RAPID LABORATORY DIAGNOSIS OF *BORDETELLA PERTUSSIS* INFECTION (WHOOPING COUGH) USING SEROLOGICAL AND DNA BASED DETECTION TECHNIQUES.

A thesis submitted by

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SUMMARY

This project was undertaken with the aim of applying newer serological and DNA based tests to the routine laboratory diagnosis of pertussis (whooping cough), a respiratory disease of humans caused by the small, gram negative bacterium *Bordetella pertussis*. At the time of commencement of the project the methods for laboratory diagnosis of pertussis included direct detection of *B.pertussis* organisms by culture and immunofluorescence and/or serological diagnosis by a bacterial agglutination assay for detection of *B.pertussis* agglutinating antibodies in patient sera. All of these techniques have their own particular disadvantages including lack of rapidity and lack of sensitivity. The bacterial agglutination assay was difficult to interpret and usually required paired sera for meaningful results. Therefore, better and more rapid methods were required, particularly for hospitalised patients where cross infection is a real problem and availability of rapid results allows appropriate isolation of infected individuals, thus reducing the risks of cross infection.

Initial experiments were aimed at developing an enzyme linked immuno-assay using killed sonicated *B. pertussis* cells as antigen, allowing detection of IgA, IgG and IgM antibodies. Tests on 1240 patient sera revealed this test to be more sensitive with 60% of the bacterial agglutination negative sera being ELISA positive. Detection of specific IgA is indicative of natural infection. Western blot analysis was performed on 10 sera from patients with culture- or serologically-proven pertussis in order to determine the nature of the human immunological response in infection. No consistent responses were observed for particular antibody classes or particular *B. pertussis* antigens, which supports the use of a whole cell antigen in the ELISA assay.

In order to increase sensitivity and rapidity of direct detection of *B. pertussis* in patient specimens of nasopharyngeal secretion, a polymerase chain reaction (PCR) assay was developed which amplifies a 153 base pair DNA sequence contained within a species specific repetitive element within the *B. pertussis* genome. This assay was very sensitive being able to detect 1 *B. pertussis* organism in *in-vitro* tests while also being specific and not amplifying DNA from other bacterial genera and human DNA. When applied to 332 patient NPA samples, 98 yielded positive results while only 66 were culture positive and 33 immunofluorescence positive.

Respiratory syncytial virus (RSV) infects young children and infants in particular and like *B. pertussis*, it is isolated from nasopharyngeal secretions. A combination reverse transcription-PCR for direct detection of RSV had also been developed. This procedure was refined such that both *B. pertussis* and RSV PCR could be performed simultaneously without interfering with the specificity or sensitivity of either assay. The use of this single PCR test allowing detection of either organism in samples of NPA is much more cost effective than performing 2 separate PCR assays.

DECLARATION

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any University and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text. More specifically, my contribution to the work published by E.Glare *et al* (1989) consisted of studies on the application of the pertussis cloning work to routine diagnosis of pertussis by way of DNA hybridization and PCR. Similarly, for the diagnosis of RSV infections by PCR where it was my responsibility to assess the usefulness of such a test in the clinical laboratory setting (see A. Paton *et al*, 1992).

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A.J.Lawrence

ABBREVIATIONS

AC	Adenylate cyclase
ACH	Adelaide Childrens Hospital
BA	Bacterial agglutination
bp	Base pair
BSA	Bovine Serum Albumin
CL Medium	Cyclodextrin liquid medium
CSL	Commonwealth Serum Laboratories
DIG	Digoxigenin
DTP	Diphtheria Tetanus, Pertussis triple antigen
ELISA	Enzyme linked immunosorbent assay
EtBr	Ethidium bromide
FHA	Filamentous haemagglutinin
GS	Goat serum
IF	Immunofluorescence
Ig	Immunoglobulin
kbp	Kilo base pair
kDa	Kilo Daltons
LB	Luria-Bertani
LB Medium	Luria-Bertani Medium
M	Molar
MeßCD	Heptakis (2,6-O-Dimethyl)ß-Cyclodextrin
mM	Milimolar
Mr	Standard relative molecular mass
NCTC	National Collection of Type Cultures

NCTC	National Collection of Type Cultures
NPA	Nasopharyngeal Aspirate
OMP	outer membrane protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline, pH 7.2
PCR	Polymerase Chain Reaction
PT	Pertussis Toxin
RSV	Respiratory Syncytial Virus
SDS	Sodium dodecyl sulfate
ST2	Serotype 2
ST3	Serotype 3
TBE	Tris Borate EDTA
TBS	Tris Buffered Saline
TCT	Tracheal cytotoxin
TE	Tris EDTA
TTBS	Tris Tween Buffered Saline
UV	Ultra violet
VTM	Viral transport medium
WCH	Women's and Childrens Hospital, Adelaide (formerly ACH)
WCS	Whole Cell Sonicate

CHAPTER ONE

INTRODUCTION

INTRODUCTION

1.1 CLINICAL DISEASE

Bordetella pertussis, a small gram negative coccobacillus, is the causative agent of an acute and chronic respiratory disease in humans called pertussis, also known as whooping cough. Pertussis typically has three clinical phases : catarrhal, paroxysmal and convalescent. Incubation time ranges from about one to three weeks. Patients in the catarrhal stage (which may last for several weeks) suffer from non-specific cold-like symptoms and the chances of recovering *B. pertussis* from nasopharyngeal secretions are greatest at this time. However, because of the lack of typical whooping symptoms in this phase, a diagnosis of pertussis is frequently not suspected and appropriate laboratory tests are not requested.

Symptoms gradually worsen over a few weeks with severe bouts of coughing, which are worse at night and may also be associated with vomiting. This signals the beginning of the paroxysmal phase, which may last for 1-4 weeks. During late catarrhal and early paroxysmal phases, patients exhibit lymphocytosis, the typical haematological feature of the disease. Total white cell counts can exceed 50,000 cells/mm^3 with a predominance of lymphocytes. Pulmonary consolidation is seen on chest radiographs in 20% of hospitalized patients (Mandel *et al.*, 1990). The typical whooping sound is caused by rapidly inspired air travelling past a partially closed (swollen) glottis during the paroxysms of coughing (Manclark and Cowell, 1984). However, not all infected patients will develop the whoop, and adults or previously immunised infants and children may only present with common cold-like symptoms (MMWR, 1984). Complications such as haemorrhagic events, hernia, pneumothorax and other pulmonary problems can occur during this acute phase. Of more concern, however, is the possibility of seizures, encephalopathy and

secondary bacterial infections, which can lead to death of the patient (Linneman, 1978). The mortality rate is highest in children under 1 year of age (particularly in developing countries) with the major cause of death being secondary bacterial infections (Friedman, 1988). Paradoxically, although symptoms are at their worst during the paroxysmal stage, isolation of *B.pertussis* is difficult because the organism has often been cleared from the respiratory tract by either the immune system and/or antibiotic treatment. Symptoms gradually resolve as convalescence continues although paroxysms of coughing may last with decreasing frequency for up to six months (Linneman, 1978).

Pertussis is highly contagious with an attack rate of over 90% in nonimmunised individuals (Friedman, 1988). Infection is spread from an infected individual to a susceptible host via the aerosol route, with highest attack rates for individuals exposed to a coughing patient at a range of 5 feet or less (Mandel, 1990). This high level of infectivity is particularly relevant to hospitalized patients who must be isolated in order to minimise cross infection.

1.2 PHENOTYPIC AND GENOTYPIC CHARACTERISTICS OF *BORDETELLA PERTUSSIS*

Although pertussis had been clinically recognised since the 16th century (Cone, 1970), it was not until 1906 that Bordet and Gengou first isolated *B. pertussis* from a patient with whooping cough, on solid synthetic media which now bears their names ie. Bordet Gengou agar (Bordet and Gengou, 1906). It is a most fastidious organism and clinical samples require meticulous handling together with special artificial media for successful isolation of the organism. Growth of *B. pertussis* is inhibited by a number of common media constituents such as fatty acids, metal ions, sulfides and peroxides. Starch, activated charcoal, serum albumin, blood or other protective substances must be added to pertussis isolation media. On special media such as charcoal agar, *B. pertussis*

forms tiny, pearl-like colonies after 3 to 5 days incubation in air at 37°C and in a humid environment. No growth occurs on ordinary blood agar.

Two other species of *Bordetella* have been isolated from humans. *B. parapertussis* causes human infections similar to whooping cough, but it is isolated infrequently. *B. bronchiseptica* causes respiratory infections in animals such as cats, dogs and rabbits, and is very occasionally found in human respiratory and wound infections (Woolfrey and Moody, 1991). Both of these species are less fastidious than *B. pertussis* and will grow on blood agar within 24 to 48 hours. The three species share some common antigens and earlier DNA hybridization studies showed marked inter-species homology (Kloos *et al* 1979, Pitman 1984, Arico 1987). Subsequently however, Arico *et al* (1987) have shown by computer analysis of sequence data that in genetic terms, *B. pertussis* strains are very homogeneous and distinct from *B. parapertussis* and *B. bronchiseptica*. They suggest that the proposition that *B. parapertussis* evolved into to *B. pertussis* is an unlikely scenario. *Bordetellae* in general are obligate aerobes, unable to attack carbohydrates and isolated only from warm blooded animals while *B. pertussis* itself is only isolated naturally from humans (Toumanen *et al.*, 1983).

B. pertussis organisms undergo a phase variation characterised by simultaneous loss of the expression of multiple virulence factors. Spontaneous variants that are no longer able to cause disease in animal models occur at a frequency of one per 10^3-10^6 organisms (Weiss and Falkow 1984, Armstrong and Parker 1986). Most importantly, these isolates are no longer able to produce pertussis toxin, adenylate cyclase, filamentous haemagglutinin, certain OMP's and other toxins such as dermonecrotic toxin and haemolysin. Avirulent phase mutants revert back to the virulent phase 1 at low frequency. If a strain of *B. pertussis* not in phase 1 was used to prepare antigen for serological tests, the sensitivity of the assay would be significantly reduced as it would not detect

antibodies to the majority of the major virulence factors, resulting in a false-negative diagnosis.

A single genetic locus encodes a positive inducer that turns on expression of the virulent-phase genes (Weiss and Falkow 1984). However, not all virulence factors are under control of this locus (eg. lipopolysaccharide and tracheal cytotoxin). A second form of regulation, phenotypic modulation, causes the virulent-phase genes to be regulated by environmental conditions. No virulence factors are produced if the organism is grown below 37°C or in medium supplemented with substances like magnesium sulphate or nicotinic acid. Expression is reinstated when the bacteria are returned to permissive growth conditions. The role of phase variation or phenotypic modulation in the disease process is unclear, although some studies suggest both may occur *in vivo*. Modulation *in vivo* may be a mechanism for an unrecognised carrier state. *B. pertussis* organisms which are not expressing virulence factors may be able to colonise individuals. It is only when conditions (such as environmental) change, that genetic expression occurs and factors such as pertussis toxin are produced, leading to frank disease (Weiss and Hewlett, 1986).

1.3 EPIDEMIOLOGY AND EFFECTS OF VACCINATION

Pertussis was a major cause of mortality and continuing morbidity in the more developed world until the introduction of a whole cell vaccine in the 1940's, which resulted in a dramatic decrease in the incidence of the disease (Cherry, 1984). There are currently two forms of vaccine available, a killed whole cell vaccine and an acellular formulation; both are usually combined with diphtheria and tetanus toxoids and delivered as the familiar triple antigen vaccine. The whole cell vaccine is most commonly used throughout the world, including Australia, at the present time. Whole cell vaccines are believed to be more than 80% efficacious in preventing pertussis, although different studies have shown

variations (0-100%) in this figure depending on the type of trial and case definition (Fine and Clarkson, 1987). Duration of protection is limited, with one study showing attack rates of ≥ 95% for individuals 12 years post immunisation (Lambert, 1965). Individuals who have been immunised and become infected will generally experience less severe disease (Grob *et al.*, 1981).

The acellular vaccine, available in some parts of the world (eg. Japan and Sweden), is composed of a mixture of purified individual *B. pertussis* antigens such as pertussis toxin (PT), filamentous haemagglutinin (FHA) and some agglutinogens, depending on the formulation. These vaccines have been in use in Japan since 1981 and have been found to be effective in reducing the incidence of pertussis and preventing infection (Mortimer *et al.*, 1990). In Japan, the use of whole cell vaccine was temporarily ceased in 1975 because of 2 fatalities attributed to vaccination. When use of the vaccine was recommenced a few months later, the acceptance rate dropped from 78% to a low of 14% in 1976 which subsequently recovered to 41% in 1977 and 64% in 1978. A huge increase in the incidence of pertussis was noted during this period of low vaccine usage, confirming the efficacy of the whole cell vaccine. Despite the increased acceptance, an attempt was made to reduce adverse affects produced by vaccination by developing an acellular formulation. Such formulations are associated with fewer side effects (predominantly, less febrile reactions) than conventional DTP and modify clinical illness even when immunised children contract whooping cough (Aoyama *et al.*, 1985).

In spite of immunisation programs, pertussis remains a significant public health problem (estimated 51,000,000 cases/year world wide) particularly in developing countries where there are high mortality rates (Walsh and Warren, 1979; Woods, 1991; Wright, 1991). In developed countries significant morbidity is associated with pertussis but there are fewer deaths. Evidence from a 10 year study in Great Britain suggests however that despite the tremendous

impact of vaccination, it alone is not sufficient to totally prevent outbreaks of pertussis, but nevertheless it attenuates the clinical severity of the disease (Jenkinson, 1988). In the USA, one study covering the four year period 1985-1988 concluded that there were an average of 3,300 hospitalisations and 25 deaths per year during this period (Sutter and Cochi, 1992). Pertussis is re-emerging as a significant problem in countries such as the United States and Great Britain, because excessive publicity given to the possible neurodegenerative side effects associated with the whole-cell vaccine has lead to a decreased acceptance of vaccination (Cody *et al.*, 1981; Miller *et al.*,1981; Miller *et al.*, 1985; Miller and Farrington, 1988). However, it appears that few of the reported cases of neurological illness were directly attributable to the vaccine itself (Cherry *et al.*, 1988). A decrease in vaccination has led to an increased incidence of the disease in the United States, United Kingdom (although the level of immunised individuals has risen over the past 10 years (Thomas *et al.*, 1989)) and Sweden (CDC 1984; Cherry, 1984; Romanus *et al.*, 1987). The Italian government is even considering making pertussis immunisation mandatory, because of the ambivalence the Italian people have toward vaccination, which is apparently not related to concerns about safety. In Italy, where fewer than 40% of children younger than 5 years are vaccinated, 25% have experienced clinical pertussis by their 5th birthday and 1 in 14 of these cases is admitted to hospital (Binkin *et al.*, 1992).

In the pre-vaccine era, pertussis was most commonly seen in children 1-5 years of age. A study in 1937 showed 60% of pertussis patients were 1-5 years, while only 19% were <1 year of age. Most adults at that time would have been exposed to pertussis at some stage and hence the adult immunity level was high. Vaccination with the whole cell vaccine has decreased the incidence of disease, but a concomitant shift in peak age of disease has also occurred in the United States. In 1982-83, 53% of pertussis cases reported were in infants

<1 year of age (MMWR 1984). It is highly likely that a similar shift in average age has occurred in Australia.

Although pertussis is a more severe disease in infancy and early childhood, adults may also be infected. One survey from Sydney, Australia, found 25.7% of cases of adults with persistent cough were infected with *B. pertussis* (Robertson *et al.*, 1987). Thus, disease can occur at any age and requires fast and accurate laboratory diagnosis in order for appropriate treatment to begin, but more importantly, to control the spread of the disease to other susceptible individuals.

In Adelaide there are still significant numbers of cases occurring despite an immunization rate of about 80% (personal communication, Dr Scott Cameron, South Australian Health Commission). **Figure 1.1** shows the number of culture confirmed cases from 1982 to 1992 by the Microbiology laboratory, WCH. It must be remembered that the culture confirmed cases probably represent less than 50% of infections in patients who have had specimens collected for culture. In addition there will be a very large number of other infants, children and adults who have been infected but not had culture or serological assays performed. These include siblings and contacts of infected patients, who are at great risk of contracting pertussis, particularly if they have not been immunised.

As can be seen from **Figure 1.2**a, a cyclical nature in the rate of infection is apparent (as represented by the number of culture positive cases) in Adelaide over the 10 year period. Epidemics usually occur in 3-5 year cycles that are attributed to the accumulation of susceptible individuals in the population (Mandel, 1990). The last major upswings in numbers of culture positive cases from the WCH were in the summers of 1985/86, near the time this project commenced, and to a lesser degree in 1989. This phenomenon is applicable to Australia in general with all states having similar outbreaks at these times (Hall, 1990; Masters *et al.*, 1990). A seasonal variation in numbers of

Figure 1.1

Culture-positive pertussis cases by year and month : 1982 - 1992.

Total numbers of culture-positive cases of pertussis from the Microbiology Department of The Women's and Children's Hospital, North Adelaide (formerly Adelaide Childrens Hospital) over the 10 year period 1982 - 1992.

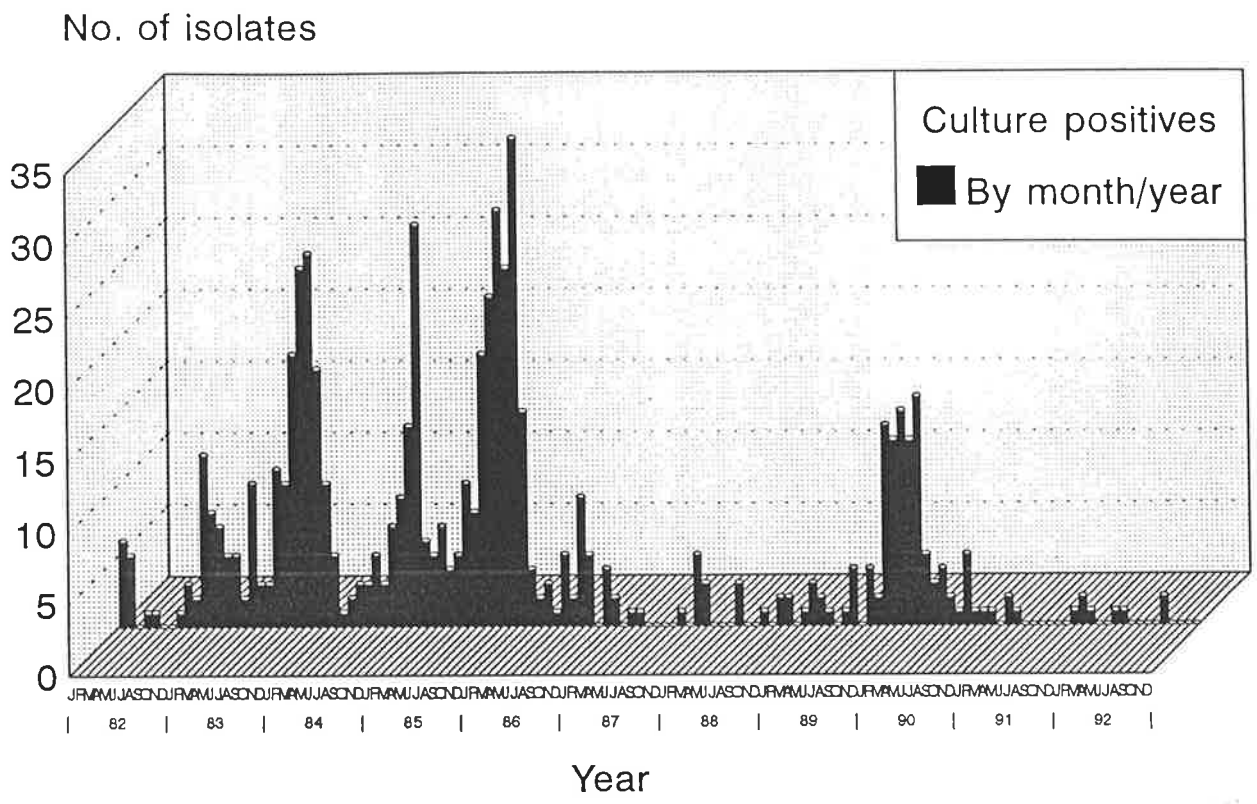

No. of isolates
Culture positives
By month/year
35
30
25
20
15
10
5
0
82
83
84
85
86
87
88
89
90
91
92
Year

Figure 1.2a

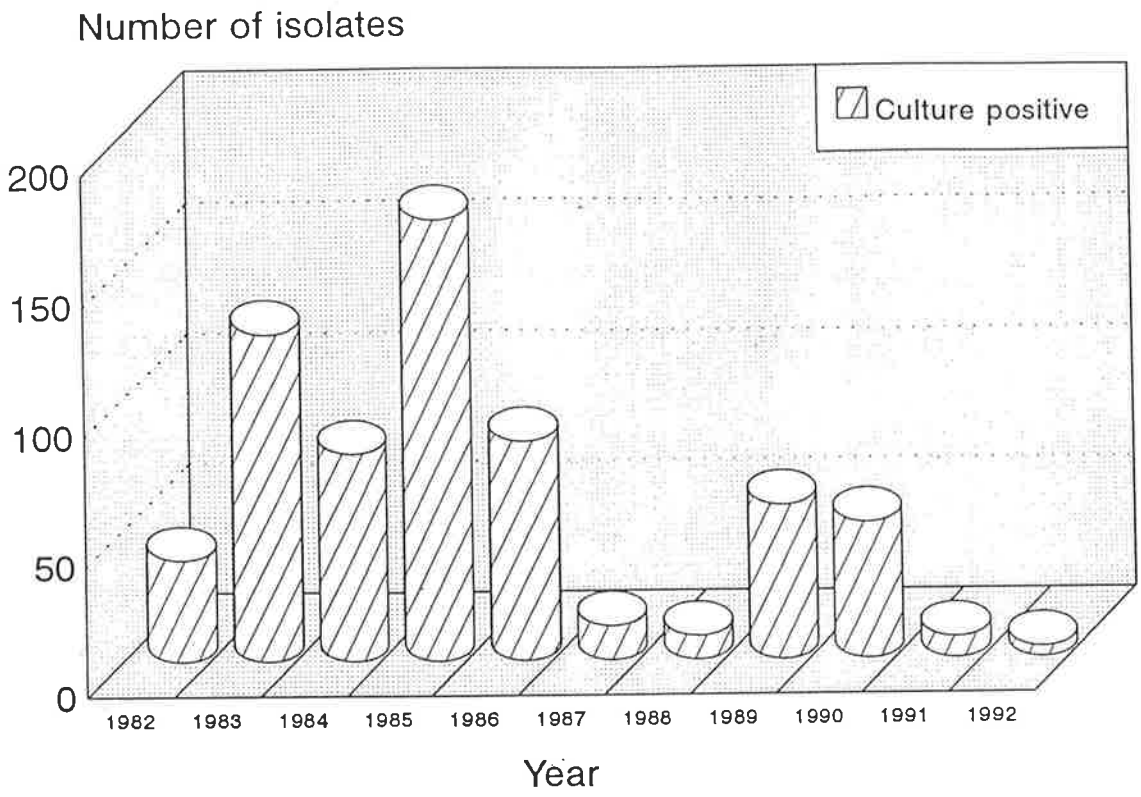
Total *B. pertussis* isolates per year : 1982 - 1992

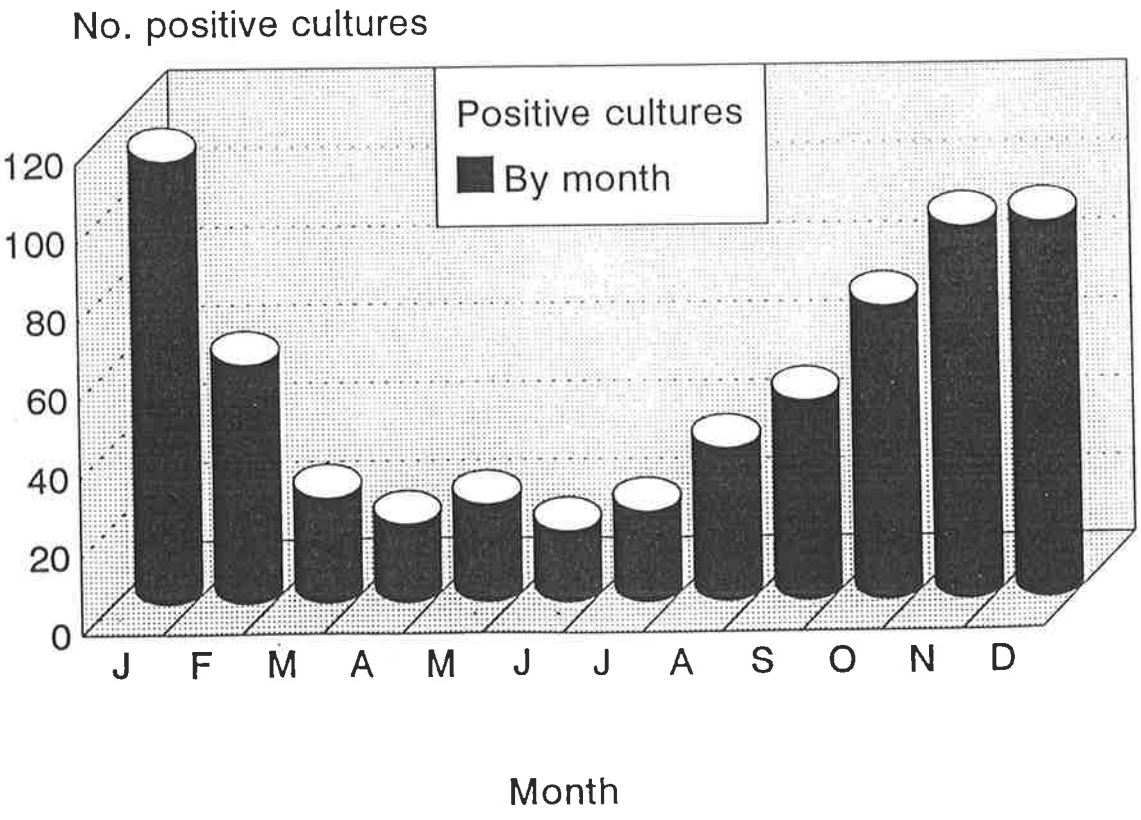
Total number of isolates of *B. pertussis* from specimens of nasopharyngeal aspirate or nasopharyngeal swabs during the 10 year period 1982 - 1992 detected at the Microbiology Department, Women's and Children's Hospital.

Figure 1.2b

Total *B. pertussis* isolates by month : 1982 - 1992 (combined)

Total numbers of culture-positive pertussis cases from the Microbiology Department of The Women's and Children's Hospital, Adelaide, for the 10 year period 1982 - 1992. The cylinders represent the cumulative total number of cases per month.


Number of isolates
Culture positive
200
150
100
50
0
1982
1983
1984
1985
1986
1987
1988
1989
1990
1991
1992
Year


No. positive cultures
Positive cultures
By month
120
100
80
60
40
20
0
J
F
M
A
M
J
J
A
S
O
N
D
Month

infections is also observed with most cases occurring in late spring and summer **(Figure 1.2b)**.

1.4 PATHOGENESIS AND VIRULENCE FACTORS

It is apparent that *B. pertussis* possesses a number of virulence factors allowing it to infect a susceptible individual. Attempts to develop animal models for investigating the pathogenesis of whooping cough, particularly in mice, rats and hamsters (not relevant to Australia) have met with limited success. However, mice have been used to identify antigens that promote protection by active and passive immunisation (Cowell *et al.*, 1984; Pittman, 1984). The disease produced in mice is not analogous to the most common respiratory manifestation of human disease (Halperin *et al.*, 1988). The rat model, however, more closely resembles human disease with respect to clinical, pathological and physiological changes.(Woods *et al.*, 1989).

The clinical manifestations of pertussis suggest it is primarily a toxin-mediated disease, which results from the release of pertussis toxin (PT) from the bacterial cells following attachment of the organisms to the cil iated respiratory tract epithelium, which are damaged in the process (Wilson *et al.*, 1991). However a number of other known and potential virulence factors are believed to be important in the establishment of an infection. These include filamentous haemagglutinin (FHA), haemolysin, endotoxin, adenylate cyclase (AC), heat-labile toxin, tracheal cytotoxin, agglutinogens and pertactin.

1.4.1 Pertussis toxin

PT is the most studied and well characterized virulence factor of *B.pertussis* and it alone accounts for the majority of the clinical manifestations of pertussis. Various names have been applied to PT (lymphocytosis promoting factor, histamine sensitising factor, islet activating factor and pertussigen)

because of its wide range of biological activities. These include leukocytosis, histamine sensitisation, lethality, mitogenicity, adjuvant effects and stimulation of insulin secretion (Pittman, 1984; Weiss and Hewlett, 1986).

The structure of PT has been found to be typical of an A-B protein toxin composed of 5 subunits. The "A" (S1) component is the active portion and catalyses the adenosine 5'-diphosphate ribosylation of a 41 kDa membrane protein in a number of mamalian cell types leading to deregulation of control on cellular adenylate cyclase, which in turn leads to enhanced activity of the mammalian cells . The "B" component is composed of subunits S2, S3, S4 and S5 arranged in 2 dimers (S2+S4) and (S3+S4), held together by S5. The B moiety mediates binding of the toxin to specific receptors on the mammalian cell surface and subsequent entry of the enzymatically active subunit into these cells (Katada and Ui, 1982; Gilman, 1984; Nicosia *et al.*, 1987).

PT is a protective immunogen in humans and animals. Granstrom *et al* (1985) demonstrated a role for PT in immunity for humans by showing a correlation between anti-PT serum titres and long term immunity to *B. pertussis* infections in patients. Studies in mice indicated that they could be protected from lethal aerosol challenge with *B. pertussis* by intraperitoneal injection of anti-PT rabbit hyperimmune serum (Sato *et al.*, 1981). Additionally mice could be protected both in the above challenge and the intracerebral challenge by either active immunisation with PT-toxoid or passive immunisation with anti-PT sera (Sato and Sato, 1984).

The genes for production of the five subunits of *B. pertussis* toxin are organised as an operon. The promoter region is only weakly active when this operon is cloned into *E.coli* with very little toxin produced in such clones. *B. parapertussis* and *B. bronchiseptica* also have this promoter region but it is again very weak (with no detectable PT mRNA) and no toxin is produced by these *Bordetellae spp.*despite the presence of the toxin genes which are 98.5% and 96% homologous to the *B. pertussis* toxin gene respectively. Efficient

transcription of the toxin genes requires at least two features : (1) a 170-bp DNA sequence upstream from the start site of transcription and (2) a trans-activating factor encoded by the *vir* locus. Non virulent, phase III *B. pertussis*, which do not produce toxin, also do not possess any of this trans activating factor (Arico and Rappouli, 1987; Nicosia and Rappouli, 1987; Gross and Rappouli, 1988).

1.4.2 Filamentous haemagglutinin

On electron microscopy, FHA appears as a fine 40-100 nm long filament like structure, extending from the cell surface, with a diameter of about 2 nm. It has been purified and has a variable M_r ranging from 220,000 to 58,000. Many of the lower molecular weight bands seen on sodium dodecyl sulphate polyacrylamide gel electrophoresis assays are probably breakdown products of higher M_r (native) FHA (Irons *et al.*, 1983; Sato *et al.*, 1983). This protein may be involved in the initial attachment and adherence of *B. pertussis* to the epithelial lining of the upper respiratory tract helping to initiate infection (Toumanen *et al.*, 1985). Mouse studies have shown that active but not passive immunisation with FHA protects against aerosol challenge and has a synergistic protective effect when used together with PT in a vaccine (Oda *et al.*, 1984; Sato and Sato, 1984). Antibody directed against FHA in humans may contribute to protection through inhibition of colonisation of the respiratory tract.

The gene encoding FHA (*fha*) has been cloned and mapped by Brown and Parker (1987) to a 6.5 kb DNA fragment. The immunoreactive FHA, as expressed in *E. coli*, was larger than that expressed in *B. pertussis*, suggesting a difference in processing of this protein between the two species of bacteria. Further analysis of a 3.5 kb nucleotide sequence encoding the amino-terminal region of the *fha* gene was carried out by Delisse-Gathoye *et al.*, (1990). This work identified the primary translation product as a protein of about 370 kDa.

The mature 220 kDa FHA polypeptide secreted by *B.pertussis* is most probably generated by proteolytic processing that eliminates a carboxy-terminal portion of about 150 kDa.

1.4.3 Adenylate Cyclase

AC is mainly associated with *B. pertussis* cell surfaces and inhibits human polymorphonuclear leukocyte function including chemotaxis and oxygen consumption. It has been hypothesized that the *B. pertussis* AC enters phagocytic cells where it is activated by calmodulin (a regulatory protein) and induces high levels of cyclic adenosine 3,5' phosphate (CAMP) which impairs polymorphonuclear leukocyte and macrophage bactericidal functions (Confer and Eaton, 1982). AC could play a role in *B. pertussis* pathogenesis by inhibiting nonspecific host defences in the respiratory tract (Friedman *et al.*, 1987). Studies have differed in their estimation of the molecular weight of AC and have M_r ranging from 43,000 to 340,000. This variation may be due to differing methods used to isolate the protein (eg. isolation from culture supernatants or cell surfaces) and whether or not it associates with calmodulin, or if it is observed in the presence of high calcium ion concentrations. It may be a high molecular weight protein which is composed of smaller sub-units or it may form aggregates (Friedman *et al.*, 1987). There is sufficient evidence however, from mouse protection and site directed mutagenesis studies, to suggest that AC is a significant virulence factor of *B.pertussis* (Friedman, 1988).

1.4.4 Agglutinogens (Fimbriae)

B. pertussis possesses a number of surface fimbriae (as do other gram-negative bacteria) which are able to cause agglutination by cross linking bacterial cells in the presence of specific antibodies. Eldering *et al.*, (1957)

used sera raised in rabbits to develop a serotyping system based on six agglutinogens. Preston *et al.*, (1982), re-evaluated the scheme and proposed agglutinogen serotypes 1,2 and 3 were the major antigens while 4, 5 and 6 were of lesser significance, because they only elicited weak serological responses. Agglutinogens are composed of helical structures formed by repeating units of monomeric fimbrial proteins of M_r 22,000 and 22,500 for agglutinogens 2 and 3 respectively, (Steven *et al.*, 1986; Ashworth *et al.*, 1988).

It is likely that these fimbriae mediate adherence, because antibodies to serotype 2 and 3 fimbriae (agglutinogens 2 and 3) inhibit binding of *B. pertussis* to Vero cells in a serotype-specific manner (Gorringe *et al.*, 1985). Furthermore, mice immunised with purified fimbriae are protected against subsequent intra-nasal challenge with virulent *B. pertussis* and patients with high agglutinogen antibody titres do not get pertussis (Manclark, 1984; Robinson *et al.*, 1985; Zhang *et al.*, 1985). These agglutinogens are therefore likely to be important virulence factors of *B. pertussis* and could be considered for inclusion in new acellular vaccines.

