No. of colonies from 20 µl at										
	Zero time	24h			48h			72h		7d	
	Undiluted	10^1	10^2	10^3	10^2	10^3	10^4	10^2	10^3	10^2	10^3
II 4	32	..	..	..	..	..	..	..	..	..	..
II 5	16, 15	..	50	3	..	48	4	..	..	..	..
II 9	48	10	1	0	216	18	..	..	..	..	..
II 12	21	20	2	..	72	6	..	..	..	..	..
II 15	60	62	4	..	±	31	..	..	..	..	..
II 16	45, 42	..	50	7	±	49	..	..	..	..	..
II 18	34	85	8	..	±	21	..	..	..	..	..
II 19	21	3	0	..	±	21	..	..	..	..	..
II 42	14	..	7	0	13	1	..	12	3	..	..
III 1	43	..	..	..	±	26	..	..	..	+	115
III 6	68	..	..	..	±	30	..	..	..	±	52
III 19	62	..	..	..	48	2	..	..	..	±	134
III 20	84	..	..	..	..	..	..	..	..	±	70
III 39	41	..	..	..	39	3	..	..	..	±	158

.. : not tested; + : growth almost confluent; ± : too many colonies to count accurately

Appendix 4b : Growth of *B. bronchiseptica* strain 5376 in CL medium : Accumulated colony count data.

Expt no.	No. of colonies from 20 µl at											
	Zero time	24h			48h			72h		7d		
	Undiluted	10^4	10^5	10^6	10^4	10^5	10^6	10^5	10^6	10^4	10^5	10^6
I 95	42, 46	±	..	5	..	..	..	..	..	..	..	..
I 96	36, 35	19	..	..	..	..	..	..	..	..	..	..
II 1	27, 40	46	..	3	..	..	..	..	..	..	..	..
II 2	15, 27	..	..	..	..	..	..	..	..	..	..	..
II 3	27, 36	..	..	..	..	..	..	..	..	..	..	..
II 4	59, 43	..	..	..	..	..	..	..	..	..	..	..
II 5	18, 19	16	..	1	++	..	48	..	..	..	..	..
II 9	44	283	..	..	..	73	..	..	..	..	..	..
II 12	25	±	67	..	+	..	27	..	..	..	..	..
II 14	30, 38	±	65	..	..	±	31	..	..	..	..	..
II 15	81	±	54	..	+	..	24	..	..	..	..	..
II 16	48, 64	±	53	..	..	±	72	..	..	..	..	..
II 17	47	±	74	..	..	+	237	..	95, 108	..	..	..
II 18	23	235	30	..	..	±	40	..	.	..	..	..
II 42	21	±	169	..	..	±	47	±	22	..	..	..
III 1	39	..	..	..	..	+	44	..	..	+	±	73
III 6	51	..	..	..	..	±	118	..	..	..	..	..
III 19	66	..	..	..	..	±	107	..	.	±	21	1
III 19	..	..	..	..	..	±	39	..	.	+	±	50
III 20	65	..	..	..	..	..	..	..	..	+	±	42
IV 3	20	..	..	..	..	±	51	..	..	..	39	1

.. : not tested; ++ : confluent growth; + : growth almost confluent; ± : too many colonies to count accurately

Appendix 4c : Growth of *B. bronchiseptica* strain 5376 in horse, sheep and dog TBW : Accumulated colony count data.

TBW species and Expt no.	Zero time	No. of colonies from 20 µl at 24h			48h			72h			7d		
	Undiluted	10^3	10^4	10^5	10^3	10^4	10^5	10^4	10^5	10^6	10^4	10^5	10^6
Horse													
II 42	20	..	..	..	++	±	92	±	83	9	..	..	..
III 1	39	..	..	..	..	±	32	..	..	..	±	209	..
III 6	57	..	..	..	..	+	276	..	..	..	±	234	..
III 19	..	..	..	..	..	+	368	..	..	..	+	±	32
		10^3	10^4	10^5	10^4	10^5		10^4	10^5	10^6	10^3	10^4	10^5
Sheep													
II 42	19	23	4	0	24	5		22	3	1	..	..	..
III 1	..	..	..	..	±	23		..	..	..	+	226	..
III 6	..	..	..	..	±	22		..	..	..	..	±	14
III 19	71	..	..	..	±	23		..	..	..	..	±	14
III 19	..	..	..	..	±	20		..	..	..	..	±	5
					10^0	10^2	10^3	10^4			10^2	10^3	10^4
Dog													
III 48	125				+	36	5	1			++	±	144
III 48	..				+	107	5	0			++	±	307
III 48	..				+	190	1	0			++	±	88

.. . not tested; ++ : confluent growth; + : growth almost confluent; ± : too many colonies to count accurately

Appendix 4d : Growth of *B. bronchiseptica* strain 5376 in mouse, rabbit and chicken TBW : Accumulated colony count data.

TBW species and Expt no.	Zero time	No. of colonies from 20 µl at 24h	48h	72h	7d
	Undiluted	10^2 10^4 10^6	10^4 10^5 10^6	10^2 10^4 10^6	10^4 10^5 10^6
Mouse					
I 72	30, 24	different dilutions tested			
I 88	54. 41	++ ± 28	± 30 ..	++ ± 37	.. .. ..
III 20	..	.. .. ..	± 68 13	.. .. ..	+ ± 13
III 20	60	.. .. ..	± 49 6	.. .. ..	+ ± 10
III 20	..	.. .. ..	± 62 10	.. .. ..	+ ± 16
		10^3 10^4 10^5	10^4 10^5	10^4 10^5 10^6	10^3 10^4 10^5
Rabbit					
II 42	26	± 35 2	70 8	54 4 ..	.. .. ..
III 1	..	.. .. ..	± 12	.. .. ..	+ 159 ..
III 6	..	.. .. ..	± 42	.. .. ..	.. ± 64
III 19	..	.. .. ..	151 7	.. .. ..	.. ± 31
		10^2 10^3 10^4 10^5 10^6		10^2 10^3 10^4 10^5 10^6	
Chicken					
III 39	53	++ ± ± 82 ..		++ + 160 9 ..	
III 39	40	++ ± ± 89 ..		++ + 199 16 ..	
III 43	46	.. .. ± 28 4		++ + ± 20 1	

.. : not tested; ++ : confluent growth; + : growth almost confluent; ± : too many colonies to count accurately

Appendix 4e : Growth of *B. bronchiseptica* strain 5376 in human TBW : Accumulated colony count data.

