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Bordetella pertussis

Vaccination and Strain Variation

by

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To the memory of the children who died of pertussis,

ABSTRACT

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***Bordetella pertussis* – Vaccination and strain variation**

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Pertussis or whooping cough is a highly contagious vaccine-preventable disease of the human respiratory tract caused by the *Bordetella pertussis* bacteria. In Finland, pertussis vaccinations were started in 1952 leading to a dramatic decrease in the morbidity and mortality. In the late 1990s, the incidence of pertussis increased despite the high vaccination coverage. Strain variation has been connected to the re-emergence of pertussis in countries with long history of pertussis vaccination. In 2005, the pertussis vaccine and the vaccination schedule were changed in Finland.

The molecular epidemiology and the strain variation of the *B. pertussis* isolates were examined in Finland and in countries with similar (France) and different (Sweden) vaccination history. Continuous evolution of the *B. pertussis* population in Finland was observed since the 1950s, and the recently circulating isolates were antigenically different from the vaccine strains. Comparison of the circulating isolates from Finland, France and Sweden did not refer to significant differences. Certain type of strains noticed in France already in 1994 mainly caused the recent epidemics in Sweden (1999) and in Finland (2003-4). On several occasions, a new type of strains first appeared in Sweden and some years later in Finland.

The *B. pertussis* isolates from the infants were shown to be similar to those from the other age groups. It is suggested that the strains originate from the same reservoir among adolescents and adults. The strain variation does not seem to have a major effect on the morbidity among recently vaccinated individuals, but it might play a role among those who are in the waning phase of immunity. The incidence of pertussis in Finland has remained low since the change of the vaccination programme. This might be related to the epidemic nature of pertussis and the near future will show the real effectiveness of the new vaccination programme. At present, many infants are infected because they are too young to be immunised with the current schedule. New strategies or vaccines are needed to protect those who are the most vulnerable.

Keywords: *Bordetella pertussis*, strain variation, vaccination, epidemiology

TIIVISTELMÄ

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***Bordetella pertussis* – Rokotukset ja kantavaihtelu**

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Hinkuyskä on erittäin tarttuva *Bordetella pertussis* -bakteerin aiheuttama ja rokottein torjuttavissa oleva tauti, jota esiintyy ainoastaan ihmisillä. Suomessa hinkuyskän sairastuvuus ja kuolleisuus laskivat huomattavasti vuonna 1952 tapahtuneen hinkuyskärokotusten aloittamisen jälkeen. Hyvästä rokotuskattavuudesta huolimatta hinkuyskän esiintyvyys lisääntyi 1990-luvun lopussa. Hinkuyskän lisääntyminen maissa, joissa hinkuyskärokotteet ovat olleet käytössä pitkään, on liitetty *B. pertussis* -bakteerin kantavaihteluun. Suomen rokotusohjelma ja käytettävä hinkuyskärokote vaihdettiin vuonna 2005.

B. pertussis -bakteerin kantavaihtelua ja molekyylitason epidemiologiaa tutkittiin Suomessa sekä samankaltaisen (Ranska) ja poikkeavan (Ruotsi) rokotushistorian maissa. Suomen *B. pertussis* -populaation jatkuva ja etenevä evoluutio havaittiin 1950-luvulta alkaen. Viime vuosina kiertäneet bakteerikannat poikkeavat rokotekannoista. Suomen, Ruotsin ja Ranskan bakteeripopulaatiot eivät poikenneet selvästi toisistaan. Sekä Ruotsin (1999) että Suomen (2003-4) edellisen epidemian aiheutti etupäässä eräs Ranskassa jo vuonna 1994 havaittu bakteerikanta. Useissa tapauksissa uudet bakteerikannat havaittiin Suomessa joitakin vuosia myöhemmin kuin Ruotsissa.

Tulokset osoittivat, että eri ikäryhmistä eristetyt *B. pertussis* -bakteerikannat ovat samankaltaisia. Näin ollen kaikissa ikäryhmissä tautia aiheuttavien bakteerikantojen oletetaan olevan lähtöisin samasta säilymöstä (reservuaarista) nuorten ja aikuisten keskuudessa. Kantavaihtelu ei näyttäisi paljonkaan vaikuttavan hiljattain rokotettujen sairastuvuuteen, mutta vaikutus saattaa ilmetä niillä, joiden immuniteetti hinkuyskää vastaan on heikentynyt. Hinkuyskää on esiintynyt Suomessa vähän rokotusohjelman vaihdon jälkeen. Tämä saattaa kuitenkin johtua taudin luontaisesta epidemiasyklistä, joten uuden rokotusohjelman todellinen tehokkuus selviää vasta lähitulevaisuudessa. Nykyisellä rokotusaikataululla useat vastasyntyneet sairastuvat hinkuyskään, koska ovat vielä liian nuoria saamaan rokotusohjelman mukaisen ensimmäisen hinkuyskärokotuksensa. Heidän suojaamisekseen tarvitaan uusia rokotusstrategioita tai rokotteita.

Avainsanat: *Bordetella pertussis*, kantavaihtelu, rokottaminen, epidemiologia

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ABBREVIATIONS

aP/p	acellular pertussis vaccine
AT	autotransporter
cfu	colony-forming unit
CyaA	adenylate cyclase toxin
D/d	diphtheria vaccine
DFA	direct fluorescent antibody
DNT	dermonecrotic toxin
EIA	enzyme immunoassay
FHA	filamentous hemagglutinin
Fim	serotypable fimbriae
GPI	Global Pertussis Initiative
Hib	*Haemophilus influenzae* type b
IDR	Infectious Diseases Register; "Tartuntatautirekisteri"
Ig	immunoglobulin
IPV	inactivated poliovirus
IS(E)	insertion sequence (element)
KTL	National Public Health Institute; "Kansanterveyslaitos"
LPS	lipopolysaccharide
NP	nasopharynx
PCR	polymerase chain reaction
PFGE	pulsed-field gel electrophoresis
prn	gene coding for pertactin
Prn	pertactin
PT	pertussis toxin
Ptl	pertussis toxin liberation
*ptx*A	gene coding for pertussis toxin S1 subunit
T/t	tetanus vaccine
T1SS to T6SS	type I to VI secretion systems
TCF	tracheal colonisation factor
TCT	tracheal cytotoxin
Th1 or 2	T-helper 1 or 2 cell
TPS	two-partner secretion
WHO	World Health Organization
wP/p	whole-cell pertussis vaccine

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals (I–IV) and some unpublished data:

I Elomaa A, Advani A, Donnelly D, Antila M, Mertsola J, Hallander H, He Q. Strain variation among *Bordetella pertussis* isolates in Finland, where the whole-cell pertussis vaccine has been used for 50 years. J Clin Microbiol 2005 Aug;43 (8):3681–7.