The genes encoding serotype 2 and serotype 3 agglutinogens have been cloned. DNA sequence analysis indicated that the gene encoding the serotype 2 antigen (ST2) has an open reading frame sufficient to encode a polypeptide of 207 amino acids, including a 26-amino-acid signal sequence. The protein deduced from the nucleotide sequence shows good agreement with the NH_2-terminal amino acid sequence, amino acid composition and molecular weight of the purified fimbrial subunit. The proposed ST2 subunit has been shown to have homology with other fimbrial subunits. Comparison of the putative promoter regions of both *fim*2 (ST2) and *fim*3 (ST3) genes reveals a conserved region which may be involved with fimbrial phase variation. A comparison of the deduced amino acid sequences of these fimbrial subunits suggests conserved, variable and hypervariable regions are present. Peptides derived from the conserved regions may be incorporated into future pertussis vaccines to induce

antibodies which confer protection against strains producing different fimbrial serotypes (Livey *et al.*, 1987; Mooi *et al.*, 1990).

1.4.5 Lipopolysaccharide (LPS)

LPS from *B.pertussis* has the same biological activities as endotoxin from other gram negative bacteria although it has a different chemical structure to that in entero-bacteria. However, the role of *B. pertussis* LPS in pathogenesis and immunity to whooping cough is unknown (Friedman 1988).

1.4.6 Haemolysin

When *B.pertussis* is grown on Bordet-Gengou agar plates (which contain 15% horse blood), zones of haemolysis are produced around isolated colonies. Studies on other bacterial pathogens have shown the importance of haemolysins as virulence factors, but its role is not yet clear in *B.pertussis* (Friedman, 1988).

1.4.7 Dermonecrotic Toxin (Heat Labile Toxin)

This toxin causes inflammation and necrotic skin lesions after sub-cutaneous injection at low doses in mice, but is lethal in high doses. It may be partially exposed on the cell surface and loses toxicity if heated to 56°C for 10 minutes (Livey and Warlaw, 1984). Differing studies have reported the M_r to be 102 and 89 kDa and may be composed of four polypeptides, two each of 30 and 24 kDa. The role and function of dermonecrotic toxin in human infection is unclear (Friedman, 1988).

1.4.8 Tracheal cytotoxin (TCT)

TCT is a small glycopeptide, probably derived from peptidoglycan in the cell wall, which causes damage to ciliated respiratory epithelial cells in *in vitro* studies using hamster tracheal ring cultures. Such cells are the site of

initial attachment of infecting organisms and failure to repair them after exposure to this toxin could provide an advantage to *B. pertussis* (Friedman, 1988). The estimated M_r of the fragments released by hydrolysis of the peptidoglycan matrix, are between 1,235 and 1,400 Da (Goldman and Herwaldt, 1985). Such compounds may be general virulence factors for gram negative bacteria.

1.4.9 Pertactin (69 kDa OMP)

This OMP has been purified, cloned and analysed and its synthesis appears to be under the control of the *vir* locus. It is an agglutinogen, localised on the outer membrane, which cross reacts with type 3 antisera of Eldering. The gene for pertactin is capable of encoding a protein of 910 amino acids with a M_r of 93,478, suggesting the 69 kDa protein is processed from a larger precursor (Charles *et al.*, 1989). Mice immunised with this OMP are protected from intracerebral challenge with virulent *B. pertussis* organisms. Antibodies reacting with the 69 kDa OMP have been detected in sera from humans recovering from disease as well as from vaccinated individuals (Eldering *et al.*, 1957; Charles *et al.*, 1989; Gould-Kostka *et al.*, 1990). These facts suggest it should also be considered as a component of future vaccines.

1.5 TREATMENT

Patients who are diagnosed as having pertussis can be treated with antibiotics such as tetracycline, erythromycin, co-trimoxazole or chloramphenicol, which are all effective in eliminating *B.pertussis* from the respiratory tract. Newer oral cephalosporins are unlikely to be of use in treating pertussis (Hoppe and Muller, 1990). Erythromycin (estolate) appears to be the most reliable because high levels are obtainable both in serum and respiratory tract tissues. Unfortunately, treatment usually has little effect on the course of disease, because by the time typical symptoms become apparent and therapy has

been commenced, the infection is well established and the neurological effects of pertussis toxin are not reversed by antimicrobial drugs. There is some evidence, however, which suggests erythromycin therapy may reduce the severity and duration of pertussis even when commenced at the beginning of paroxysmal stage (Bergquist *et al.*, 1987; Hoppe, 1992). Antibiotics such as erythromycin will certainly clear any viable organisms from the respiratory tract of the infected patient thus preventing spread of infection to close contacts such as siblings. Common cough suppressants are not effective in controlling the paroxysms of coughing.

1.6 LABORATORY DIAGNOSIS

The laboratory diagnosis of pertussis, which includes detection of *B. pertussis* in nasopharyngeal secretions or serological techniques, presents a number of problems for the clinical microbiology laboratory. These relate to the fastidious nature of *B. pertussis* growth requirements, the slow growth of the organism on artificial media and the relatively low recovery rate from infected patients. In addition the disease does not always present in a typical form (Linneman and Nasenberry, 1977; Nelson 1978; Robertson *et al.*, 1987).

At the time of commencement of the project (1986) three different methods for laboratory diagnosis of whooping cough were in use at the ACH.

1.6.1 Culture

Isolation of *B. pertussis* by culture is the diagnostic "Gold Standard" for pertussis and confirms the diagnosis of whooping cough. By definition, the specificity of culture is 100% since the organism is not considered normal flora of the nasopharynx of humans. *B. pertussis* is a very fastidious and slow growing organism, which requires special artificial media for growth. Charcoal-horse blood agar supplemented with cephalexin (40 μg/ml) has been shown to be the most effective solid medium for primary isolation of *B.*

pertussis from nasopharyngeal secretions (Hoppe and Schwaderer, 1989). Collection of appropriate specimens is crucial and nasopharyngeal aspirate samples (NPA) must be cultured onto appropriate solid media as soon as possible after collection. At the WCH, nasopharyngeal aspirates are collected by laboratory staff and processed within 10 minutes. Growth of the organism from primary culture may take from 2 to 7 days of incubation at 37°C, after which small pearl like colonies are visible. The identity of these colonies is confirmed by a slide agglutination reaction with specific *B. pertussis* antiserum. Culture is not a sensitive method for detecting the organism in nasopharyngeal aspirates and evidence suggests that positive laboratory results may be obtained in only 30% - 60% of clinical cases (Combined Scottish Study 1970; Granstrom *et al.*, 1982 (a) and 1982 (b); Viljanen *et al.*, 1982; Hakansson *et al.*, 1984; Mertsola *et al* 1983 (a) and 1984 (b); Granstrom *et al.*, 1991). There are good reasons for this, particularly in the group of patients who present at hospitals such as the WCH. These patients are often in the paroxysmal cough or even convalescent (later) stage of the disease and have probably had symptoms for several weeks or even months, and may also have been treated with antibiotics. At this stage of the disease the chances of recovering viable organisms from the nasopharynx are significantly reduced. This is despite the presence of typical whooping cough symptoms, which are a result of neurological activities of the toxin produced by the organism when it was infecting the patient, but which is no longer necessarily present in the respiratory tract. The likelihood of obtaining a positive culture is generally inversely proportional to the period of time symptoms have been present.

1.6.2 Immunofluorescence (IF)

An alternative method for detection of *B. pertussis* in NPA's is IF. It has a theoretical advantage in that it can detect the presence of viable or dead *B. pertussis* cells, but IF suffers from poor sensitivity and additionally sometimes

specificity is a problem (Chalvardjian 1966, Field and Parker 1975, Gilligan and Fischer 1984, Halperin *et al* 1989). Experience at WCH indicates that only NPA specimens containing large numbers of viable organisms, sufficient to produce a heavy growth on charcoal agar, give positive results using direct immunofluorescence. Notwithstanding this, IF is useful in diagnosing pertussis particularly in young infants less than 3 months of age. The disease tends to be clinically more severe and progresses more rapidly in this group compared to older children and adults. Thus, these infants are often admitted to hospital at an earlier stage of the disease, when there are likely to be larger numbers of viable organisms present in the nasopharynx.

It is important to have a technologist with considerable experience in immunofluorescent microscopy to process all specimens. This helps to reduce the high proportion of false positive results (estimated to range from 7-40%) which is associated with this method (Holwerda and Eldering, 1963; Broome *et al.*, 1978; CDC, 1984). The principal advantages of IF lie with the rapidity of the test (same day results are possible) and the relatively low cost of consumables, but these do not fully compensate for the lack of sensitivity and specificity.

1.6.3 Sero-diagnosis of Pertussis

Serological diagnosis of pertussis, based on specific antibody responses to *B. pertussis* antigens which occur during infection, is an important adjunct to diagnostic methods centered around detection of organisms. Such methods are particularly valuable for patients who present late in the course of illness. Moreover, serum antibodies are much more stable than are *B. pertussis* organisms in nasopharyngeal secretions. A bacterial agglutination assay (BA) was used at the WCH for serological diagnosis of whooping cough at the time of commencement of the project. This assay detected agglutinating antibodies present in serum to *B. pertussis*. Again, from personal experience, this was not

a reliable assay and there were often difficulties in interpreting the agglutination reactions. It was also not known which class of antibodies were being detected and against which antigens they were directed. The test was slow, requiring overnight incubation as well as relatively large amounts of serum. Optimal analysis required paired samples which were usually collected 14 days apart. The results gave some indication of levels of clinical protection against infection, but diagnosis of current infection was difficult, particularly if paired samples were not received.

1.7 PROJECT AIMS

The rapid, sensitive and specific laboratory diagnosis of *B.pertussis* infection in children and adults is an important function of the clinical microbiology laboratory, particularly in a paediatric hospital where prevention of cross infection is a priority. The methods for laboratory diagnosis (serological and bacteriological) in use at the WCH prior to the commencement of this study were neither sensitive nor rapid.

Enzyme linked immunosorbent assays (ELISA) are sensitive tests which can be applied to serological diagnosis of infectious diseases, by quantitating class-specific antibody responses to particular antigens which are bound to the walls of 96-well microtitre trays. Appropriately diluted patient serum is added to each well. Any specific antibody will then bind to its respective antigen and anti-human antibody conjugated with an enzyme such as horse radish peroxidase is added. After addition of appropriate chromogenic substrate, any bound conjugate will produce a colour change which can be measured spectrophotometrically. Use of such an assay for detection of specific antibody to *B. pertussis* antigens offered a means of greatly improving the serological diagnosis of whooping cough. Additionally, there now existed the possibility for diagnosis on a single serum sample if the assumption that natural infection

will elicit an IgA response was used (Nagel and Poot-Scholtens 1983). Therefore the potential for more rapid result generation existed because there may be no need for a second, paired specimen to be collected 2 weeks after the first.

In addition to serological diagnosis, rapid detection of the organism itself in NPA specimens was a high priority. Recent developments in molecular biological detection techniques, particularly the advent of polymerase chain reaction (PCR) also provided the possibility of improving methodology for direct detection of *B. pertussis* organisms in NPA specimens.

The aims of this project were therefore to improve the routine laboratory diagnosis of *B. pertussis* infection by :

a) Developing a rapid, sensitive and specific ELISA based diagnostic test for semi-quantitative detection of class specific antibodies directed against antigens of *B. pertussis* in sera from patients suspected of having whooping cough.

and

b) Developing a rapid DNA hybridization and/or PCR based test for detection of *B. pertussis* specific DNA sequences in samples of nasopharyngeal aspirate from patients with symptoms of whooping cough.

CHAPTER 2

GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

2.1 MICRO-ORGANISMS

2.1.1 Bacterial strains

B. pertussis NCTC 10908. (serotype 1,2,3,4), *B. pertussis* NCTC 10911(serotype 1,2,5,6), *B. bronchiseptica* NCTC 8344 and *B. parapertussis* NCTC 5952 were all obtained from the National Collection of Type Cultures (NCTC, Pubic Health Laboratory Service, 61 Colindale Ave., London).

B. pertussis and *B. parapertussis* clinical laboratory isolates from patient samples of nasopharyngeal secretion were cultured and collected from the WCH, Microbiology Department. All cultures were stored at -75°C in serum broth (10% horse serum in meat extract broth) until required, at which time they were plated onto charcoal agar plates and incubated in a moist environment for at least 48 hours.

96 different species of bacteria other than bordetellae were collected from clinical specimen cultures in the routine diagnostic laboratory of the Microbiology Department, WCH. These strains were used to test the specificity of a *B. pertussis* DNA probe and polymerase chain reaction assays. The group of organisms consisted of 45 different species representing 24 genera. Included in the group were members of the most closely related genera to *Bordetella*, ie. *Pasteurella, Alcaligenes* and *Haemophilus* species.

Escherichia coli K 12 strain JM109 has been described by Yanisch-Perron *et al* (1985).

2.1.2 Respiratory syncytial virus (RSV)

a) Long strain was kindly provided by Dr. I Gust, Fairfield Hospital, Melbourne.

b) Clinical isolates (present in RSV culture positive nasopharyngeal secretions) were provided by Dr. P Hallsworth, Clinical Microbiology

Department, Flinders Medical Centre. Further RSV-positive NPA's were obtained from patients admitted to the WCH during 1992. These samples had been found to be culture positive, after routine culturing for RSV by the Division of Medical Virology, Institute of Medical and Veterinary Science, Adelaide.

2.2 MEDIA

2.2.1 Media for growth of *B. pertussis*

a) Solid media. Isolates were routinely grown on charcoal agar (Oxoid Ltd., Basingstoke UK) containing 10% defibrinated horse blood (CSL, Parkville, Victoria). Clinical isolates of *B. pertussis* and *B. parapertussis* were routinely isolated from primary culture on charcoal agar as above and charcoal agar supplemented with cephalexin (40μg/ml, supplied as Ceporex, Glaxo (Australia), Boronia, Victoria). These solid media were sterilized by autoclaving at 121°C for 15 minutes and after cooling to 45°C, defibrinated horse blood was added as well as cephalexin where appropriate. Cultures were routinely incubated in air at 37°C and in a humid atmosphere for at least 48 hours before harvesting.

b) Liquid media. Cultures of *B. pertussis* were also grown in liquid media primarily for preparation of antigen for use in Western blotting. This medium was a modification of the Stainer-Scholdt (SS) medium and designated CL medium. This medium has been shown to stimulate cell growth and yield 100 times more PT compared to SS media. (Stainer and Scholte, 1971; Imaizumi *et al.*, 1983). The formula of CL medium in grams/litre is as follows; sodium L-glutamate 10.7, L-proline 0.24, NaCl 2.5, KH_2PO_4 0.5, KCl 0.2, $MgCl_2.6H_2O$ 0.1, $CaCl_2$ 0.02, Tris 6.1, L-cysteine 0.04, $FeSO_4.7H_2O$ 0.01, Niacin 0.004, glutathione reduced 0.15, ascorbic acid 0.4, casamino acids (certified) 10.0, Heptakis (2,6-O-Dimethyl)ß-Cyclodextrin (MeßCD) 1.0.

2.2.2 Media for growth of *E coli*

E. coli strains were grown in Luria-Bertani (LB) medium (Maniatis *et al.*, 1982) with or without 1.5% bacto-agar (Difco Laboratories, Detroit, Michigan). LB medium consists of 1% bacto-tryptone (Difco), 0.5% bacto yeast extract (Difco), 1% NaCl, pH 7.5. The media was sterilized by autoclaving at 121°C for 15 minutes. When appropriate, ampicillin (Commonwealth Serum Laboratories, Melbourne, Australia) was added to the media at a concentration of 50μg per ml after it had been autoclaved and cooled to 45°C.

2.2.3 Media for growth of other bacteria

All other bacteria to be tested in the hybridization experiments were grown overnight on horse blood agar (heated blood agar for *H. influenzae*). This solid medium consisted of blood agar base No. 2 (Oxoid) supplemented with 2.5% defibrinated horse blood (CSL). All cultures were grown overnight in 95% air/5% CO_2 at 37°C .

2.3 Routine Chemicals

Tris (Hydroxymethyl) aminomethane (Tris) was purchased from Boehringer Mannheim, Sydney, Australia. Sodium dodecyl sulfate (SDS) was obtained from the Sigma Chemical Co, St Louis, Missouri, USA.

All other general chemicals and solvents were purchased from Ajax Chemicals, Auburn, NSW and were at least A.R. grade. All solutions were made with water which had passed through a High Pure de-ionization water system (Permutit, Australia). Filter sterilization (if required) was achieved by passaging a solution through a sterile 0.2 μm filter.

2.4 STORAGE OF BACTERIAL AND VIRUS STRAINS

2.4.1 *B. pertussis* NCTC strains

These strains were stored using the Protect system (STL, Sydney, Australia). Organisms were harvested from fresh charcoal agar plates and suspended in the Protec liquid medium which also contained 25 beads. Excess fluid medium was removed and the beads, impregnated with bacteria, remained. All procedures were performed according to the manufacturer's instructions. The vials containing 25 beads were kept at -80°C until required, when a single bead was removed and inoculated onto a charcoal plate.

2.4.2 Clinical isolates of *B. pertussis* and other organisms

Cells from fresh 48 hour charcoal agar plates or fresh overnight 2.5% horse blood agar plates, respectively, were harvested and inoculated into 2 ml aliquots of serum broth. The serum broth consisted of 90ml of meat extract broth and 10ml of filter sterilized horse serum (Commonwealth Serum Laboratories, Melbourne, Australia). The meat extract broth consisted of 10% peptone (BDH Limited, UK), 5% NaCl and 6% Lab Lemco Powder (Oxoid), pH7.4. This broth was incubated at 90°C for 60 minutes after which any precipitate which had formed was filtered out, and then autoclaved at 121° for 15 minutes. After addition of the sterile horse serum, 2ml aliquots were dispensed into disposable, sterile plastic vials. A moderately dense suspension of the organisms was made in this broth and placed at -80°C without added glycerol.

2.4.3 RSV strains

RSV strains were stored as freeze-thawed lysates of infected HEp2 cell cultures, and were kept at -80°C in Dulbeco's modified Eagles medium, until

thawed for use as test samples or positive controls, either in enzyme immunoassays or polymerase chain reaction assays.

2.5 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR SPECIFIC *B. pertussis* ANTIBODIES

2.5.1 Antigen preparation and coating of microtitre plates

Freshly harvested bacteria were suspended in 0.1M sodium carbonate (pH 9.6) such that the Absorbance at 600 nm wavelength filter (A_{600}) was approximately 1.0. The suspension was incubated at room temperature overnight with 0.1% (vol/vol) formaldehyde and then sonicated for 60 seconds at 100 watts, pulsed at 50% duty cycle (Branson Sonifier, Model B30, Danbury, CT, USA). Samples of the sonic extract (100μl) were then added to alternate columns of wells of flat bottomed 96-well polystyrene microdilution plates (Dynatech Laboratories Inc, Plochingen, Germany). An equal volume of 0.1M sodium carbonate (pH 9.6) was added to the wells in the remaining columns. After incubation at 4°C overnight, the plates were washed 3 times with phosphate buffered saline containing 0.05% (vol/vol) Tween 20 (PBS-Tween) and then dried. The coated plates could be stored in sealed plastics bags at 4°C for up to four weeks before use.

2.5.2 Serum specimens

Single acute-phase serum specimens were obtained on initial presentation from 1240 patients (age range 1 month to 73 years; median age 6 years) who had clinical signs of pertussis (eg. chronic or paroxysmal cough with or without associated vomiting, etc.). A second serum specimen was also obtained from 129 of these patients approximately 2 weeks after collection of the first. These second sera allowed testing of paired specimens.

Serum was also collected from 100 patients who had been admitted to the Adelaide Children's Hospital for reasons unrelated to respiratory complaints (generally this was either elective or non-elective surgery) (age range 1 month to 21 years; median age 9 years). These sera were collected at about the same time as the above samples and were tested to determine the level of pertussis antibody in normal ie. uninfected, individuals. All sera were stored at -20°C before being tested.

2.5.3 ELISA

Immediately before use, the antigen coated plates were blocked by the addition of 150μl of PBS-Tween supplemented with 1% (wt/vol) bovine serum albumin and 1% (vol/vol) normal goat serum (PBS-Tween-BSA-GS) to each well. Plates were incubated at 37°C for 2 hours and then washed in PBS-Tween. Test sera were routinely diluted 1:400, 1:200 and 1:100 in PBS-Tween-BSA-GS for determination of anti *B. pertussis* IgG, IgM and IgA, respectively. Duplicate 100 μl samples of the diluted test sera were added to both antigen-coated and uncoated (control) wells for each immunoglobulin class to be assayed. Reference standards (see below) and negative control sera were also included in each plate. The plates were incubated for 2 hours at 37°C in a humid atmosphere and then washed 3 times in PBS-Tween. Horseradish peroxidase-conjugated goat antihuman -IgG, -IgM and -IgA were obtained from KPL Laboratories, Gathersberg, MD., and were routinely diluted 1:2000, 1:1000, and 1:500, respectively, in PBS-Tween-BSA-GS before use. Diluted conjugate (100μl) was added to the appropriate wells and the plates were incubated at 37°C for 2 hours and washed 3 times in PBS-Tween. 100μl peroxidase substrate solution (Bio-Rad Laboratories, Richmond, CA. USA.) was then added to all wells and after incubation for 30 minutes at 30°C, the A_{415} of each well was measured with a Uniscan ELISA reader (Labsystems, Helsinki, Finland).

A test result for a particular serum specimen was considered acceptable only if the A_{415} of the respective non-antigen-coated well was less than 0.35 and A_{415} of the reference standard sera were ≥ 1.5. The reference standard sera for IgG, IgM and IgA assays were pooled human sera which had also been tested previously (by Dr. P. Robertson, Serology Department, Childrens Hospital, Camperdown, Sydney) and found to be strongly positive for the respective anti *B. pertussis* immunoglobulin class, but negative for the other two classes. Initially, the negative control consisted of pooled, human sera also provided by Dr Robertson, Sydney. Subsequently, negative sera from our own laboratory were pooled and calibrated against the original control. Negative controls were tested with every plate and the A_{415} were always less than 0.05. For each test serum the A_{415} was expressed as a percentage of the A_{415} for the respective reference standard sera. An ELISA result was defined as positive if this value exceeded the mean value for the normal population by more than 2 standard deviations. By this criterion, the critical cut-off values for positive IgG, IgM and IgA results were ≥ 51%, ≥ 67% and ≥ 31% respectively (the mean values for the normal population were 20%, 35%, and 12% respectively). The mean plus or minus standard deviation values for ELISA positive sera from patients were 88% ± 9%, 105% ± 23%, and 68% ± 27% for IgG, IgM and IgA, respectively.

2.5.4 Bacterial agglutination test

The BA test was performed by adding serial two fold dilutions of test sera (prepared in saline) to an equal volume of fresh *B. pertussis* suspension (A_{600} = 0.5). Tubes were observed for agglutination after overnight incubation at 37°C in a water bath. A test was defined as positive if the end point agglutination titre of an individual serum specimen was greater than or equal to 1:256 or if there was a four fold or greater change (increase or decrease) in titre between acute and convalescent phase sera.

2.6 WESTERN BLOT ANALYSIS

2.6.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed by SDS-polyacrylamide gel electrophoresis as described by Laemmli (1970) except that slab gels were used instead of tube gels. A Protean-II gel electrophoresis apparatus (Bio-Rad) was used.

The gels consisted of a separating gel, 15 cm wide x 11cm long, of 12.5% (weight per volume) acrylamide, topped by a stacking gel, 15 cm wide x 2.5 cm long of 3% (weight per volume) acrylamide and were 1.5 mm thick. The electrophoresis buffer consisted of 20 mM Tris, 0.2M glycine, 0.1% SDS (pH 8.3). Samples were suspended in a loading buffer consisting of 10% (volume per volume) glycerol, 0.005% (weight per volume) bromophenol blue, 2% SDS, 62.5 mM Tris-HCl (pH 6.8), 5% ß-mercaptoethanol, and were boiled for five minutes before loading on to the gel.

2.6.2 Gradient polyacrylamide gel electrophoresis

The same plates and buffers were used as for SDS-PAGE (described in 2.6.1), but the separation gel acrylamide solutions were prepared in two concentrations, 7.5% and 20%. These were then fed into the gel plates via a mixing apparatus, which constructed an acrylamide gradient from top to bottom of 7.5% to 20%.

2.6.3 Western Immunoblotting

Proteins were electrophoretically transferred from SDS-polyacrylamide gels onto nitrocellulose filters (Schleicher Schuell, Dassel,West Germany), as described by Towbin *et al.*, (1979), at 250 milliamps for 2 hours using a Bio-Rad transblot apparatus fitted with a water cooled coil. The transblot buffer consisted of 25 mM Tris, 200 mM glycine, 5% (vol/vol) methanol, pH 8.3. After the transfer of proteins was complete, the filter was agitated gently for 20

minutes at room temperature in 200 ml of TTBS (0.05% vol/vol Tween 20, 20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 5% (wt/vol) instant skim milk powder. The filter was then incubated overnight with gentle shaking at room temperature in 50ml TTBS containing 0.02% (wt/vol) skim milk, and 200 μl of serum from patients with suspected pertussis. The filter was then washed in 3 changes of TTBS at room temperature over a 30 minute period. Each patient's filter was then cut in 3 and labelled for identification purposes and each strip added to 3 separate containers. 50 ml TTBS (plus skim milk, 0.02% wt./vol) were added to each container along with 100 μl of goat anti-human IgA-, IgG- or IgM- horseradish peroxidase conjugate. These containers were rocked for 1 to 2 hours at room temperature. The filters were then washed at room temperature through 4 changes of 100ml of TTBS and then 2 changes of 100 ml TBS (TTBS without Tween-20) over a 30 minute period. Immunoreactive bands were visualized by the addition of HRP colour development reagent, prepared immediately before use by addition of 9.9 mg of 4-chloro-1-napthol (Bio-Rad Laboratories) to 3.3 ml of cold methanol (kept at -15°C) and 6.7 ml TBS.

2.7 AGAROSE GEL ELECTROPHORESIS

Electrophoresis through agarose gel was used to separate, identify and purify DNA fragments. The gel apparatus consisted of a BRL (Bethesda Research Lab, Cambridge, UK), horizontal gel apparatus series 1087, model H5. Tris-borate-EDTA (TBE) (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA) was used as the running buffer. To visualise the DNA, 0.5μg per ml of ethidium bromide (Sigma) was added to the running buffer. Agarose (Agarose NA, Pharmacia, Uppsala, Sweden) was suspended in TBE, melted in a mocrowave oven, and poured into a gel former to produce a gel with dimensions of 110 mm x 140 mm, with wells 2 mm x 4 mm. The concentration of agarose used was 2% (wt/vol) for PCR products and 1% for restricted

chromosomal DNA. 0.025% bromopheno-blue with 5% glycerol was used in the loading buffer. This was made up as a 10-fold concentrated solution and added to the sample just prior to loading. The gels were electrophoresed at 5-10 volts per centimetre. The ethidium bromide stained DNA was visualised by placing the gel on a UV transilluminator model TM-15 (UVP Incorporated, San Gabriel, California). This transilluminator has a peak UV wave length of 302 nm. Stained gels were photographed with Polaroid type 57 film with exposures of 1 or 1½ seconds. The camera used was Polaroid MP4 land camera (Polaroid, Cambridge, Mass.) DNA molecular weight markers were purchased from Bresatec (Adelaide, South Australia) and consisted of pUC19 DNA cut with *Hpa*II which results in production of DNA fragments ranging in size from 500 to 26 base pairs.

2.8 DNA ISOLATION AND ANALYSIS

2.8.1 Extraction of *B. pertussis* chromosomal DNA

Chromosomal DNA was extracted from *Bordetella* species by a modification of the method of Brown and Parker, (1987). Bacteria grown on four charcoal agar plates were harvested and washed in 150 mM NaCl, 10 mM Tris-HCl (pH 7.5) and resuspended in a volume of 20 ml of the same buffer. The suspension was frozen at -20°C. One volume of 1% SDS, 100 mM NaCl, 100 mM Tris-HCl (pH 8.8) containing 100 μg of pronase (Boehringer Manheim, North Ryde, New South Wales, Australia) (previously self digested at 37°C for one hour) was used to thaw the cell suspension by gentle inversion. The lysate was digested overnight at 37°C. Nucleic acids were precipitated with 2.5 volumes of chilled ethanol, suspended in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0), and digested again with pronase overnight. The solution was extracted with phenol chloroform (1:1) and then with chloroform and was precipitated again. *B. pertussis* DNA was further purified on a CsCl

gradient. TE buffer-CsCl (735 μg/ml) was added to 8ml centrifuge tubes and underlayed with 2.4ml of DNA solution to which 4.2 grams of CsCl and 400 μl of ethidium bromide (10μg/ml) had been added. After centrifugation at 180,000 x g for 42 hours, DNA bands were removed under UV illumination and cleaned as described by Maniatis *et al*, (1982).

2.8.2 Southern Hybridization Analysis

Digestion of genomic DNA with restriction endonucleases was conducted under the conditions recommended by the supplier (Boehringer Mannheim). Restricted DNA was electrophoresed in 1% agarose gels as described in Section 2.7. Electrophoresed DNA was transferred to nitrocellulose by the method of Southern, (1975) and then baked at 80°C in vacuo. Fixed membranes were pre-hybridized in 6 X SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate), 5 X Denhardt's solution (100 X Denhardt's is 2% BSA, 2% Ficoll (Pharmacia LKB), 2% polyvinyl-pyrolidone), 1% SDS, 50% formamide, 100 μg/ml herring sperm DNA for at least 1 hour at 42°C. Filters were then hybridized and autoradiographed as described by Maniatis *et al.*, (1982). Hybridization was carried out overnight at 42°C in 6 x SSC , 1 x Denhardt's solution, 1% SDS, 100 μg herring sperm DNA per ml, 100 μg heparin per ml, 50% formamide. Filters were washed twice in 2 x SSC for 15 minutes at 65°C and twice in 0.2 x SSC, 0.1% SDS for 5 minutes at 65°C. Probe DNA was labelled with α-[^{32}P]-dCTP (3000 Ci/mmol; Amersham, North Ryde, New South Wales, Australia) by the method of Feinberg and Volgelstein, (1983).

2.8.3 Lysate blots

Light suspensions of bacterial cells from agar plates were made in 200μl of TE buffer. Cells were then lysed by the addition of SDS to a final

concentration of 1%. After the suspension was vortexed briefly, 0.8 M NaOH was added to a final volume of 1.2 ml. Samples of each lysate were diluted 1:100 with 0.8 M NaOH and applied to nitrocellulose by loading 50μl into the wells of a Biodot apparatus (Bio-Rad). The samples were washed with 100 μl of 20 x SSC per well before the membrane was removed and baked for 2 hours *in vacuo* at 80°C.

2.9 DETECTION OF *B. PERTUSSIS* BY CULTURE

The NPA collection tubing was flushed out by aspiration of 1 ml of sterile phosphate buffered saline (pH 7.2) and the entire specimen then vortexed. Specimens were cultured (100-200 μl of sample per plate) immediately after collection, on charcoal agar as well as on charcoal agar supplemented with 40 μg of cephalexin per ml and charcoal agar supplemented with 2.5 μg of flucloxacillin per ml, and incubated for up to seven days at 37°C in air and in a humid environment. Plates were examined daily and suspect colonies (small and pearl like) were subcultured and identified by slide agglutination using *B. pertussis* and *B. parapertussis* antisera obtained from Wellcome Diagnostics (Dartford, United Kingdom).

2.10 DETECTION OF *B. pertussis* BY IMMUNOFLUORESCENCE

A direct immunofluorescence technique was used to detect *B. pertussis* in samples of NPA. 100 μl samples of each aspirate were dried on slides, fixed in 10% methanol and stained with 15 μl of undiluted fluorescein-conjugated rabbit anti *B. pertussis* antibody (Difco). The slides were stained for 1 hour at 37°C and then washed three times in PBS followed by a final rinse in distilled water. Slides were examined under UV light using a Leitz Dialux 20 UV microscope (Leitz, Germany)(mean excitation wavelength 490 nm, mean emission wavelength 525 nm) for the presence of fluorescing coccobacilli.

2.11 POLYMERASE CHAIN REACTION ASSAY FOR DETECTION OF *B. pertussis*

2.11.1 Sample preparation

After removal of the samples of NPA for culture and direct immunofluorescence analysis as described above, approximately 50 μl samples of the remainder were digested by the addition of 1 M Tris hydrochloride, pH 7.6, to 12 mM and proteinase-K to 0.2 mg/ml and incubated at 65°C for 1 hour. The proteinase-K was then inactivated by boiling the extracts for 20 minutes.

2.11.2 Ethanol precipitation

A single ethanol precipitation, after the proteinase-K digestion, was performed in order to eliminate potential inhibitory factors in NPA samples. All NPA samples which continued to inhibit the PCR reaction were subjected to the ethanol precipitation. 500μl of 70% ethanol was added to the 50μl of NPA digested with proteinase-K. After mixing, the reaction tubes were kept at -80°C for 30 minutes, thawed, and centrifuged at 13,500 rpm in a Clements microfuge for 15 minutes and the supernatants carefully removed leaving small pellets. After drying at 65°C for about 10 minutes, the PCR reaction mix was added directly to the tubes.

2.11.3 PCR amplification

PCR amplification was conducted in 50 μl reaction mixtures containing 20 μl of NPA extract, 200 μM deoxynucleoside triphosphates, 2 mM $MgCl_2$, approximately 1 μM of each primer, one unit of Taq polymerase and 5 μl of 10x PCR buffer, which were initially obtained from IBI, Newhaven, Connecticut. Taq polymerase was subsequently purchased from Bresatec, Adelaide, South Australia for use in later experiments. *B. pertussis* specific

oligonucleotide primers were synthesized on an Applied Biosystems DNA synthesizer, Model no. 391 and their sequences are as follows ; 5'-GATTCAATAGGTTGTATGCATGGTT-3' and 5'-AATTGCTGGACCATTTCGAGTCGACG-3 '. Samples were subjected to 30 PCR cycles, each consisting of 1 minute denaturation, annealing and elongation steps at temperatures of 94°C, 57°C and 72°C, respectively. The 153 base pair amplified product was detected by electrophoresing 20 μl aliquots through 2% agarose gels in the presence of ethidium bromide for approximately 30 minutes at 10 V per centimetre, followed by photography under UV illumination. Positive (*B. pertussis* DNA) and negative (proteinase-K treated saline) control extracts were included in each PCR run. The PCR reaction was performed on a Hybaid intelligent heating block (Integrated Sciences) or a Perkin Elmer DNA thermal cycler. For the latter instrument, the following modifications to the amplification protocol were required : One cycle consisting of denaturation at 95°C for three minutes, annealing at 55°C for two minutes and elongation at 72°C for 1 minute. Then followed 28 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. A final single cycle of 55°C for 1 minute, 72°C for 10 minutes and 30°C for 5 minutes finished off the reaction.

2.11.4 Hot start utilising Ampliwax PCR Gems

An upper and lower reaction mix are required for this PCR test. The lower mix consisted of 10.8 μl sterile distilled water, 6.75 μl 10x Taq buffer II (Bresatec), 107.7 μl dNTP's mix (10 mM), 3.4 μl (10-25 mM) primers and 32.3 μl $MgCl_2$ (25 mM). The total reaction mix volume was 11.7 μl. One PCR gem wax bead was added to each reaction tube which was heated to 75°C for 10 mins. and then allowed to cool to room temperature at which the wax sets and forms a seal. The bulk upper reaction mix consisted of 176.1 μl distilled water, 60.6 μl 10x Taq buffer and 3.4 μl (2.5 units/reaction) Taq polymerase (Bresatec). 17.1 μl of this reaction mix was added to each tube

together with 20 μl of test solution such as a pertussis positive control containing approx 120 *B. pertussis* cells. All tubes were kept on ice while the reagents were added and the heating block of the thermal cycler was kept at 94°C until the tubes were ready, at which time the standard PCR program was initiated.