TBW species and Expt no.	No. of colonies from 20 µl at								
	Zero time	48h				7d			
	Undiluted	10^0	10^4	10^5	10^6	10^0	10^4	10^5	10^6
Human 1									
III 62	52	++	±	146	..	..	±	55	8
IV 3	26	..	±	110	5	..	±	50	5
Human 2									
III 62	..	0	..	..	..	0	..	..	..
IV 3	47	0	..	..	..	0	..	..	..
Human 3									
IV 3	21	++	..	40	3	..	±	109	11

Appendix 5a : Growth of *B. avium* strain P4091 in PBS : Accumulated colony count data.

Expt no.	No. of colonies from 20 µl at										
	Zero time	24h			48h			72h		7d	
	Undiluted	10^1	10^2	10^3	10^2	10^3	10^4	10^2	10^3	10^2	10^3
II 42	65	..	8	0	±	43	..	±	62	..	..
II 49	13	10	0	..	1	0	..	1	1	..	..
III 6	27	..	..	..	22	9	..	..	..	±	37
III 17	140	..	..	..	22	3	..	..	..	0	0
III 17	..	..	..	..	10	1	..	..	..	12	1
III 20	25	..	..	..	..	..	.	..	.	15	0

.. : not tested; + : growth almost confluent; ± : too many colonies to count accurately

Appendix 5b : Growth of *B. avium* strain P4091 in CL medium : Accumulated colony count data.

Expt no.	Zero time	No. of colonies from 20 µl at: 24h					48h				72h			7d		
	Undiluted	10^2	10^3	10^4	10^5	10^6	10^3	10^4	10^5	10^6	10^4	10^5	10^6	10^4	10^5	10^6
I 72	26, 21	----------other dilutions used----------														
I 77	26, 37	++	++	±	138	..	++	..	529	80	..	+	11	..	..	..
I 80	18, 16	++	±	..	..	..	..	+	..	52	..	..	..	..	..	..
I 85	18, 9	46	4	0	..	..	..	±	..	27	+	..	49	..	..	..
I 89	43, 43	++	++	..	..	..	++	..	..	125	+	..	113	..	..	..
I 93	26, 19	..	..	++	+	16	..	..	+	70	..	±	87	..	..	..
II 42	78	..	+	±	13	..	..	..	7	0	..	±	23	..	..	..
II 49	20	..	+	303	..	..	..	+	561	..	..	±	31	..	..	..
III 6	40	..	..	..	..	..	..	..	±	42	..	..	..	..	31	3
III 17	38	..	..	..	..	..	..	..	±	39	..	..	..	±	47	5
III 17	..	..	..	..	..	..	..	..	±	41	..	..	..	±	26	1
III 20	27	..	..	..	..	..	..	..	..	..	..	..	..	..	32	4

.. : not tested; + : growth almost confluent; ± : too many colonies to count accurately

Appendix 5c : Growth of *B. avium* strain P4091 in horse, sheep and dog TBW : Accumulated colony count data.

TBW species and Expt no.	No. of colonies from 20 µl at															
	Zero time	24h			48h				72h				7d			
	Undiluted	10^3	10^4	10^5	10^2	10^3	10^4	10^5	10^4	10^5	10^6		10^2	10^3	10^4	10^5
Horse																
II 42	56	67	2	0	..	++	+	238	±	±	40		..	..	..	..
II 49	21	13	0	..	..	..	±	117	..	36	1		..	..	..	..
III 6	24	..	..	..	..	..	±	157	..	..	..		..	..	48	4
III 17	..	..	..	..	..	..	±	59	..	..	..		..	..	50	4
III 17	..	..	..	..	..	..	±	96	..	..	..		..	..	64	10
Sheep																
II 42	57	66	4	1	..	..	±	63	±	38	..		..	..	..	..
II 49	14	38	5	..	..	..	74	9	89	13	..		..	..	..	..
III 6	..	..	..	..	..	..	24	2	..	..	..		..	..	7	1
III 17	..	..	..	..	..	±	24	3	..	..	..		96	9	0	..
III 17	57	..	..	..	..	±	42	3	..	..	..		±	20	0	..
Dog																
III 48	100	..	..	..	90	2	0	..	..	..	..		++	±	319	..
III 48	..	..	..	..	22	0	0	..	..	..	..		++	±	79	..
III 48	..	..	..	..	13	3	0	..	..	..	..		++	±	128	..

.. : not tested; + : growth almost confluent; ± : too many colonies to count accurately

Appendix 5d : Growth of *B. avium* strain P4091 in mouse, rabbit and chicken TBW : Accumulated colony count data.

TBW species and Expt no.	Zero time	24h			48h				72h			7d		
		No. of colonies from 20 µl at												
	Undiluted	10^3	10^4	10^5	10^3	10^4	10^5	10^6	10^4	10^5	10^6	10^2	10^3	10^4
Mouse														
I 72	39, 32	----------different dilutions used----------												
I 85	17, 11	±	893	..	..	±	..	11	106	0	0	..	..	..
I 89	58, 49	++	..	168	++	..	127	13	80	14	..	..	..	..
II 49	13	+	389	..	..	±	64	..	..	19	2	..	..	..
III 20	..	..	..	..	..	..	..	..	..	..	..	..	..	4
III 20	..	..	..	..	..	..	..	..	..	..	..	..	..	7
III 20	32	..	..	..	..	..	..	..	..	..	..	..	..	11
Rabbit														
II 42	68	±	65	0	..	113	10	2	±	15	0	..	..	..
II 49	21	±	30	..	..	26	3	..	27	2	..	..	..	..
III 6	..	..	..	..	..	±	20	..	..	..	..	..	..	0
III 17	..	..	..	..	..	84	7	..	..	..	..	±	±	14
III 17	..	..	..	..	..	45	7	..	..	..	..	±	33	5
III 20	..	..	..	..	..	..	..	..	..	..	..	±	±	9
Chicken														
III 42	13	..	..	..	±	77	10	..	..	..	..	44	10	1
III 42	..	..	..	..	±	70	7	..	..	..	..	64	3	0
III 42	..	..	..	..	±	61	9	..	..	..	.	66	6	0

.. : not tested; + : growth almost confluent; ± : too many colonies to count accurately

Appendix 5e : Growth of *B. avium* strain P4091 in human TBW : Accumulated colony count data.