II Caro V, Elomaa A, Brun D, Mertsola J, He Q, Guiso N. *Bordetella pertussis*, Finland and France. Emerg Infect Dis 2006 Jun;12 (6):987–9.

III Elomaa A, Advani A, Donnelly D, Antila M, Mertsola J, He Q, Hallander H. Population dynamics of *Bordetella pertussis* in Finland and Sweden, neighbouring countries with different vaccination histories. Vaccine 2007 Jan 15;25 (5):918–26.

IV Elomaa A, He Q, Tran Minh NN, Mertsola J. Pertussis in Finland before and after the introduction of acellular pertussis vaccines. Submitted.

1 INTRODUCTION

Pertussis or whooping cough is a highly contagious vaccine-preventable disease of the human respiratory tract caused by the *Bordetella pertussis* bacteria. The first whole-cell pertussis (wP) vaccines were introduced already during the 1940s. Since 1974, pertussis vaccine has been recommended by the World Health Organization (WHO). In the prevaccine era, whooping cough was one of the main causes of death in infants and more than half of the children suffered from pertussis before beginning school (Halperin 2007). With the widespread use of pertussis vaccines, the incidence of pertussis dropped dramatically. Still, the disease is one of the leading causes of vaccine-preventable deaths in the world today (WHO 2003).

In Finland, the wP vaccine combined with diphteria (D) and tetanus (T) toxoids has been included in the national vaccination programme since 1952. The immunisation schedule with DTwP vaccinations was stable since the 1970s until an acellular booster vaccine (dtap) was introduced in 2003 for children at 6 years of age, and the immunisation schedule was totally changed in 2005. The vaccination coverage has traditionally been high in Finland (Leino et al. 2007), but still there was a significant increase in the incidence of pertussis during the late 1990s. Re-emergence of pertussis has also been noticed in other countries with decades of pertussis vaccinations with high coverage (Celentano et al. 2005; Mattoo and Cherry 2005; WHO 2007).

B. pertussis produces many virulence factors that are responsible for the clinical disease (Cherry et al. 2005; Locht et al. 2001). Pertussis infection is a complex interaction between the bacterial virulence factors and the immune system of the human host. One of the mechanisms in the bacteria to avoid or weaken the immune reaction of the host is antigenic variation of virulence factors. This strain variation observed in *B. pertussis* populations has been connected to the pertussis re-emergence in countries with a long history of pertussis vaccination (Gzyl et al. 2001; Mooi et al. 1998; Poynten et al. 2004; van Amersfoorth et al. 2005; Van Loo and Mooi 2002; Weber et al. 2001).

The aims of this thesis were to study the molecular epidemiology and the strain variation of the *B. pertussis* population in Finland during the five decades of pertussis vaccination, to compare the bacterial populations between countries with similar and different vaccination histories, and to evaluate the effects of the change of vaccination programme on the bacterial population and pertussis epidemiology especially among young children.

2 REVIEW OF THE LITERATURE

2.1 Pertussis disease

Pertussis, also known as whooping cough, is an acute infectious disease of the human respiratory tract caused by the Gram-negative bacterium *Bordetella pertussis* (Locht 2007; Tozzi et al. 2005). Estimated by WHO, 300 000 deaths and 50 million cases of pertussis occur every year (WHO 2003).

2.1.1 Pertussis infection and symptoms

Pertussis is mainly transmitted by *B. pertussis* containing airborne droplets from cough or sneeze during the first three weeks of infection (Crowcroft and Pebody 2006; Heininger 2001; Kerr and Matthews 2000; Loeffelholz 2003). The household members are the source of infection in 73–82% of infants with pertussis (Wendelboe et al. 2007). The disease is highly contagious evidenced by the secondary attack rate among family members being over 80% (Long et al. 1990; Mertsola et al. 1983). However, as much as 46% of the secondary cases are asymptomatic and not expected to be diseased (Mertsola et al. 1983; Schellekens et al. 2005). It has been estimated that every primary case of symptomatic pertussis leads to approximately five secondary cases of asymptomatic or clinically insignificant infection (Ward et al. 2006).

The clinical symptoms are mainly caused by virulence factors such as bacterial toxins that *B. pertussis* secretes (Kerr and Matthews 2000). The disease typically lasts 6 to 12 weeks or longer with three stages: catarrhal, paroxysmal and convalescent (Loeffelholz 2003). First, after exposure to the bacteria, the incubation period of 7–10 days precedes the development of symptoms. During the catarrhal stage, non-specific symptoms appear with rhinitis and mild cough lasting approximately 7 to 14 days. At this stage, the disease is highly communicable but rarely suspected. The cough gradually becomes paroxysmal with severe coughing spells followed by whooping and possibly post-tussive vomiting. The patients may also become cyanotic and require airway support. This paroxysmal cough (the sound can be heard in the homepages of Dr. Jenkinson (Jenkinson)) is the typical symptom of pertussis, but the whoop may be absent in the infants younger than 6 months, who may only present apnoea or even sudden death (Heininger et al. 2004b). Other characteristic signs during the paroxysmal stage in infants include leucocytosis, lymphocytosis, and weight loss due to the post-tussive vomiting. After 2 to 8 weeks, the frequency and severity of the paroxysms decrease referring to the transition of the illness to the convalescent or recovery stage. The paroxysms are gradually improved but may, however, recur during other respiratory infections leading to a misdiagnosis of prolonged cough or asthma (CDC 2000; Greenberg et al. 2007). Co-infections such as respiratory syncytial virus infection may modify the symptoms and thus complicate the diagnosis at all stages of the disease (Crowcroft et al. 2003a; Korppi and Hiltunen 2007).

Severe pertussis and complications such as cyanosis and pneumonia primarily occur among infants too young to be vaccinated, and over 80% of the fatal cases are among infants

younger than 4 months (Crowcroft et al. 2002; Crowcroft et al. 2003a; Greenberg et al. 2005; Vitek et al. 2003). Older patients often have less severe or asymptomatic infections that are more difficult to be diagnosed (Gregory 2006; Hewlett and Edwards 2005; von Konig et al. 2002).