2.11.5 Preparation of digoxigenin labelled oligonucleotide probe

A 22-mer oligonucleotide (5'-GGCGATTTCGAGTTTCTCAAAT-3'), internal to the 153 bp specific *B. pertussis* amplified segment and complimentary to positions 45-66 of that fragment was used as a probe (synthesized by Dr. C.P. Morris, Chemical Pathology, Women's and Children's Hospital, using an Applied Biosystems DNA Synthesizer Model no. 391). This oligonucleotide was labelled with digoxigenin (DIG) using a DIG DNA tailing kit (Boehringer Mannheim, Sydney, Australia). The tailing reaction was set up on ice and consisted of 4μl tailing buffer (5 x potassium cacodylate, ph 6.6), 6 μl cobalt chloride (5 mM), 1 μl oligonucleotide (approximately 0.25 μg), 2 μl DIG-11-dUTP (1mM), 2μl dATP (0.25 mM), 4 μl sterile water and 1U of terminal transferase. After all reagents were added, the reaction was incubated at 37°C for 10 minutes, diluted to 100 μl with sterile distilled water and frozen at -20°C until required.

2.11.6 Detection of PCR product by dot-blot hybridization

Nylon filters (Hybond-N+, Amersham Ltd) were prepared and 5 or 10 μl samples of the PCR product were spotted onto the filter in pre-recorded areas. The nylon strip was laid on a moistened Whatman No 1 filter paper which had been saturated with 0.4 M NaOH / 0.6 M NaCl, DNA side up for 15 minutes. The above step was repeated using 20 x SSC to saturate the filter and left for 20 minutes. The filters were air dried and placed DNA side down on a transilluminator covered with gladwrap. The filter was irradiated with UV for 3

minutes. UV-fixed filters were agitated in 20 ml of pre-hybridization mix (DNA side up) for at least 1½ hours at 55°C. The pre-hybridization mix consisted of 5ml of 20x SSC, 2ml of 10% SDS, 0.01 g of polyvinyl pyrolidone, 1 g of skim milk powder and 12.7 ml of sterile distilled water. Filters were then placed in 20ml hybridization mix together with 100μl of DIG-labelled oligonucleotide and agitated at 55°C overnight in a shaking water bath. This probe solution was reclaimed and stored at -20°C for re-use. The hybridization mix consisted of 5 ml of 20 x SSC, 2 ml of 10% SDS, 333 μl Bovine Serum Albumin, 0.1 gm of polyvinyl pyrolidone, 200 μl of 0.2 M sodium pyrophosphate and 12.7 ml of sterile distilled water. The filters were washed with 5 x SSC at 55°C for 30 minutes each and once in 2 x SSC at room temperature for 30 minutes. A further 1 minute wash occurred in DIG buffer 1 (0.1M Tris-HCl, 0.15 M NaCl, pH 7.5). The filters were incubated for 30 minutes on a shaker at room temperature in 100 ml of DIG buffer 2 (1 g of blocking reagent dissolved in 100 ml DIG buffer 1) and after a 1 minute wash in DIG buffer 1, 6μl of anti-digoxigenin alkaline phosphatase conjugate in 30 ml of DIG buffer 1 was added to the filters which were agitated at room temperature for 30 minutes. Filters were then subjected to two further 15 minute washes in 100ml of buffer 1 and a rinse for 2 minutes in 20ml of buffer 3 (0.1 M Tris-HCl, 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5). 10 ml of colour substrate reagent was then added to the filter which was shaken for up to 12 hours at room temperature. The colour reagent consisted of 45 μl of Nitro blue tetrazolium solution (75 mg/ml dissolved in 70% dimethyl formamide (DMF)), 35 μl of 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in DMF (X-phosphate), 10ml of buffer 3. When the colour had developed the reaction was stopped by rinsing for 5 minutes in 50ml of TE buffer. Developed filters were then photographed.

2.12 RAPID DETECTION OF *B. pertussis* AND RESPIRATORY SYNCYTIAL VIRUS CONCURRENTLY IN NASOPHARYNGEAL ASPIRATES BY REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION

2.12.1 Sample preparation

Nucleic acid preparations were performed on all nasopharyngeal aspirates prior to testing by PCR. Latex gloves were worn when dealing with the preparations to minimise the degradation of viral RNA present in specimens by RN'ases potentially present on bare hands. Sterile tips and tubes were always used. After thawing, 400 μl of NPA samples were vortexed and added to 1.5ml microcentrifuge tubes containing 3 μl proteinase-K mix (0.5 μl 20 mg/ml proteinase-K, 0.6 μl 1 M Tris-HCl pH 7.6, 1.9 μl sterile deionised water). 20 units of RN'ase inhibitor (Perkin Elmer Cetus, Norwalk, USA) was immediately added. The tubes were heated at 65°C for 1 hour. A single phenol extraction (300 μl) was followed by chloroform extraction (300 μl). After careful removal of the supernatant and addition of 1/10 volume sodium acetate (3 M), nucleic acids were precipitated by addition of an equal volume of isopropanol, freezing at -20°C for 30 minutes and centrifugation. The resulting pellets were washed in 70% ethanol, centrifuged, dried and finally resuspended in 10 μl water. These preparations were either used immediately or stored at -20°C until required.

2.12.2 Reverse transcription-Polymerase chain reaction (RT-PCR)

The RT reaction was carried out using an RNA PCR kit obtained from Perkin-Elmer Cetus, Norwalk, Conn. The final reaction volume contained 20 μl 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM (each) dATP, dCTP, dGTP and dTTP, approximately 1 μM of each of the pertussis primers and the RSV primers, 1 unit RN'ase inhibitor, 3 μl specimen preparation and

2.5 units of avian myeloblastosis virus reverse transcriptase. The reaction tubes were overlaid with 50 μl of mineral oil, incubated at 42°C for 15 minutes and then heated at 99°C for 5 minutes. This sequence of events was performed on a DNA thermal cycler (Perkin Elmer). Reaction tubes were then kept at 4°C until used in the PCR assay.

For the PCR step, the reaction volume was made up to 100 μl with 10 mM Tris-HCl (pH 8.3), 50 mM KCl and the final $MgCl_2$ concentration was adjusted to 2 mM. Taq polymerase (2.5 U, Bresatec, Adelaide, Sth Aust.) was added to each tube, which was subjected to 35 amplification cycles. A cycle consisted of the following segments ; Segment 1, 60 sec. transition to 94°C; segment 2, 94°C for 1 min. 30 sec. (denaturation); segment 3, 60 sec. transition to 50°C; segment 4, 50°C for 3 min. (annealing); segment 5, 60 sec. transition to 72°C (elongation); segment 6, 72°C for 4 minutes. A ten second extension to the final segment of each cycle was programmed into the machine (ie. an additional 10 seconds automatically added to the final segment of each cycle in a compounding fashion) and this file was linked to a another file, holding the tubes at a temperature of 10°C following completion of 35 cycles.

The sequences of the pertussis primers were described previously in this thesis and the RSV primer sequences were as follows : 5'-TTAACCAGCAAAGTGTTAGA-3' and 5'-TTTGTTATAGGCATATCATTG-3' (Paton *et al.*, 1992). These primers direct amplification of a 243 bp segment of the RSV genome encoding the F1 subunit of the fusion protein. Detection of the 153 bp pertussis PCR product and the 243 bp RSV product was performed by electophoresing 20 μl aliquots through 2% agarose gels in the presence of ethidium bromide for approximately 30 minutes at 10 V/cm and photographing the gel under UV illumination.

CHAPTER 3

EFFICACY OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE RAPID SEROLOGICAL DIAGNOSIS OF *BORDETELLA PERTUSSIS* INFECTION (WHOOPING COUGH).

EFFICACY OF AN ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE RAPID SEROLOGICAL DIAGNOSIS OF *BORDETELLA PERTUSSIS* INFECTION (WHOOPING COUGH).

3.1 INTRODUCTION

During the early (catarrhal) stage of pertussis, large numbers of *B.pertussis* organisms may be present in the nasopharynx. However, by the time the classical symptoms of whooping cough become apparent, the numbers of viable organisms are decreasing and examination of nasopharyngeal smears by immunofluorescence or culture for *B. pertussis* frequently yields negative results. Failure to diagnose pertussis early in the course of illness has important consequences for infection control, because there is a high probability that undiagnosed individuals could transmit the infection to siblings and other close contacts, such as children in schools or day care centres, or nearby patients, in the case of hospitalised individuals.

Since symptoms sufficiently serious to warrant attendance at or admission to a hospital may develop only after the infection has been established for several weeks, a measurable humoral response might already have occurred by the time of presentation. Thus sensitive serological tests have the potential to provide rapid diagnosis of whooping cough. Ideally, a serological test should have the capacity to enable a diagnosis to be made from a single serum specimen collected at the time of presentation. It also needs to be able to distinguish antibodies present in serum as a consequence of current or recent infection from those resulting from past infection with *B. pertussis* or immunisation. Previous studies have demonstrated that following infection or vaccination, both pertussis-specific IgG and (to a lesser extent) IgM antibodies are formed, whereas significant levels of IgA antibodies only arise following

natural infection (McCaulay, 1981; Viljanen *et al.*, 1982; Burstyn *et al.*, 1983; Nagel and Poot-Scholtens, 1983; Mertsola *et al.*, 1984).

The bacterial agglutination test was previously the only method available for serological diagnosis of whooping cough at the WCH and had several problems associated with it, such as difficulty in observing agglutination and reproducibility. A new method was clearly required and an ELISA-based system, with its superior sensitivity and specificity, was an ideal candidate. An ELISA could also measure semi-quantitatively the class-specific serum antibody responses, which might enable a differential diagnosis of current or past infection (or previous vaccination) to be made on single serum samples. Such an assay would also enable results to be obtained on the same day as collection in many cases.

There were several reports in the literature of ELISA assays capable of detecting class-specific antibodies to *B. pertussis*, but no commercial kit was available at the time this study commenced (Ruuskanen *et al.*, 1980; Vilganen *et al.*, 1982; Granstrom *et al.*, 1982; Mertsola *et al.*, 1983).

Hence an in-house ELISA was developed based on these studies and was used to measure specific anti-*B. pertussis* IgA, IgG and IgM. The performance of the new ELISA was compared to the old bacterial agglutination test using serum specimens from a large number of patients (infants, children and adults) suspected of having pertussis.

3.2 RESULTS

3.2.1 Establishment and Optimisation of ELISA

3.2.1.1 *Antigen preparation*

Two strains of *B. pertussis* (NCTC 10908 and NCTC 10911) were selected as appropriate sources of antigen. These organisms were always used

in their phase 1 (virulent) form and belonged to serotype 1,2,3,4 and 1,2,5,6, respectively, which accounts for all the serotypes of wild type strains infecting patients in Australia at that time (Dr A Blaskett, Commonwealth Serum Laboratories, personal communication). Initial experiments were aimed at determining the optimal method of antigen preparation (ie. harvesting organisms from either solid or liquid media).

Two methods for production of *B. pertussis* antigen from broth culture were used. (1) CL medium was seeded with a primary inoculum and then shaken on an orbital shaker at 180 rpm at 35° for 40 hours. (2) CL medium was inoculated as before and incubated at 35°C without shaking for 5 days. The primary inoculum was prepared by inoculating 100 ml of CL medium with *B. pertussis* strains harvested from a charcoal agar plate which was no more than 2 days old. These cultures were then incubated at 35°C whilst shaking at 180 rpm for 24 hours. Flasks containing 500 ml of CL medium were then seeded with 25 ml of this primarily inoculum . Half of the flasks were then shaken as described in method 1 and the other half were not shaken. After an appropriate time of incubation, cells were centrifuged at 3000 x *g* (Clements GS200 bench centrifuge) for 30 minutes and the pellet was re-suspended in 20 ml CL medium. The supernatants were collected and concentrated by ultrafiltration (10,000 molecular weight exclusion) (Amicon, Sydney Australia) to a final volume of 5 ml. The resultant fluids were sonicated (see methods) and then frozen at -20°C for future testing for the presence of FHA.

Organisms were also grown on solid media and harvested directly. Plates were inoculated with a loopful of *B. pertussis* and incubated at 37°C in air and in a moist environment for 48 hours. Growth from 4 plates of each strain was harvested into vials containing 15 ml of phosphate buffered saline (pH 7.2), sonicated and centrifuged as above. The supernatant was retained at 4°C until tested.

Any antigen preparation to be used for detecting *B. pertussis* antibodies must contain significant amounts of the major *B. pertussis* antigens such as FHA and PT, both of which have been shown to be important virulence factors. By definition, phase 1 *B. pertussis* cells will produce toxin however the content of FHA in the preparations used for these experiments was unknown. Prior to the availability of commercially prepared FHA, an attempt was made to estimate the content of FHA from cultures of *B. pertussis* by direct harvesting from charcoal plates after 24-48 hours incubation and from CL broth culture. FHA can be detected by mixing solutions containing this protein with chicken erythrocytes and observing for agglutination. Ten serial 2 fold dilutions of the respective supernatants were made. 70 μl of PBS was used to dilute 70 μl of supernatant in microtitre trays and 70 μl of 0.7% fresh chicken erythrocytes (courtesy of Mr Ken Lee, MedVet, South Australia) suspension (in PBS) added to each well. Trays were initially shaken and then incubated at room temperature for 1 hour. Each well was observed for the presence of agglutination of the erythrocytes. No agglutination of chicken erythrocytes could be detected in the supernatant of either broth culture as prepared above. Titres of 1/8 and 1/32 for NCTC 10908 and 10911 respectively were observed for the supernatant of sonicated, spun cells harvested from solid media (charcoal agar). The experiment was repeated with new stock cultures from -80°C freezer, with the same result.

It has been shown that a 0.5 M sodium chloride solution is able to inhibit the haemagglutinating activity of FHA, (Granstrom *et al.*, 1982). To confirm that the haemagglutinating activity present in the above supernatants was due to FHA, the tests were repeated using 0.5 M sodium chloride in place of PBS as diluent. Agglutination was inhibited under these conditions, confirming the presence of FHA .

This result, together with the result of SDS PAGE analysis of antigen harvested from solid media showing the presence of good levels of pertussis toxin (Figure 4.10 a and b, Chapter 4), suggest *B. pertussis* organisms harvested

in this way are a better source of antigens than broth cultures and are therefore suitable for use in a diagnostic ELISA.

3.2.1.2 *Microtitre Plates*

A number of different micro-titre plates were tested in order to establish which polystyrene would bind the antigen most efficiently. These included plates from Disposable Products, (Adelaide, South Australia), Nunc, (Roskilde, Denmark) and Dynatech Laboratories (Plochingen, Germany). Tests were performed using known positive controls and optical density readings were compared for the three brands of plates tested under identical conditions. Results from these tests indicated Dynatech plates gave consistently higher OD's for the control serum and it was therefore decided to use these in future tests.

3.2.1.3 *Serum and Conjugate Dilutions*

Checkerboard titrations of serum and conjugate dilutions (for each class of antibody) were performed using a known positive control serum and optical density readings analysed. The range of serum dilutions used were ; neat, 1:10, 1:50, 1:100, 1:200, 1:400, 1:800, 1:1000, 1:2000 while conjugate dilutions were 1:100, 1:250, 1:500, 1:1000, 1:1500, 1:2000, 1:2500, 1:3000. Optimal dilutions (ie. the greatest dilution which is within the linear portion of the graph when OD is plotted against dilution) of serum were found to be 1:100 for IgA, 1:400 for IgG and 1:200 for IgM. The optimal results for conjugates were achieved at dilutions of 1:500, 1:1000 and 1:2000 for IgA-,IgG- and IgM- respectively.

3.2.1.4 *Negative Controls*

In order to determine the normal range of *B. pertussis* antibody levels in uninfected individuals and hence define a negative cut-off value for this ELISA, sera were collected from 100 patients who had been admitted to the Adelaide

Children's Hospital for reasons unrelated to respiratory complaints. These patients had been admitted for elective or non-elective surgery and their ages ranged from one month to 21 years with a median age of 9 years. All sera were stored at -20°C before being tested. These were tested by the ELISA methodology described previously in Section 2.5.1. The mean values plus 2 standard deviations for these sera were found to be ≥51, ≥67 and ≥31% of the standard reference sera for IgG, IgM, and IgA, respectively.

3.2.2 Analysis of single serum samples by ELISA and Bacterial Agglutination assay

Single acute phase serum specimens were obtained on initial presentation from 1240 patients whose ages ranged from one month to 73 years and whose median age was 6 years. They all had clinical signs of pertussis, which included chronic or paroxysmal cough, with or without associated vomiting. All 1240 acute phase serum specimens were assayed for antibodies to *B. pertussis* by bacterial agglutination and ELISA and scored as positive or negative, as described previously in Section 2.5.1. A total of 255 specimens yielded positive BA results and 242 (95%) were also positive by ELISA. However, a further 591 (60%) of the 985 BA negative specimens were positive by ELISA. Remarkably, 284 of these ELISA-positive specimens had undetectable BA titres (less than 4). Of the ELISA-positive, BA-negative specimens, 66% were positive by ELISA for at least two immunoglobulin classes, whereas of the ELISA positive BA positive specimens, 86% were ELISA positive for at least two classes. Of the 833 ELISA positive specimens, 83% had positive IgG values, (either alone or in combination with positive IgM or IgA values) 73% had positive IgA values and 46% were positive for *B. pertussis* IgM (see **Table 3.1**).

Table 3.1

ELISA and BA analysis of single (acute-phase) sera for antibodies to *B. pertussis* antigens.

	Number of ELISA results						
				Positive for :			
BA Result	**NEG**	**POS**	**TOTAL**	**IgG only**	**IgM only**	**IgA only**	**>= 2 Ig classes**
Negative	394	591	985	106	51	43	391
Positive	13	242	255	26	3	5	208
Total	407	833	1240	132	54	48	599

3.2.3 Analysis of paired serum samples

The results described above highlight the superior sensitivity of the ELISA for diagnosis of pertussis from a single serum specimen. However, serologic diagnosis by BA is also made on the basis of a four-fold change (increase or decrease) in titre between acute and convalescent phase sera and by this criterion, neither titre need necessarily exceed 128 for the result to be positive. Of the 1240 patients tested above, 129 also had a second paired sera available for analysis both by ELISA and BA. A total of 100 of the paired specimens were ELISA positive and of these 87 had positive first specimens. However, the BA test detected only 64 positive paired specimens by all criteria and diagnosis could have been made with the first serum alone in only 25 of these. Of 65 paired specimens which yielded negative BA results 41 (63%) were positive by ELISA (29 of these were ELISA positive for both serum

specimens). Conversely, of the 29 paired specimens which were both negative by ELISA only 5 yielded positive BA results (see **Table 3.2**).

Table 3.2

ELISA and BA analysis of 129 paired sera from patients suspected of having pertussis.

	Number of ELISA Results				
BA result	**Both sera negative**	**Serum 1 only positive**	**Serum 2 only positive**	**Both sera positive**	***n***
Negative	24	7	5	29	65
Significant change in titre	1	1	2	5	9
Positive titre in either serum	1	1	2	17	21
Significant change and positive titre	3	0	4	27	34
Total	29	9	13	78	129

3.2.4 BA and ELISA in culture proven cases of pertussis

ELISA and BA results for sera from 58 patients with culture proven pertussis are shown in **Table 3.3**. For the 45 patients over four months of age, 34 specimens were ELISA positive but only 10 of these were BA positive. Serologic diagnosis was less effective in children under four months of age however, as only three of the 13 specimens were ELISA positive and BA titres were positive for only 2 patients.

Raw data expressed in table 3.4 demonstrates that for patients older than 4 months who were also culture positive, testing sera for IgA alone would have missed (unacceptably) 6 of 45 (13%) of genuine pertussis cases. This is not the case for babies who were culture positive and less than 4 months old where the only detectable responses in culture proven cases was due to raised IgA levels. The numbers however are small (3 out of 13 babies) and clearly indicates that serology alone is not adequate for diagnosis in this age group.

Table 3.3 Bacterial agglutination and ELISA test results for 58 patients with culture proven pertussis.

	Number of ELISA results			
BA Result	**< 4 mnth. of age (n = 13)**		**>= 4 mnth. of age (n = 45)**	
	Negative	**Positive**	**Negative**	**Positive**
Negative	9	2	11	24
Positive	1	1	0	10

Table 3.4 Class specific antibody ELISA results for 58 *B.pertussis* culture positive patients

Age	IgA	IgG	IgM	All Ig Negative	n
≤ 4 months	*3	0	0	10	13
> 4 months	24	15	16	16	45
n	27	15	16	26	58

* The figures refer to the number of positive ELISA test results for a particular antibody class.

3.3 DISCUSSION

This chapter describes a simple and rapid ELISA for detection of class specific immunoglobulins directed against *B. pertussis* in sera from children (and adults) suspected of having whooping cough. In this study a high proportion (67%) of acute phase serum specimens collected from patients with suspected pertussis were ELISA positive by the test criteria. Such a high rate of positive specimens is consistent with the distinctive clinical picture of pertussis and may also have been contributed to by the fact that there was a small epidemic of whooping cough in Adelaide at the time most of the sera were collected. The critical positive ELISA value (mean optical Absorbance value of aged matched normal sera plus 2 standard deviations) was set such that statistically, only 2.5% false positives would be expected. similar high positive rates have also been detected in other smaller studies which used similar criteria

(Granstrom *et al* 1982, Viljanen *et al* 1982). In this study the ELISA, as would be expected, had vastly superior sensitivity to that of the BA test, which detected only 21% of positive acute phase specimens. When paired specimens were tested, the number of positive pertussis cases detected by BA increased to 50% whereas testing a second (convalescent) phase serum specimen by ELISA increased the proportion of specimens detected as positive by that method from 67% to 77%.

One potential problem with calculating the positive cut-off ELISA values during a mini epidemic of pertussis (such as during the workup for this ELISA) is that the herd immunity of our negative controls may be high because of the increased overall incidence of disease and hence increased exposure to the organism by the general population. These circumstances could lead to artificially high positive and negative cut-off ELISA values. Normal range controls for this study were collected over the same time period as the test sera in order to avoid this problem. As an epidemic passes the overall levels of specific *B. pertussis* antibody in individuals will probably diminish and hence to avoid false negative results, a set of negative controls needs to be tested every couple of years. This should ensure the appropriate positive cut-off values for each class of antibody are maintained.

Some previous studies have reported the use of the ELISA for IgA to certain pertussis antigens (particularly the fimbrial haemagglutinin) for the diagnosis of whooping cough. Natural infection has been shown to induce production of anti-pertussis IgA, whereas vaccination does not (Granstrom *et al.*, 1982 (a) and 1982 (b); Nagel and Poot-Scholtens, 1983; Burstyn *et al.*, 1983). Thus by using the ELISA for detection of IgA, people with genuine pertussis, as opposed to those recently vaccinated, could be distinguished. However, in the present study, use of the IgA ELISA values alone would have missed 27% of the positive cases. Background IgG and IgM levels in the general population as a result of prior immunization have been adequately

accounted for by the test criteria and did not contribute to an unacceptably high level of false positive specimens. Thus, measurement of IgA, IgG and IgM is clearly justified. This study has demonstrated that the ELISA, as described, has considerable potential for the rapid diagnosis of *B. pertussis* infections. An encouragingly high proportion of positive cases were detected by testing a single serum specimen collected at the time of presentation or admission. Under optimal circumstances a result would be available on the day the serum was collected. A culture result on the other hand would not be available for at least three to four days.

The source of antigen in this study was a formalin killed, whole *B. pertussis* cell sonicate, with the cells being harvested from fresh charcoal agar plates. Initially the only method available for confirming the presence of FHA in the preparation was haemagglutination of chicken erythrocytes. In an attempt to confirm the presence of FHA in 2 preparations of *B. pertussis*, this assay was used. A preparation from broth culture appeared to have no haemagglutinating activity while cells harvested from fresh charcoal plates did agglutinate the red cells. This agglutination was inhibited when 0.5 M sodium chloride was used as diluent, thus confirming the presence of FHA (Granstrom *et al.*, 1982). It appears therefore that harvested *B.pertussis* cells from fresh charcoal plates contain sufficient FHA components to act as an appropriate antigen to be bound to the wells of microtitre plates for a diagnostic ELISA. Subsequently, specific antibody against FHA and PT was obtained and the presence of these antigens in whole cell preparations was confirmed using Western blot assays (see Chapter 4).

Culture of nasopharyngeal secretions was, however, essential, particularly in children less than four months of age, who had poor serologic responses in the present study (of 13 culture proven cases, only 3 had positive ELISA results from the serum specimens collected at the same time as the cultured nasopharyngeal secretions). In older infants and children (age > 4

months) 35 of 45 culture proven cases yielded positive results from serum specimens collected on the same day as the nasopharyngeal secretions. Trends similar to this have been reported in other studies (Viljanen 1982). It has been known for many years that the probability of successfully isolating *B. pertussis* is greatest in the early stage of infection at which time a serologic response may not yet be detectable. Thus, ELISA and culture should be seen as complementary. Notwithstanding the above, the ELISA used in this study is highly sensitive, simple to perform and in many cases the most rapid and only means of diagnosing pertussis in the laboratory.

Subsequent to the results of this survey, the ELISA test as described was introduced routinely for serological diagnosis of pertussis at the Microbiology Department, Womens and Childrens Hospital, Adelaide, and has been in use since that time. A decline in the incidence of positive cases of pertussis (both culture-proven and serologically-diagnosed) has been observed since about 1988 and continued until about the middle of 1992. From that time onwards there has been a steady increase in the number of laboratory diagnosed whooping cough cases.

One further issue which has to be considered when any new diagnostic test is introduced into routine practice, is that of cost. Since the completion of this project, a relatively expensive commercial kit (produced in Australia) has become available (PanBio, Brisbane) which measures the IgA response only. The "in house" ELISA for pertussis is very cheap (<$1.00/test) compared to the commercial counterpart (approx $4.00/test) and is ideally suited to batch-testing and automated reading. Using a whole cell sonicate as the antigen keeps the cost of this component to a minimum when compared to purified individual antigens (eg FHA or PT) which are relatively difficult to prepare in pure form in a routine diagnostic laboratory, and expensive to purchase commercially.

The ELISA as described is more rapid with greatly improved sensitivity and specificity over the bacterial agglutination test (in use at the commencement of this project) for serological diagnosis of whooping cough.

CHAPTER 4

WESTERN BLOT ANALYSIS OF THE HUMAN IMMUNE RESPONSE TO *B.PERTUSSIS* ANTIGENS DURING NATURAL INFECTION.

WESTERN BLOT ANALYSIS OF THE HUMAN IMMUNE RESPONSE TO *B.PERTUSSIS* ANTIGENS DURING NATURAL INFECTION.

4.1 INTRODUCTION

ELISA tests for antibodies directed against specific (purified) antigens of *B. pertussis* have been described in recent years. Such antigens include PT, (Granstrom *et al.*, 1985), FHA (Granstrom *et al.*, 1982), AC (Weiss and Falkow, 1984), 69 kDa OMP associated with AC (Seddon *et al.*, 1987) and other outer membrane proteins (Ashworth *et al.*, 1983). One test using only FHA as the antigen coating the solid phase in a diagnostic ELISA has been described (Granstrom *et al.*, 1982). Detection of pertussis specific IgA by ELISA in nasopharyngeal secretions had been used as an indicator of recent infection (Goodman *et al.*, 1981) and similarly for serum (Nagel and Poot-Scholtens, 1983). A potential advantage of such monospecific ELISA's might be the improved capacity to distinguish serological response to current infection from that resulting from prior immunisation or past infection.

The Western blot technique offers a means whereby the immune response to all *B. pertussis* antigens during a natural infection in humans can be examined. This information is potentially important for refining the sensitivity and specificity of serodiagnostic techniques such as ELISA, as well as identification of potential targets for vaccination. At the commencement of this work (late 1987) there were no such data in the literature for *B. pertussis*.

In Chapter 3, a diagnostic ELISA for serodiagnosis of pertussis was described using sonicated, formalin-treated, whole cells as the antigen source.

In this chapter, the antigen specificity of immune responses in infection was examined using Western immunoblotting. These analyses were performed using a variety of human sera, primarily from children, in order to determine the class specific serum antibody responses to antigens contained in a whole cell sonicate of *B. pertussis*.

4.2 RESULTS

4.2.1 Western blot analysis sera from three culture-positive cases of pertussis

Initial experiments examined the *B. pertussis* antigen specificity of IgA, IgG and IgM antibodies in acute and convalescent sera from 3 patients with culture-confirmed pertussis **(Fig 4.1)**.

Patient 1 was male and 6 years old at the time of presentation. IgA antibodies directed against a 98 kDa antigen and a 21 kDa antigen in the convalescent phase serum were observed. No such antibodies could be detected in the acute phase serum. IgG antibodies against the same 98 kDa antigen were present in both sera while antibodies to 39, 26 and around 69 kDa (together with a very weak response to a 21 kDa) antigens were only detectable in the convalescent serum. IgM antibodies against 24 kDa and <20 kDa antigens were seen in the acute serum, with an increase in intensity observable in convalescent serum. Additionally, IgM activity was seen to be directed at higher molecular weight antigens including those of M_r of approximately 69 kDa, 98 kDa and some weak reactions with antigens >116 kDa.

Patient 2, also male, was 9 years old at the time of presentation. IgA directed against a 98 kDa antigen was present in the acute phase serum and increased in intensity in the convalescent phase serum together with acquisition of IgA directed against some extra higher molecular weight proteins and antigen

species within the range 40 kDa to 98 kDa. IgG was directed against a large number of components in both sera with the only addition in convalescence being a band at about 120 kDa. IgM response was directed at a large number of antigens with molecular weights ranging between 20 and >116 kDa in both sera. An increase in intensity was noticed with the 98 kDa antigen in convalescent serum.

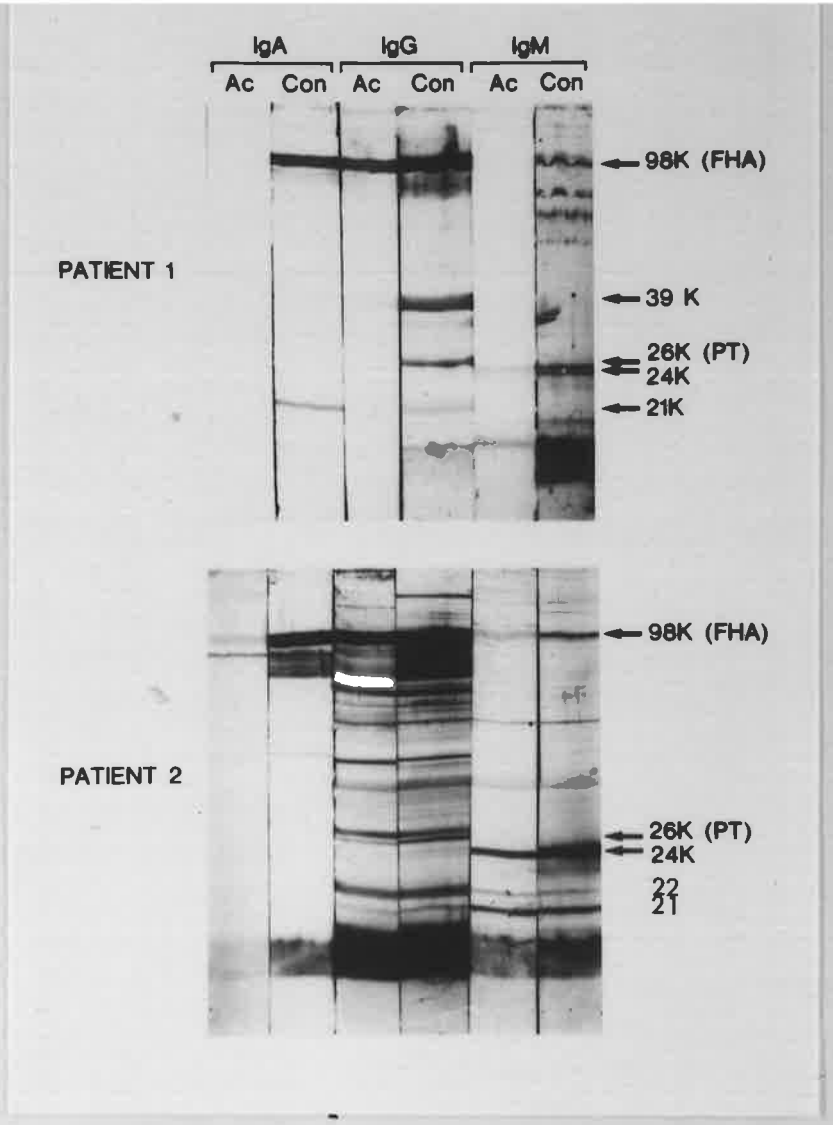
Patient 3 was female and 6 years old at the time of presentation. Very weak IgA activity (the bands appear very faint in the photographic reproduction) was directed towards a 30 kDa antigen in both acute and convalescent sera and to larger molecular weight antigens (>116 kDa) in convalescence serum only. IgG antibodies to a number of antigens with molecular weights ranging from 20 to >116 kDa antigens were observed in both sera whilst 2 (ie antigens with molecular weights around 45 and 33 kDa) appeared only in the convalescence serum. Intense IgM activity was directed at a 21 kDa antigen and very faint activity was directed against antigens of 69 and >116 kDa in both sera **(Figure 4.1)**.