TBW species and Expt no.	Zero time	48h						7d					
	No. of colonies from 20 µl at												
	Undiluted	10^0	10^1	10^2	10^3	10^4	10^5	10^0	10^2	10^3	10^4	10^5	
Human 1													
IV 4	49	++	++	++	..	..	..	++	±	±	39	6	
IV 4	32	..	..	..	+	±	13	..	..	..	..	..	
Human 2													
IV 4	..	0	..	..	..	..	0	0	..	..	..	..	
Human 3													
IV 4	52	++	++	++	..	..	..	++	±	27	2	0	
IV 4	30	..	..	..	+	±	13	..	..	..	..	..	

.. : not tested; + : growth almost confluent; ± : too many colonies to count accurately

Appendix 6a : Growth of *B. parapertussis* strain 10520 in PBS : Accumulated colony count data.

Expt no.	No. of colonies from 20 µl at				
	Zero time	24h	48h	72h	7d
	Undiluted	Undiluted	Undiluted	Undiluted	Undiluted
II 28	42	0	0	0	0
II 44	31	5	0	0	0
II 49	32	10	3	0	0
III 11	31	..	20, 26	0	0
III 21	28	..	..	..	0
III 45	35	..	..	..	0

Appendix 6b : Growth of *B. parapertussis* strain 10520 in CL medium : Accumulated colony count data.

		No. of colonies from 20 µl at														
	Zero time	24h			48h					72h				7d		
Expt no.	Undiluted	10^0	10^1	10^2	10^0	10^1	10^2	10^3	10^4	10^0	10^2	10^3	10^4	10^4	10^5	10^6
I 83	97, 52	±	..	6	+	..	43	..	0	++	351	..	4	..	..	..
I86	22, 21	---------- contaminated----------														
I 87	30, 19	..	..	2	±	..	9	..	..	+	79	..	1	..	..	..
I 90	38, 37	±	42	2	++	±	171	..	..	..	..	..	..	..	..	..
II 28	45	±	34	1	++	..	±	20	..	..	..	..	..	..	..	..
II 44	38	±	66	6	++	+	401	..	..	..	..	..	..	..	..	..
II 49	45	..	102	14	..	..	±	78	..	..	..	±	183	..	..	..
III 11	47	..	..	..	..	..	±	123	12	..	..	..	..	+	±	..
III 21	45	..	..	..	..	..	..	..	..	..	..	..	..	..	±	39
III 21	..	..	..	..	..	..	..	..	..	..	..	..	..	..	±	66
III 45	44	..	..	..	..	..	+	±	17	..	..	..	..	++	±	101
III 49	55	..	..	..	..	..	±	244	..	..	..	..	..	..	±	..
III 60	100	..	..	..	..	..	±	±	25	..	..	..	..	..	..	..
IV 4	71	..	.	..	..	..	147	12	..	..	..	..	..	..	..	..

Appendix 6c : Growth of *B. parapertussis* strain 10520 in horse, sheep and dog TBW : Accumulated colony count data.

TBW species and Expt no.	Zero time	24h			48h				72h				7d		
	No. of colonies from 20 µl at														
	Undiluted	10^0	10^1	10^2	10^0	10^1	10^2	10^3	10^0	10^2	10^3	10^4	10^0	10^2	10^3
Horse															
II 44	34	0	0	0	0	0	0	..	0	0	..	..	0	..	..
II 49	21	0	..	..	0	..	..	..	0	..	..	..	0	..	..
III 11	37	0	..	..	0	..	..	..	0	..	..	..	0	..	..
Sheep															
II 44	28	±	19	0	+	±	25	..	++	627	..	..	±	72	..
II 49	44	±	27	..	..	..	±	56	..	..	±	17	..	..	..
III 11	..	..	..	..	..	..	66	1	..	..	..	..	..	28	..
III 11	..	..	..	..	..	..	..	..	..	..	..	..	..	46	..
Dog															
III 45	41	..	..	..	..	0	0	0	..	..	..	..	0	..	..
III 45	..	..	..	..	..	4	1	0	..	..	..	..	0	..	..
III 45	..	..	..	..	..	0	0	0	..	..	..	..	0	..	..
III 49	31	..	..	..	12	..	..	..	..	..	..	..	0	..	..
III 49	..	..	..	..	23	..	..	..	..	..	..	..	0	..	..
III 49	..	..	..	..	13	..	..	..	..	..	..	..	0	..	..

Appendix 6d : Growth of *B. parapertussis* strain 10520 in mouse, rabbit and chicken TBW : Accumulated colony count data.

TBW species and Expt no.	No. of colonies from 20 µl at																			
	Zero time	24h				48h						72h				7d				
	Undiluted	10^0	10^1	10^2	10^3	10^0	10^1	10^2	10^3	10^4		10^0	10^2	10^3		10^0	10^1	10^2	10^3	
Mouse																				
I 83	79, 78	+	..	75	5	±	..	12	0	0		8	1	0		..	..	..	..	
I 86	10, 16	+	..	153	15	++	..	±	..	19		..	19	0		..	..	..	..	
I 87	27, 18	+	..	92	7	++	..	±	..	34		±	56	0		..	..	..	..	
II 28	47	+	..	66	5	++	..	..	±	19		++	213	..		±	±	24	1	
II 44	29	140	14	1	..	++	±	68	..	..		++	409	..		..	..	..	..	
II 49	40	+	332	..	..	---------- contaminated----------														
III 21	72	---------- contaminated----------																		
III 21	..	---------- contaminated----------																		
Rabbit																				
II 44	50	±	25	5	..	++	+	262	..	..		++	418	..		±	..	39	..	
II 49	38	..	30	..	..	..	..	138	7	..		..	..	146		..	..	..	..	
III 11	..	..	..	..	..	..	..	14	4	..		..	..	..		..	..	24	4	
III 21	..	..	..	..	..	..	..	..	..	..		..	..	..		..	..	46	2	
Chicken																				
III 45	49	..	..	..	..	..	±	24	2	..		..	..	..		..	±	12	0	
III 45	..	..	..	..	..	..	±	68	3	..		..	..	..		..	±	21	2	
III 45	..	..	..	..	..	..	±	124	14	..		..	..	..		..	±	46	3	

Appendix 6e : Growth of *B. parapertussis* strain 10520 in human TBW : Accumulated colony count data.