The individuals with partial immunity – such as infants with some maternal antibodies, incompletely vaccinated children, or adolescents and adults with waning immunity – may show atypical symptoms with less severe paroxysms and shorter course of illness causing misdiagnosis during the early stages of pertussis (Tozzi et al. 2005). As these patients are not diagnosed and treated during the most contagious stage of the illness, they frequently serve as sources of infection and expose the susceptible infants to the disease (Hewlett and Edwards 2005).

2.1.2 Diagnostics

Clinical suspicion of pertussis is most important for the appropriate and early diagnosis of pertussis. Other pathogens – such as respiratory synctial virus, adenovirus, *Mycoplasma pneumoniae*, *Chlamydia trachomatis* and *C. pneumoniae* – cause similar cough illnesses (Hallander 1999; Korppi and Hiltunen 2007; Wheeler and Simmons 2005). Prolonged cough caused by pertussis is sometimes also confused with and misdiagnosed as asthma (Weinberger and Abu-Hasan 2007). *Bordetella parapertussis* also causes a pertussis-like illness with milder symptoms (Hallander 1999; He et al. 1998; Hoppe 1999). For the differential diagnosis of the cough illnesses, the clinical diagnosis based on the symptoms should be completed with laboratory methods to assure the accurate diagnosis and to avoid the underdiagnosis of pertussis due to the unawareness of the clinicians (Greenberg et al. 2007).

During an outbreak, the clinical diagnosis of pertussis is adequate when linked in time and place to a confirmed pertussis case. In practice, the patient with pertussis-like symptoms has been in close contact (such as family or school class) with a pertussis patient within three weeks prior to the symptoms. The early suspicion and diagnosis is even more important during outbreaks to limit the transmission and, finally, stop the outbreak (CDC 2000; KTL 2007; WHO 2003).

The laboratory diagnostics of pertussis rely on microbiological findings of the bacteria or the serological changes caused by the bacteria. The microbiological methods are based on culture, nucleic acid amplification by polymerase chain reaction (PCR), or direct fluorescent antibody (DFA) test (McGowan 2002; Muller et al. 1997; Tozzi et al. 2005). Due to the poor sensitivity of the DFA testing (Tilley et al. 2000), it is currently not recommended and not offered for the pertussis diagnosis in Finland. Current recommendations in Finland refer to the microbiological diagnosis by culture or PCR if the patient has coughed less than one month – after that, serological diagnosis is recommended (KTL 2007).

The specimen collection for the microbiological testing is a critical part of the diagnosis. As *B. pertussis* binds to the ciliated epithelial cells in the human nasopharynx (NP) of the upper respiratory tract (Crowcroft and Pebody 2006; Kerr and Matthews 2000; Loeffelholz

2003; Mattoo and Cherry 2005), the specimens from mouth or throat are improper. The specimen of choice is a nasopharyngeal aspiration, wash or swab. In a hospital setting, aspirates should be preferred over swabs due to the larger amount of material obtained (McGowan 2002). When taking the swab specimens, calcium alginate, Dacron or rayon swabs are recommended – cotton swabs should never be used due to their toxic effects on *B. pertussis* (Cloud et al. 2002; Loeffelholz 2003; McGowan 2002). For the optimal result, the swab has to be taken very precisely from the back of the NP (Figure 1) – it is an unpleasant experience for the patient, but the major cause of false negative results is that the swab was not taken from far enough (Cherry et al. 2005).

Culture is the basic method for the diagnosis of pertussis, and it provides isolates for the further analysis of the bacterial strain. However, its downsides are varying sensitivity and the challenges to maintain the viability of the bacteria after the specimen collection. The specimen should be placed directly on the culture plate or in a suitable transport medium – such as Bordet-Gengou or Regan-Lowe medium (Regan and Lowe 1977; Sutcliffe and Abbott 1972) – immediately after the collection. The optimum culture conditions for *B. pertussis* are in ambient air at 35°C with high humidity, elevated CO_2 is not needed. Most colonies become visible after 3 to 4 days of incubation, but an extended incubation time as long as 12 days has been shown to improve the sensitivity by 16% (Katzko et al. 1996). However, usually laboratories answer the results after 7 days unless earlier defined positive (HUSLAB 2008; UTULab 2008). Bacterial culture is recommended for the public health purposes to obtain isolates for the epidemiological strain typing, monitoring of emerging antigenic variants, and long-term follow-up of the antimicrobial resistant strains and the bacterial genome, even though its role as a sole diagnostic tool has lessened (KTL 2007; Mooi et al. 2000).

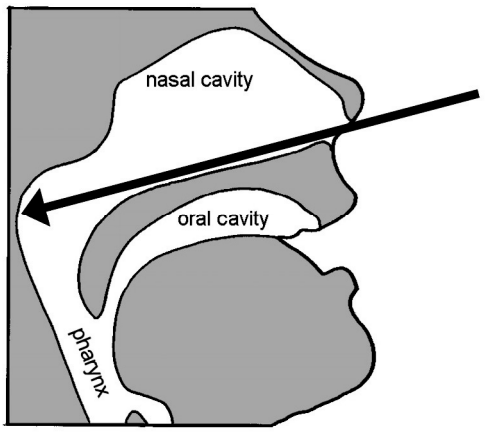

Figure 1. ***The nasopharyngeal specimen should be taken by passing the swab through the nostril until it touches the lining of the nasopharynx (arrow).***

Nucleic acid amplification by PCR is a fast and sensitive test for the diagnosis of pertussis. Its advantages over culture are detecting bacterial fragments and dead bacteria in addition to the viable ones and the ability to amplify the target genes. These features increase the sensitivity significantly when testing patients already treated with antibiotics (Edelman et al. 1996). If the PCR is used for the diagnosis, the specimen is defined as positive or negative usually within a day or even in hours. The method is based on the amplification of a defined region of the genome (Boehm 1989). The amplified region is defined by primers, which are oligonucleotides designed to recognize specific target sequences in the genome of the defined organism. In addition to the primers, specific probes may be included in the amplification reaction to confirm the amplified products and to distinguish the different alleles of the genes. In the case of *B. pertussis*, the target sequences mostly used in the diagnosis are insertion sequence element 481 (IS481) and the promoter region of the gene coding for pertussis toxin (PT) (Fry et al. 2004; Muyldermans et al. 2005; Riffelmann et al. 2005). However, the IS481-based PCR has a disadvantage of not being able to differentiate *B. pertussis* from *B. holmesii* and *B. bronchiseptica*, because IS481-like sequences are found in their genomes (Reischl et al. 2001). Still, as the two species rarely cause pertussis-like symptoms in humans, the IS481 specific PCR may well be used for the diagnosis of *B. pertussis* (Antila et al. 2006). The downsides of PCR are that the false positive results may occur due to contamination or unspecificity (Lievano et al. 2002), and the method has not yet been standardised.