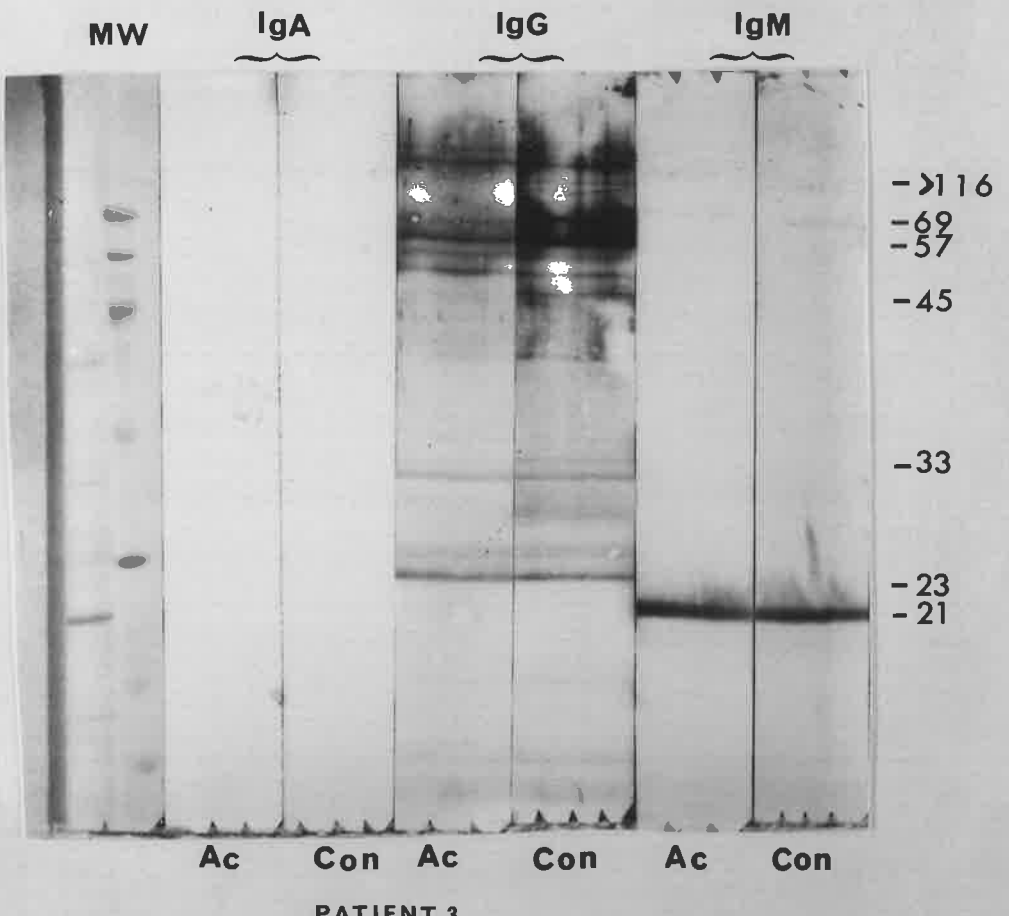
In summary, no single consistent antibody response to any given antigen in any immunoglobulin class was observed. Two of the three culture positive pairs of sera (ie from patients 2 and 3) examined displayed reactivity for IgA antibodies in both acute and convalescent sera. The 3rd serum (ie patient 1) was negative in acute, but positive in the convalescent serum. IgM responses were detected in 2 out of the 3 acute phase sera while all 3 convalescent sera contained specific IgM reactivity. IgG class antibodies to many antigens were detected in both acute and convalescent sera in 2 of the 3 cases with the third displaying reactivity mainly in the convalescent serum specimen. A summary of the total responses for patients 1,2 and 3 against the various antigens are seen in **Figures 4.2, 4.3** and **4.4** and the corresponding ELISA results for these sera are shown in **Table 4.1**.

Figure 4.1

Western blot analysis using acute and convalescent phase sera from 3 patients all of whom were culture positive for *B. pertussis*.

The source of antigen was a whole cell sonicate of phase 1 *B. pertussis* organisms separated by electrophoresis in a 12% SDS-polyacrylamide gel and transblotted to nitrocellulose. Filters labelled Ac and Con have been probed with acute or convalescent phase patient sera, respectively, followed by goat anti human-IgA,-IgG or IgM-horseradish peroxidase conjugate, as indicated. The molecular size of major immunoreactive bands, calculated frojm the mobility of marker proteins (not shown) is indicated on the right hand margins in kDa. The labels FHA and PT refer to bands with a size consistent with that of filamentous haemagglutinin and pertussis toxin, respectively (see text).


IgA
IgG
IgM
Ac
Con
Ac
Con
Ac
Con
98K (FHA)
PATIENT 1
39 K
26K (PT)
24K
21K
98K (FHA)
PATIENT 2
26K (PT)
24K
22
21


MW
IgA
IgG
IgM
>116
69
57
45
33
23
21
Ac
Con
Ac
Con
Ac
Con

PATIENT 3

Figure 4.2

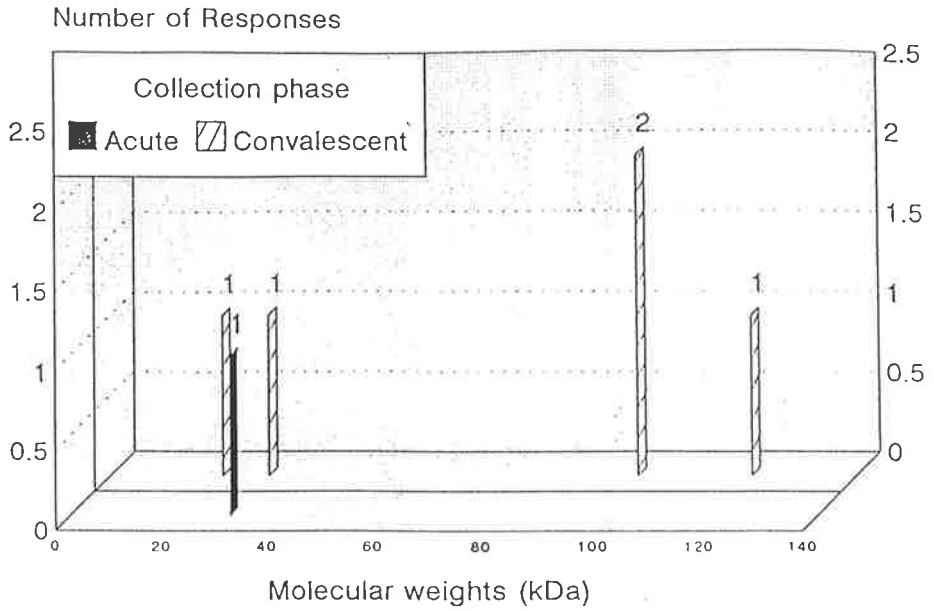
Western blot IgA analysis using a whole *B. pertussis* cell sonicate as antigen source and transblot filters probed with sera from 3 culture positive patients.

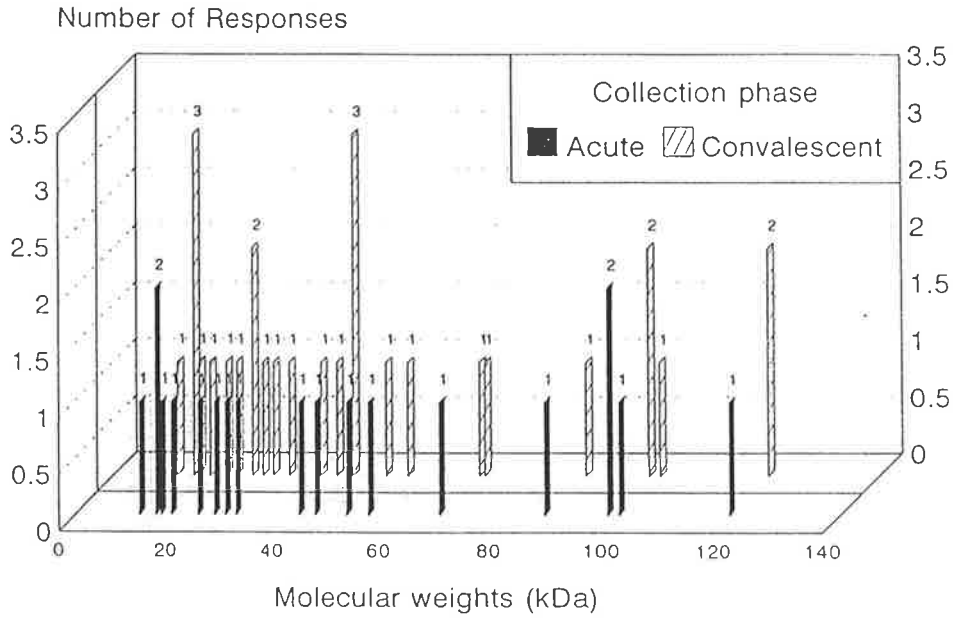
Figure 4.3

Western blot IgG analysis using a whole *B. pertussis* cell sonicate as antigen source and transblot probed with sera from 3 culture positive patients.

Figure 4.4

Western blot IgM analysis using a whole *B. pertussis* cell sonicate as antigen source and transblot filters probed with sera from 3 culture positive patients.


Number of Responses
Collection phase
Acute
Convalescent
Molecular weights (kDa)


Number of Responses
Collection phase
Acute
Convalescent
Molecular weights (kDa)

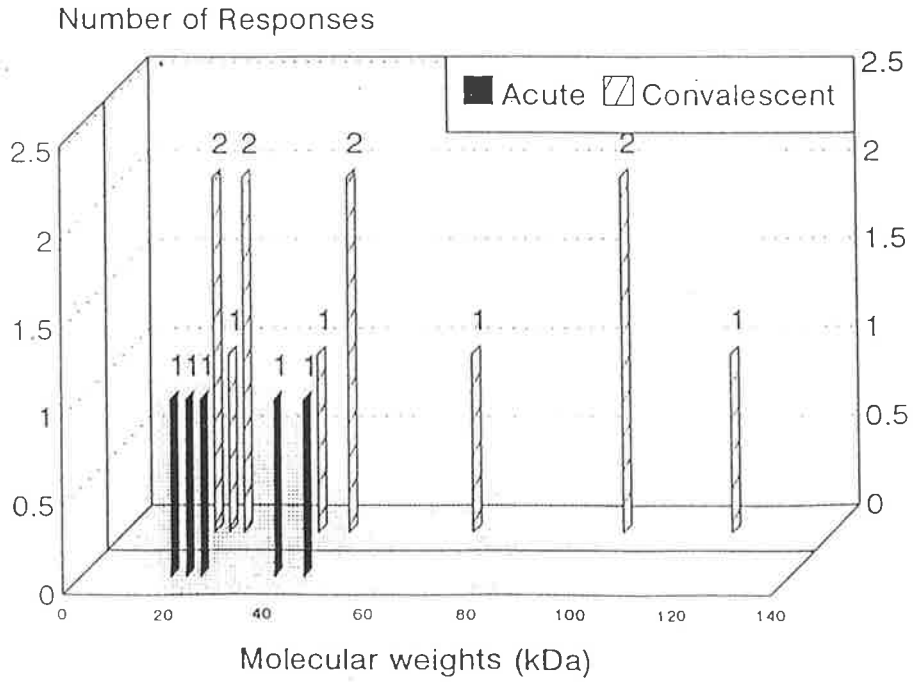

Number of Responses
Acute
Convalescent
Molecular weights (kDa)

Table 4.1

***B. pertussis* specific antibody class ELISA results for 3 culture positive patients.**

	IgA		IgG		IgM	
Patient	**Ac**	**Conv**	**Ac**	**Conv**	**Ac**	**Conv**
1	20	45	33	83	85	99
2	56	68	79	78	57	61
3	39	46	61	84	70	68

IgA positive cutoff value : 30

IgG positive cutoff value : 50

IgM positive cutoff value : 70

Note : The above cut-off figures represent the percentage OD relative to a reference control (as described in Chapter 2).

4.2.2 Western Blot Analysis of IgA ELISA positive paired sera from seven patients

Western blot data for the 7 paired sera assayed correlated well with the ELISA results (not shown) for IgG and IgM but less so for IgA class antibodies. The seven pairs of sera examined from this group of patients all contained raised

levels of IgA antibodies in the ELISA test in convalescent phase only, suggesting they were all cases of recent pertussis. Results for two of these patients are shown in **Fig 4.5**. 3 of the 7 contained raised IgM levels in both sera, 2 became positive in convalescence with the remaining 2 going from positive to negative. For IgG, 6 of the 7 pairs became positive in convalescence with the one remaining positive in both acute and convalescent phase sera.

Examples of acquisition and loss of class specific antibodies were observed. Acquisition of IgG antibody to a number of antigens ranging in size from 15 up to >116 kDa was observed in one patient (**Fig. 4.5a**, lane 4), while loss of IgM antibody directed toward a 21 kDa antigen was also be seen in the convalescent sera of the same patient (lane 6). A similar picture emerged for a second patient. IgA antibody was acquired in convalescent sera directed predominantly at 116 kDa antigen (**Fig. 4.5b**, lane 2). IgG antibody was seen to be directed at various antigens with molecular weights ranging from 14 up to 116 kDa in the convalescent sera only (lane 4). IgM antibody (a weak reaction) to a 69 kDa antigen was acquired in convalescence while some other antibodies diminished in intensity suggesting a decrease in titre in the serum (lane 6).

Only 3 of the 7 pairs of sera showed IgA classs antibody responses in the Western blot. This activity was directed against a 69 kDa antigen. IgG antibody response was directed at many different antigens of *B. pertussis*, often present in both acute and convalescent phase sera. Where responses were present in convalescent sera and not in acute phase sera, the responses were directed primarily against lower molecular weight antigens (14 to 28 kDa) together with a 45 kDa antigen. A number of reactivities were also observed in the 50 - 55 kDa range. The majority of responses to 69 kDa and 98 kDa antigens were present in both acute and convalescent sera including many bands with molecular weights of ≥116 kDa. IgM antibodies against many antigens in sera from both phases were also observed but the total number was much less than for IgG. Responses to 21, 102 and ≥116 kDa antigens were particularly

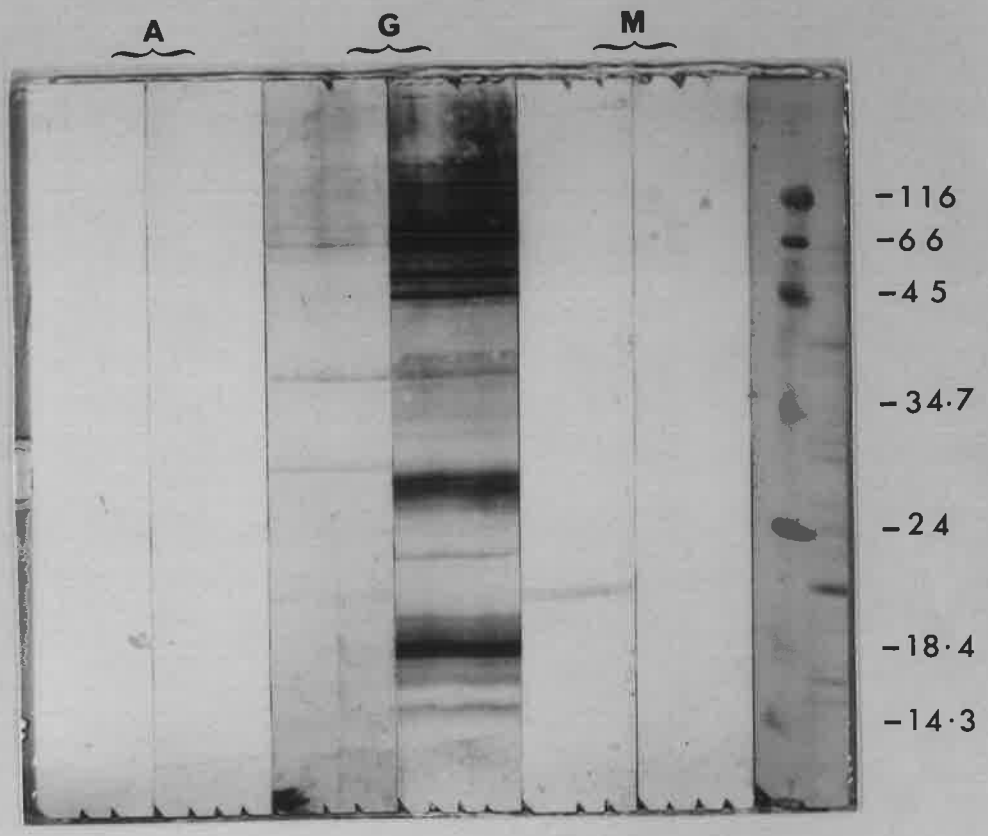
Figure 4.5

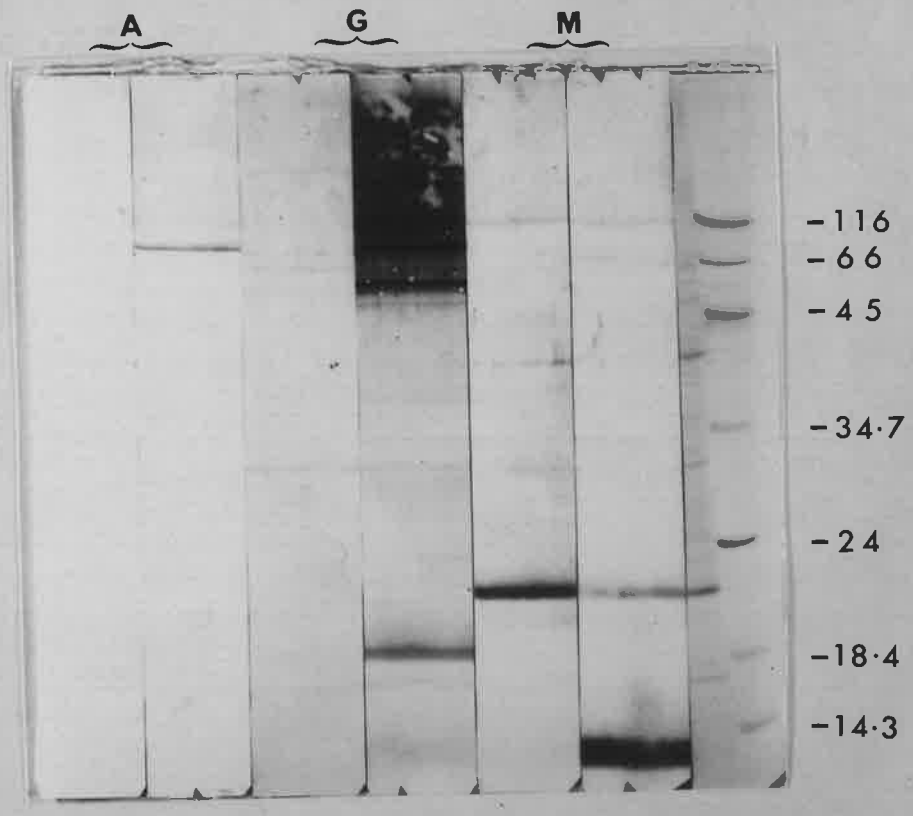
Western blot filters showing class specific antibody reactions with convalescent and acute phase sera from 2 patients with serologically diagnosed pertussis.

The antigen source was a formalin killed whole cell sonicate of *B. pertussis* organisms in phase 1. The left hand side of each pair of filters was tested with acute phase patient sera while the right hand side filters were tested with convalescent phase sera.

Upper photograph, patient 1. From left to right : Filters 1&2, 3&4, 5&6 probed with anti-IgA, IgG and IgM horseradish peroxidase conjugate respectively. Filter 7 shows molecular weight markers (in kDa) and an amido black stained portion of the whole cell antigen.

Lower photograph, patient 2. From left to right : Filters 1&2, 3&4, 5&6 probed with anti-IgA, IgG and IgM horseradish peroxidase conjugate respectively. Filter 7 shows molecular weight markers (in kDa) and an amido black stained portion of the whole cell antigen.


A
G
M
-116
-66
-45
-34·7
-24
-18·4
-14·3


A
G
M
-116
-66
-45
-34·7
-24
-18·4
-14·3

noticeable in both acute and convalescent sera. In 3 cases reactivities present in the acute phase disappeared in convalescence (30, 40 and 45 kDa). The reverse occurred in 2 other cases (12, 69 kDa). A summary of responses to *B. pertussis* antigens for these patients can be seen in **Figures 4.6** and **4.7**.

As was the case for sera from culture positive patients, no consistent responses to specific antigens or immunoglobulin class was observed in the seven pairs of IgA ELISA-positive sera. There were however a number of antigens for which specific antibodies were often present in convalescent sera or both acute and convalescent sera including those with molecular weights as follows ; 21, 26, 45, 69, 98, ≥116 kDa . These antigens which elicit detectable immune responses, may play important roles in establishing and maintaining infection.

4.2.3 Purified PT and FHA compared with whole cell sonicate as a source of antigen in immunoblots

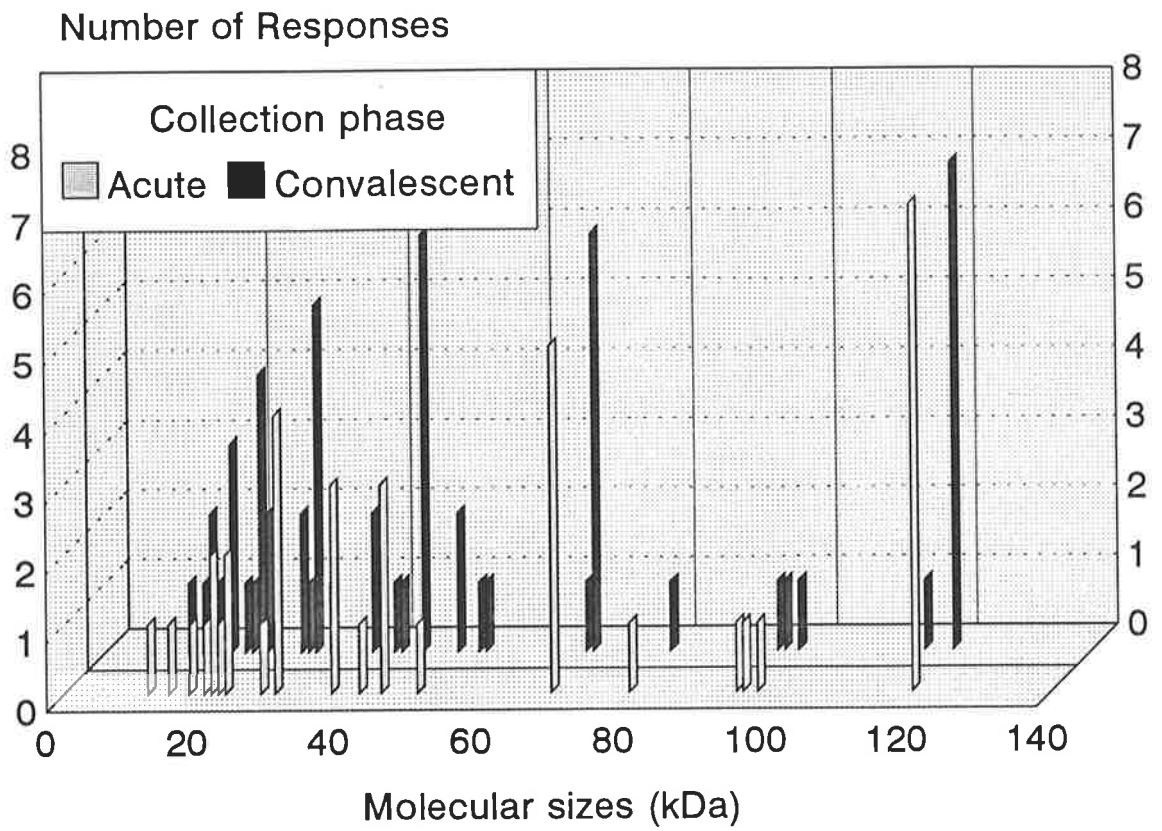
In an attempt to confirm the identity of some of the immuno-reactive antigens observed previously, Western blot analysis was performed using purified PT and FHA (obtained from List laboratories, Campbell, USA, and Commonwealth Serum Laboratories, Parkville, Victoria (CSL), respectively) as antigen. Comparisons were made with the whole cell sonicate (derived from *B. pertussis* NCTC 10908 and 10911) used routinely as antigen source for ELISA and Western blots. All filters were probed with mouse anti-PT and mouse anti-FHA antibody (also provided by CSL) following electrophoretic transfer to nitrocellulose. Transblots were performed after elctrophoresis both in 12.5% acrylamide gels and 7-20% gradient acrylamide gels. Results from the two different gel types were similar in terms of protein separation and mobilities, and because of the extra difficulty in preparation of gradient gels, they were not used for further tests.

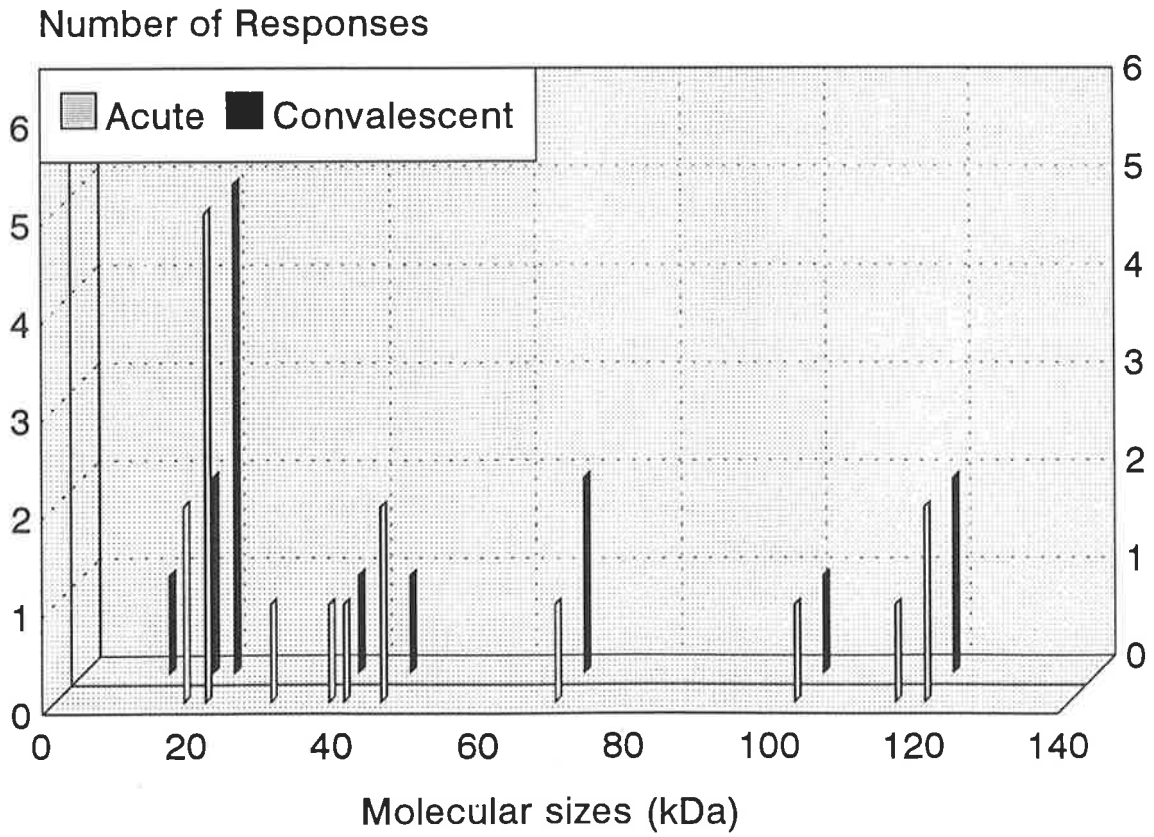
Figure 4.6

Western blot IgG analysis using a whole *B. pertussis* cell sonicate as antigen source and transblot filters probed with sera from 7 patients with positive IgA ELISA assays.

Figure 4.7

Western blot IgM analysis using a whole *B. pertussis* cell sonicate as antigen source and transblot filters probed with sera from 7 patients with positive IgA ELISA assays.


Number of Responses
Collection phase
Acute
Convalescent
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
140
Molecular sizes (kDa)


Number of Responses
Acute
Convalescent
0
1
2
3
4
5
6
0
20
40
60
80
100
120
140
Molecular sizes (kDa)

A major reactivity was observed with a 24 kDa antigen on filters transblotted from a gel containing the commercial PT and culture sonicate after PAGE and probing with mouse anti-PT antibody. The culture sonicate filter also showed weak reactivity with antigens of molecular weights around 30, 60 and >116 kDa when probed with same antibody (**Figure 4.8b**).

When purified FHA (CSL) was subjected to Western blotting, a number of distinct bands >116k Da (approx. 180 and 146 kDa) as well as others at 98, 74, 45 and 40 kDa were observed on the filters when probed with mouse anti-FHA antibody. Western blots using whole cell sonicate as antigen source revealed a number of bands on the filters after probing with the same anti-FHA antibody. These included antigens with molecular weights ranging from 22 to >116 kDa (**Fig 4.8a**).

In order to confirm the presence of anti-FHA antibody in patient sera, Western blots were performed for each antibody class using both purified FHA and the whole cell sonicate as antigen sources and filters were probed with sera from serologically positive patients. Two sera, P788 and P124 were selected to use in these experiments. IgG and IgM antibodies (**Figure 4.9 a. and b.,** respectively) were seen to be directed at antigens with molecular weights of 116 kDa and 98 kDa as well as proteins with molecular weights in excess of 116 kDa. The IgM reactivity was additionally directed at antigens with MW of 45 and 41 kDa (present in the purified FHA blot) in both sera. IgA antibodies were directed at higher MW antigens, as for the other class antibodies, but the reactions were faint, except for patient 1, whose serum reacted strongly with 98 kDa antigen in the whole cell sonicate (**Figure 4.10**).

Figure 4.8a

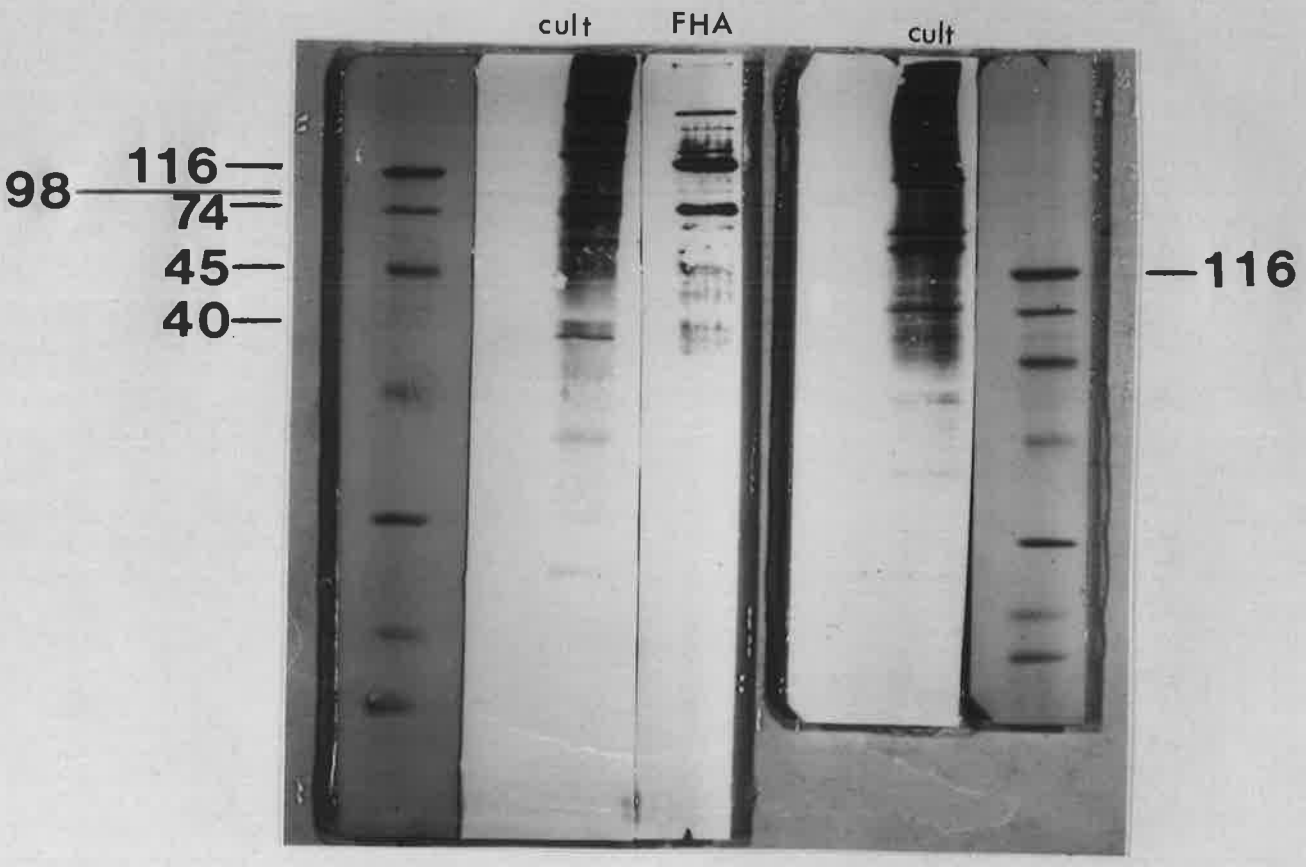
Western blot analysis of a whole *B. pertussis* cell sonicate and purified FHA preparation. PAGE was performed in 12.5% acrylamide or 7%-20% acrylamide gradient gels and filters probed with murine anti-FHA antibody. Filters from left to right are as follows :

Filter 1, Molecular weight markers (116, 45 kDa are labelled); Filter 2, whole cell sonicate; Filter 3, purified FHA; Filter 4, whole cell sonicate; Filter 5 MW markers. Filters 1,2 and 3 were transblotted from 12.5% acrylamide gel, while filters 4 and 5 were blotted from a gradient gel.

Figure 4.8b

Western blot analysis of a whole *B. pertussis* cell sonicate and purified pertussis toxin preparation. PAGE was performed in 12.5% acrylamide and a 7%-20% acrylamide gradient gels and filters probed with murine anti-PT antibody.

Filter 1, Molecular weight markers (116 and 24 kDa are labelled); Filter 2, whole cell sonicate; Filter 3, purified PT; Filter 4, molecular weight markers (24 kDa marker labelled); Filter 5, whole cell sonicate; Filter purified PT. Filters 1,2 and 3 were transblotted from 12.5% acrylamide gel while filters 4, 5 and 6 were blotted from a gradient gel.


cult
FHA
cult
98
116
74
45
40
116

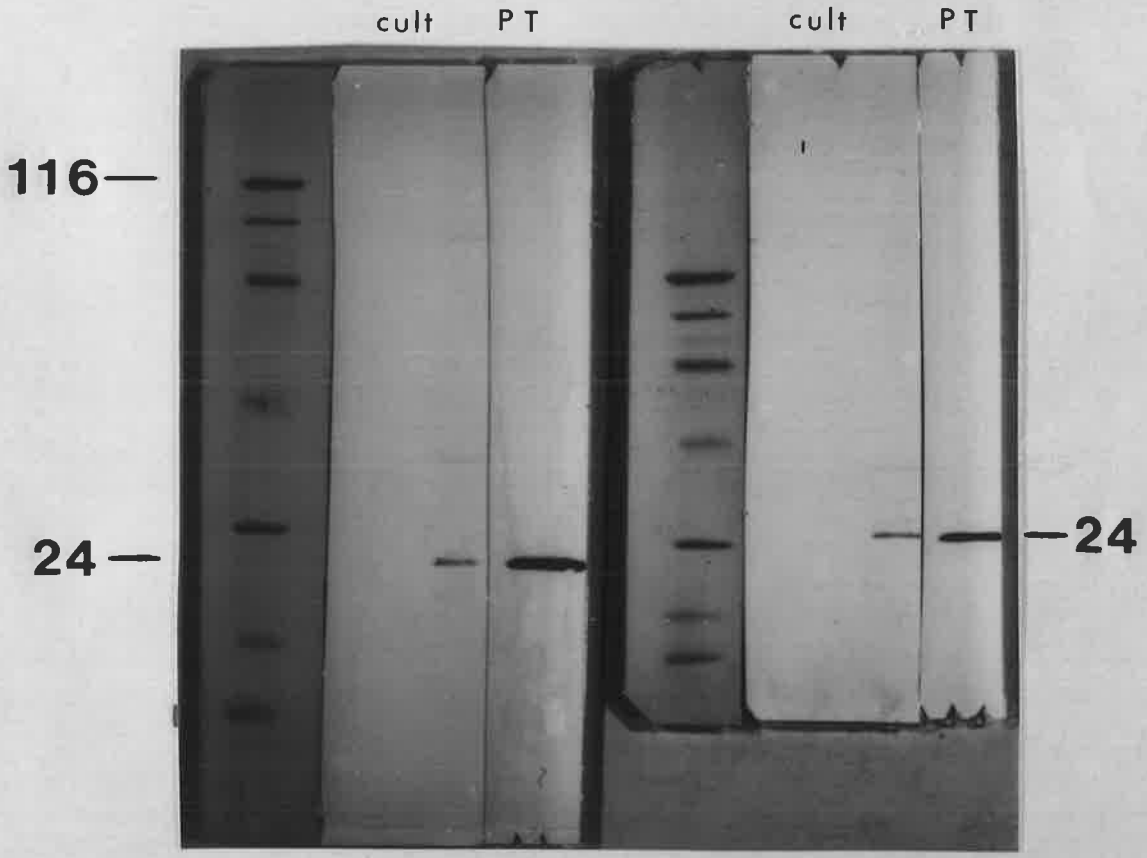

cult
PT
cult
PT
116
24
24

Figure 4.9a

Western blot analysis using whole cell sonicate (WCS) and purified FHA as antigen source.