TBW species and Expt no.	No. of colonies from 20 µl at								
	Zero time	48h			7d				
	Undiluted	10^0	10^2	10^3	10^0	10^3	10^4	10^5	10^6
Human 1									
IV 4	..	++	±	70	..	..	0	0	0
IV 4	64	..	..	..	43	0	..	..	..
Human 2									
III 60	117	0	..	..	0	..	..	..	..
IV 4	..	0	..	..	0	..	..	..	..
Human 3									
IV 4	75	++	±	26	..	..	0	0	0
IV 4	54	..	..	..	36	0	..	..	..

Appendix 7a : Growth of *B. pertussis* strain 18334 in PBS and CL medium : Accumulated colony count data.

Fluid and Expt. no.	No. of colonies from 20 µl at									
	Zero time	48				7d				
	Undiluted	10^0	10^1	10^2	10^3	10^0	10^4	10^5	10^6	10^7
PBS										
III 37	9	0	..	..	..	0	..	..	..	.
III 46	163	2	..	..	..	0	..	..	..	..
III 50	..	0	..	..	..	0	..	..	..	..
IV 4	25	0	..	..	..	0	..	..	..	..
CL										
III 37	10	±	104	10	..	..	++	+	270	..
III 46	178	++	±	99	5	..	..	±	±	39
III 46	..	++	±	±	22	..	..	±	102	6
III 50	71	..	±	34	3	++	..	..	..	..
III 56	78	++	±	99	..	..	..	..	±	13
IV 4	37	+	..	60	6	..	..	±	±	18

Appendix 7b : Growth of *B. pertussis* strain 18334 in TBW from various vertebrate species : Accumulated colony count data.

Fluid and Expt. no.	No. of colonies from 20 µl at				
	Zero time	48			7d
	Undiluted	10^0	10^1	10^2	Undiluted
Horse					
II 46	..	0	..	..	0
II 46	..	0	..	..	0
Sheep					
II 37	20	4	0	0	0
II 46	..	19	0	0	0
Dog					
II 46	139	0	..	..	0
II 46	..	0	..	..	0
II 50	85	0	..	..	0
Mouse					
II 37	20	0	0	0	0
II 46	..	0	..	..	0
II 50	71	0	..	..	0
Rabbit					
II 37	22	0	0	0	0
II 46	..	19	..	..	0
Chicken					
II 50	74	0	..	..	0
II 50	..	0	..	..	0
II 50	..	0	..	..	0

Appendix 7c : Growth of *B. pertussis* strain 18334 in human TBW : Accumulated colony count data.

Fluid and Expt. no.	No. of colonies from 20 µl at Zero time	48	7d
	Undiluted	Undiluted	Undiluted
Human 1			
III 56	98	0	0
IV 4	..	0	0
Human 2			
III 56	92	0	0
IV 4	..	0	0
Human 3			
IV 4	11	0	0

Appendix 8 : Absorbance at 540 nm, in the Drabkin test, of dilutions of mouse blood (%, v/v) in PBS as recorded in 11 assays.

Assay no.	Absorbance at 540 nm with mouse blood (%, v/v) of :						
	0.015625	0.03125	0.0625	0.125	0.25	0.5	1.0
1	0.003	0.007	0.011	0.022	0.047	0.095	0.198
2	0.003	0.007	0.015	0.020	0.045	0.086	0.183
3	0.002	0.004	0.008	0.022	0.042	0.090	0.181
4	..	0.005	0.011	0.022	0.040	0.079	0.165
5	..	..	0.005	0.012	0.023	0.079	0.184
6	..	..	..	..	0.044	0.090	0.183
7	..	..	0.011	0.021	0.042	0.081	0.159
8	0.003	0.005	0.016	0.021	0.050	0.091	0.189
9	..	..	..	0.017	0.035	0.081	0.182
10	..	..	..	..	0.042	0.104	0.194
11	..	..	..	0.016	0.038	0.082	0.172
Mean	0.003	0.006	0.011	0.019	0.041	0.087	0.181
SD	0.0005	0.001	0.004	0.003	0.007	0.008	0.012
SEM	0.00025	0.0006	0.001	0.001	0.002	0.002	0.004

Appendix 9 : Blood contamination of the cell pellet from centrifuged horse, rabbit and sheep TBW and the membrane-filtered supernates. Results are expressed as % (v/v) mouse blood equivalents.

Expt no.	Fraction	% (v/v) mouse blood equivalent (mean + SEM)				
		Horse	Rabbit 1	Rabbit 2/3	Sheep 1	Sheep 2
III 8	Pellet	0.19	..	0.06	0.14	0.07
III 8	Pellet	0.14	..	0.07	0.16	0.06
III 9	Pellet	0.11	..	0.02	0.10	0.02
III 9	Pellet	0.10	..	0.01	0.12	0.04
III 12	Pellet	0.11	..	..	0.12	0.08
III 12	Pellet	0.10	..	..	0.15	0.03
III 13	Pellet	0.11	0.14	0.07	..	..
III 13	Pellet	0.12	0.13	0.07	..	..
III 13	Pellet	0.13	0.14	0.05	..	..
III 14	Pellet	..	0.13	..	..	..
III 14	Pellet	..	0.10	..	..	..
III 14	Pellet	..	0.12	..	..	..
		(0.12 ± 0.01)	(0.12 ± 0.01)	(0.12 ± 0.01)	(0.12 ± 0.01)	(0.12 ± 0.01)
III 8	Supernate	..	0.09	0.07	0.02	0.01
III 8	Supernate	..	0.09	0.07	0.04	0.01
III 8	Supernate	..	0.10	0.08	0.09	0.01
III 12	Supernate	0.12	0.07	0.07	0.02	0.00
III 12	Supernate	0.11	0.08	0.05	0.01	0.01
III 12	Supernate	0.13	0.05	0.06	0.01	0.00
III 14	Supernate	0.09	..	..	..	..
III 14	Supernate	0.10	..	..	..	.
III 14	Supernate	0.11	..	..	..	..
III 15	Supernate	0.14	..	..	..	..
III 15	Supernate	0.15	..	..	..	..
III 15	Supernate	0.13	..	..	.	.
		(0.12 ± 0.01)	(0.08 ± 0.01)	(0.07 ± 0.004)	(0.03 ± 0.01)	(0.007 ± 0.002)

Appendix 10 : Blood contamination of the cell pellet from centrifuged mouse, chicken, and dog TBW and the membrane-filtered supernates. Results are expressed as % (v/v) mouse blood equivalents.