Serology is rarely useful for the diagnosis of pertussis in the acute phase. However, serological methods are practical for epidemiological purposes and for confirmation of the diagnosis in adolescents and adults (Cherry et al. 2005). Serological tests are based on the detection of the antibodies that are induced by the exposure to the bacteria. Enzyme immunoassays (EIA) are currently the methods of choice because they are sensitive and easy to perform in microwell plate formats (Loeffelholz 2003). The levels of immunoglobulins A, G and M (IgA, IgG and IgM) to different virulence factors such as pertussis toxin (PT), filamentous hemagglutinin (FHA) or pertactin (Prn) can be measured with different EIAs. The results of the serological anti-pertussis antibody assays are, however, difficult to interpret, because both pertussis infection and vaccination can cause similar IgG and IgM antibody responses. The formation of IgA antibodies in patient serum sample refers to a natural infection. The greatest specificity is achieved by the measurement of IgG and IgA antibodies to PT (Ward et al. 2006). Paired sera including the pre-infection or acute-phase sera and the convalescent-phase sera showing the increase of the anti-pertussis antibodies would be the most reliable method for the serological diagnosis. Significant antibody increase between the two sera should be demonstrated, but high levels of IgG or IgA in a single serum sample may also indicate pertussis in adolescents and adults, who have not been immunised against pertussis since childhood, and thus, are not expected to have high levels of anti-pertussis antibodies without the infection. However, the pertussis booster vaccinations of adults and adolescents make the serological diagnosis even more difficult to interpret (Cherry et al. 2005; Locht 2007; Loeffelholz 2003).

2.1.3 Treatment

Symptomatic treatment for the cough caused by pertussis has been systematically reviewed (Pillay and Swingler 2003). However, cough reducing agents such as corticosteroids, antihistamines and salbutamol were not found to be effective on the coughing spells caused by pertussis. Furthermore, due to their potential side effects, they are not recommended.

Current recommendations for the treatment of pertussis include azithromycin and clarithromycin – or sulfa/trimethoprim in the case of macrolide allergy – for the patients who have had cough less than three weeks (KTL 2007). After that, the antibiotics have no effect on the recovery from the disease. Recent review showed that the treatment with antibiotics is effective in eliminating *B. pertussis* from the NP and thus limiting the transmission of the disease, but do not have significant effect on the clinical course of the disease (Altunaiji et al. 2007). This may be due to the delayed treatment because of the delayed diagnosis. Thus, the antibiotic treatment should be considered on the basis of a clinical diagnosis already before the laboratory confirmation (Tozzi et al. 2005). Antibiotic susceptibility testing of *B. pertussis* is not routinely performed, because macrolide-resistant strains have very seldom been reported (Bartkus et al. 2003; Korgenski and Daly 1997).

Antibiotic prophylaxis is recommended for family members and close contacts of a diagnosed pertussis patient if there is an infant less than 6 months of age or a pregnant mother (gestational week over 36) in the family (KTL 2007). Even though there is insufficient evidence to justify the prophylactic treatment of pertussis contacts (Altunaiji et al. 2007), it is recommended due to the high risk of morbidity and mortality in infants less than 6 months of age who are incompletely immunised. In the case of an outbreak at a nursery school, school or a working community, the risk groups and symptomatic cases should be identified and treated. The symptomatic patients that have coughed for less than three weeks should stay at home for five days since the beginning of the treatment to prevent the transmission of the disease (KTL 2007).

As explained above, the symptomatic treatment and the antibiotics are not effective during the convalescent phase. The treatment of severe cases in hospital is most important for infants who may suffer from dehydration and malnutrition because of the post-tussive vomiting and their inability to eat (Hewlett and Edwards 2005; Tozzi et al. 2005).

2.2 Genus *Bordetellae*

Bordetellae are strictly aerobic Gram-negative coccobacilli and β-proteobacteria within the family Alcaligenaceae. Nine species are known: *B. pertussis*, *B. bronchiseptica*, *B. parapertussis*, *B. avium*, *B. hinzii*, *B. holmesii*, *B. trematum, B. petrii* and *B. ansorpii* (Locht 2007). *B. parapertussis* is further divided to two subspecies by their hosts: *B. parapertussis*$_{hu}$ of the human host and *B. parapertussis*$_{ov}$ of the sheep host. The first report of whooping cough epidemic was given by de Baillou, who described it in Paris in 1578. The modern history of whooping cough starts in the beginning of the 20th century: in 1904, Bordet and Gengou were able to culture the bacterium causing whooping cough, and they published their findings in 1906 (Gerlach et al. 2001). The classification of the

Bordetella species has been eventful. For example, the name for the species *B. bronchiseptica* was changed five times – *Bacillus bronchicanis* (1910), *Bacterium bronchisepticus* (1913), *Alcaligenes bronchisepticus* (1925), *Brucella bronchiseptica* (1929), *Alcaligenes bronchicanis* (1935) and *Haemophilus bronchisepticus* (1946) – before the genus *Bordetellae* and the current name was described in 1952 (Goodnow 1980).

The most widely studied species are *B. bronchiseptica*, *B. pertussis* (Figure 2) and *B. parapertussis*. *B. bronchiseptica* is suggested to be the evolutionary ancestor of the other two (Bjornstad and Harvill 2005; Parkhill et al. 2003). Most *Bordetellae* have been isolated from humans: some of them are very strictly human pathogens (*B. pertussis*, *B. parapertussis*$_{hu}$) but others have a wider host range including both animals and humans (*B. bronchiseptica, B. hinzii*). Only one member of the genus, *B. petrii*, has been isolated from the environment and has shown ability to grow anaerobically (von Wintzingerode et al. 2001). The genomes of *B. pertussis*, *B. parapertussis*$_{hu}$, *B. bronchiseptica*, *B. petrii* and *B. avium* have been sequenced (Table 1) (Parkhill et al. 2003; Preston et al. 2004; Sebaihia et al. 2006; von Wintzingerode et al. 2001).