The first antibodies were sera from 2 sero-positive pertussis patients and filters were probed with anti-human IgG-horseradish peroxidase conjugate. Filters described from left to right are as follows :

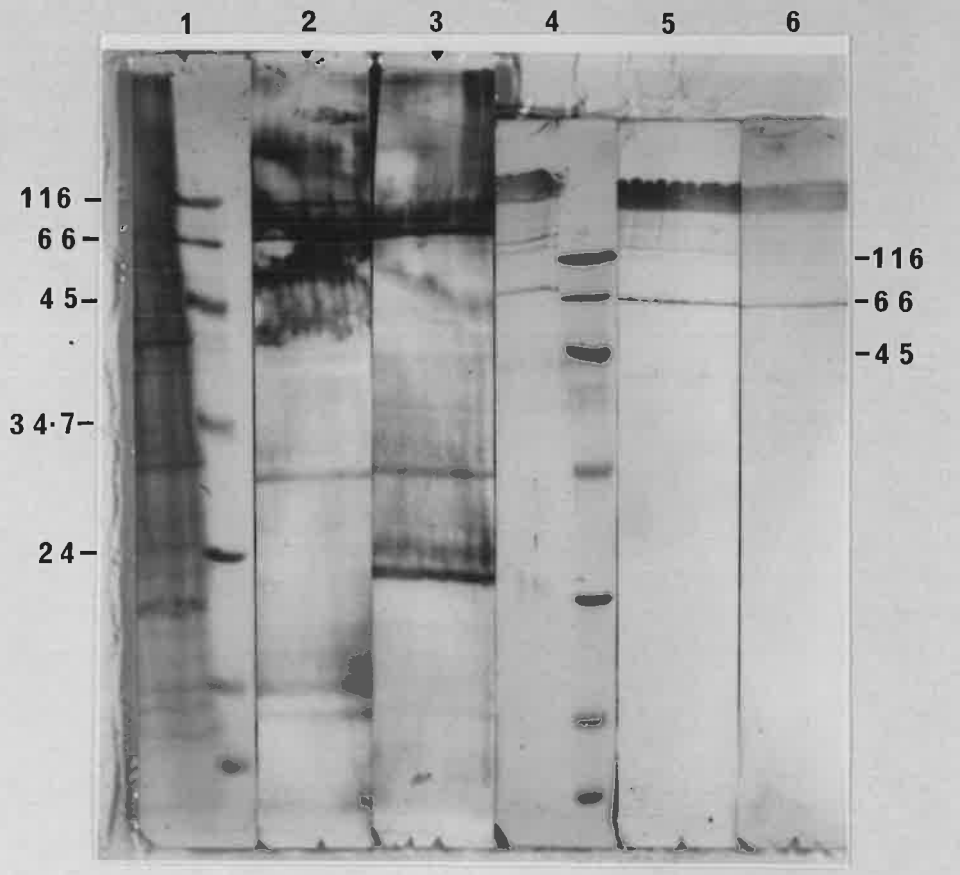
Filter 1, WCS without antibody (amido black stained) plus molecular weight markers (kDa); Filter 2, WCS/sera patient 1; Filter 3, WCS/sera patient 2; Filter 4, purified FHA without antibody plus molecular weight markers (kDa)(amido black stain); Filter 5, FHA/sera patient 1; Filter 6, FHA/sera patient 2.

Figure 4.9b

Western blot analysis using whole cell sonicate (WCS) and purified FHA as antigen source.

The first antibodies were sera from 2 sero-positive pertussis patients and filters were probed with anti-human IgM-horseradish peroxidase conjugate.

Filter 1, WCS without antibody (amido black stained) plus molecular weight markers (kDa); Filter 2, WCS/sera patient 1; Filter 3, WCS/sera patient 2; Filter 4, purified FHA without antibody plus molecular weight markers (kDa)(amido black stain); Filter 5, FHA/sera patient 1; Filter 6, FHA/sera patient 2.


1
2
3
4
5
6
116
66
45
34·7
24
116
66
45

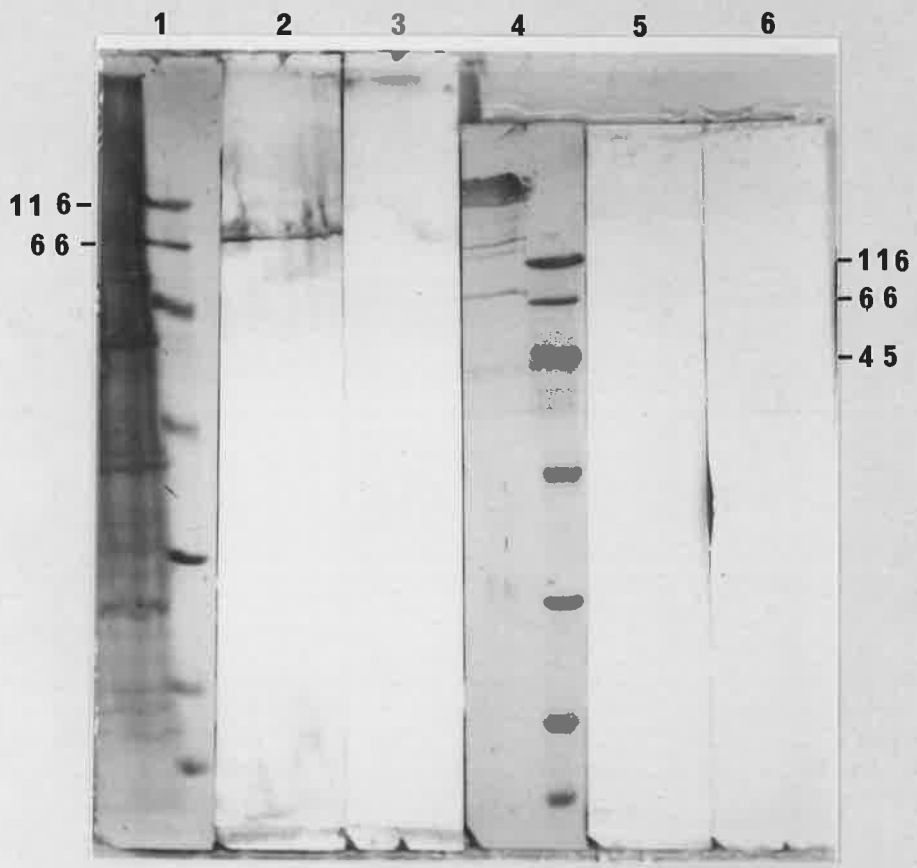

1
2
3
4
5
6
116
66
45
34·7
24
116
66
45
34·7

Figure 4.10

Western blot analysis using whole cell sonicate (WCS) and purified FHA as antigen source.

The first antibodies were sera from 2 serologically positive patients and filters were probed with anti-human IgA-horseradish peroxidase conjugate.

Filter 1, WCS without antibody (amido black stained) plus molecular weight markers (kDa); Filter 2, WCS/sera patient 1; Filter 3, WCS/sera patient 2; Filter 4, purified FHA without antibody plus molecular weight markers (kDa)(amido black stain); Filter 5, FHA/sera patient1; Filter 6, FHA/sera patient2.;


1
2
3
4
5
6
116
66
116
66
45

4.3 DISCUSSION

In 1987 there was a paucity of information concerning use of Western blot for detection of antibodies to *B. pertussis*. A study was reported shortly thereafter however, which described the presence of serum antibodies to two proteins (molecular weights of 75 and 84 kDa) which were associated both with natural infection and vaccination in children and adults, as well as responses to a number of other antigens, particularly high molecular weight FHA (Redd *et al.*, 1988). Later, Thomas *et al.*, (1989) and Arciniega *et al.*, (1991) examined the serum antibody response to various antigens (PT, FHA, AC, 69kDa protein) however these tests were not class specific although the latter study did consider a larger range of cell envelope proteins, which may also elicit an antibody response. The results of Anwar (1991), who used Western blot analysis to detect antibodies to *B. pertussis* antigens in human patient convalescent sera only, showed that a 69 kDa protein, a 40 kDa protein and agglutinogens 2 and 3 and a number of unknown OMP's were surfaced-exposed and able to stimulate the immune system. All of these data were reported after completion of my work in 1987-88.

Isolation of *B. pertussis* organisms from patients is still regarded as the "Gold Standard" in laboratory diagnosis of whooping cough, but most patients who attend an emergency service of a hospital, with symptoms of pertussis, are culture negative. Serological tests, although often retrospective, have been shown to be important in confirmation of the diagnosis of pertussis and even able to give rapid results (see Chapter 3). Paired sera (acute and convalescent) are very useful samples for observing rises and falls in specific antibody over time. In the hospital setting it is not easy to obtain paired samples of serum from patients who are suspected of having pertussis since their stay is usually only a few days. When pairs are available however, useful information on development of the immune response to various antigens can be obtained using

techniques such as Western immunoblotting. It is for this reason that a number of paired sera from patients with culture and/or serologically proven pertussis were chosen for study in these experiments.

The second (convalescent) sera were generally collected 14 days after the first specimen. Previous studies suggest an IgA response is elicited in natural disease (Burstyn *et al.*, 1983, Nagel and Poot-Scoltens, 1983). Western blot data from the 3 culture-positive patients (all of whom were IgA positive by ELISA) in this study, suggest IgA activities are probably directed against pertussis toxin and FHA in convalescence. The intensity of reaction to antigens which are probably FHA components were much greater than some antigens which are probably components of PT. This might be expected because FHA is thought to be involved in attachment of *B.pertussis* to the epithelial lining and hence should be exposed to the immune system very early on in the disease process resulting in quantitative differences in serum antibody levels with time. These results are also consistent with previous studies using ELISA assays which identified increases in anti FHA IgA in the late stage of convalescence (Steketee *et al.*, 1988). The ELISA results for these same 3 patients were all positive for IgA and all showed rises in IgA levels in convalescent sera. The Western blot data in this study reflects the ELISA results and suggests the IgA response may be due primarily to antibody directed at FHA and to a lesser extent PT.

A marked increase in serum IgG ELISA titre occurred in patient 1. This was reflected in the Western blots where an intense band at 26 kDa, with lesser ones at 21 kDa and 45 kDa, appearing in convalescent sera. Conversely, an extra, very high molecular weight band appeared in the convalescent sera of patient 2 without any concurrent increase in ELISA titre. These high molecular weight antigens may not bind as efficiently to the wells of microtitre trays compared to smaller molecular weight antigens and in such circumstances serum antibodies directed at them will not be detected in ELISA assays. Many IgG-

reactive bands were present for all patients in both phases, suggesting probable past immunization in addition to current or recent infection. One interesting observation was that a consistent and intense IgM reaction was directed at a 24 kDa antigen while no IgG response for this band was observed. There was however an equally intense IgG reaction to a larger protein (26 kDa) but no IgM activity. These results suggest these antigens may be presented to the immune system at different times during infection. Serum IgM antibodies appear earlier in the course of disease and their level diminishes more quickly than IgG antibodies. The fact that there is a very strong IgG reaction to the 26 kDa antigen suggests this antigen is immunodominant, but it's importance in longer term immunity and ability to confer protection to susceptible individuals, is unknown. The 24 kDa antigen probably presents to the immune system at an earlier stage of infection, given the large IgM antibody reaction. Although it is not possible to determine the function of such a protein in this experiment it is possible that because of the timing of the antibody response, it may be involved in the early stages of establishing infection.

All patient sera contained anti-pertussis IgM antibody although the titres in patient 2's sera (both acute and convalescent) were just under the positive cut-off level in the ELISA test despite reacting to several antigens by WB. This observation suggests patient 2 may have presented later in the course of disease and therefore would be expected to have a diminishing IgM response together with a positive IgG and IgA response, which is the case (**Table 4.1**). Alternatively these antibodies could be present because of previous immunisation although this seems less likely given the fact that this patient was culture positive. The only IgM antibody to appear in convalescent sera was a 69kDa protein which may have given rise to the slight increase in ELISA titre and would be due to current or recent infection. Patient 1 showed the greatest increase in ELISA antibody level and this was reflected by increased intensity of the higher molecular weight proteins in convalescent serum and 69 kDa protein.

Patient 3 was borderline ELISA positive in both sera and displayed a very intense IgM reaction to an antigen (21 kDa) which on the basis of its size, may be a pertussis toxin subunit, both in acute and convalescent sera. The antibody detected by ELISA could therefore have been anti-toxin antibody in this case.

Only 3 of the 7 paired sera showed detectable IgA bands on the filters after probing. These reactions were primarily directed at a 69 kDa antigen and only were present in the convalescent sera. All 4 sets of paired sera showing no reaction for IgA displayed significant ELISA rises for this class of antibody in convalescence indicative of current or recent infection. One explanation of the lack of reactivity in the Western blot may be changes in conformation of antigens following PAGE and immunoblotting. The physical conformation of antigens bound to the walls of a microtitre plate prepared for ELISA testing may be different to those bound to a filter following boiling in SDS and immunoblotting and both of these may differ from the native protein. Small conformational changes could prevent IgA antibodies recognised in the ELISA from binding to the filter-bound antigens.

There was good correlation between ELISA and Western blot data for IgG, with antibody directed at a large number of antigens both in acute and convalescent sera. It is likely that the majority of these patients were also vaccinated, because of the strength of the response in acute phase sera. Interestingly, in the cases where antibody appeared in convalescence, it was predominantly directed at lower molecular weight antigens (14 - 18 kDa) and a 22 kDa antigen. Anwar (1990) described agglutinogen 2 as an antigen with a molecular weight of 22 kDa although the antigen with the equivalent mobility in this study cannot be confirmed as being agglutinogen2 given the accuracy of SDS-PAGE. IgG antibodies were also directed at a 45 kDa antigen in a number of convalescent sera only. Other studies have described an antigen with a MW of 45 kDa as being adenylate cyclase, however specific identification of the 45 kDa antigen observed in the current study, was not attempted.

Pertussis vaccination will induce formation of specific IgM and IgG antibodies. This needs to be considered when interpreting Western blot data. In this study there were however a number of specific antibodies which appeared only in convalescent sera suggesting current or recent natural infection in these patients. These antibodies were directed at a number of antigens including 18, 24, 45, 69 ,98 and ≥116 kDa in size. A number of specific antibodies were seen to disappear in convalescent sera (particularly a 30 kDa antigen). This may be expected for IgM in infected patients because although it rises in titre first, levels also fall away relatively quickly. ELISA data support this because serologically positive patients with raised IgG and IgA anti-*B. pertussis* antibody levels often have normal IgM values, suggesting IgM has fallen away quickly.

The relative concentrations of various antigens in whole *B. pertussis* cell sonicate preparations may vary depending on environmental factors during growth. For ELISA analysis of patient sera for diagnostic purposes, there must be adequate concentrations of at least the major virulence factor proteins (PT and FHA) present in the antigen preparations used to coat microtitre wells. Western blot data confirmed the presence of PT and FHA in the whole cell sonicate prepared by harvesting the growth from fresh charcoal agar plates (≤ 48 hours old) thus confirming it's suitability as a source of antigen in diagnostic tests such as the ELISA described in Chapter 3.

The presence of bands below 50 kDa in both the sonicate and the purified CSL FHA preparation, suggest the antibody (obtained from CSL) used to probe the filter may have been prepared with antigen containing small amounts of other *B. pertussis* antigens. FHA components are usually in the range 50-200 kDa although breakdown products of the FHA macromolecule could also be present. Likewise when anti-PT antibody (also courtesy CSL) was used to probe electrophoresed, transblotted filters of purified PT and the whole cell sonicate, a number of unexpected, higher MW bands were observed. A

reason for this observation may be that the antigen used to prepare this antibody also contained small amounts of other pertussis antigens such as FHA or AC.

The major point to be made from these experiments however is that whole phase 1 *B. pertussis* cells harvested and prepared (including sonication) from fresh charcoal agar plates contain sufficient amounts of FHA and PT for use as a source of antigen in diagnostic ELISA tests or Western blot assays.

Western blot does not lend itself to routine testing of sera for diagnostic purposes and ELISA should be used in this situation. This study has used immunoblotting to confirm the premise that a number of antigens should be used for coating the solid phase in a diagnostic ELISA for detecting *B. pertussis* antibody in serum. There were large numbers of different antigens against which specific antibodies were directed. No singularly consistent antibody response in any patient to particular *B. pertussis* antigens was observed. Therefore, in order to maintain good sensitivity in *B. pertussis* ELISA tests, a number of antigens need to be present in the coating antigen preparation. If a diagnostic ELISA was based on only a single purified antigen, a number of positive cases would be missed. One other factor which must be considered in ELISA tests or WB assays for antibodies to bacterial pathogens such as *B. pertussis*, is that there may be cross reactivity with certain antibodies raised against antigens which may also be present in the cell walls of other gram negative organisms. An example of such an antigen could be the group of proteins known as porins.

A significant IgG response to a 22 kDa antigen was noted. This antigen may therefore be a useful component of an acellular vaccine for use in Australia since it appears to elicit a response in the later stages of disease and probably remains in the serum for a long period of time. Data from the 3 culture positive cases, where it can be assumed the response is relatively early in the infective process, indicates a total lack of response to the 22 kDa antigen. This is in stark contrast to the data from 7 IgA positive patients where a response to it

predominated. Whether or not the 22 kDa antigen has significant ability to protect (or help protect) individuals from infection needs to be established. Anwar (1990) described an antigen with this MW as being agglutinogen 2, although the identity of this antigen was not confirmed in the present study. Another study found that mice immunised with purified type 2 fimbriae (agglutinogen 2) were protected from aerosol challenge of *B. pertussis* (Zhang *et al* 1985).

In summary, the Western blot data support the view expressed in the last chapter that semi-quantitative determination of the 3 major classes of antibody levels should be performed in diagnostic ELISA tests rather than just IgA alone. The source of antigen for the solid phase must either be a whole cell sonicate or a mixture of a number of antigens possibly including PT, FHA, AC, 69kDa OMP, agglutinogen 2 (and possibly agg. 3) and possibly some other OMP's (eg. 40kDa protein). No single, identifiable *B. pertussis* antigen consistently elicited increases in titre for any particular antibody class after natural infection. However, detecting the presence of and quantifying *B. pertussis* specific IgA, as an indicator of infection, remains a useful and important diagnostic test.

CHAPTER 5

APPLICATION OF THE POLYMERASE CHAIN REACTION FOR RAPID DETECTION OF *BORDETELLA PERTUSSIS* IN SPECIMENS OF NASOPHARYNGEAL SECRETION.

APPLICATION OF THE POLYMERASE CHAIN REACTION FOR RAPID DETECTION OF *BORDETELLA PERTUSSIS* IN SPECIMENS OF NASOPHARYNGEAL SECRETION.

5.1 INTRODUCTION

In recent years there has been much interest in the application of DNA probes for laboratory diagnosis of infectious disease, particularly because of the increasing availability and ease of use of non-radioactive labelling techniques. Detection of slow growing and fastidious organisms such as *B. pertussis* is ideally suited to this newer DNA technology. Current techniques for isolating and identifying *B. pertussis* in nasopharyngeal aspirates from patients suspected of suffering from whooping cough can take up to seven days and false negative culture results are obtained in a significant proportion of cases (Linneman, 1978).

The presence of species-specific reiterated chromosomal DNA sequences for a number of bacterial species had been reported in the literature as early as 1981 (Kleckner, 1981; Higgins *et al.*, 1982; Clark-Curtis and Docherty, 1989). Similar reports also began to appear in 1988 for *B. pertussis* (McPheat and McNally, 1987 (a) and (b); McClafferty *et al.*, 1988; Park *et al.*, 1988; Alsheikhly and Lofdahl, 1989). Reiterated sequences are ideal targets for diagnostic DNA probes because multiple targets enhance the sensitivity of probe-based assays. For *B. pertussis* the repeat is present in 50-100 copies per cell and consensus sequences up to approximately 1.1 kb long have been determined by comparing sequence data from different clones containing portions of the repeating element. (McClafferty 1988, Park 1988).

McLafferty *et al.*, (1987), had described a DNA probe for detection of *B. pertussis* in NPA's with a sensitivity of 1,000 organism however there was some cross reaction with *Pseudomonas aeruginosa* and other *Bordetella spp.* The lack of sensitivity reported, combined with the observed cross hybridization with *Pseudomonas* suggests this hybridization assay is not suitable for use as a routine diagnostic test.

A concurrent study in the ACH Department of Microbiology had resulted in the cloning of the reiterated sequence for *B. pertussis*. This was shown to be tandemly repeated in the genome (Glare *et al*, 1990). The DNA sequence of the 1046-bp repeating unit was also determined.

This chapter initially describes the analysis of the sensitivity and specificity of a DNA probe based on the repeat fragment for detection of *B. pertussis*. During the period of this work, it was realised that the reiterated sequences might be suitable targets for DNA amplification assays such as the polymerase chain reaction (PCR) which had recently been described by Saiki *et al.*, (1988). This technology, using specific DNA primers and a heat-stable DNA polymerase, was potentially able to amplify target sequences by a factor of 10^9 and therefore could possibly be applied to routine detection of *B. pertussis* in NPA specimens. This chapter describes the development and application of a PCR assay for direct detection of *B. pertussis* in specimens of NPA.

5.2 RESULTS

5.2.1 Specificity of the repeated sequence.

Studies carried out by E. Glare in the Microbiology Department, WCH, resulted in the isolation of 2 *E.coli* clones, (with recombinant plasmids designated pJCP601 and pJCP602) which contained reiterated *B. pertussis*-

specific DNA sequences cloned in pUC19 (Glare *et al*, 1990). pJCP601 contained a 188 bp insert of which 168bp were derived from one of the copies of the reiterated sequence in the *B. pertussis* genome. This was used as a probe to isolate and clone a 1046-bp *Cla*I fragment which contained a complete repeat unit of the reiterated sequence (pJCP602).

To assess the suitability of the reiterated sequence as a diagnostic DNA probe specific for *B. pertussis*, the insert of pJCP601 was labelled with ^{32}P and hybridized to dot blots of lysates from 96 different bacteria isolated from clinical specimens. These bacteria included 45 different species representing 24 genera including organisms likely to be present in nasopharyngeal secretions (**Table 5.1**). While the probe hybridized strongly to *B. pertussis* strains, it did not hybridize to any other bacteria including members of the most closely related genera (*Pasteurella, Alcaligenes* and *Haemophilus*). Furthermore no hybridization was observed with *B. parapertussis* lysates even when autoradiography at -80°C was extended considerably. However, under these conditions some hybridization could be detected to the lysate blot of *B. bronchiseptica*. (**Figure 5.1**.)

Table 5.1

List of bacterial isolates used in a dot blot hybridization assay with a ^{32}P-labelled, cloned insert of pJCP601

Haemophilus spp.	*Escherichia spp.*	*Vibrio spp.*	*Staphylococcus spp.*
Alcaligenes sp.	*Klebsiella spp.*	*Enterobacter spp.*	*Streptococcus spp.*
Pasteurella spp.	*Proteus spp.*	*Citrobacter spp.*	*Coynebacterium spp*
Neisseria spp.	*Flavobacterium spp.*	*Serratia spp.*	*B. pertussis*
Acinetobacter spp.	*Salmonella spp.*	*Bacteroides sp.*	*B. parapertussis*
Moraxella spp.	*Aeromonas spp.*	*Eikenella corrodens*	*B. bronchiseptica*

Figure 5.1

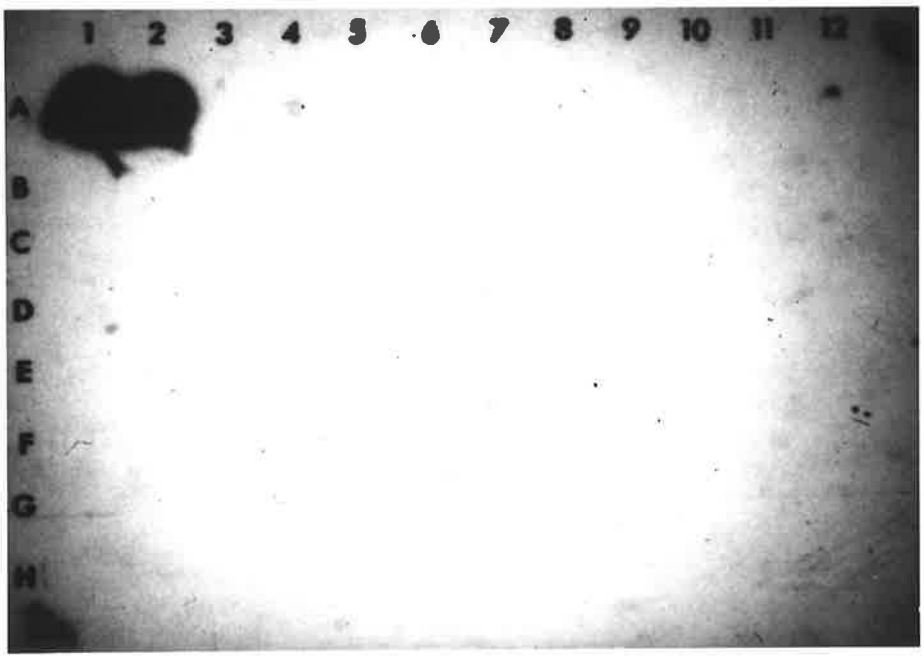
Autoradiograph of lysate blots of 96 different bacteria isolated from clinical material including NPAs, and probed with a ^{32}P-labeled insert of pJCP601.

Row A, Lanes 1 and 2 are *B. pertussis* NCTC 10908 and 10911 respectively.

Row A, Lane 3 is *Bordetella parapertussis*

Row A, Lane 4 is *Bordetella bronchiseptica*.

The remaining positions were lysate blots of 45 different bacterial species from 24 genera (see text).


1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

The nature of the homology between the *Cla*I fragment and the genomic DNA of the other *Bordetella* species was determined by Southern hybridization analysis of various restriction digests of DNA (**Figure 5.2**). Multiple bands were observed for *B. pertussis* after only 4 hours of autoradiography. After prolonged autoradiography (48 hours at -80°C), hybridization to one or two bands in each restriction digest of *B. bronchiseptica* DNA was detected, but was not detected with *B. parapertussis* DNA. The strength of the hybridization suggests that single copy sequences that have at least partial homology to the repetitive *Cla*I fragment found in *B. pertussis* exist in *B. bronchiseptica*.

To determine whether the *Cla*I fragment (contained in plasmid pJCP602) is present in all strains of *B. pertussis*, the insert was hybridized to lysate blots of 100 clinical isolates of *B. pertussis*. Hybridization to all *B. pertussis* isolates was detected, but was not observed with any of the control samples (DNA from other bacteria and human DNA) (87 shown in **Figure 5.3**).

5.2.2 Sensitivity of the insert of pJCP602 as a probe for *B.pertussis* sequences.

In order to test the sensitivity of the insert of pJCP602 as a probe for pertussis sequences, a lysate blot was performed using 10-fold serial dilutions of a *B. pertussis* culture. The emulsified *B. pertussis* cells were lysed and fixed to nitrocellulose and probed with ^{32}P-labelled insert. After autoradiographing over-night at -80°C, it was apparent that approximately 1000 cells could be detected, with only faint hybridization observed at this level of organism concentration (**Figure 5.4**).

5.2.3 PCR Primer Selection.

The sensitivity of direct hybridization for detection of *B. pertussis* was considered too low to be diagnostically useful. Therefore it was decided to employ PCR amplification to increase sensitivity. As a first step in this, the

Figure 5.2

Southern blot analysis of DNA from other *Bordetella* species.

DNA from *B. pertussis* (A), *B. bronchiseptica* (B), or *B. parapertussis* (C) was digested with *Bam*HI (lanes 1), *Cla*I (lanes 2) or *Eco*RI (lanes 3). Southern blots of electrophoresed DNA were then probed with the insert of pJCP602. Filter A was autoradiographed for 4 hours while filters B and C were autoradiographed for 4 days.

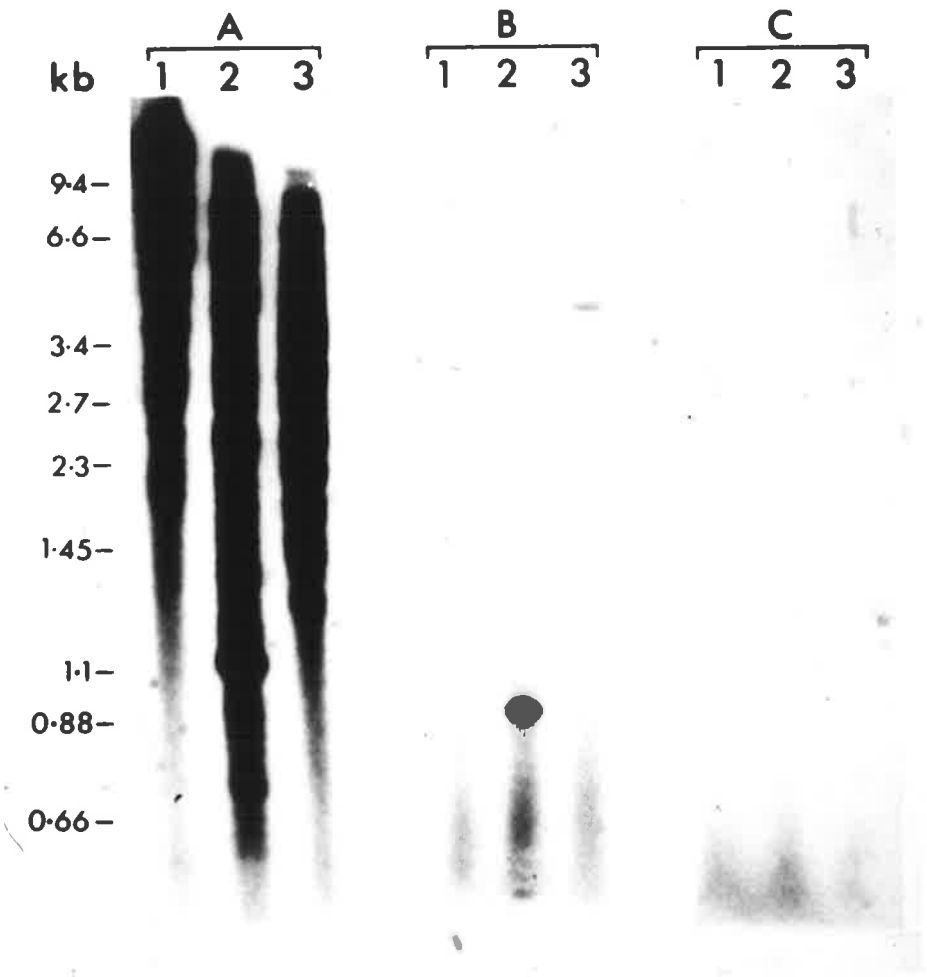

A
B
C
kb
1 2 3
1 2 3
1 2 3
9·4–
6·6–
3·4–
2·7–
2·3–
1·45–
1·1–
0·88–
0·66–

Figure 5.3

Dot blot hybridization of lysates of 87 *B. pertussis* strains isolated from patients attending the WCH, other bacterial genera and human DNA, using a ^{32}P-labeled insert of pJCP602 (see text) as probe.

Row A, lanes 1,2 and 3 are *B. pertussis* strains NCTC 10908, 10911 and 10387, respectively.

Row A, lanes 4-11 are bacterial strain lysates including *Pasteurella*, *Alcaligenes* and *Haemophilus sp*.

Row A, lane 12 is human DNA.

The remaining positions are clinical strains of *B. pertussis* isolated from patients with pertussis isolated at the WCH.

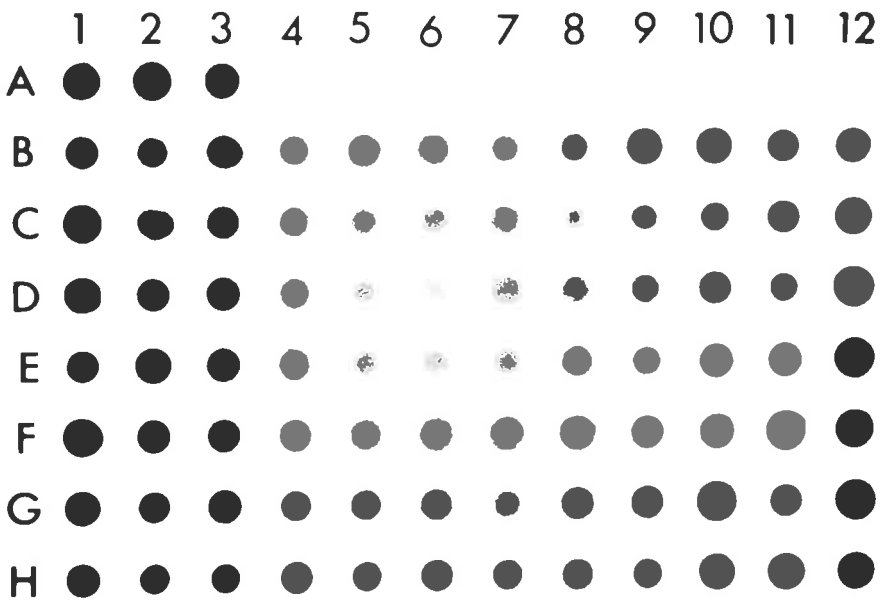
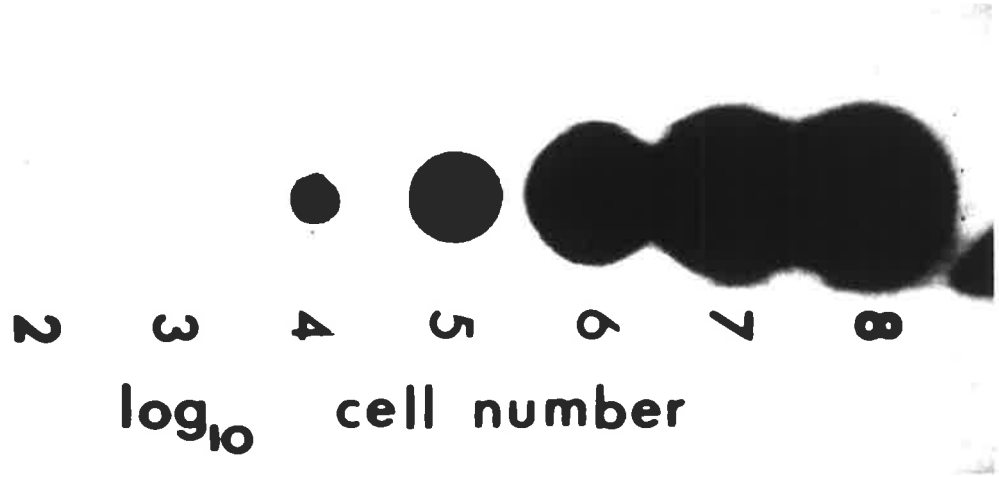

1 2 3 4 5 6 7 8 9 10 11 12
A
B
C
D
E
F
G
H

Figure 5.4

Sensitivity of dot-blot hybridization with the repetitive sequence for direct detection of *B. pertussis*.