Expt no.	Fraction	% (v/v) mouse blood equivalent (mean + SEM)				
		Mouse (I57)	Mouse (I58)	Mouse (I65)	Chicken	Dog
III 7	Pellet	..	..	0.20	..	..
III 7	Pellet	..	..	0.23	..	..
III 8	Pellet	0.31	..	0.34	..	..
III 8	Pellet	0.31	..	0.38	..	..
III 9	Pellet	0.20	..	0.15	..	..
III 9	Pellet	0.20	..	0.22	..	..
III 13	Pellet	..	0.15	..	..	..
III 13	Pellet	..	0.15	..	..	..
III 13	Pellet	..	0.15	..	..	..
III 13	Pellet	..	0.16	..	..	..
III 13	Pellet	..	0.17	..	..	..
II 13	Pellet	..	0.17	..	..	..
III 14	Pellet	..	..	0.43	..	..
III 14	Pellet	..	..	0.40	..	..
III 14	Pellet	..	..	0.46	..	..
III 28	Pellet	..	..	..	0.04	..
III 28	Pellet	..	..	..	0.04	..
III 28	Pellet	..	..	..	0.05	..
III 30	Pellet	..	..	..	0.006	..
III 30	Pellet	..	..	..	0.006	..
III 54	Pellet	..	..	..	..	0.12
III 54	Pellet	..	..	..	..	0.12
III 54	Pellet	..	..	..	..	0.12
III 54	Pellet	..	..	..	..	0.14
III 54	Pellet	..	..	..	..	0.12
III 54	Pellet	..	..	..	..	0.09
		(0.26 ± 0.03)	(0.16 ± 0.004)	(0.31 ± 0.04)	(0.038 ± 0.01)	(0 12 ± 0.01)

Appendix 10 (continued)

Expt no.	Fraction	% (v/v) mouse blood equivalent (mean + SEM)				
		Mouse (I57)	Mouse (I58)	Mouse (I65)	Chicken	Dog
III 7	Supernate	..	..	0.14	..	..
III 7	Supernate	..	..	0.17	..	..
III 8	Supernate	..	..	0.16	..	..
III 13	Supernate	..	0.13	0.14	..	..
III 13	Supernate	..	0.14	0.17	..	..
III 13	Supernate	..	0.12	0.13	..	..
III 13	Supernate	..	0.11	..	..	..
III 13	Supernate	..	0.14	..	..	..
III 13	Supernate	..	0.11	..	..	..
III 15	Supernate	0.19	..	..	..	..
III 15	Supernate	0.24	..	..	..	..
III 15	Supernate	0.19	..	..	..	..
III 15	Supernate	0.25	..	..	..	..
III 15	Supernate	0.21	..	..	..	..
III 15	Supernate	0.25	..	..	..	..
III 28	Supernate	..	..	..	0.01	..
III 28	Supernate	..	..	..	0.03	..
III 28	Supernate	..	..	..	0.02	..
III 30	Supernate	..	..	..	0.00	..
III 30	Supernate	..	..	..	0.00	..
III 54	Supernate	..	..	..	..	0.012
III 54	Supernate	..	..	..	..	0.012
III 54	Supernate	..	..	..	..	0.012
III 54	Supernate	..	..	..	..	0.017
III 54	Supernate	..	..	..	..	0.023
III 54	Supernate	..	..	..	..	0.005
		(0.22 ± 0.01)	(0.13 ± 0.01)	(0.15 ± 0.04)	(0.01 ± 0.005)	(0.014 ± 0.002)

Appendix 11 : Blood contamination of the cell pellet from centrifuged human TBW and the membrane-filtered supernates. Results are expressed as % (v/v) mouse blood equivalents.

Expt no.	Fraction	% (v/v) mouse blood equivalent (mean + SEM)		
		Human 1	Human 2	Human 3
IV 6	Pellet	0.88	0.44	0.02
IV 6	Pellet	0.81	0.50	0.02
IV 6	Pellet	0.88	0.56	0.03
		(0.86 ± 0.02)	(0.50 ± 0.03)	(0.02 ± 0.003)
IV 6	Supernate	0.00	0.16	0.00

Appendix 12 : Absorbance of standard leucine concentrations (mM) at 570 nm for 7 assays of amino acid content of TBW.

Assay no.	Absorbance at 540 nm with leucine mM of :			
	0.25	0.5	1.0	2.0
	0.052	0.100	0.199	0.430
1	0.032	0.097	0.208	0.413
	0.036	0.103	0.186	0.430
2	0.054	0.071	0.188	0.413
3	0.045	0.107	0.209	0.400
4	0.028	0.059	0.132	0.286
	0.031	0.056	0.132	0.283
5	0.050	0.092	0.185	0.365
6	0.038	0.102	0.162	0.387
	0.052	0.070	0.190	0.364
7	0.046	0.084	0.174	0.336
	0.044	0.088	0.178	0.330
Mean	0.042	0.086	0.178	0.370
SD	0.009	0.018	0.026	0.052
SEM	0.003	0.005	0.007	0.015

Appendix 13 : Amino acid content of horse, rabbit and sheep TBW expressed as mM leucine equivalents (based on at least 3 observations). The TBW supernate was membrane-filtered before analysis.

Expt no.	Amino acid concentration as mM leucine equivalents (mean ± SEM) in				
	Horse	Rabbit 1	Rabbit 2/3	Sheep 1	Sheep 2
II 37	0.83	0.11	0.19	..	..
II 41	0.69	..	..	..	..
II 51	0.54	..	..	..	..
III 5	0.88	0.25	0.15	0.22	0.07
III 16	..	0.26	0.08	0.13	0.11
III 16	..	0.19	0.05	0.11	0.11
III 16	..	..	..	0.14	0.08
	(0.74 ± 0.08)	(0.20 ± 0.03)	(0.12 ± 0.03)	(0.15 ± 0.02)	(0.09 ± 0.01)

Appendix 14 : Amino acid content of mouse, chicken and dog TBW expressed as mM leucine equivalents (based on a least 3 observations). The TBW supernate was membrane-filtered before analysis.