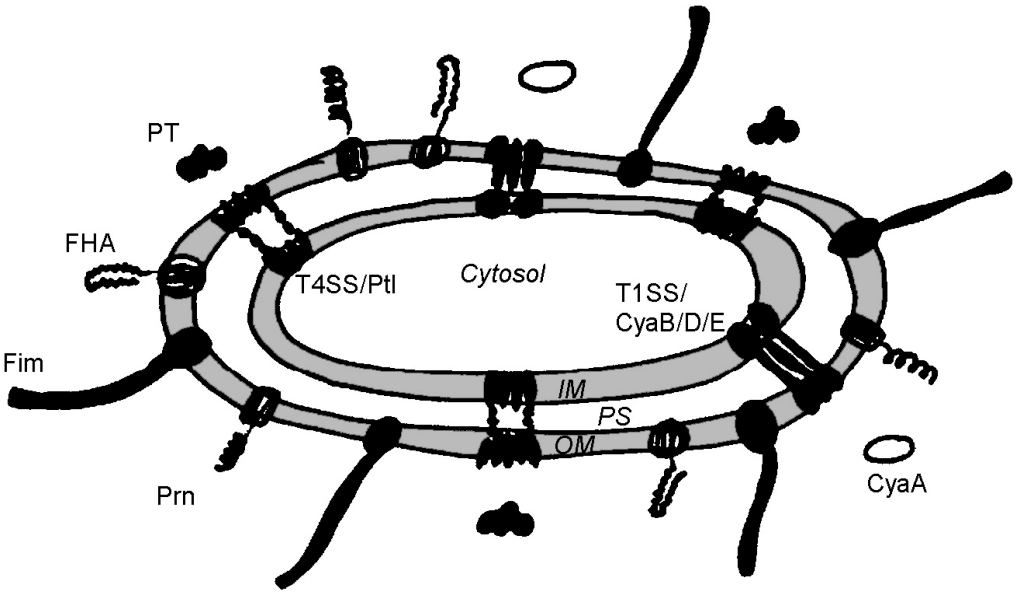

Figure 2. ***Simplified schematic presentation of Bordetella pertussis with inner and outer membranes (IM and OM), periplasmic space (PS) and cytosol. The virulence factors shown in the figure: adenylate cyclase toxin (CyaA), filamenteous hemagglutinin (FHA), fimbria (Fim), pertactin (Prn) and pertussis toxin (PT). Type I and IV secretion systems for CyaA and PT are shown (T1SS/CyaB/D/E and T4SS/Ptl). Modified from (Locht et al. 2001).***

2.2.1 Human pathogens

B. pertussis, *B. parapertussis*$_{hu}$, *B. hinzii*, *B. holmesii*, *B. trematum*, *B. petrii* and *B. ansorpii* have been isolated from humans (Locht 2007). *B. bronchiseptica* is mainly an animal pathogen still capable of zoonotic infection in human, but a human-associated lineage of the species has also been identified (Diavatopoulos et al. 2005). This chapter will concentrate on the other human pathogens of the genus, and *B. bronchiseptica* and *B. pertussis* will be discussed later.

The host specificity of *B. parapertussis* is quite unique: it appears in humans and in sheep, with two subsets of strains – *B. parapertussis*$_{hu}$ and *B. parapertussis*$_{ov}$ – that only attack either human or sheep, respectively (van der Zee et al. 1997). The host adaptation of *B. parapertussis* is suggested to interlock with the inactivation and loss of genes compared to its suggested multi-hosted progenitor, *B. bronchiseptica* (Brinig et al. 2006b). As *B. pertussis*, *B. parapertussis*$_{hu}$ first described in 1937 and thoroughly reviewed by Hoppe (Hoppe 1999) can cause whooping cough in humans but with milder symptoms. One study in Finland among the whooping cough patients show that as much as 32% of the cases are caused by *B. parapertussis*$_{hu}$, and in addition to that, 7% of the patients suffer from mixed infections with both *B. pertussis* and *B. parapertussis*$_{hu}$ (He et al. 1998). Another commonality is the presence of insertion element IS1002; it is found in *B. pertussis* and *B. parapertussis*$_{hu}$, but not in *B. parapertussis*$_{ov}$ (van der Zee et al. 1997). The main differences between *B. pertussis* and *B. parapertussis*$_{hu}$ are the lack of PT production and the presence of insertion element IS1001 – characteristics that have been used for the differential diagnostics of the two (Fry et al. 2004; Lind-Brandberg et al. 1998). In addition, the structure of lipopolysaccharide (LPS) of *B. pertussis* differs from *B. parapertussis* (Parkhill et al. 2003). It seems that the immunity induced by *B. parapertussis*$_{hu}$ infection also protects from *B. pertussis* infection, but surprisingly, the cross-immunity is asymmetrical as immunity caused by *B. pertussis* does not prevent *B. parapertussis*$_{hu}$ infections (Wolfe et al. 2007).

B. hinzii was first isolated from poultry and humans (Vandamme et al. 1995). The isolates from chicken were found in respiratory tracts, but the human isolates were from sputum and blood culture. Human infection of *B. hinzii* is rare, and it is believed to be an opportunistic pathogen.

B. holmesii is another recently found opportunistic human pathogen. In the first publication, *B. holmesii* was isolated from blood cultures of patients from the United States, Saudi Arabia and Switzerland (Weyant 1995). Later it has been isolated from blood, pleural fluid and sputum as well as NP specimens in several countries such as UK, Australia and Germany (Dorbecker et al. 2007; Greig et al. 2001; Russell et al. 2001). The genome of *B. holmesii* was found to contain IS481 and IS1001. Thus, *B. holmesii* can be a possible factor causing unspecificity in the diagnostic PCR based on IS481 for *B. pertussis* or IS1001 for *B. parapertussis*, which cannot distinguish *B. holmesii* from the other two species (Reischl et al. 2001). However, a large study of clinical specimens submitted for the diagnosis of *B. pertussis* and *B. parapertussis* suggested that *B. holmesii* does not substantially confound the diagnostics in practice, as it was not detected in any of the analysed samples from the patients with pertussis-like symptoms (Antila et al. 2006).