Lysates of serial 10-fold dilutions of b. pertussis cultutres containing the indicated number of viable bacteria were hybridized with ^{32}P labelled insert of pJCP602, washed and autoradiographed overnight as described in section 2. . A faint hybridization signal detectable on the original autoradiograph for the 10^3 spot has been lost during photographic reproduction.


2
3
4
5
6
7
8
log10 cell number

DNA sequence of the insert of pJCP602 was determined by R.Premier, Commonwealth Serum Laboratories, Parkville, Victoria. The sequence of the insert of pJCP602 is compared with the two previously published sequences for the *B. pertussis* repeated element (McClafferty *et al.*, 1988; Park *et al.*, 1988) in **Figure 5.5**. Park's sequence however, included a further 5 base differences (3 deletions and 2 substitutions) with respect to both the present study and that of McClafferty. Thus it appears that the DNA sequence of the repeated element of *B. pertussis* is not absolutely conserved. The existence of this sequence variation, particularly the deletions, makes it extremely unlikely that the repeated element encodes a protein product. This sequence data was then used to select suitable primers for PCR amplification. A section of DNA which was identical in pJCP601, pJCP602 and the sequence of McLafferty *et al* (1988) was chosen for amplification. The two oligonucleotide primers (see Chapter 2.11.3) which were selected (see **Fig. 5.5**) would be expected to give rise to a 153-bp PCR product after amplification.

5.2.4 Detection of *B.pertussis* by PCR amplification of part of the repeated element

Oligonucleotides flanking a 153 bp region of the repeated element (see **Figure 5.5**) were synthesized and used as primers for PCR amplification as described in the Section 2.11.3. In initial experiments, a Hybaid intelligent heating block was used for the amplifying process and proteinase-K treated suspensions of various bacteria were tested. PCR product was detected by agarose gel electrophoresis and ethidium bromide staining. This is a rapid technique which is suitable for analysing large numbers of samples and photography under UV illumination provides a permanent record. Assessment of the size of the PCR product is also an indirect check on specificity. PCR-amplified extracts of *B. pertussis* culture showed the presence of an intensively stained DNA band of the expected size (153 bp) (see **Figure 5.6**). When a new

Figure 5.5

Location of PCR primers within the DNA sequence of the *B. pertussis* repeated element.

The complete DNA sequence shown (B) is that of the insert of pJCP602, aligned with data of McLafferty et al (McLafferty 1988) (A) and data of Park et al (Park 1988) (C). For sequences A and C a dot indicates base identity with sequence B, whereas a dash in any of the sequences indicates a base deletion. The location of the region amplified by PCR is indicated by the arrows, which underscore the position of annealing of the two primer oligonucleotides. The *ClaI* recognition site at position 620 is underlined.

```
                                                    50                                                  100
A   CTAGGTGTGA AGATTCAATA GGTTGTATGC ATGGTTCATC CGAACCGGAT TTGAGAAACT GGAAATCGCC AACCCCCCAG TTCACTCAAG GAGCCCGGCC
B   CTAGCTGTGA AGATTCAATA GGTTGTATGC ATGGTTCATC CGAACCGGAT TTGAGAAACT GGAAATCGCC AACCCCCCAG TTCACTCAAG GAGCCCGGCC

                                                   150                                                  200
A   GGATGAACAC CCATAAGCAT GCCCGATTGA CCTTCCTACG TCGACTCGAA ATGGTCCAGC AATTGATCGC CCATCAAGTT TGTGTGCCTG AAGCGGCCCG
B   GGATGAACAC CCATAAGCAT GCCCGATTGA CCTTCCTACG TCGACTCGAA ATGGTCCAGC AATTGATCGC CCATCAAGTT TGTGTGCCTG AAGCGGCCCG

                                                   250                                                  300
A   CGCCTATGGG GTCACCGCGC CGACTGTGCG CAAATGGCTG GGCCGCTTCC TGGCTCAGGG CCAGGCGGGC TTGGCCGATG CGTCGTCGCG CCCGACGGTC
B   CGCCTATGGG GTCACCGCGC CGACTGTGCG CAAATGGCTG GGCCGCTTCC TGGCTCAGGG CCAGGCGGGC TTGGCCGATG CGTCCTCGCG CCCGACGGTC
C                                                                   AGG_ CCAGGCGG_C TTGGCCGATG CGTCCTCGCG CCCAGCG_TC

                                                   350                                                  400
A   TCGCCCCGAG CGATTGCGCC GGCCAAGGCG CTGGCTATCG TGGAGCTGCG CCGCAAGCGG CTGACCCAAG CGCGCATCGC CCAGGCGCTG GGCGTGTCAG
B   TCGCCCCGAG CGATTGCGCC GGCCAAGGCG CTGGCTATCG TGGAGCTCCG __G_AAGCGG CTGACCCAAG CGCGCAT_GC CCAGGCGCTG GGCGTGTCAG
C   TCGCCCCGAG CGATTGCGCC GGCCAAGGCG CTGGCTATCG TGGAGCTCCG __G_AAGCGG CTGACCCAAG CGCGCATCGC CCAGGCGCTG GGCGTGTCAG

                                                   450                                                  500
A   CCAGCACCGT CAGCCGCGTC CTGGCCCGCG CCGGTCTGTC GCACCTGGCC GACCTGGAGC CGGCCGAGCC GGTGGTGCGC TACGAGCATC AGGCCCCCGG
B   CCAGCACCGT CAGCCGCGTC CTGGCCCGCG CCGGTCTGTC GCACCTGGCC GACCTGGAGC _GGCCGAGCC GGTGGTGCGC TACGAGCATC AGGCCCCCGG
C   CCAGCACCGT CAGCCGCGTC CTGGCCCGCG CCGGTCTGTC GCACCTGGCC GACCTGGAGC _GGCCGAGCC GGTGGTGCGC TACGAGCATC AGGCCCCCGG

                                                   550                                                  600
A   CGATCTGCTG CACATCGACA TCAAGAAGCT GGGACGTATC CAGCGCCCTG GCCACCGGGT CACGGGCAAC CGACGCGATA CCGTTGAGGG GGCCGGCTGG
B   CGATCTGCTG CACATCGACA TCAAGAAGCT GGGACGTATC CAGCGCCCTG GCCACCGGGT CACGGGCAAC CGACGCGATA CCGTTGAGGG GGCCGGCTGG
C   CGATCTGCTG CACATCGACA TCAAGAAGCT GGGACGTATC CAGCGCCCTG GCCACCGGGT CACGGGCAAC CGACGCGATA CCGTTGAGGG GGCCGGCTGG

                                                   650                                                  700
A   GACTTCGTCT TCGTGGCCAT CGATGACCAC GCCCGCGTGG CCTTCACCGA CATCCACCCC GACGAGCGCT TCCCCAGCGC CGTCCAGTTC CTCAAGGACG
B   GACTTCGTCT TCGTGGCCAT CGATGACCAC GCCCGCGT_G CCTTCACCGA CATCCACCCC GACGAGCGCT TCCCCAGCGC CGTCCAGTTC CTCAAGGACG
C   GACTTCGTCT TCGTGGCCAT CGATGACCAC GCCCGCGTGG CCTTCACCGA CATCCACCCC GACGAGCGCT TCCCCAGCGC CGTCCAGTTC CTCAAGGACG
                      ClaI

                                                   750                                                  800
A   CAGTGGCCTA CTACCAGCGC CTGGGCGTGA CCATCCAGCG CTTGCTCACC GACAATGGCT CGGCCTTTCG CAGCCGCGCC TTCGCCGCGC TGTGCCATGA
B   CAGTGGCCTA CTACCAGCGC CTGGGCGTGA CCATCCAGCG CTTGCTCACC GACAATGGCT CGGCCTTTCG CAGCCG_GCC TTCGCCGCGC TGTGCCATGA
C   CAGTGGCCTA CTACCAGCGC CTGGGCGTGA CCATCCAGCG CTTGCTCACC GACAATGGCT CGGCCTTTCG CAGCCG_GCC TTCGCCGCGC TGTGCCATGA

                                                   850                                                  900
A   GCTGGGCATC AAGCACCGCT TTACCCGACC TTACCGCCCA CAGACCAATG GCAAGGCCGA ACGCTTCATC CAGTCGGCCT TGCGTGAGTG GGCTTACGCT
B   GCTGGGCATC AAGCACCGCT TTACCCGACC TTACCGCCCA CAGACCAATG GCAAGGCCGA ACGCTTCATC CAGTCGGCCT TGCGTGAGTG GGCTTACGCT
C   GCTGGGCATC AAGCACCGCT TTACCCGACC TTACCGCCCA CAGACCAATG GCAAGGCCGA ACGCTTCATC CAGTCGGCCT TGCGTGAGTG GGCTTACGCT

                                                   950                                                 1000
A   CACACCTACC AGAACTCCCA ACACCGAGCC GATGCCATGA AATCCTGGCT ACACCACTAC AACTGGCATC GACCCCACCA AGGCATCGGG CGCGCTGTAC
B   CACACCTACC AGAACTCCCA ACACCGAGCC GATGCCATGA AATCCTGGCT ACACCACTAC AACTGGCATC GACCCCACCA AGGCATCGGG CGCGCTGTAC
C   CACACCTACC AGAACTCCCA ACACCGAGCC GATGCCATGA AATCCTGGCT ACACCACTAC AACTGGCATC GACCCCACCA AGGCATCGGG CGCGCTGTAC

                                                  1050
A   CCATCTCCAG ACTCAACCTG GACGAATACA ACCTATTGAA TCTTCACAGC TAG
B   CCATCTCCAG ACTCAACCTG GACGAATACA ACCTATTGAC AGTTCACAGC TAG
C   CCATCTCCAG ACTCAACCTG GACGAATACA ACCTATTGAC AGTTCACAGC TAGC
```

Perkin Elmer DNA thermal cycler became available the protocol was modified to suit the new machine and all further tests were carried out with it. More rapid results could be obtained in this way because the total time required for the PCR reaction was significantly reduced to a total of about 2 hours compared to 4 hours using the original protocol.

5.2.5 Specificity.

A very weak band of approximately the same size (153 bp) was seen in amplified extracts of *B. bronchiseptica,* but no bands were seen in amplified extracts of *B. parapertussis* (results not shown). Additionally, no DNA bands were detected in the amplified extracts of any of the other bacteria tested earlier by hybridization or with human DNA (results not shown) thus making the test suitable for application in clinical laboratories. The bacterial isolates tested include members of the genera *Pasteurella, Alcaligenes a*nd *Haemophilus* which are all closely related to *B. pertussis.* Thus, the specificity of the PCR amplification, was the same as that shown for direct hybridization to the insert of pJCP602.

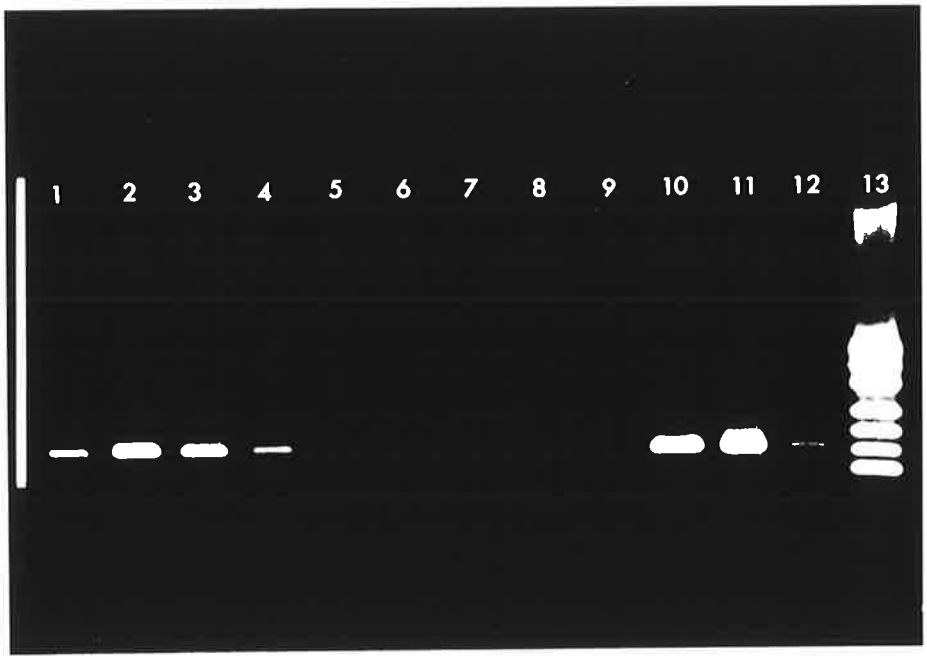
5.2.6 Sensitivity

The sensitivity of the PCR assay for detection of B. *pertussis w*as examined in vitro. A fresh (24hr) culture of *B. pertussis w*as serially diluted in tryptic soya broth (Difco) and 50μl aliquots were immediately plated on charcoal agar for determination of the number of viable bacteria. 50μl samples from each dilution were also treated with proteinase-K, followed by PCR amplification. PCR products were electrophoresed and visualised under UV light in an agarose gel containing ethidium bromide as described in Materials and Methods **(Figure 5.6, lanes 1-7).** A 153bp DNA band was still detectable in the reaction mixture that initially contained approximately 3 viable bacteria (lane 5) and was faintly discernible in the reaction mixture supposedly

Figure 5.6

PCR amplification of repetitive DNA sequences for direct detection of *B. pertussis*.

Samples were treated with proteinase-K K, PCR amplified, electrophoresed and stained with ethidium bromide, as described in the text. Lanes : 1, amplified purified *B. pertussis* DNA (positive control); 2-6, amplified *B. pertussis* culture extracts containing (approximately) 3,000, 300, 30, 3, and 0.3 cfu, respectively, in the total PCR mix; 7, amplified diluent (negative control); 8 and 9, culture negative and IF negative NPA; 10 and 11, culture and IF positive extracts; 12, culture positive, IF negative extract; 13, DNA size markers (500, 404, 242, 190, 147, and 110 bp, respectively, from top to bottom).


1
2
3
4
5
6
7
8
9
10
11
12
13

containing 1/10th of this amount (lane 6). This is difficult to visualise in the photograph presented in Fig. 5.6, possibly because this was reproduced from a 35mm slide and has lost resolution in processing No band was seen when proteinase-K-treated diluent was amplified (lane 7) or when further dilutions of the bacterial suspension were similarly treated (not shown).

A system which utilises a hot start technique coupled with the use of commercially prepared wax beads (Ampliwax Gems, Perkin Elmer) was used to determine whether or not the sensitivity of the PCR reaction could be increased (see Section 2.11.4), as suggested by the product insert of Ampliwax Gems. No difference in sensitivity was seen when 10 fold serial dilutions (from 10^1 to 10^{10}) of a *B. pertussis* control (NCTC 10908) used as target DNA were subjected to normal and hot start (with Ampliwax beads) PCR protocols. A viable count of the undiluted inoculum showed 5.9 x 10^8 cfu/ml were present which equates to 11 viable organisms per assay at a 10^6 dilution. This dilution was the greatest one still showing a visible ethidium bromide stained PCR product **(Figure 5.7a).** Both the hot start and the routine PCR were identical with respect to their sensitivity.

Using the same positive control samples as in (b) above, a standard PCR test was run for 25, 28 or 35 cycles with detection of 1200, 12 and <1 cfu present in the inoculum respectively (see **Figure 5.7b, 5.7c).** Increasing the time allowed for denaturation and annealing from 30 secs. each to 45 secs. in each cycle made no difference to the sensitivity (detection of 12 cfu's) but increased the overall time of the PCR considerably (result not shown).

An attempt at halving the quantity of oligonucleotide primers used in each reaction was made in order to confirm that the original quantity of primers used per reaction was optimal. Results of these tests indicated that a ten fold decrease in product occurred if the quantity of primers used per reaction was halved **(Figure 5.7d).**

Figure 5.7 (a), Upper left photograph

Comparison of mineral oil and Ampliwax Gems (Perkin Elmer) as an overlay in the standard pertussis PCR.

Serial 10 fold dilutions of a *B. pertussis* culture containing 5.9 x 10^8 cfu/ml were prepared (at a dilution of 10^6, this equates to approximately 11 viable cells). All dilutions were subjected to the PCR as described in the text using either mineral oil (upper tracks) as overlay or a hot start technique (lower tracks) using Ampliwax gems (Perkin Elmer) as overlay. PCR products were visualised by ethidium bromide staining after electrophoresis in 2% agarose gels. Lanes 1-10 are results from amplification of 10^{-1} to 10^{-10} dilutions of the culture respectively. Lane 11 was a negative control and lane 12 DNA markers. The expected mobility of a 153-bp PCR product is indicated.

Figure 5.7 (b), Upper right photograph.

Determination of the sensitivity of the pertussis PCR assay using 25 and 35 cycles of the DNA-thermal cycler.

Lanes 1-10 show PCR products from amplification of serial 10 fold dilutions (10^{-1} to 10^{-10},) of a *B. pertussis* culture containing 5.9 x 10^8 cfu/ml. Lane 11 is a negative control and lane 14 molecular weight markers. The upper track shows PCR products after 25 amplification cycles while the lower tracks received 35 cycles. The standard protocol for the PCR as described in the text were followed with the exception of cycle number.

Figure 5.7 (c), lower left photograph.

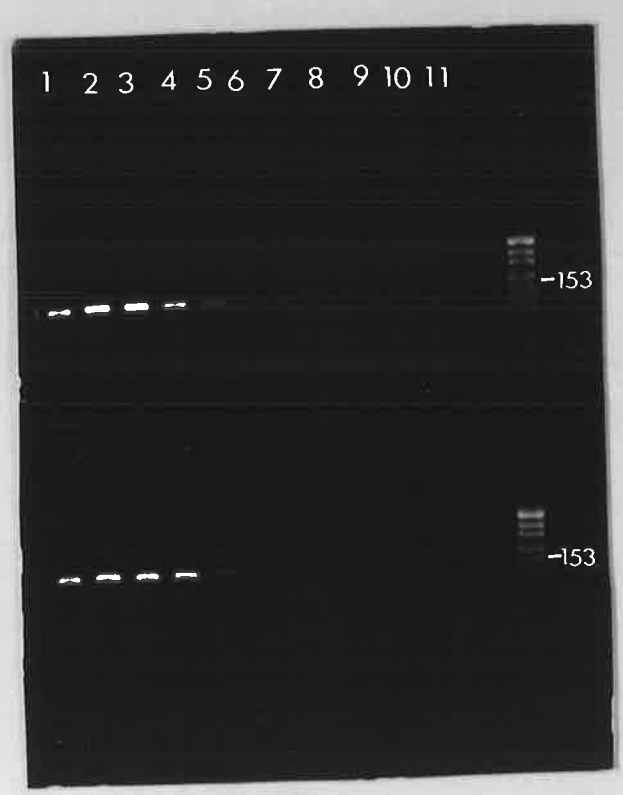
Sensitivity of the pertussis PCR using 28 cycles of amplification.

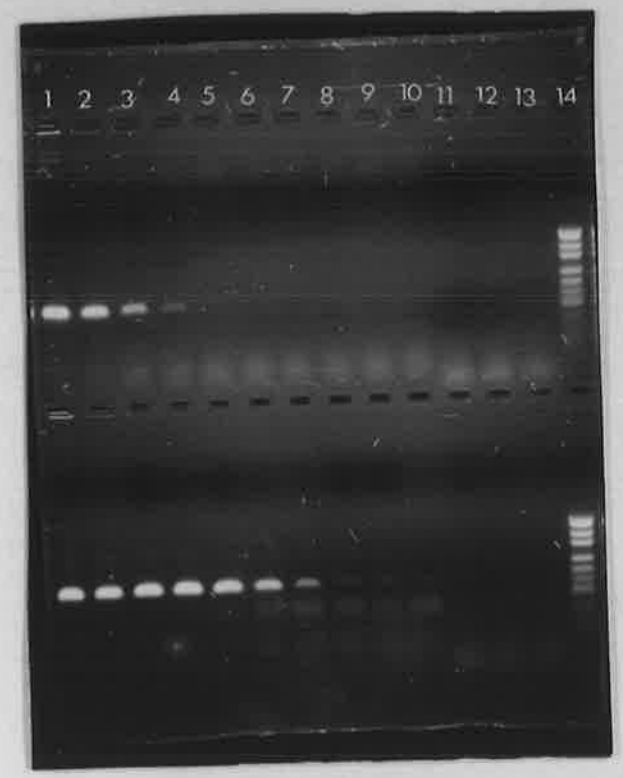
Lanes 1-10 show PCR products from amplification of serial 10 (10^{-1} to 10^{-10}) fold dilutions of a *B. pertussis* culture containing 5.9 x 10^8 cfu/ml. Lane 11 is a negative control. The PCR reaction was identical to the standard protocol described in the text with the exception that 28 cycles of amplification were used.

Figure 5.7 (d)

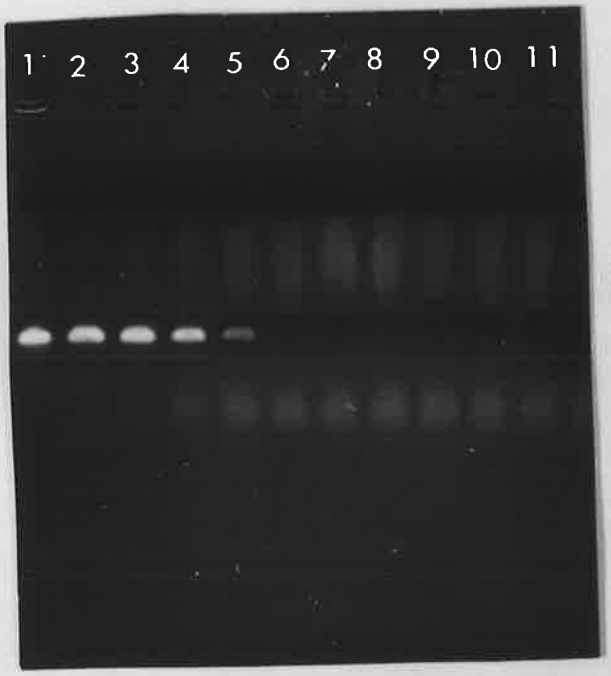
Comparison of the sensitivity of the pertussis PCR with the standard quantity of primers compared to using only half that amount.

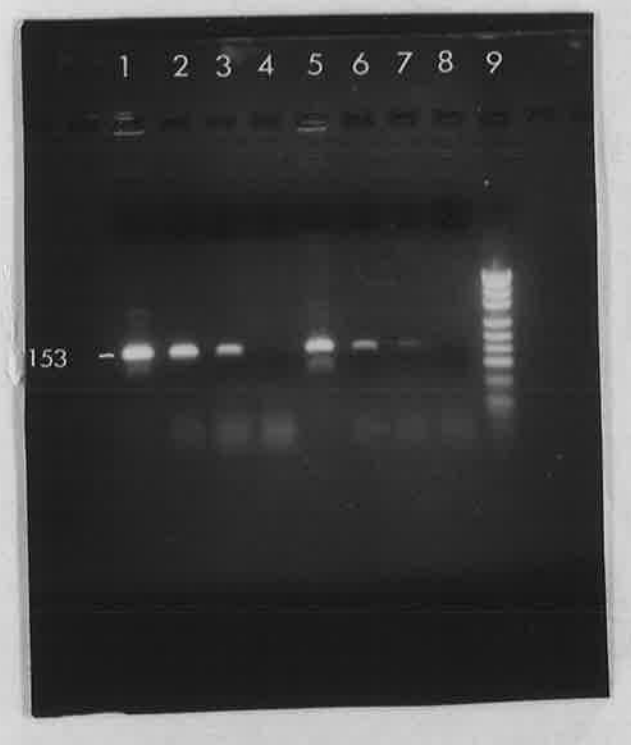
Lanes 1-4 show the 153 bp pertussis specific PCR product produced under the standard conditions while lanes 5-8 show the results obtained using only half that quantity of primers in the reaction mix. The following inocula were used in the reaction mixtures : Lanes 1 & 5, neat *B. pertussis* positive control, lanes 2 & 6, 10^{-4} dilution, lanes 3 & 7, 10^{-5} dilution, lanes 4 & 8, negative controls.


1 2 3 4 5 6 7 8 9 10 11
153
153


1 2 3 4 5 6 7 8 9 10 11 12 13 14

153 -


1 2 3 4 5 6 7 8 9 10 11


1 2 3 4 5 6 7 8 9
153

5.2.7 Clinical specimen (NPA) comparative trial

The PCR assay was then used to detect *B. pertussis* in NPAs collected from children with suspected pertussis. A total of 332 nasopharyngeal aspirates were obtained from children aged ten days to 16 years (median age approximately 18 months) with suspected pertussis (patients had a persistent cough with or without vomiting or paroxysms). Specimens collected over a 12 month period were tested but most of these were obtained during the last three months of 1989, which was a period of high incidence of pertussis in Adelaide. The results obtained are summarized in **Table 5.2.** while **Figure 5.6, lanes 8-12** show examples of PCR amplification of a number of NPA specimens. The PCR assay results were compared with those obtained for immunofluorescence and culture in terms of rapid, direct detection of *B. pertussis* organisms. In general, aspirates yielding a moderate to heavy growth of *B. pertussis* were strongly positive by PCR while those yielding scanty growth (which were usually negative by IF) resulted in a less intense but nevertheless clearly positive PCR result. A subset of NPA samples (6 positive and 14 negative) was retested in another laboratory (R.Premier, CSL, Parkville, Victoria) with identical results demonstrating the reproducibility of the PCR assay.

The PCR test yielded positive results in a total of 98 samples compared with 66 for culture and 33 for direct IF. All of the IF positive samples were PCR positive as were 63 of the samples from which *B. pertussis* was eventually cultured. The three culture-positive, PCR-negative samples grew fewer than five colonies each. 231 specimens which were negative by IF and culture were also negative in the PCR assay. However 33 culture and IF negative specimens were positive by PCR assay; at least three of these specimens were collected from close contacts of culture proven pertussis patients and two were follow-up specimens from recent culture confirmed patients. Sera were available from ten of the remaining patients and when these were tested by ELISA (as described in

Chapter 3) for IgA, G, and M antibodies directed at *B. pertussis,* five showed serological evidence of recent infection.

The PCR test was introduced as a routine, diagnostic test on 21 May, 1990. From this date until December 30, 1990, a total of 14 cases yielded positive cultures and PCR detected all 14 together with 5 additional NPAs which did not grow *B. pert ussis* but yielded a PCR product of the predicted size for this organism. During 1991 the number of culture positive cases were 8 while PCR detected 30 positives and the figures for 1992 were 4 and 7, respectively **(Fig 5.8)**. The trend of the PCR assay detecting significantly more positives than culture has continued at the WCH.

TABLE 5.2

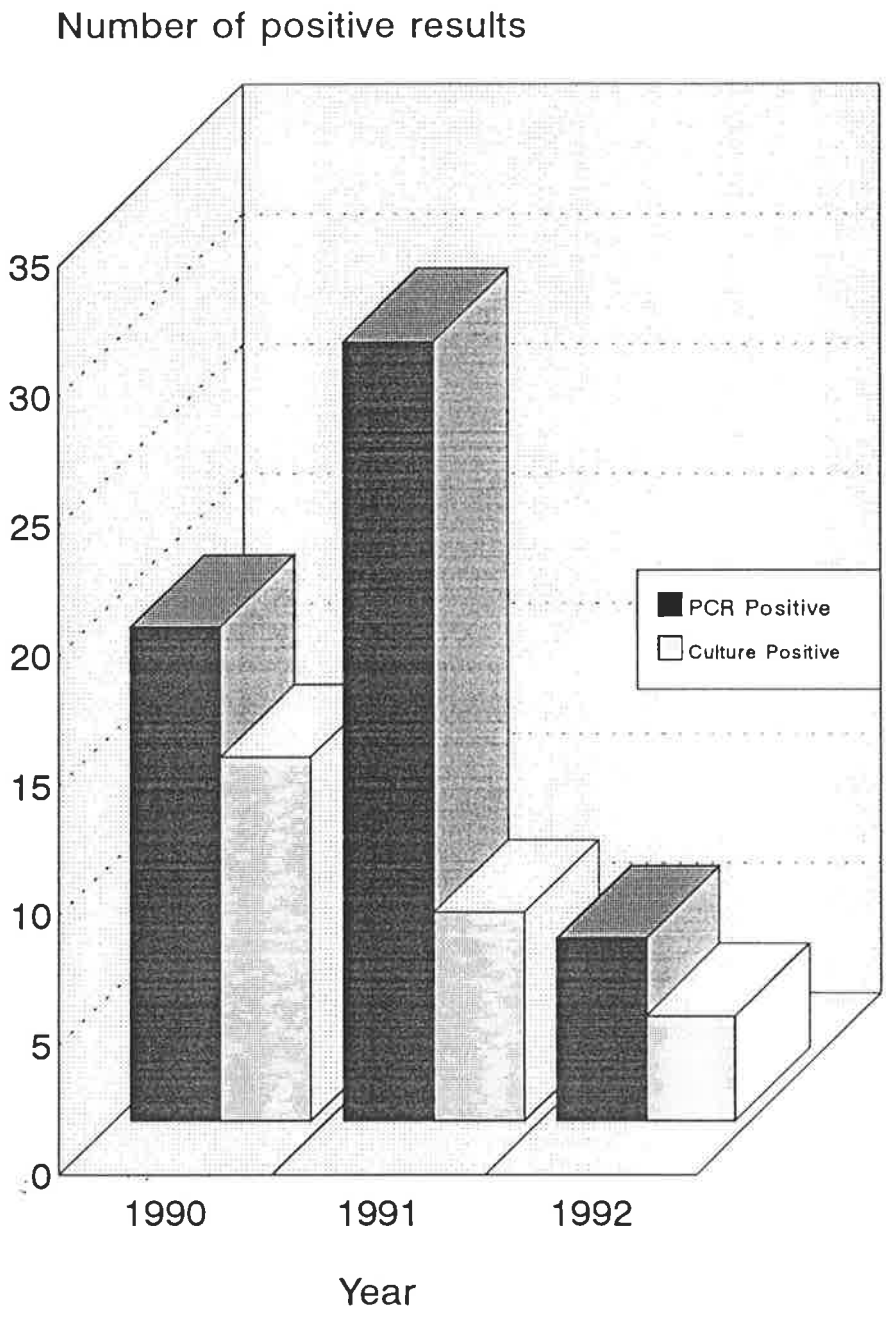
Detection of *B.pertussis* in NPA by PCR assay compared with culture and immunofluorescence (IF).

	IF + CULT +	IF - CULT +	IF + CULT -	IF CULT-	*n*
PCR positive	31	32	2	33	98
PCR negative	0	3	0	231	234
n	31	35	2	264	332

NPA were treated with proteinase K and amplified by PCR after samples had been withdrawn for culture and IF analysis. The numbersof specimens yielding a positive (+), or negative (-) result for the various assays are indicated.

Figure 5.8

Numbers of culture-positive and PCR-positive NPA samples detected at the WCH between 21 May 1990 and December 31, 1992.


Number of positive results
35
30
25
20
15
10
5
0
PCR Positive
Culture Positive
1990
1991
1992
Year

5.2.8 PCR Product Detection

In the initial development and routine use of the PCR assay, ethidium bromide staining of agarose gels and visualisation under UV light was used to detect the 153-bp PCR product. This system was simple to perform, was fast and provided a permanent photographic record.

In order to verify that the amplified products visualised (including very weakly stained bands) were derived from the *B. pertussis*-specific reiterated sequence, dot-blot hybridization experiments using an oligonucleotide probe were performed. The probe, a 22mer oligonucleotide (5'-GGCGATTTCGAGTTTCTCAAAT-3'), internal to the 153-bp specific *B. pertussis* amplified segment and complimentary to positions 45-66 of that fragment, was labelled with digoxigenin (DIG) using a DIG DNA tailing kit. Aliquots of PCR product were spotted onto nylon filter, fixed and hybridized as described in Section 2.11.5. Hybridization was carried out at 55°C.

Experiments were performed to compare the sensitivity of PCR product detection using either ethidium bromide (EtBr) staining of gels or a DIG-labelled oligonucleotide probe. The sensitivity of detection was compared by observing PCR products produced from amplifying serial 10-fold dilutions of a positive *B. pertussis* culture (NCTC 10908) in the 2 detection systems. A viable count on the positive control culture indicated that it contained 8.9×10^8 viable *B. pertussis* cells per ml. This equates to 3 viable organisms present in the PCR reaction using the 10^{-6} dilution of the culture and 0.3 organisms using the 10^{-7} dilution given that 3μl of each dilution was added to the reactions as target DNA. Comparison of the two detection systems showed that a faint band was visible in the gel with an inoculum of 3 organisms while dot blot hybridization with the DIG labelled oligo-probe was able to detect product at 0.3 organisms (presumably because *B. pertussis* DNA was present in the inoculum which had been released from dead cells and the fact that each *B. pertussis* genome contains approximately 100 copies of the reiterated sequence)**(Figure 5.9).** This

Figure 5.9

Comparison of EtBr staining and DIG labelling for detection of *B. pertussis* specific PCR product.