Expt no.	Amino acid concentration as mM leucine equivalents (mean ± SEM) in				
	Mouse (I57)	Mouse (I58)	Mouse (I65)	Chicken	Dog
II 37	..	..	0.42	..	..
II 41	1.08	..	..	..	..
III 16	0.70	0.49	0.39	..	..
III 16	0.72	0.44	0.38	..	..
III 16	0.74	0.46	0.38	..	..
III 16	..	0.50	..	..	..
III 29	..	..	..	0.07	..
III 29	..	..	..	0.16	..
III 29	..	..	..	0.07	..
IV 12	..	..	..	..	0.26
IV 12	..	..	..	..	0.25
IV 12	.	..	..	..	0.18
	(0.81 ± 0.09)	(0.47 ± 0.01)	(0.39 ± 0.09)	(0.10 ± 0.03)	(0.23 ± 0.02)

Appendix 15 : Amino acid content of human TBW expressed as mM leucine equivalents. The TBW supernate was membrane-filtered before analysis.

Expt no.	Amino acid concentration as mM leucine equivalents (mean ± SEM) in	
	Human 2	Human 3
IV 7	0.68	0.20
IV 7	0.80	0.22
IV 7	0.72	0.22
	(0.73 ± 0.04)	(0.21 ± 0.01)

Appendix 16 : Nicotinic acid assay : Volume of NaOH (ml) required for acid neutralization, in relation to standard concentrations of nicotinic acid in 48h *Lactobacillus* cultures.

Assay no.	Volume of NaOH for acid neutralization at nicotinic acid concentrations of (µg/ml) :				
	0.01	0.02	0.04	0.08	0.16
1	1.5	2.1	6.2	11.4	18.5
	1.5	1.9	5.7	11.0	19.9
2	1.0	2.7	4.7	8.9	16.6
	1.2	3.0	4.5	9.2	16.7
3	1.4	2.9	5.6	12.0	23.7
	1.5	2.9	6.0	12.0	24.3
Mean	1.35	2.58	5.45	10.75	19.95
SD	0.21	0.47	0.69	1.37	3.37
SEM	0.08	0.19	0.28	0.56	1.38

Appendix 17 : Nicotinic acid content[a] of various vertebrate TBW (based on at least 2 observations). The TBW was centrifuged and membrane-filtered before analysis.

Expt no.	Nicotinic acid concentration expressed as µg/ml (mean ± SEM) in					
	Horse	Rabbit 2/3	Sheep 1	Mouse (I57)	Chicken	Dog
III 26	0.96	0.16	0.48	1.04	..	..
III 26	1.04	0.28	0.44	0.88	..	..
III 32	1.20	0.20	0.20	0.72	0.20	..
III 32	1.04	0.28	0.20	0.72	0.20	..
IV 12	..	..	..	..	0.00	0.54
IV 12	..	..	..	..	..	0.54
IV 12	..	..	..	..	..	0.54
	(1.06 ± 0.05)	(0.23 ± 0.03)	(0.33 ± 0.08)	(0.84 ± 0.08)	(0.20 ± 0.00)	(0.54 ± 0.00)

[a] expressed as µg/ml

Appendix 18 : Nicotinic acid content[a] of Human TBW (based on at least 2 observations). The TBW was centrifuged and membrane-filtered before analysis.

Expt no.	Nicotinic acid concentration expressed as µg/ml (mean ± SEM) in	
	Human 2	Human 3
IV 12	0.54	0.54
IV 12	0.54	0.80
IV 12	0.80	0.66
	(0.63 ± 0.09)	(0.67 ± 0.08)

[a] expressed as µg/ml

Appendix 19 : Protein content[a] of various vertebrate TBW (based on at least 2 observations). The TBW was centrifuged and membrane-filtered before analysis.

Expt no.	Protein concentration expressed as µg/ml (mean ± SEM) in					
	Horse	Rabbit 2/3	Sheep 1	Mouse (I57)	Chicken	Dog
III 34	1360	264	890	970	116	..
III 34	1520	296	894	1102	..	..
III 35	1338	356	679	1016	62	..
III 35	957	358	613	952	85	..
IV 5	..	..	..	..	..	762
IV 5	..	..	..	..	..	436
	(1294 ± 119)	(318 ± 23)	(769 ± 72)	(1010 ± 33)	(88 ± 16)	(599 ± 113)

[a] expressed as µg/ml

Appendix 20 : Protein content[a] of human TBW (based on 2 observations). The TBW was centrifuged and membrane-filtered before analysis.

Expt no.	Protein concentration expressed as µg/ml (mean ± SEM) in	
	Human 1	Human 2
IV 5	2074	1788
IV 5	2270	1942
	(2172 ± 98)	(1865 ± 77)

[a] expressed as µg/ml

Appendix 21 : Growth of *B. bronchiseptica* strain no. 5376 in PBS: a) prepared from analytical grade salts in distilled water (PBS/DW); b) prepared from analytical grade salts in reagent grade water (PBS/RGW) and c) passed through a charcoal filter (PBS/CH), and reagent-grade water (RGW) alone during 24 and 48h at 37°C.

Expt no. and Fluid	Zero time	Colony count and GI from 20 µl sample at 24h 10^0	10^1	10^2	10^3	GI	48h 10^0	10^1	10^2	10^3	10^4	GI
RGW												
II 14	21, 28	269, 252	..	..	..	1.0	560	..	..	..	..	1.4
II 15	70	102	12	..	..	0.2	165	21	..	..	..	0.5
II 16	42, 41	118	12	..	..	0.5	123	15	..	..	..	0.5
PBS/DW												
I 95	39, 40	++	±	±	18	2.6	..	..	..	..	..	..
I 96	28, 46	++	..	±	21	2.8	..	..	..	..	..	..
II 1	39, 35	++	±	27	3	1.9	..	..	..	..	..	..
II 14	33, 32	..	+	168	15	2.7	..	..	±	70	..	3.3
II 15	58	..	±	35	2	1.8	..	..	±	39	..	2.8
II 16	45, 42	..	..	50	7	2.1	..	..	±	49	..	3.0
PBS/RGW												
II 5	16, 15	++	..	50	3	2.5	++	+	..	48	4	3.5
II 9	48	..	10	1	0	0.3	..	..	216	18	..	2.6
II 12	21	++	10	2	..	0.9	..	.	72	6	..	2.5
II 15	60	..	62	4	..	1.0	..	..	±	31	..	2.7
II 18	34	..	85	8	..	1.4	..	..	±	21	..	2.8
PBS/CH												
II 12	22	++	+	629	..	3.4	.	..	+	137	..	3.8
II 15	50	..	..	±	78	3.2	..	..	+	178	17	3.5

Appendix 22 : Effect of serial transfer of *B. bronchiseptica* strain no. 5376 in PBS and CL medium at 37°C.