Table 1. ***Genomes of the sequenced species of Bordetellae.***

	B. pertussis	***B. parapertussis*$_{hu}$**	***B. bronchiseptica***	***B. petrii***	***B. avium***
Host	human	human	mammals	environmental	avian
Strain	Tohama I	12822	RB50	DSM 12804	197N
GenBank	BX470248	BX470249	BX470250	AM902716	AM167904
Genome size (bp)	4 086 189	4 773 551	5 339 179	5 287 950	3 732 255
GC content	67%	68%	68%	65%	61%
Genes (N)	3 816	4 404	5 007	?	3 417
Coding percentage (%)	82	86	91	90	88
Pseudogenes	358	217	12	4	40
References	Parkhill, GenBank	Parkhill, GenBank	Parkhill, GenBank	GenBank	Sebaihia, GenBank

Eleven isolates of *B. trematum* have been described (Daxboeck et al. 2004; Vandamme et al. 1996), most of them in ulcers of the extremities. The pathogenic significance of the species is unknown, and as *B. hinzii* and *B. holmesii*, it is considered as an opportunistic pathogen. Only two isolates of *B. ansorpii* have been reported. The first was isolated in Korea (Ko 2005) from an epidermal cyst of a 19-year-old woman, and the second from a blood culture of an 88-year-old man in UK (Fry et al. 2007). They both were treated for cancer at the time of the isolation, and thus *B. ansorpii* is also perceived as an opportunistic pathogen.

B. petrii is the first and only species of the genus isolated from the environment (von Wintzingerode et al. 2001). It was found in an anaerobic bioreactor. Later, it has been isolated from humans in UK and Australia (Fry 2005; Stark et al. 2007).

2.2.2 Species with other hosts

B. bronchiseptica, *B. parapertussis*$_{ov}$, *B. avium*, *B. hinzii*, and *B. petrii* have been found in either animals or environment. As *B. hinzii* and *B. petrii* are discussed above, this chapter will concentrate on the other three species.

B. bronchiseptica is recognised as the progenitor of the other *Bordetella* species causing respiratory diseases in numerous mammals such as swine, cats and laboratory animals (Goodnow 1980). Human infections caused by *B. bronchiseptica* are rare and mostly detected in severely compromised hosts (reviewed in Woolfrey and Moody 1991). The wide host range of *B. bronchiseptica* may be explained by its large gene repertoire compared to *B. pertussis* and *B. parapertussis* (Parkhill et al. 2003). Another difference between *B. bronchiseptica* and the other two is that *B. bronchiseptica* can cause chronic

infections, whereas the host usually clears *B. pertussis* and *B. parapertussis* (Preston et al. 2004). There is a notable disease burden of *B. bronchiseptica* among the livestock, companion and laboratory animals (Goodnow 1980) and vaccines have been developed to protect animals against it (Mann et al. 2007; Sekiya et al. 1983). However, there seem to be differences between the vaccine-induced and infection-induced immunity against *B. bronchiseptica* as the natural infection prevents subsequent infections better than vaccination (Gopinathan et al. 2007).

B. parapertussis$_{ov}$ is in many ways different from its human-associated sibling (Porter et al. 1996; van der Zee et al. 1996a). Two different evolutionary paths have been suggested: (i) *B. parapertussis*$_{ov}$ evolved from *B. bronchiseptica* earlier than *B. parapertussis*$_{hu}$ inferring to two separate *B. bronchiseptica*-like ancestors (van der Zee et al. 1996a), or (ii) *B. parapertussis*$_{ov}$ and *B. parapertussis*$_{hu}$ evolved from an unidentified common ancestor between them and *B. bronchiseptica* (Brinig et al. 2006b). The host adaptation of *B. parapertussis* is seen in the genome, transcriptional, and disease level. Based on a microarray analysis and an inoculation study on sheep, the genome of the ovine-associated subset is larger than the human-associated one, sequence differences exist, several toxins and virulence factors are differently regulated in the two subsets, and the persistence of *B. parapertussis*$_{ov}$ bacteria in the nasal cavity is significantly longer lasting and on a higher level than that of *B. parapertussis*$_{hu}$ (Brinig et al. 2006b).

B. avium causes a respiratory disease called bordetellosis in avian species. It was isolated from the respiratory tracts of turkeys in the 1970s and finally classified in 1984 (Kersters 1984). The genome of *B. avium* has been sequenced, and it was perceived having the smallest genome and being the most distant of the sequenced *Bordetellae*, still having 1100 genes absent in *B. bronchiseptica* (Sebaihia et al. 2006). *B. avium* being an avian pathogen and *B. bronchiseptica* a mammal pathogen, the genetic differences are most likely related to the host adaptation. Interestingly, *B. avium* has no genes for PT and adenylate cyclase toxin (CyaA), which are often attributed to the symptoms of *Bordetellae*-caused illnesses (Sebaihia et al. 2006). Thus, the cause of pathology and clinical symptoms of bordetellosis is still untangled.

2.2.3 Genetics and genomics

Whereas microbiology studies the microscopic forms of life, molecular biology is a branch of biology studying the molecular basis of inheritance and protein synthesis. Genetics deals with the heredity and variation of organisms, and further, molecular genetics studies the structure and activity of genetic material at the molecular level. Genome, however, is a functional unit composed of chromosomes, genes, pseudogenes resembling the sequences of known genes but lacking a genetic function, and DNA sequences with unknown function (Blundell 2007). Genomics, again, is a technology that applies the techniques of genetics and molecular biology to the genetic mapping and DNA sequencing of the genes or genomes of selected organisms (2008).

Genetic modification of *B. pertussis* by plasmid transfer was published in 1982 (Weiss and Falkow 1982). Four years later, PT was the first virulence-associated gene of *B. pertussis* being sequenced (Locht and Keith 1986; Nicosia et al. 1986). Several molecular techniques

such as multilocus enzyme electrophoresis and sequence typing (Diavatopoulos et al. 2005; van der Zee et al. 1997) and pulsed-field gel electrophoresis (Khattak and Matthews 1993) have been used when trying to elucidate the molecular evolution of *B. pertussis*. Since the genome sequence of *B. pertussis* was revealed in 2003 (Parkhill et al. 2003), exceeding amount of research has been done for the understanding of the relationship between the genetics and pathogenesis of the bacteria. The most modern technique utilising the genome data of *B. pertussis* is the comparative genomic hybridisation by DNA microarray (Brinig et al. 2006b; Caro et al. 2006; Cummings et al. 2004; Diavatopoulos et al. 2005; Heikkinen et al. 2007). The methodology for the strain typing and molecular epidemiology is further discussed in chapter 2.6.