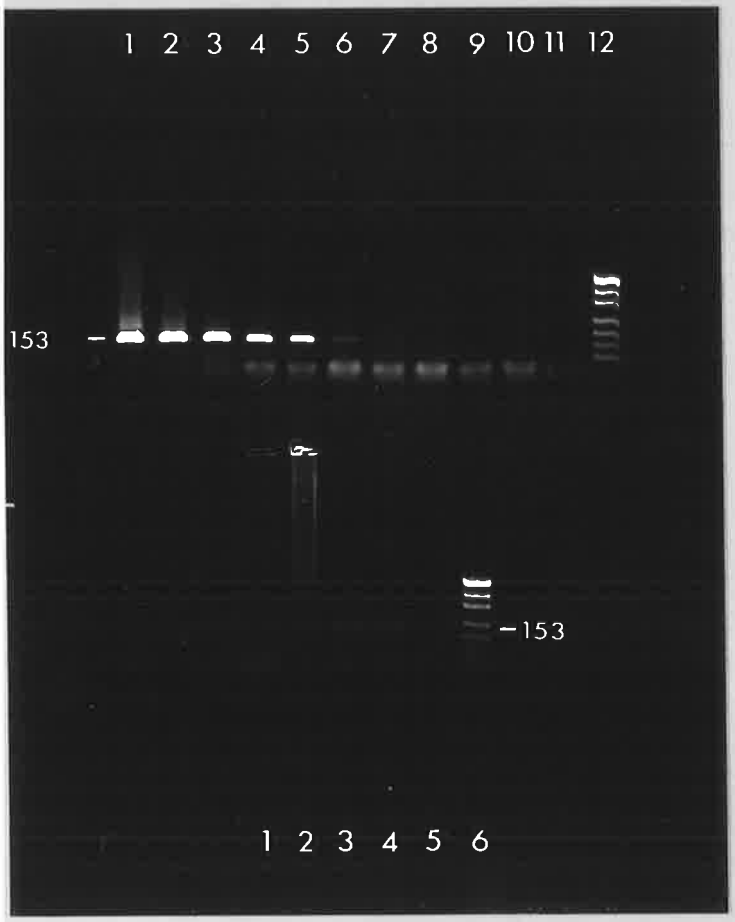
Upper track

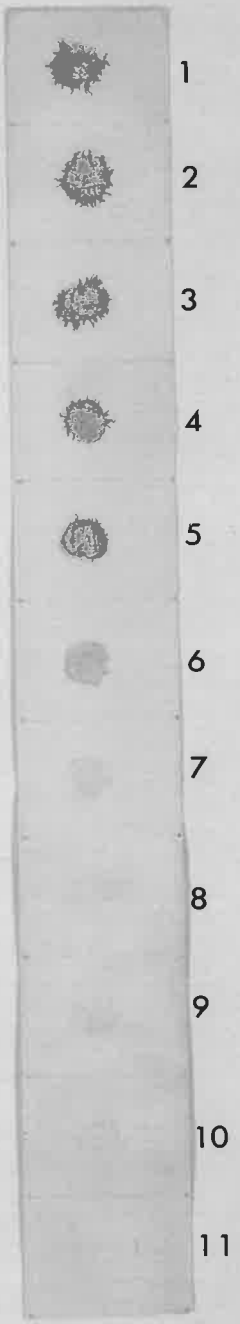
Ethidium bromide stained 2% agarose gel showing the results of PCR-amplification of 10-fold dilutions of a *B. pertussis* culture, containing 8.86 x 10^8 cfu/ml. Lanes 1-10 show the results of PCR amplification of 10^{-1} to 10^{-10} dilutions of the positive control, respectively. Lanes 11 and 12 are a negative control and DNA size marker, respectively.

Filter 1 (labelled 1-11) is the result of lysate blot hybridization of the reaction mixtures from the corresponding tubes run in lanes 1-11 of the gel. The probe was a DIG labelled, 22 mer specific oligonucleotide directed at a sequence contained within the 153 bp PCR product.

Lower track

Ethidium bromide staining of PCR products in 2% agarose gels after subjecting 5 NPA samples from patients suspected of having whooping cough, to PCR analysis. Lanes 1,2 and 5 are the results from 3 culture-negative NPA samples. Lanes 3 and 4 were from PCR analysis of 2 culture-positive NPAs with only slight growths of the organism. The filter labelled 1-5,+ and - represents the corresponding lysate blots after hybridization to the DIG-labelled probe described above. The positive control (+) was PCR product from amplification of a 10^{-1} dilution of the control (lane 1 above) while the negative control (-) was sterile distilled water.


1 2 3 4 5 6 7 8 9 10 11 12
153
153
1 2 3 4 5 6


1
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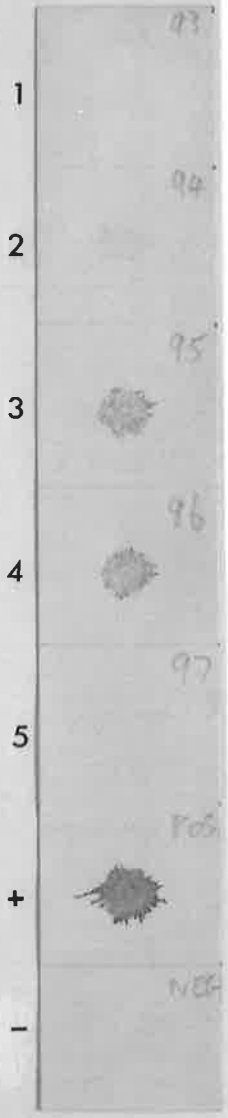
3
4
5
6
7
8
9
10
11


1
2
3
4
5
+
−

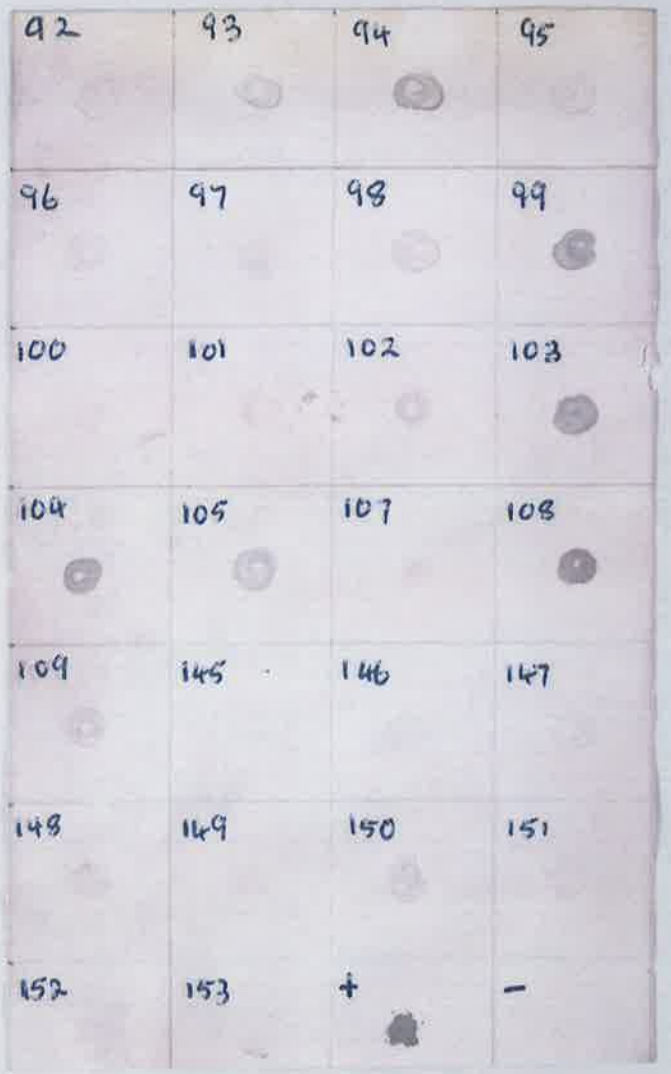
represents an approximate 10-fold increase in sensitivity of detection of PCR product in this set of experiments.

In order to compare the two methods of PCR product detection further, 229 NPA specimens from patients suspected of having pertussis or viral illness, which had been previously tested by PCR and had been kept at -20°C for up to six months, were retested. The resulting PCR products were analysed by agarose gel electrophoresis and EtBr staining or by filter hybridization with the probe, as described above. An example of a test filter can be seen in **Figure 5.10.** A range of staining intensities was observed, with the positive control being most intense together with NPAs 108, 103 and 99. Three other NPAs ie. 104, 105 and 94, stained moderately intensely while 93 was faint. **Figure 5.11** compares the results of a gel stained with ethidium bromide, and a hybridization filter with the same PCR products, probed with DIG labelled, pertussis specific oligonucleotide. A range of intensities were observed using both detection systems. Lower track 1 (filter no. 85) is strongly positive while lower track, lane 2 (filter no. 84) is weakly staining. An example of background staining was observed with the hybridization reaction in filter position number 69 which equates to the clearly negative, lower gel track lane 3. Some background reaction can be seen in the DIG labelled filter, which is a potential problem when using this type of detection system.

Of the 229 NPAs tested, 37 were positive by EtBr staining and 45 positive by hybridization with the DIG labelled probe. There were 11 (23%) samples where EtBr staining was negative and probe positive while 34 (92%) of the 37 positive by EtBr staining were detected also. 3 (2.7%) were positive by EtBr staining and negative by oligo-probe hybridization. 5 faintly staining bands were confirmed as positive by the probe. There was a single specimen yielding a moderately intense band in EtBr, close to but slightly larger than 153 bp which did not react with the DIG-labelled probe. Intotal, 181 (79%) NPAs gave negative results in both detection systems **(Table 5.3).**

Figure 5.10

Lysate dot blot filter resulting from hybridization of PCR product from amplification of 28 NPA samples from patients suspected of having pertussis, with a DIG labelled oligonucleotide probe internal to a 153bp DNA sequence contained within the *B. pertussis* specific repeat unit of this organism's genome. The numbers refer to codes given to NPA samples prior to testing and identify individual patients. Positive (+) and negative (-) controls were included consisting of *B. pertussis* cells and water respectively.


92
93
94
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96
97
98
99
100
101
102
103
104
105
107
108
109
145
146
147
148
149
150
151
152
153
+
-

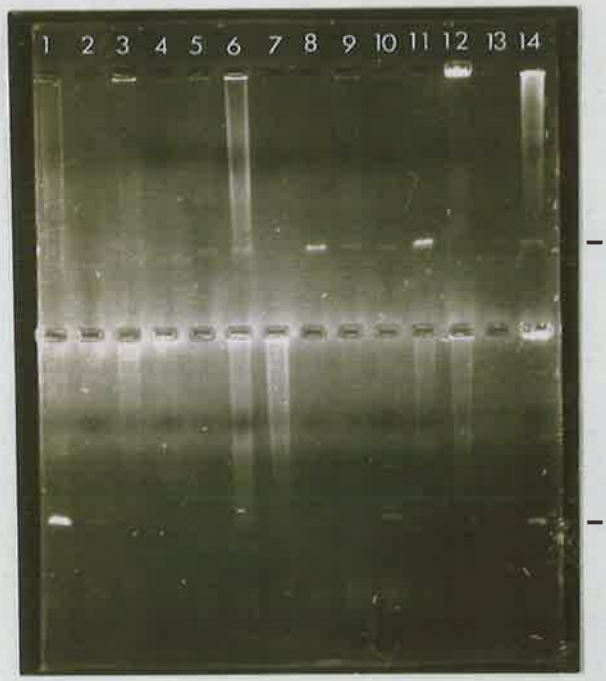
Figure 5.11

Comparison of ethidium bromide stained 2% agarose gel and DIG-labelled dot blot filter for specific pertussis PCR product detection, in 26 samples of NPA (labelled with their serology laboratory number) from patients suspected of having whooping cough. The 153-bp band indicating a positive result in the gel and it's corresponding position on the filter appear below :

upper track lanes : 2, 4, 5, 6, 8, 9, 10, 11, 14
filter position no. : 87, 82, 83,76, 75, 79, 77, 74, 64

lower track lanes : 1, 2, 6, 10
filter position no. : 85, 84, 59, 65

The negative and positive controls appear in lower track lanes 13 and 14 respectively and labelled NEG and POS on the filter.


1 2 3 4 5 6 7 8 9 10 11 12 13 14
- 153
- 153

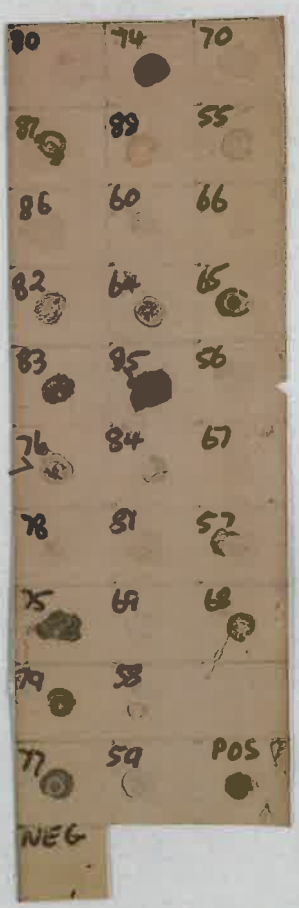

80 74 70
81 89 55
86 60 66
82 64 65
83 85 56
76 84 67
78 81 57
75 69 68
79 58
77 59 POS
NEG

Table 5.3

Comparison of EtBr and hybridization analysis as a means of detecting *Bordetella pertussis* specific PCR product in 229 nasopharyngeal aspirates.

	DIG Positive	DIG Negative	*n*
EtBr Positive	34	3	37
EtBr Negative	11	181	192
n	45	184	229

5.2.9 PCR Inhibition

PCR assays are known to be inhibited by a number of substances. Tests were therefore conducted to determine if any substances present in NPAs would inhibit PCR assays, particularly in the absence of a DNA purification step. A total of 118 NPA specimens which had been routinely cultured for *B. pertussis* and assayed by PCR and found to be negative, were tested for their ability to inhibit the PCR reaction. Routinely requested NPAs were collected using suction catheters which had been flushed through with either water, phosphate buffered saline (pH 7.2)(PBS) or viral transport medium (VTM). PCR reactions (as previously described except the *Taq* polymerase was now supplied by Bresatec, Adelaide, South Australia) were set up and 3μl of a 1000 fold dilution of a *B. pertussis* control culture (NCTC 10908) containing approximately 12 viable cell equivalents, was added to the NPA sample prior to the proteinase-K digestion. Positive controls were set up in either distilled water, PBS or VTM. Inhibition of the PCR reaction was indicated by failure of

the reaction to produce the 153-bp product when visualised in agarose gels after electrophoresis and EtBr staining under UV illumination. An example of inhibition can be seen in **Figure 5.12 (c)** where 24 NPA's were tested. Inhibition of the PCR assay is observed in lanes 1,5,6,8,9,12(partial) (upper track) and 2,4,5,6,11 (partial) 12 (lower track). Lane 13 (lower) is a positive control suspended in viral transport medium initially which shows no inhibitory effect.

Of the 118 specimens of NPA seeded with *B. pertussis* organisms subjected to PCR, a total of 19 (16%) showed total inhibition of the PCR reaction while a further 7 (6%) showed partial inhibition giving an overall inhibition rate of 22%. A particular NPA, number 1313, lost it's ability to inhibit the PCR when the test for inhibitory activity was repeated. When the remaining 24 NPA's previously positive for inhibition were retested, a total of 8 showed no inhibition. Inhibition was however apparent in 16 NPA'S (upper track lanes 1,2,3,6,8,10,12,13 and lower track lanes 2,3,4,5,6,7,9,10) which reduces the overall inhibition rate to 14% **(Figure 5.12 (a))**. Viral transport medium, phosphate buffered saline (pH 7.2) or sterile distilled water were seeded with *B. pertussis* organisms and subjected to PCR in order to determine if these substances, all of which may be used to flush out the nasopharyngeal catheters used to collect secretions from patients, would themselves inhibit the PCR assay. Intensely staining 153 bp *B. pertussis* specific PCR products were observed after PCR assay for all three solutions suggesting no inhibitory activity could be ascribed to them **(Figure 5.12b).**

The effect of magnesium ion concentration (which is known to affect PCR) on the PCR reaction was considered for one NPA (1313) which displayed total inhibition on initial testing. NPA 1313 was seeded with approximately 12 *B. pertussis* cells equivalents (as above). The total volume of the PCR mix was adjusted such that five reaction mixes contained magnesium ion concentrations of 2mM, 4mM, 6mM, 8mM and 10mM. An identical set of mixes was

Figure 5.12a (Upper left photograph)

PCR inhibition studies on 24 NPAs previously found to inhibit the PCR assay. Lanes 1 - 12 (upper and lower tracks) represent results of PCR analysis on the 24 NPAs after seeding with *B. pertussis* cells. The *B. pertussis* specific 153bp PCR product is observed in a number of lanes.

Figure 5.12b (Upper right photograph)

Upper track

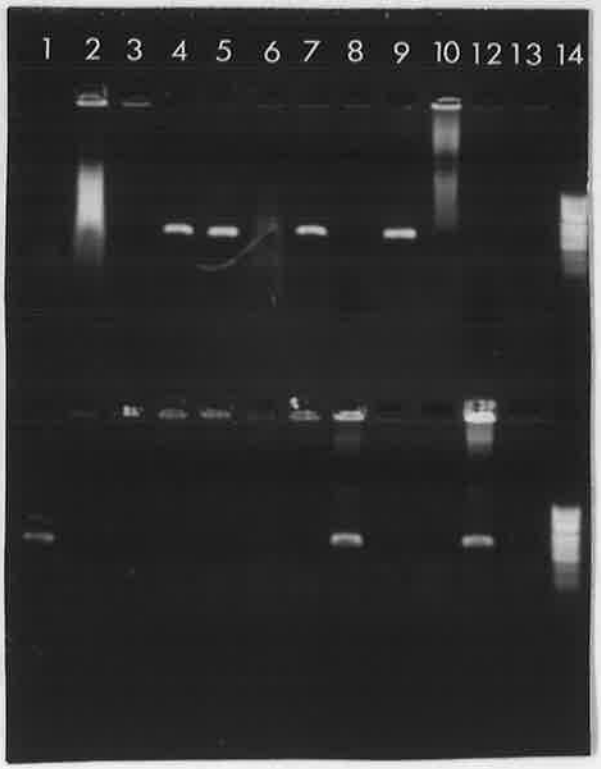
Results of PCR analysis on *B. pertussis*-seeded samples of distilled water, phosphate buffered saline (PBS) and viral transport medium (VTM) as substitutes for NPA samples. Lanes 9,10 and 11 (distilled water, PBS and VTM respectively) show the presence of 153bp PCR products after electrophoresis in 2% agarose gel and ethidium bromide staining. Lanes 1 - 8 represent results of earlier PCR assays using *B. pertussis* seeded NPAs.

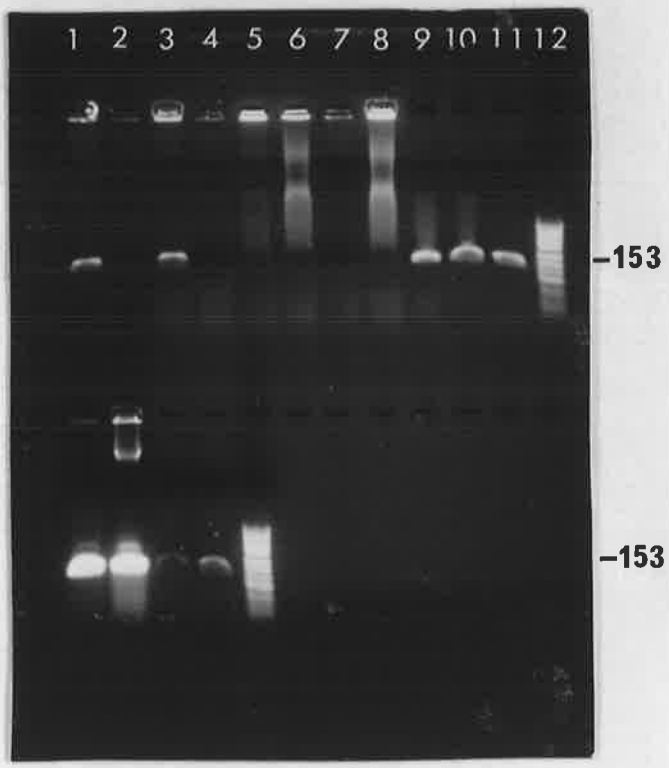
Lower track

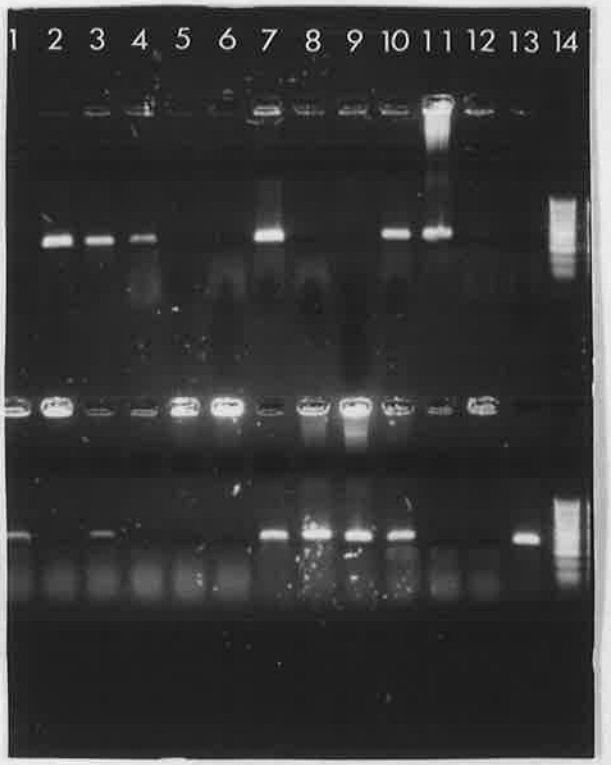
Lanes 1 and 2 show results of PCR assay of a *B. pertussis* positive control using the standard quantity of newly synthesized *B. pertussis* specific primers while lanes 3 and 4 show an identical assay using the same primers initially diluted 1000-fold.

Figure 5.12c (Lower photograph)

Inhibition of the pertussis PCR by components present in patient samples of nasopharyngeal aspirate. Lanes 1 - 10 (upper and lower tracks) represent results of PCR on 24 NPA samples seeded with approximately 12 cfu's in each reaction tube. Lane 13 (lower track only) is a positive *B. pertussis* control suspended in viral transport medium. Each sample was subjected to a standard pertussis PCR and products visualised in a 2% agarose gel after electrophoresis.


1 2 3 4 5 6 7 8 9 10 12 13 14
153–
153–


1 2 3 4 5 6 7 8 9 10 11 12
–153
–153


1 2 3 4 5 6 7 8 9 10 11 12 13 14
153–
153–

prepared for use with sterile water as the sample. No inhibition was observed when the standard concentration of magnesium ions (2mM) was used in the PCR reaction. The effect of increasing magnesium ion concentration did not improve the formation of PCR product and the reaction ceased to function at a concentration of 10mM in the NPA sample and 8mM in the water sample. This suggested that very high concentrations (> 8-10mM) of magnesium ions are inhibitory (as may be expected) and that also in this NPA at least, the presence of substances able to chelate magnesium leading to reduction in the efficiency of amplification were not responsible for the observed inhibition.

In order to remove the inhibiting substances from NPA samples, a simple ethanol precipitation was included immediately after the proteinase-K lysis step. Both standard proteinase-K lysis and the single ethanol precipitation coupled with proteinase-K lysis were compared in a series of experiments. 12 of 19 NPAs described above were used because they had previously been found to completely inhibit the standard PCR assay. Fresh aliquots of these 12 NPA's were subjected to proteinase-K lysis, followed by a single ethanol precipitation before proceeding with PCR (see Chapter 2.11.2). In brief 47μl of each NPA and 3μl of water containing approximately 12 *B. pertussis* cells were subjected to the proteinase-K lysis step, mixed with 500μl ethanol per tube and frozen at -70°C for 30 minutes. After centrifugation the pellet was dried and the standard PCR protocol followed. 4 of the 12 produced mainly weakly staining 153bp bands on standard retesting (lanes 4,5,9 and 12, (upper track) **figure 5.13a**). All seeded samples (including these 4) produced an intensely staining 153bp PCR product when the ethanol precipitation step was included. 153 bp PCR product was observed from the remaining 8 NPA samples which was not present following standard PCR assay (lanes 1,2,3,6,7,8,10 & 11 (lower track and upper tracks respectively) **figure 5.13a**). **Figure 5.13b** also demonstrates the removal of inhibition where PCR products are visible in lanes 3 and 8 (lower track) after but not before (upper track) DNA extraction followed by PCR.

Figure 5.13 (a)

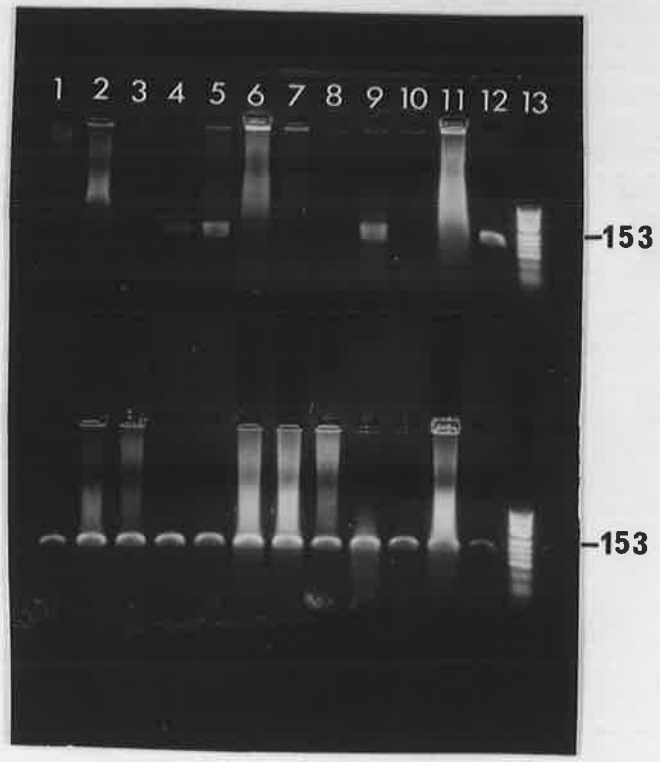
Comparison of standard proteinase-K lysis and ethanol precipitation following proteinase-K lysis as methods of preparing NPA samples for pertussis PCR assay.

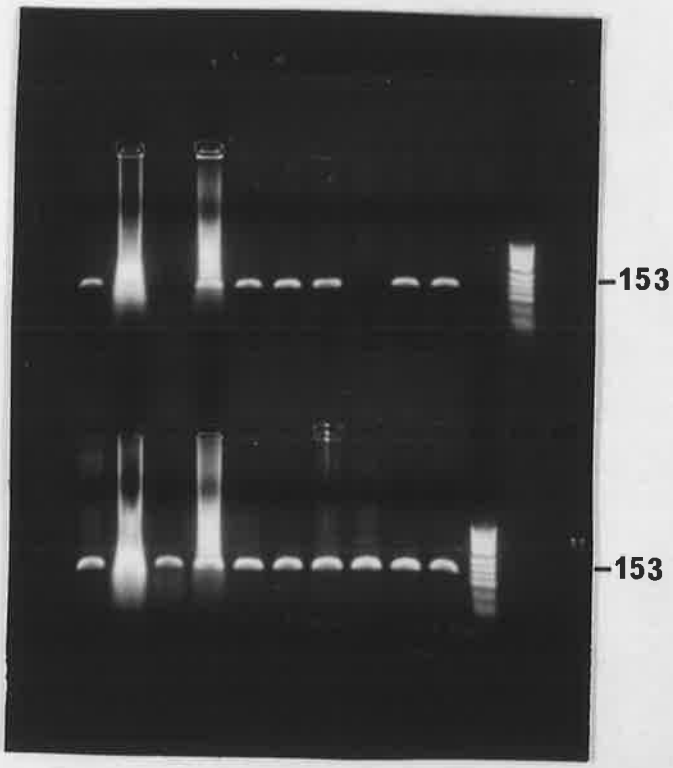
NPAs previously inhibitory to PCR were seeded with *B. pertussis* cells (approx. 12 cfu) and retested under standard preparation procedures (upper track) or incorporating ethanol precipitation (lower track). Lanes 1 - 12 (upper track) result from standard PCR assay on *B. pertussis* seeded NPAs previously shown to be inhibitory. Lanes 1 - 12 (lower track) are the same seeded NPAs subjected to PCR after crude DNA purification by ethanol precipitation. Lane 13 (upper and lower) are DNA size markers.

Figure 5.13 (b)

Comparison of standard proteinase-K lysis and ethanol precipitation following proteinase-K lysis as methods of preparing NPA samples for pertussis PCR assay using 10 *B. pertussis* seeded NPA samples.

Lanes 1 - 10 show results of gel electrophoresis and ethidium bromide staining of PCR products before (upper lanes) and after (lower lanes) ethanol precipitation of the NPA samples. Lanes 13 (upper) and 12 (lower) are DNA size markers.


1 2 3 4 5 6 7 8 9 10 11 12 13
153
153


153
153

This suggests that a crude DNA purification such as ethanol precipitation is able to adequately overcome the inhibitory effect of unknown substances in some NPA's samples.

5.3 DISCUSSION

The complete repetitive DNA sequence in the *B. pertussis* genome has been shown to be 1046-bp long with unique *Cla*I and *Acc*I restriction endonuclease sites and the many of the repeats arranged in tandem. Our group cloned the complete repetitive unit as a *Cla*I fragment (pJCP602) after isolating the repetitive DNA band from electrophoresed *Cla*I-digested genomic DNA (Glare *et al.*, 1990).

The chromosomal arrangement of the repeat appears to be conserved between strains of *B. pertussis*. Unpublished work of E. Glare showed that only minor differences in restriction fragment lengths and band intensities were observed in genomic DNA from 7 clinical isolates and 3 NCTC strains of *B. pertussis* after restriction fragment length polymorphism analysis. In particular, all strains had repetitive 1-kbp bands of consistent intensities in *Cla*I digests. The high degree of conservation of fragments bearing the repetitive sequence implies that the repeat is not involved in DNA rearrangements as has been suggested elsewhere (McPheat and McNally, 1987; Alsheikhly and Lofdahl, 1989).

All isolates of *B. pertussis* tested in the present study contained the repeated DNA sequence which (apart from a very weak hybridization with *B. bronchiseptica*) was absent from all other species of bacteria tested. *B. bronchiseptica,* however, is a very rare human pathogen, accounting for only 0.1% of all isolated *Bordetellae* (Lautrop, 1960; Goodfellow, 1980; Woolfrey

and Moody, 1991). Therefore, this sequence was likely to be a good target for detection of *B. pertussis* using a DNA probe.

Other DNA-based tests to detect specific *B. pertussis* sequences in clinical samples have been reported. These tests have a theoretical advantage of rapidity over culture because they can probe the specimen directly with results available within 1 to 2 days. One such test used a cloned *Bam*HI fragment of genomic *B. pertussis* DNA which also recognised the repeat sequence present in the *B. pertussis* genome. A detection limit in specimens of NPA was reported as 5 x 10^3-10^4 cfu, which is not likely to be sensitive enough for routine testing, particularly where only very low numbers of organisms are present in the late acute and convalescent stages of whooping cough. When this fragment was used to probe replica blots of culture plates however, the sensitivity improved. Membranes were placed on the culture plates and then probed but only after sufficient growth had occurred (after 3 days) did the sensitivity increase to 10 cfu. This hybridization step however negates the advantage of rapidity which a direct DNA-based test should have, and decreases it's usefulness as a front line rapid diagnostic test (Reizenstein *et al.*, 1990). The authors suggested that the sensitivity of hybridization would be adequate only if coupled with a more rapid amplification system.

In this study, the limit of detection when the ^{32}P-labelled repeated sequence was used to probe dot blots of *B. pertussis* suspensions was approximately 1000 organisms after overnight autoradiography and this limit is also clearly inadequate for a diagnostic test in the clinical setting. However, the use of synthetic oligonucleotide primers for PCR amplification of part of the repeated element although vastly increasing the sensitivity could conceivably be complicated by variations in the precise DNA sequence from one strain of *B. pertussis* to another. Indeed a total of 12 base differences (5 substitutions and 7 deletions) were detected between the sequence of the insert of pJCP602 and that reported by McClafferty *et al.*, (1988). In the present study the region of the

repeated element from bases 12 to 164 (inclusive) was chosen for amplification by PCR because it is a region of absolute sequence identity between the inserts of pJCP601 and pJCP602 and that of McClafferty *et al.*, 1988. Furthermore, Southern hybridization experiments using the inserts of pJCP601 and pJCP602 as probes indicated that virtually all copies (complete or otherwise) of the reiterated sequences in the *B. pertussis* genome included this region.

Under the conditions used in this study, the PCR assay for direct detection of *B. pertussis* had an apparent sensitivity limit of less than one viable organism (i.e. < 1 cfu). It is possible however that the diluted cell suspensions tested included non-viable *B. pertussis* cells and therefore it is uncertain whether less than one *B. pertussis* genome (which would contain approximately 100 copies of the target sequence) could have been detected.

Use of the hot start technique with Ampliwax gems made no difference to the sensitivity of the PCR test. Additionally, hot start is probably not warranted in this PCR because very few spurious bands occur using our standard amplification protocol. The theoretical limit of detection by PCR assay is one copy of the target sequence and therefore it should be possible to increase the sensitivity of the assay. This could be achieved by increasing the number of amplification cycles or by increasing the sensitivity of PCR product detection compared with ethidium bromide staining. The latter may be achieved by hybridization with a labelled oligonucleotide probe specific for a section of amplified sequence which did not overlap with primer sequences (see below). It was decided initially, however, that these steps would increase the time required to complete the assay to a point at which same day results would not be achievable in the clinical laboratory setting. Thus the balance between speed and sensitivity needs to be considered.

The performance of the PCR assay was influenced by the type of thermal cycler used. Amplification conditions optimised for the Hybaid intelligent heating block were unsuitable for the Perkin Elmer DNA thermal

cycler. Modifications to the original parameters restored the sensitivity when using the Perkin Elmer machine and faster heating/cooling rates reduced the overall run time from 41/2 to 2 hours. This is of considerable benefit in the clinical laboratory setting because more time is available for collection of specimens in the wards and transport to the laboratory while still enabling a result to be available by late afternoon, even with the incorporation of the quick DNA purification step.

In an attempt to increase the sensitivity of the assay and to confirm the identity of weak bands, ethidium bromide staining of agarose gels was compared with DNA hybridization using a specific DIG-labelled oligonucleotide for detection of PCR product. Hybridization was observed to be approximately 10-fold more sensitive. 229 NPA samples from patients suspected of having pertussis were tested by PCR and both methods of PCR product detection were used. A 23% increase in sensitivity was observed when hybridization was used, but a potential problem was the level of background staining. Occasionally, a low level of background staining could be seen in negative controls, particularly negative NPAs. The true positives however stand out clearly. Hybridization did confirm that some faint bands were indeed PCR products of the correct sequence and that occasional bands of incorrect size seen in ethidium bromide stained gels were not *B.pertussis*-specific sequences. However, hybridization requires an additional day's work which negates much of the rapidity of PCR and hence may not be suitable for routine testing. It is likely that even greater sensitivity could be achieved using Southern blotting methodology (together with the oligoprobe previously described), but the extra time involved in this procedure is again too great. Southern blotting would have the advantage that specificity is confirmed both by size and specific hybridization, compared to size only with ethidium bromide stain and hybridization only for the dot blot.

Other PCR product detection systems such as colourmetric detection (Kemp *et al.*, 1989) could also be applied to routine testing allowing the use of

automated ELISA readers in the process. The use of a commercial product, Captagene (Amrad Corporation, Victoria), could be applied to routine testing of large numbers of samples. It requires one of the oligonucleotide primers to be biotinylated and the other to have incorporated a specific 12 bp recognition sequence for the double stranded DNA binding protein GCN4. Amplified dsDNA containing this specific recognition sequence is captured in the wells of a microtitre plate via an immobilised GCN4 fusion protein. The presence of amplified DNA can then be detected by the biotin-streptavidin peroxidase colourimetric reaction. These systems are ideally suited to large batch testing but can also be applied to the smaller runs which are more likely to be the case in routine clinical diagnostic laboratories.