Expt no. and Transfer no.	Fluid	Colony count per 20 µl at Zero time	10^0	10^1	10^2	24h 10^3	10^4	10^5	10^6
I 95									
1	PBS	39, 40	++	±	..	18	..	..	..
1	CL	42, 46	++	..	..	..	±	..	5
2	PBS	450	++	±	..	28	..	..	..
2	CL	125 x 10^5	++	..	..	..	+	..	±
3	PBS	700	++	±	..	85	..	..	..
3	CL	..	++	..	..	..	++	..	117
4	PBS	2125	++	±	..	50	..	..	..
4	CL	2925 x 10^3	++	..	..	..	..	..	61
I 96									
1	PBS	28, 46	++	..	±	21	..	..	..
1	CL	36, 35	++	..	±	..	19	..	..
2	PBS	525	++	..	±	47	..	..	..
2	CL	4750	++	..	..	..	+	..	..
3	PBS	1175	++	..	±	47	..	..	..
3	CL	..	++	..	..	..	+	..	..
4	PBS	1175	++	..	±	63	..	..	..
4	CL	..	++	..	..	..	+	..	..
II 1									
1	PBS	39, 35	++	±	27	3	..	..	..
1	CL	27, 40	++	..	..	..	46	..	3
2	PBS	75	++	+	±	7	..	..	..
2	CL	11500	..	..	..	..	+	..	95
3	PBS	175	++	+	±	55	..	..	..
3	CL	2375 x 10^3	..	..	..	..	+	..	92
4	PBS	1375	++	+	±	33	..	..	..
4	CL	23 x 10^5	..	..	..	..	±	..	96

The 0h counts for transfers 2-4 were calculated as a 1/40th of the previous 24h count.

Appendix 23 : Effect of serial transfer of *E. coli* strain no. JM 83 in PBS and CL medium at 37°C.

Expt no. and Transfer no.	Fluid	Colony count per 20 µl at Zero time	24h 10^0	10^1	10^2	10^3	10^4	10^5	10^6
I 95									
1	PBS	7, 7	10	0	..	0	..	..	..
1	CL	10, 6	++	..	..	..	+	..	29
2	PBS	0, 0	0	0	..	0	..	..	..
2	CL	7.25×10^5	++	..	..	..	+	..	32
3	PBS	0, 0	0	0	..	0	..	..	..
3	CL	8×10^5	++	..	..	..	++	..	68
4	PBS	0, 0	0	0	..	0	..	..	..
4	CL	17×10^5	++	..	..	..	..	..	70
I 96									
1	PBS	9, 14	7	0	..	0	..	..	..
1	CL	24, 12	..	..	..	..	..	±	32
2	PBS	0, 0	0	0	..	0	..	..	..
2	CL	8×10^5	..	..	..	..	..	±	27
3	PBS	0, 0	0	0	..	0	..	..	..
3	CL	675×10^3	..	..	..	..	..	±	33
4	PBS	0, 0	0	0	..	0	..	..	..
4	CL	825×10^3	..	..	..	..	..	±	20
II 1									
1	PBS	47, 32	36	4	..	0	..	..	..
1	CL	39, 41	..	..	..	..	++	..	15
2	PBS	0, 0	0	0	0	0	..	..	..
2	CL	375×10^3	..	..	..	..	+	..	11
3	PBS	0, 0	0	0	0	0	..	..	..
3	CL	275×10^3	..	..	..	..	++	..	248
4	PBS	0, 0	0	0	0	0	..	..	..
4	CL	62×10^5	..	..	..	..	±	..	13

The 0h counts for transfers 2-4 were calculated as a 1/40th of the previous 24h count.

Appendix 24 : Growth of *B. bronchiseptica* strain no. 5376 in dilutions of CL medium during 24 and 48h at 37°C.

Expt no. and Dilution	Colony count and GI from 20 µl sample at												
	Zero time	24h						GI	48h					GI
		10^0	10^1	10^2	10^3	10^4	10^5		10^2	10^3	10^4	10^5	10^6	
II 9														
10^0	44	..	..	++	..	254	..	4.8	..	++	..	430	5	6.0
10^2	40	..	..	++	±	283	..	4.8	++	++	.	73	.	5 3
10^4	43	..	..	2	0	0	..	0.7	+	239	..	4	..	3.7
10^6	49	..	..	4	1	0	..	0.9	±	30	..	0	..	2.8
10^8	40	..	..	3	1	0	..	0.7	±	25	..	0	..	2.8
10^{10}	43	..	..	3	0	0	..	0.8	±	30	..	0	..	2.8
10^{12}	54	..	..	2	0	0	..	0.8	±	35	..	0	..	2.8
II 12														
10^0	25	..	..	..	..	±	67	5.4	..	..	+	..	27	6.0
10^2	16	..	..	..	..	+	32	5.3	..	..	+	96	..	5.8
10^4	21	±	166	..	..	..	..	1.9	..	±	28	..	..	4.1
10^5	18	±	80	..	..	..	..	1.6	..	42	..	..	..	3.4
10^6	27	±	100	..	..	..	..	1.6	±	32	..	..	..	3.1
10^8	22	±	14	..	..	..	..	0.8	139	14	..	..	..	2.8

Information for PBS was taken from previous experiments.

Appendix 25 : Growth of *B. bronchiseptica* strain no. 5376 in pond and sea water during 24 and 48h at 37° C

Expt no. and Water type	Zero time	Colony count and GI from 20 µl sample at 24h 10^0	10^1	10^2	10^3	GI	48h 10^0	10^1	10^2	10^3	GI
Pond											
II 3	33, 29	++	+	286	18	2.8	..	..	..	..	..
II 5	21, 29	++	±	±	13	2.7	++	+	424	..	3.2
II 12	25	++	+	±	49	3.3	..	..	+	74	3.5
II 15	58	..	..	±	118	3.3	..	..	±	155	3.4
Sea											
II 3	31, 34	79	4	1	2	0.4	..	..	..	..	..
II 5	34, 22	±	9	2	..	0.5	+	78	5	..	1.4
II 12	20	134	12	..	..	0.8	±	27	..	..	1.1
II 15	61	57	1	..	..	0	156	10	..	..	0.4

Appendix 26 : Growth of *B. bronchiseptica* strain no. 5376 in fresh water samples from various lakes during 24 and 48h at 37° C