The comparative analysis of the genome sequences of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* suggests that *B. pertussis* evolved from *B. bronchiseptica* and adapted to the human host through a loss and rearrangements of genes (Parkhill et al. 2003). The number of pseudogenes increased and insertion sequence elements (ISEs) appeared in the genome of *B. pertussis* (Parkhill et al. 2003). Further, many genes lost their functioning ability because of the pseudogene formation and ISEs (animated figure in Friesen and Woolridge 2005); e.g. the non-motile nature of *B. pertussis* is due to the pseudogenes and ISEs within the flagellar operon coding for the flagella, the organ of motion of many microorganisms such as *B. bronchiseptica* (Parkhill et al. 2003; West et al. 1997). Virulence increasing genomic changes appeared in the promoter region of PT coding operons, many of them at the BvgA binding sites (Parkhill et al. 2003). The sequence is conserved in *B. parapertussis* and *B. bronchiseptica*, which do not produce PT, and mutations appear in *B. pertussis* producing PT (Parkhill et al. 2003). Another *B. pertussis* specific change occurred in the locus for the biosynthesis of O-antigens (polysaccharide side chains) that are involved in the evasion of host immunity mechanisms (reviewed in Wang et al. 2008). Unlike *B. parapertussis* and *B. bronchiseptica*, *B. pertussis* does not express O-antigen due to the ISEs in the O-antigen biosynthesis locus (Parkhill et al. 2003).

2.2.4 Lipopolysaccharides

Lipopolysaccharides (LPSs) are components of the outer membrane of Gram-negative bacteria such as *B. pertussis*. They consist of a hydrophobic lipid backbone known as lipid A and an oligosaccharide core. In many species, an external polysaccharide side chain referred as O-antigen is also present (Raetz and Whitfield 2002). The LPSs are characterised as smooth and rough by the presence and absence of the O-antigen, respectively (Hitchcock et al. 1986). LPSs are responsible for the severe sepsis and septic shock during the infection of Gram-negative bacteria by the Toll-like receptor 4 (TLR4)-mediated inflammatory response (Palsson-McDermott and O'Neill 2004; Raetz and Whitfield 2002).

In the case of *Bordetellae*, the LPSs differ between *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*: unlike the other two, *B. pertussis* LPS does not contain O-antigen (Parkhill et al. 2003), which may explain the poor cross-immunity between *B. pertussis* and *B. parapertussis* (Wolfe et al. 2007). Similar variation on the LPS structure within the genus has been shown in the genus Yersinia, where the expression of the LPS O-antigen is

detected in some species such as *Y. enterocolitica* but not in others such as *Y. pestis* (reviewed in Skurnik and Bengoechea 2003).

The LPSs have shown to be essential for the virulence of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* (Harvill et al. 2000). Further, the oligosaccharide core of *Bordetellae* LPSs seems to protect the bacteria against the clearance of infection by the pulmonary surfactant proteins responsible for the first innate immune response in the respiratory tract (Schaeffer et al. 2004). The expression of LPS in *B. pertussis* is dependent on the environmental conditions such as temperature rather than phase variation, which is an important strategy to evade the immune system of the host in many other Gram-negative pathogens (Lukácová et al. 2008; van den Akker 1998).

2.2.5 Secretion systems from type I to VI

The Gram-negative bacterial pathogens secrete proteins across the inner membrane, periplasm and outer membrane to the extracellular space or into the host cells via several secretion systems; six of those have been described and named as type I to VI secretion systems (T1SS to T6SS) (Economou et al. 2006). Within these secretion systems, proteins are translocated across the inner membrane in the unfolded form via the general secretion route, Sec-pathway, or in the folded form via the twin-arginine translocation, Tat-pathway (Natale et al. 2007).

Via direct secretion by T1SS, CyaA is secreted from the bacterial cytoplasm to the extracellular space. The T1SS for CyaA secretion is composed of three proteins, CyaB, CyaD and CyaE (Figure 2, page 18) (Glaser et al. 1988). T2SS is a Sec-dependent pathway, where the proteins are first taken to the periplasm and then fold before becoming secreted through large channels formed in the outer membrane by proteins termed secretins (Economou et al. 2006). This secretion system is, however, absent in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, but present in *B. avium* (Sebaihia et al. 2006).

T3SS is a complex export structure delivering the effector proteins directly from the bacterial cytoplasm into the host cell cytosol and thus altering the function of the host cell. The gene encoding T3SS has been identified in *Bordetellae* (Parkhill et al. 2003), but the protein expression of T3SS in *B. pertussis* was only recently presented (Fennelly et al. 2008). It was shown that a functional T3SS is expressed by the clinical *B. pertussis* isolates but not by the laboratory strains suggesting that the long-term laboratory culture has significantly changed the gene expression and the protein production. T3SS was also shown to participate in the bacterial colonisation and survival in the host by targeting the innate immune system. The role of T3SS in the host-pathogen interaction has been widely studied among species other than *Bordetellae* (Stavrinides et al. 2008). Both in animal models and in human infection, T3SS activity is well correlated with the infection progression and outcome (Coburn et al. 2007). Thus, the recent findings of the functionally active T3SS in *B. pertussis* by Fennelly and colleagues (Fennelly et al. 2008) will improve our understanding of the immune responses to *B. pertussis* infection and especially to this important immunomodulator and virulence factor. (Galán and Collmer 1999)

PT is secreted via T4SS named as pertussis toxin liberation (Ptl) composed of nine proteins (Figure 2, page 18) (Craig-Mylius and A. Weiss 1999). PT is assembled from the subunits in the periplasmic space, and the Ptl proteins are predicted to form a large complex spanning both the inner and outer membranes. It seems that the Ptl T4SS mediates the secretion of PT to the extracellular space, but is not involved in the host cell translocation of PT. (Backert and Meyer 2006)

T5SS is the simplest and most common of the secretion systems. Two Sec-dependent T5SS pathways are known: the autotransporter (AT) pathway and the two-partner secretion (TPS) pathway. In the AT system, the protein itself mediates the secretion across the outer membrane. Among the *Bordetella* AT proteins, Prn is the most widely studied, and in fact, suggested as the archetype of the AT proteins of all bacteria (Junker et al. 2006). In the TPS, an additional pore-forming transporter protein is required for the secretion. A *Bordetella* virulence factor, FHA, is used as a generic model for the TPS pathway (Hodak et al. 2006). (Henderson et al. 2004; Kostakioti et al. 2005)

T6SS is the most newly described secretion system, and it has been proposed to form an apparatus that punctures the target cell membranes delivering the bacterial effector proteins directly to the target cell cytosol (Pukatzki et al. 2007). The role of T6SS in *B. pertussis* is not yet known, but the T6SS gene clusters were recently found in *B. bronchiseptica* and *B. parapertussis* (Bingle et al. 2008).