Formal assessment of the specificity and sensitivity of new methods for diagnosis of pertussis is complicated by the absence of a satisfactory gold standard with which comparison can be made. Culture, for example, is highly specific (by definition 100%) but for *B. pertussis* it's sensitivity is very poor, with positive results obtained in at most 60% of cases (Combined Scottish Study, 1970; Linneman *et al.*, 1978; Granstrom *et al.*, 1982; Granstrom *et al.*, 1982; Hakansson *et al.*, 1984; Mertsola *et al.*, 1983 and 1984; Viljanen *et al.*, 1982; Granstrom *et al.*, 1991). In the present study, immunofluorescence, whilst specific, was even less sensitive than culture. Also, serological studies may yield false negative results early in the course of infection and in chidren under 4 months of age, as described in Chapter 3. The only clinical symptom with high positive predictive value (92%) for clinical diagnosis is the presence of whoops (Granstrom *et al.*, 1991). However, some pertussis cases present atypically and the classical symptoms can also be produced by other infectious agents such as adenovirus or *Mycoplasma pneumoniae.* Therefore consideration should be given to developing gold standard criteria based on an appropriate combination of laboratory and clinical data.

Notwithstanding the limitations described above, the PCR assay appears to perform well in comparison with the other diagnostic techniques currently in use. Under optimal conditions the PCR result was available within five hours of collection of the NPA, whereas culture took 3 days or more. The PCR results were negative in only 3 of 66 samples from which *B. pertussis* was cultured and these samples grew fewer than 5 colonies each. NPA samples were submitted for PCR assay only after samples had been removed for culture and IF analysis. For both these techniques, parts of the specimen containing mucus were considered more likely to contain the organism and were sampled preferentially. Also a much larger inoculum was used for culture than for PCR (100-200μl for each of three culture plates compared with 20 μl for PCR). Thus the PCR assays were conducted on much smaller and probably inferior quality samples. Given the small number of *B. pertussis* colonies which grew from the three aspirates yielding apparently false negative PCR results, it is possible that the samples assayed by PCR did not actually contain any *B. pertussis* at all. The PCR assay was positive, however, in a further 35 samples which were negative by culture; two of these were positive by IF. Classification of the remaining 33 culture and IF negative PCR positive samples, as true positive or false positive, is difficult. For several of these specimens there is strong circumstantial evidence that the PCR result was genuine as they were collected either from symptomatic family contacts of culture-proven pertussis patients, or were follow-up specimens from patients who were previously culture positive (and PCR positive) but were culture negative at the time of the follow-up sampling. The maximum duration between collection of the two NPAs was six weeks. In addition, appropriate serum specimens for assessment of serological response to *B.pertussis* by ELISA were available for 10 of the remaining PCR-positive, culture- and IF- negative patients and 5 of these yielded positive results. Unfortunately sera were not available from the remaining patients. Thus, PCR amplification of reiterated *B. pertussis* specific DNA sequences has the potential

to be a rapid, highly sensitive and specific method for laboratory diagnosis of pertussis. The test, with some modifications for specimen preparation (see Materials and Methods) is now in routine use at WCH for the rapid detection of specific *B. pertussis* DNA sequences in specimens of nasopharyngeal aspirate. Since the assay was introduced into routine use in the laboratory, the number of PCR positives has exceeded the number of isolations of *B. pertussis* and is continuing to be of use for rapid diagnosis of whooping cough.

The PCR test may be subject to interference caused by components present in the NPA samples being tested. Inhibition may occur both in clinical samples and environmental specimens and some substances such as haemoglobin have been implicated previously (Clewley, 1989; Skakni *et al.*, 1992). One possible solution to this problem is to partially purify DNA from patient samples prior to testing by PCR. Skakni *et al.*, (1992), found that when amplifying *M. pneumoniae* sequences from NPAs, DNA purification was important and inhibition (detected by using internal ß-globin gene amplification primers) was caused by trace contaminants present. Indeed it was shown that even with a DNA purification step, 25% of NPA samples tested were found to inhibit the PCR assay.

A number of different methods of nucleic acid preparation from clinical samples could be used, however a very rapid and simple method which fits into routine laboratory procedures is required. A single ethanol precipitation was therefore selected for trial. This simple technique was found to remove partial or total inhibition in all 12 samples tested which had previously been able to inhibit the PCR. This fact suggests a cleaner initial DNA sample extracted from the NPA leads to increased production of PCR product per reaction and therefore sensitivity may be increased. It only takes 45 minutes to perform the extraction and hence does not significantly extend the overall time required to complete the procedure.

The results from initial studies comparing culture and PCR suggested very little inhibition was occurring. There were only three cases out of 63 which were PCR negative and culture positive. One of these was a bloodstained NPA and hence could easily be explained because of the haemoglobin contamination. The failure to detect *B. pertussis* in the other 2 may have been due to inadequate sampling (see above). The actual isolates of *B. pertussis* from each of these NPAs gave positive PCR reactions when tested. It may be that there were substances present in these NPAs which inhibited the PCR. Another explanation may lie in the source of polymerase enzyme. Work at CSL in Victoria by R.Premier (personal communication) showed that IBI's Taq polymerase was more efficient than other brands in situations where impure DNA was used as template. The early work in this study was all performed using IBI Taq polymerase, while the latter experiments (including the inhibition studies) used Bresatec polymerase. During 1990, 1991 and 1992 there have been very few isolates of *B. pertussis* and hence on the surface there seemed to be no problem with the PCR in terms of inhibition or decreased sensitivity. There were no further cases of culture-positive, PCR-negative specimens.

CHAPTER SIX

RAPID DETECTION OF *BORDETELLA PERTUSSIS* AND RESPIRATORY SYNCYTIAL VIRUS CONCURRENTLY IN NASOPHARYNGEAL ASPIRATES BY REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION.

RAPID DETECTION OF *BORDETELLA PERTUSSIS* AND RESPIRATORY SYNCYTIAL VIRUS CONCURRENTLY IN NASOPHARYNGEAL ASPIRATES BY REVERSE TRANSCRIPTION AND POLYMERASE CHAIN REACTION.

6.1 INTRODUCTION

Respiratory syncytial virus (RSV) is an important cause of acute lower respiratory tract infections in humans, with infants and young children being particularly susceptible, often developing bronchiolitis. This group of patients is also very susceptible to infection by *B. pertussis*. Dual RSV/*B. pertussis* infection has been described and simultaneous infection should be considered in young children hospitalised for presumed viral respiratory illness (Nelson *et al.*, 1986). Although the peak rate of RSV infection in Adelaide occurs primarily in the middle of winter (Goldwater *et al.*, 1991), there is sufficient overlap in seasonal prevalence such that often both RSV and *B. pertussis* detection are requested together, by the attending physicians, as part of a "respiratory pathogen screen". Doing both tests in the one reaction will save both time and money leading to an increase in laboratory cost efficiency. Traditionally, laboratory diagnosis of RSV infection has centred around culture of the virus from nasopharyngeal secretions. However cell culture techniques are slow and may take a week before a result is available and sensitivity may be affected by lability of RSV (Welliver, 1988).

The need for more rapid diagnostic tests and availability of anti-viral therapeutic agents has prompted the development of immunochemical techniques

such as direct immunofluorescence and antigen capture immunoassays (EIA)(Welliver, 1988). Although much faster, these techniques have variable sensitivity and specificity (Welliver, 1988; Johnston and Collins., 1990; Rothbarth *et al.*, 1991; Takimoto *et al.*, 1991; Thomas and Book, 1991). Cross infection rates (for both RSV and *B. pertussis*) may decrease if a faster, more sensitive and more specific test were available.

A rapid method for detection of RSV in NPA's based on reverse transcription (RT) and amplification of viral nucleic acid sequences by the PCR had been developed at the WCH Microbiology laboratory (Paton *et al.*, 1992). Because the RSV genome is composed of RNA, a reverse transcription step must take place prior to the PCR assay. The RT-PCR assay employs oligonucleotide primers specific for the region of the RSV genome which encodes the F1 subunit of the fusion (F) glycoprotein. The RT-PCR assay performed on RSV-positive NPA results in production of a 243-bp DNA fragment, while other viruses do not give a positive reaction. This assay was tested on 202 NPA samples collected from children with clinical signs of infection and the results were compared with those obtained from virus culture and enzyme immunoassay. RT-PCR results were positive in 118 of 125 samples from which RSV was cultured, as well as in 4 of 7 samples which were culture negative but EIA positive, suggesting that culture may not be an adequate gold standard for laboratory diagnosis of RSV infection. Chapter 5 of this thesis described a similar situation for *B. pertussis*. 2 NPAs were EIA and culture negative/RT-PCR positive for RSV, which probably reflects the greater sensitivity of PCR compared to EIA (unpublished data, Lawrence 1992).

An important requirement for the reverse transcription reaction to proceed efficiently is to have relatively clean nucleic acid templates. Hence a nucleic acid purification step is essential, which unfortunately adds considerable time to the test, but is of paramount importance for reliable results.

The capacity to perform both *B. pertussis* and RSV PCR assays in the same tube would result in considerable savings in staff time and reagents. The specificity and sensitivity of the RSV RT-PCR assay were high (94.6% and >97% respectively) and it was decided to combine amplification of both the 153 bp *B. pertussis* sequence and the 243 bp RSV specific sequence in one PCR reaction. This chapter describes testing of such a bivalent PCR and its application to diagnosis of both whooping cough and RSV infection in children.

6.2 RESULTS.

6.2.1 Clinical specimen trial

The amplification protocols originally described for the *B. pertussis* and RSV assays were not identical and experiments were initially aimed at determining whether suitable common amplification conditions could be employed without compromising sensitivity or specificity of either assay. In addition, a more extensive nucleic acid purification process was required before proceeding with RT-PCR.

The initial set of tests involved analysis of 22 RSV-positive (both by culture and PCR) and 7 *B. pertussis*-positive (both by culture and RT-PCR) NPAs. The suction catheters from RSV-positive patients were flushed through with 2 ml of viral transport medium (VTM), and approximately 1.0 ml stored at -80°C until tested. The 7 other NPA's from culture proven whooping cough patients had been stored in phosphate buffered saline at -80°C for up to six months.

Ten NPAs which were RSV RT-PCR-positive were seeded with 10μl of a 100-fold dilution of a *B. pertussis* NCTC 10908 culture suspension in distilled water (harvested from 24hr charcoal agar plates) which was equivalent to approximately 1100 viable organisms. The remaining 12 RSV-positive NPAs

were mixed in approximately equal proportions with *B. pertussis* culture-positive NPAs.

Positive controls consisted of the *B. pertussis* culture suspension as above, RSV culture-positive NPAs, *B. pertussis* culture/PCR positive NPAs (or 153-bp *B. pertussis* PCR product) and various combinations of these NPAs. An internal reverse transcriptase control (part of the RNA PCR kit, Cetus-Perkin Elmer) was also used to confirm that the RT reaction had indeed taken place. Negative controls consisted of a further 14 NPAs (found to be culture negative for RSV and *B. pertussis*). A sample of sterile distilled water was always included as a negative control to ensure no contamination of the reagents (eg. with PCR product) had occurred.

PCR assays were initially carried out on a Hybaid intelligent heating block using 2 ml screw capped tubes. 35 cycles were used for amplification, with 1.5 minute denaturation, annealing and elongation times at temperatures of 94, 47 and 72°C, respectively.

8 of the 10 *B. pertussis*-seeded RSV-positive NPAs gave moderate to strong dual 153 bp (*B. pertussis*) and 243 bp (RSV) bands in ethidium bromide stained gels after RT-PCR, as described in Section 2.12.2. Of the remaining 2, one gave no bands at all despite being previously positive (suggesting that tube may have missed out on a critical reagent) and the other gave a strong pertussis band (153 bp) but only a weak RSV band (243 bp). Results for six of these samples are shown in **Figure 6.1**. The remaining 12 RSV positive NPAs were mixed (in equal volumes) with pertussis positive NPAs and then subjected to the RT/PCR. All of them produced dual bands of the correct size (six are shown in **Fig. 6.1**) and in only one case were there a number of bands of incorrect size. There was however some variation in intensity of EtBr staining (**Figure 6.1**).

Fourteen culture negative (for RSV and *B. pertussis*) NPAs yielded negative RT/PCR test results with the appropriate positive controls giving bands of the predicted size in these reactions (results not shown).

Figure 6.1

Initial experimentation to determine if both RSV and *B. pertussis* specific sequences could be detected concurrently in samples of NPA following reverse transcription and PCR.

The assays were performed on a Hybaid intelligent heating block.

Upper portion

Lanes 1-6 show results of RT-PCR on 6 different NPA specimens, all culture and ELISA positive for RSV, seeded with approximately 1,100 cfu of *B. pertussis*. Lane 7 resulted from mixing an NPA positive for RSV with one positive for *B. pertussis*. Lane 8 is DNA size markers and expected mobility of 153bp and 243bp fragments is indicated.

Lower portion

The amplified products visible in lanes 9-13 result from RT-PCR on mixtures (in equal proportions) of NPA samples positive for RSV and others positive for *B. pertussis*. Lane 14 resulted from a mixture of positive controls for RSV and pertussis and lane 15 was a negative control. Lane 16 is DNA size markers and expected mobility of 153bp and 243bp fragments is indicated.


1 2 3 4 5 6 7 8
- 243
- 153
- 243
- 153
9 10 11 12 13 14 15 16

In an effort to simplify the addition of separate RSV and *B. pertussis* positive controls, one single control containing both targets was made. A set of experiments were performed using a number of dilutions of a positive RSV PCR product with a diluted culture of *B. pertussis*, containing approximately 120 viable organisms per 20μl sample. Testing serial dilutions of both controls showed it was necessary to dilute the RSV control 10,000 times in order to prevent interference with the efficacy of amplification of the pertussis template. Without such dilution, presumably the vast excess of RSV target over *B. pertussis* target template would result in exhaustion of reactants such as nucleotides before a sufficient number of amplification cycles had occurred to permit visualization of the less abundant *B. pertussis* PCR product in EtBr stained agarose gels.

6.2.2 Optimisation of PCR conditions

As previously mentioned, the initial RSV PCR experiments were performed on a Hybaid Intelligent Heating Block. Using this machine and the conditions outlined above, adequate amounts of RSV PCR product were observed in an ethidium bromide stained gel (lane 2, **figure 6.2a**). If the cycle number was reduced to 30 (as used in the pertussis PCR described in chapter 5), only a very faint RSV specific band was visible in the gel (lane 4, **figure 6.2a**). For reasons of efficiency and standardisation with the pertussis PCR, it was decided to continue the work on a Perkin Elmer DNA Thermal Cycler. A series of experiments were required to optimise conditions on this machine (the optimal conditions are described in the Methods Section, Chapter 2.12.2). If identical conditions to those established on the Hybaid thermal cycler were applied directly to the Perkin Elmer machine, very little specific DNA PCR products were produced with only a faint 253bp (RSV specific) band visible (lane 6, **Figure 6.2a**).

Figure 6.2 (a)

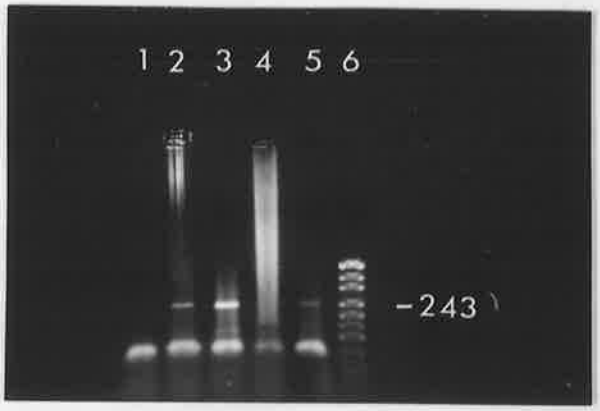
Optimization of RSV PCR conditions using a Perkin Elmer DNA Thermal Cycler.

Lanes 1,3 and 5 are negative controls while lane 2 shows the 243 bp intensely staining product produced after RT-PCR analysis of a pooled RSV culture-positive NPA, using the original set of PCR conditions on the Hybaid thermal cycler (35 cycles). Lane 4 shows the result of decreasing the number of cycles from 35 to 30 on the same machine. Lane 6 shows the result of amplification using the same sample and identical conditions (35 cycles) originally defined, but using the Perkin Elmer DNA Thermal Cycler. The products are visualised in an ethidium bromide-stained 2% agarose gel following electrophoresis.

Figure 6.2 (b)

This figure shows the results of RSV-*B. pertussis* RT-PCR assays performed on the Perkin Elmer DNA thermal cycler under the following conditions : 94^{o}C for 1:50, 47^{o}C for 3:00, 72^{o}C for 4:00 mins. with a ramp transition time of 60 secs. for each step. Lane 1 was a negative control, lanes 2 and 5 represent RSV positive NPA samples, lane 3 a positive RSV control and lane 4 a positive *B. pertussis* control.


1 2 3 4 5 6
– 243


1 2 3 4 5 6
–243

The following conditions were found to successfully amplify the RSV specific sequence on the Perkin Elmer machine but not the *B. pertussis* sequence ; 94°C for 1:50 secs, 47°C for 3:00 mins, 72°C for 4:00 mins. with a ramp transition time of 60 seconds for each step (lane 3 **Figure 6.2b**). The *B. pertussis* positive control produced non specific DNA amplicons following PCR under these conditions, possibly because of the lower annealing temperature used (lane 4 **Figure 6.2b**). 2 previously RSV positive NPA samples produced an appropriate PCR product (lanes 2 and 5 **Figure 6.2b**) although the band was very faint for the second NPA (lane 5). No definitive band was observed for the pertussis positive control however. and another set of tests were commenced. The other notable feature in these experiments was the excessive amounts of unutilised primers which are observed in all lanes except lane 4.

In order to establish the optimal annealing temperature for a dual RSV/pertussis PCR assay, tests were performed using 3 different temperatures. The positive RSV controls used in these experiments were diluted PCR product and 2 culture-positive NPAs (v414, v534), while the positive pertussis control was a diluted culture of NCTC 10908 (as previously described). Reactions were run on consecutive days with the only difference being the annealing temperatures which were either 47°C, 50°C or 55°C. The pertussis PCR product was clearly visible as a distinct and bright band when 50°C or 55°C for annealing were used (a DNA smear was observed at 47°C). A 253 bp band of RSV PCR product was observed for NPA v414 and the RSV positive control when 47°C was used for annealing however only a very faint band was observed when 55°C was used. When the temperature of annealing was set at 50°C, all samples, including NPA v534, gave the appropriate size PCR products (**Figure 6.3a**).

It was necessary to use a sixty second transition time for each segment of the program in order to produce a band of high intensity. A series of experiments were performed utilising the same set of controls as above but using

Figure 6.3 (a)

RSV-*B. pertussis* RT-PCR performed on the Perkin Elmer DNA thermal cycler using 2 different annealing temperatures.

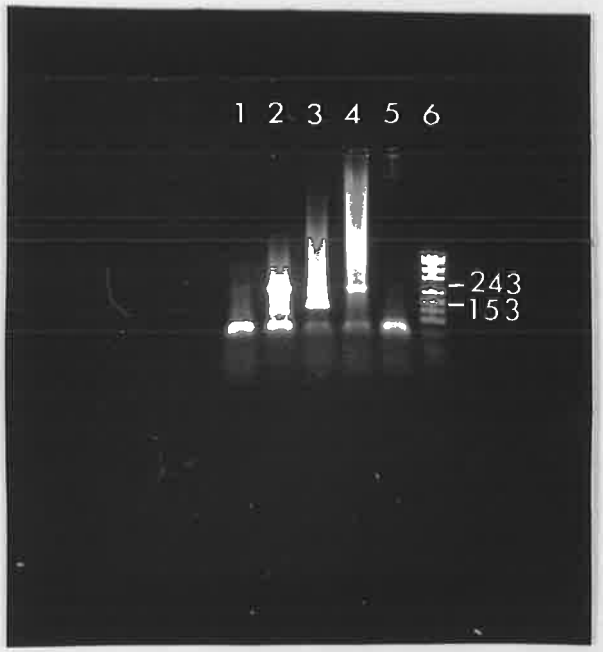
Two sets of PCR assays on the same 5 samples were performed using annealing temperatures of 50°C (lanes 7-11) or 55°C (lanes 1-5). Lanes 1 and 7 are negative controls, lanes 2 & 8 are RSV positive controls, lanes 3 & 9 are pertussis positive controls. Lanes 4 & 10 and 5 & 11 are RSV positive NPA samples, v414 and v534 respectively. Lane 12 is DNA size markers and the expected mobility of 153bp and 243bp fragments is indicated.

Figure 6.3 (b)

RSV-*B. pertussis* RT-PCR without the 10 second autoextension to the final step (elongation) of each cycle on the Perkin Elmer DNA thermal cycler.

Lane 1, negative control; lane 2, RSV positive control; lane 3, pertussis positive control; lane 4, NPA 414; lane 5, NPA 534; lane 6, DNA size markers and the expected mobility of 153bp and 243bp fragments is indicated.


1 2 3 4 5 6 7 8 9 10 11 12
243
153


1 2 3 4 5 6
243
153

an annealing temperature of 50°C. A 10 second extension on the final segment of the PCR cycle was programmed into the thermal cycler as part of the standard set of parameters controlling the machine. This means that 10 seconds is added to each of the cycles in a cummulative manner in order to account for any decrease in enzyme activity and availability of nucleotides as the reaction proceeds. If this 10 second extension removed from the program, the 253 bp band normally produced by the weakly RSV positive NPA v534 disappeared (indicating a decrease in sensitivity), and the intensity of the band from v414 diminished. The positive pertussis and RSV controls remained positive, but with some non-specific amplification products, observed as a slight smearing in the gel, was also noted **(figure 6.3b)**.

In an attempt to decrease the cost of the RT/PCR, a series of tests using only half (1.5 units) of the usual amount of reverse transcriptase (the most expensive reagent) were performed. Eight serial ten fold dilutions of the positive RSV control (previously RSV-positive, diluted NPA) were made in sterile distilled water and these were used as the sample for RT/PCR. The PCR proceeded as previously described and a photographic record was made. Results of the above PCR-RT assays showed a decrease in sensitivity of 2-3 log if only half of the usual amount of RT was used. **(Figure 6.4)**

6.3 DISCUSSION

Simultaneous infection by *B. pertussis* and RSV is an uncommon occurrence and the RT-PCR assay as described would usually only be detecting either one of these pathogens. However, these two organisms are the cause of serious acute respiratory infection, particularly in young infants, and are both highly contagious with potential for cross infection in a hospital setting. There is significant overlap in the seasonal prevalence of these two pathogens (RSV-winter/spring, *B. pertussis* spring/summer) and simultaneous requests for RSV

Figure 6.4

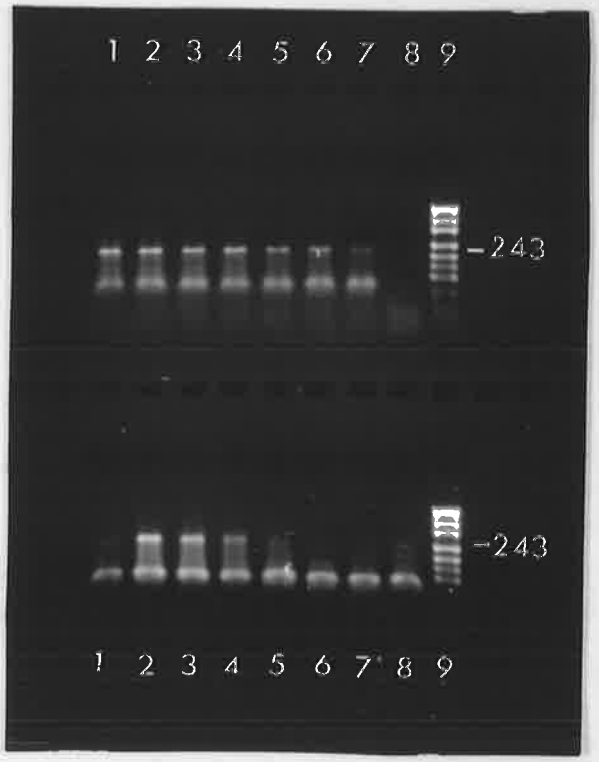
Effect of reduction of reverse transcriptase concentration on the sensitivity of the RSV-*B.pertussis* RT PCR.

Upper Track

Lanes 1-8 (upper track) show serial 10 fold dilutions (10^0 - 10^7) of an RSV-positive control (pooled RSV positive NPAs) used as the template in a standard RT-PCR reaction (2.5 units RTase/tube).

Lower Track

Lanes 1-8 (lower track) show the same sample treated as above, but with 1.75 units RTase/tube.


1 2 3 4 5 6 7 8 9
-243
-243
1 2 3 4 5 6 7 8 9

and *B. pertussis* detection are frequently received for a given patient, particularly in late winter/early spring period.

Separate PCR assays have been developed for RSV and *B.pertussis* and both have been shown to be superior to existing diagnostic procedures. Thus, modification of any protocols such that both PCR-based assays can be performed together in the same tube would provide significant cost advantages and be of great benefit to the individual patient as well as to hospital infection control management.

In the present study, these two assays have been combined and conditions optimised for performing the tests on a Perkin Elmer DNA Thermal Cycler have been developed. Following amplification, specific PCR products for both *B. pertussis* (153 bp) and RSV (243 bp) were distinguished on ethidium bromide stained agarose gels. Combining the two sets of primers in the PCR appeared to have no effect on the ability to amplify specific targets in clinical samples of nasopharyngeal secretion and control samples, and no spurious bands were apparent, as long as template was not present in vast molar excess over the other (an unlikely natural occurence).

The RT-PCR as described has one problem in that it takes about one and a half days to obtain a result compared to a few hours for the pertussis PCR. Longer specimen preparation time is required for the RT process which means results are available the next morning rather than the afternoon of the same day. The time required from the beginning of sample preparation to the beginning of PCR is approximately 5 hours with the PCR itself taking about 8 hours (usually run overnight). Although taking longer than an enzyme immunoassay for RSV antigen detection (4 hour test) currently used, the PCR reaction is more specific and more sensitive (A.Lawrence, unpublished data) and has the benefit of detecting *B. pertussis* as well. As a rule, during the summer months or during pertussis epidemics (when very little or no RSV infection occurs), only the more rapid pertussis PCR would be performed.

RT-PCR assays were performed on 22 NPA samples where 12 out of 12 pertussis- and RSV-positive mixed NPA samples initially tested gave dual bands of the predicted size. 9 of 10 RSV-positive NPAs seeded with *B. pertussis* also gave dual bands. In one sample both bands were missing, possibly because the tube may not have received a critical reagent. Bands of the correct size were present on repeat testing of this specimen, resulting in sensitivity and specificity of 100%.

When using the Perkin Elmer Thermal Cycler it was necessary to include a sixty second extension to the heating/cooling rate on each segment to ensure adequate amplification. It is likely that by doing this, the rates of temperature increase and decrease closely resemble those achieved on the Hybaid machine originally. Results showed that 47°C was too low for specific annealing of the pertussis primers. At this temperature many different sized products were amplified leading to a DNA smear rather than formation of a single molecular weight product. At 55°C no RSV product formed, presumably because this temperature was too high for proper annealing of the primers with their targets while a compromise temperature of 50°C worked well for both pertussis and RSV sequence amplification. The length of RSV primers could not be increased to enable annealing at higher temperatures because they were based on limited consensus sequences of the A and B subgroups of RSV.

A 10 second extension on the elongation step was necessary to achieve adequate sensitivity. This is probably important in the latter stages of the amplification reaction when the activity of the Taq polymerase enzyme and the concentration of dNTP's are decreased.

The cost of a diagnostic test is an important consideration for the clinical laboratory. The newer DNA detection based systems such as PCR are expensive and any means of cutting material costs need to be considered. One such way may be to decrease the usage of expensive reagents such as reverse transcriptase. Attempts at halving the amount of RT required in this reaction as

described in the methods were disappointing. A substantial loss of sensitivity (2-3 log) was observed which is not acceptable for a routine diagnostic test. The unit cost of a bivalent PCR test (such as the one described) which can be used to detect two pathogens in the one tube is however significantly reduced (almost halved) and is an important advantage of a dual PCR.

This chapter has documented a reverse transcription reaction coupled to a PCR for concurrent detection of both *B. pertussis* and RSV in specimens of NPA. It could be used as a first line diagnostic test to obtain rapid, specific and sensitive results for these slow growing, difficult to culture organisms.

CHAPTER SEVEN

FINAL DISCUSSION

7.1 FINAL DISCUSSION

Pertussis remains an important disease, primarily of childhood, which is associated with considerable morbidity (symptoms may persist for many months) in our society, despite high rates of immunisation. It is necessary for the clinical microbiology laboratory to be able to accurately and rapidly identify patients who have pertussis, particularly in the early stages of the disease when cross infection can easily occur. In the hospital situation this is particularly important because patients need to be isolated in order to reduce the risks of cross infection, which are considerable with pertussis. Equally important are negative results because symptomatic patients with infections other than pertussis (eg. RSV infection) may be grouped together in a ward thus saving staff time and ward space which equate to a dollar saving for the hospital. The total cost of diagnostic tests (particularly new expensive ones) to the laboratory is also important when consideration is given to introducing them into routine diagnostic service .

From the early 1980's, much work has been published, primarily concerning the serological diagnosis of whooping cough using ELISA based tests. A variety of individual pertussis antigens or whole cell sonicates have been used to coat the solid phase (Friedman, 1988). Some effort has been put into other ELISA's such as those for detection of anti-PT and anti-FHA IgA antibodies in saliva (Zackrisson *et al.*, 1990). IgA antibodies both to FHA and PT were detected, but the authors suggest there is little value in this being used as a routine diagnostic test because only one third to a quarter of patients in their test group developed significant salivary antibody titres.

A colony immuno-blot assay using monoclonal antibodies to FHA and lipopolysaccharide of *B. pertussis* was developed in an attempt to speed up the identification of *B. pertussis* growing on agar plates cultured from patient NPA's (Gustafsson and Askelof, 1989). The system was specific and able to detect colonies of *B. pertussis* before they became visible but still required an incubation time of 40 hours.

DNA-based tests to detect specific *B. pertussis* sequences in clinical samples (such as the repeat sequence described in Chapter 5 of this thesis) have also been reported elsewhere but lacked sensitivity with a limit of detection of 5 x 10^3-10^4 cfu (Reizenstein *et al.*, 1990). This level of detection is not sensitive enough for routine testing, particularly where low numbers of organisms may be present in the late acute and convalescent stages of pertussis. The authors suggest that the sensitivity of hybridization would be adequate only if coupled with a more rapid amplification system.

PCR offers a means by which very low numbers of organisms can be detected (by amplification of specific sequences) directly from samples of NPA and then subjected to hybridization with specific probes or visualization of specific DNA bands in ethidium bromide stained agarose gels. PCR assays other than that described in this thesis, for detecting *B. pertussis*, have been reported in the literature recently. One amplifies a 191-bp sequence found in the regulatory region of the *B. pertussis* toxin gene or a 121-bp *B. pertussis* specific insertion sequence. These assays were found to be sensitive and could possibly be used for routine testing, but were not subjected to a clinical trial (Houard *et al* 1989). A second article (Olcen *et al* 1992) reports amplification of a 400 bp DNA sequence contained within the repetitive sequence of the *B. pertussis* genome. This PCR assay detected the specific PCR product in 19/25 culture positive cases and 5/50 culture negative children initially suspected of having pertussis. These results are comparable to those described in chapter 5 of this thesis, but the assay is less sensitive. The point was made, however, that

diagnosis was possible within one day of sampling, which is critical if a rapid test is to be used in routine laboratory diagnosis.

More recently, He *et al*, (1993), compared PCR with culture and ELISA for diagnosis of pertussis. The primers (one of which was identical to BP1 described in Chapter 5 of this thesis) used in this study were based on the reiterated sequence present in the *B. pertussis* genome. Two small outbreaks of pertussis were investigated using all three techniques as described above. Their assay was similar to that detailed in Chapter 5 in terms of sensitivity and specificity suggesting (as in chapt. 5) that PCR will detect a number of cases of pertussis which are not detectable by other means (ie. serology or culture). Grimpel *et al* (1993) have used a PCR assay to detect the PT promoter sequence of *B. pertussis* in samples of NPA collected from 24 infants and children with pertussis and 13 adult contacts. They chose the PT promoter sequence for an amplification target because it has been detected in all strains of *B. pertussis* so far tested and is not detectable in *B. bronchiseptica* or *B. parapertussis*. Although this will ensure 100% specificity, the sensitivity is likely to be diminished compared to the PCR assay described in Chapter 5 because the chosen target sequence is not reiterated (ie. only one copy compared with 100 copies per genome for the reiterated target sequence). In this group of infants, the sensitivities of culture and PCR were 54.1% and 95.8% respectively. In the adult contacts, the sensitivities of the 2 methods were 15.4% and 61.5% respectively. Their conclusion was that PCR, in association with serology, promises to be a valuable tool for epidemiological studies.

It appears that *B. pertussis* infection will continue to be a cause of considerable morbidity in Australia and other developed communities, despite generally good levels of immunisation. The problem is far worse in developing countries with an unacceptably high level of infant mortality of 600,000 per year (UNICEF, State of the Worlds Children 1993, Summary). The relatively high cost of diagnostic tests such as PCR, and to a lesser extent ELISA, means

that the probability of using these sensitive technologies in developing countries is remote. There would be a place however for very cheap ELISA based tests which could be used in the field. Use of different solid phases for ELISA's, such as antibody coated polystyrene balls, may provide increased sensitivity with less cost. Increasing sensitivity would be useful when detecting low levels of antibody to individual antigens of pertussis such as FHA or pertussis toxin.

An area of work which also deserves much attention in the future is an extension of the RSV/Pertussis PCR. There would be great diagnostic and cost advantages in development of a multivalent PCR capable of detecting a number of different bacterial/virus-specific sequences in the one reaction tube. The unit cost per test would be greatly reduced in this type of assay, making it more applicable to screening larger numbers of samples in the routine laboratory. Whether this can be achieved without sacrificing specificity and/or sensitivity remains to be seen.

There is also scope for some improvement in the serologic diagnosis of pertussis. The work described in chapters 3 and 4 confirm the need for diagnostic ELISA-based tests to be able to detect antibody responses towards a large number of *B. pertussis* antigens. Now that a number of these antigens have been identified both in terms of structure and function, it may be possible to design an ELISA using a cocktail of purified antigens rather than a whole cell preparation for coating the solid phase. This may result in improved binding of serologically important antigens and hence greater sensitivity. The increased cost of purified antigens however, may be a limiting factor in introducing such a test into the routine laboratory (assuming specificity and sensitivity would be better).

In summary, this project has been concerned with improving the routine laboratory diagnosis of whooping cough using ELISA and DNA based assays. It is apparent that no one test alone, will be able to successfully diagnose all

cases of pertussis. Appropriate combinations of culture and/or serology and/or specific DNA sequence detection still needs to be performed routinely. Evidence suggests, however, that culture could be replaced by PCR, although with current PCR's the serotype distribution of isolates could not be determined and antimicrobial drug susceptibilities could not be performed.

The successful application of these newer technologies to routine testing has greatly improved the sensitivity and rapidity of laboratory diagnosis of whooping cough for children and adults using the services of the Microbiology Department, WCH.

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