Expt no. and Water type	Colony count and GI from 20 µl sample at							
	Zero time	24h 10^0	10^1	10^2	GI	48h 10^2	10^3	GI
Milngavie Reservoir								
II 17	39	±	109	10	1.4	209	25	2.8
II 19	41	..	32	0	0.9	±	18	2.6
Loch Katrine								
II 17	26	±	117	6	1.4	292	34	3.1
II 19	42	..	38	2	0.9	±	88	3.3
Loch Achray								
II 17	46	±	131	10	1.4	±	39	2.9
II 19	14	..	28	0	1.3	±	46	3.5
Loch Ard								
II 17	54	+	±	26	1.7	190	33	2.8
II 19	25	..	58	0	1.4	±	30	3.1
Loch Lomond								
II 17	53	++	±	101	2.3	±	45	2.9
II 19	31	..	290	9	2.0	±	88	3.4
Lake of Menteith								
II 17	43	+	±	31	1.8	±	37	2.9
II 19	25	..	83	0	1.5	±	56	3.4

Appendix 27 : Comparison of *B. bronchiseptica* strains 5376, 452 and 10541 for their ability to grow in PBS and fresh water from Loch Lomond over 48h at 37°C.

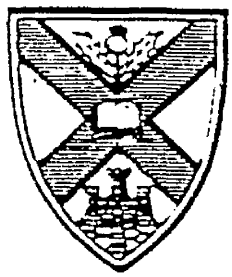
Strain and Expt no.	PBS	Loch Lomond	Colony count from 20 µl at Zero time	48h 10^0	48h 10^1	48h 10^2	48h 10^3	GI
5376								
II 5	Yes	..	16, 15	++	+	..	48	3.5
II 9	Yes	..	48	..	..	216	18	2.6
II 12	Yes	..	21	..	..	72	6	2.5
II 15	Yes	..	60	..	..	±	31	2.7
II 18	Yes	..	34	..	..	±	21	2.8
II 17	..	Yes	53	..	..	±	45	2.9
II 19	..	Yes	31	..	..	±	88	3.4
452								
II 36	Yes	..	53	..	..	±	32	2.8
II 36	Yes	..	58	..	..	±	19	2.5
II 36	..	Yes	61	..	..	±	23	2.6
II 36	..	Yes	71	..	..	±	26	2.6
10541								
II 36	Yes	..	31	..	..	±	20	2.8
II 36	Yes	..	26	..	..	±	53	2.8
II 36	..	Yes	23	..	..	±	65	3.4
II 36	..	Yes	25	..	..	±	45	3.2

Appendix 28 : Comparison of *B. bronchiseptica* strains 11, 6353 and 13325 for their ability to grow in PBS and fresh water from Loch Lomond over 48h at 37°C.

Strain and Expt no.	PBS	Loch Lomond	Colony count from 20 µl at Zero time	48h 10^0	48h 10^1	48h 10^2	48h 10^3	GI
11								
II 30	Yes	..	7, 7	++	±	±	40	3.8
II 38	Yes	..	15	..	..	60	7	2.9
II 30	..	Yes	10, 6	++	+	±	50	3.8
II 38	..	Yes	26, 17	..	..	±	64	3.5
6353								
II 30	Yes	..	9, 6	++	±	±	18	3.4
II 38	Yes	..	15	..	..	35	6	2.4
II 30	..	Yes	6, 4	++	+	±	69	4.1
II 38	..	Yes	9	..	..	±	28	3.5
13325								
II 30	Yes	..	23, 20	++	+	±	42	3.3
II 38	Yes	..	15	..	..	±	32	2.6
II 30	..	Yes	30, 30	++	+	±	87	3.5
II 38	..	Yes	71, 77	..	..	±	56	2.9

Appendix 29 : Long-term survival of three strains of *B. bronchiseptica* in PBS and fresh water from Loch Lomond at 37°C (A) and 10°C (B) over a period of six months.

Strain and Fluid	Zero time	1wk			1mth			3mth			6mth			
	Undiluted	10^0	10^2	10^3	10^0	10^2	10^3	10^0	10^2	10^3	10^0	10^1	10^2	10^3
452														
PBS A	46	++	54	8	++	±	23	++	±	11	++	±	±	12
PBS B	..	82	1	0	+	9	1	++	±	13	++	+	±	24
LL A	58	++	±	30	++	52	6	++	±	11	+	±	11	1
LL A	..	±	8	0	+	69	2	++	14	2	±	19	2	1
6353														
PBS A	6	++	+	124	++	±	38	++	±	19	++	+	±	13
PBS B	..	++	41	9	++	±	28	++	±	100	++	+	±	41
LL A	9	++	±	55	+	35	4	++	±	24	++	+	±	25
LL B	..	++	±	16	+	±	35	++	±	73	++	+	±	71
11														
PBS A	6	++	±	19	++	±	11	++	±	51	0	0	0	0
PBS B	6	++	43	6	++	±	126	++	±	73	++	±	33	11
LL A	7	++	±	60	++	±	19	++	±	294	0	0	0	0
LL B	8	+	17	4	++	±	73	++	±	56	++	+	±	167



SOCIETY FOR GENERAL MICROBIOLOGY

Abstracts Booklet

(119th Meeting)

University of Edinburgh

9-12 April, 1991

1445 GROWTH OF BORDETELLA SPECIES IN MAMMALIAN TRACHEOBRONCHIAL WASHINGS.

LF Porter, R. Parton and A.C. Wardlaw.
Department of Microbiology, School of Biological Sciences, University of Glasgow, Glasgow G12, 8QQ.

Although there have been numerous studies on the growth of bordetellae in a variety of artificial culture media, there appears to be no report on the *in vitro* growth of the bacteria in respiratory-tract secretions of host species.

We have therefore examined tracheobronchial washings (TBW) from a variety of animals and one bird species for ability to support bordetella growth from washed inocula of 10^3 CFU/ml. *B. bronchiseptica* and *B. avium* grew in all six species of TBW and *B parapertussis* in most of them. *B. pertussis* failed to grow in any.

Phosphate-buffered saline (PBS) was used as a non-nutrient control fluid in these experiments. To our surprise, it supported some growth of *B. bronchiseptica* and *B. avium*, but not *B. parapertussis* or *B. pertussis*. The trace nutrients in lake and pond water also supported growth of *B. bronchiseptica* which suggested that this species may not be an obligate respiratory-tract parasite, but may have the capacity to exist in aquatic enviroments.