2.3 *Bordetella pertussis*

2.3.1 Microbiology

The bacterium causing whooping cough, later named as *B. pertussis* (Figure 2, page 18), was first cultured by the scientists Jules Bordet and Octave Gengou in the beginning of the 20th century. In 1909, a thorough study of the bacterium was published by Martha Wollstein – the first female member of the American Pediatric Society. She summarises the findings of Bordet and Gengou: "The *Microbe de la Coqueluche* is short, poled, ovoid, a trifle larger than *Bacillus influenzae* [today called *Haemophilus influenzae*], not motile, very regular in size and shape, arranged singly within groups, occasionally in pairs, either end to end or parallel. It is negative to Gram's stain, but colors especially well with toluidine-methylene blue". In addition, she already reported that "the bacillus of Bordet and Gengou is present in the sputum in early cases of pertussis, and in the lungs at autopsy in fatal cases of the disease. After the second week it is not present in the sputum in sufficiently large numbers to be readily isolated." (Wollstein 1909)

Human is the sole host and reservoir of *B. pertussis*. It is the most fastidious of the *Bordetellae* being inhibited by several agents such as fatty acids, metal ions and peroxides. Thus, it needs a special culture medium containing protective agents such as charcoal, blood or starch. Cephalexin is often added to the culture medium to suppress the growth of other bacteria. The most commonly used media are Bordet-Gengou (Sutcliffe and Abbott 1972) and Regan-Lowe (Regan and Lowe 1977), where *B. pertussis* grows as round, mercury-silver colored and shiny colonies. In addition, *B. pertussis* shows haemolytic

activity on Bordet-Gengou agar. Both *in vivo* and *in vitro*, *B. pertussis* produces virulence factors such as adhesins and toxins (Figure 2, page 18). (Crowcroft and Pebody 2006; Loeffelholz 2003)

2.3.2 Toxins

The established toxins produced by *B. pertussis* are pertussis toxin (PT), adenylate cyclase toxin (CyaA), dermonecrotic toxin (DNT) and tracheal cytotoxin (TCT). CyaA, DNT and TCT are expressed in *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. PT is only expressed in *B. pertussis* even though the genes are present in all three species.

There is wide variation in the roles of the toxins in the infection and virulence of *B. pertussis*. PT is the only one of the four toxins known to contribute to the attachment of the bacterium to the epithelium of the host. PT, CyaA and DNT cause systemic effects; PT, CyaA and TCT have a role in the evasion of the host defence. In addition, all four toxins induce local effects on the respiratory epithelium of the host. The two most widely studied toxins PT and CyaA are discussed below (Figure 2, page 18). (Kerr and Matthews 2000; Preston 2005)

The PT gene, *ptx*, consists of genes for five subunits, S1-S5, forming a hexameric structure. The subunits are encoded by the genes *ptx*A to *ptx*E, respectively, and their expression is regulated via the promoter *ptx*P. The S1 subunit contains the catalytically active site, and the binding site consists of two dimers, S2S4 and S3S4, joined by S5 (Tamura et al. 1982). The genetic alteration of the S2 and S3 suggest that the two subunits can substitute for each other to a certain degree, but their main biological activities are different. If S3 is replaced by S2, the toxin production is significantly enhanced; if S2 is replaced by S3, the ADP-ribosylation activity of the toxin is increased (Raze et al. 2006). PT is a soluble 106-kDa toxin secreted to the extracellular space by T4SS named as Ptl (Backert and Meyer 2006). Both the *ptx* and *ptl* genes are located in the same operon and expressed from the same *ptx*P promoter enabling the efficient production and secretion of PT (Kotob et al. 1995).

PT has an early and wide yet not thoroughly defined role in the *B. pertussis* infection. The systemic lymphocytosis has long been known to be caused by PT (Munoz et al. 1981). One of PT's molecular mechanisms of action is the ADP-ribosylation of G-proteins, which are signal transducers between extracellular signals and intracellular events (Kaslow and Burns 1992). Recently, PT has been shown to be an important colonisation factor and to target the airway macrophages, the phagocytic white blood cells responsible for the antigen presentation, as one of the earliest events in establishing the infection (Carbonetti et al. 2007). Due to the protective role of anti-PT antibodies, inactivated PT is included in all currently available acellular pertussis vaccines either alone or combined with other antigens (WHO 2005b).

CyaA is a single 200-kDa polypeptide secreted by T1SS (Bellalou et al. 1990) and composed of two protein moieties: an adenylate cyclase enzyme and cytolysin (Glaser et al. 1988). It has a dual role in manipulating the physiology of the host cell as it intrudes into both the cellular signalling pathways and the ion homeostasis of the host cell. After the CyaA is secreted, it binds to the target cell membrane in two conformational isoforms with

different functions: the membrane translocator promotes the release of the adenylate cyclase domain to the target cell cytosol, and the cation-selective pore composed of two CyaA molecules represents the cytolysin activity of CyaA (Basler et al. 2007). CyaA is known to target neutrophils, phagocytic cells that migrate to the site of infection (Harvill et al. 1999). It is suggested that CyaA acts in proximity to the bacteria producing it – contrary to the freely soluble PT – and that it intoxicates the neutrophils on the site of infection: in the early stage of *B. pertussis* infection the influx of neutrophils is delayed by PT, and after that, CyaA promotes the infection by depleting the neutrophils (Carbonetti et al. 2005). The role of CyaA and its mechanisms of action are well reviewed by Jana Vojtova and colleagues (Vojtova et al. 2006).

2.3.3 Adhesins

Bacterial components mediating the adherence of the bacteria to the host are called adhesins. They are often located on the surface of the bacteria, and are important for the attachment and colonisation. The main adhesins of *B. pertussis* are filamentous hemagglutinin (FHA) (Kimura et al. 1990), fimbria (Fim) (Blom et al. 1983) and an autotransporter protein pertactin (Prn) (Charles et al. 1989). Other autotransporters – proteins that mediate their secretion through the outer membrane themselves – such as tracheal colonisation factor (TCF) (Finn and Stevens 1995; van Gent et al. 2007), BrkA (Fernandez and Weiss 1994) (encoded by *brkA* gene in the locus called the *Bordetella* resistance to killing, *brk*) and Vag8 (Finn and Amsbaugh 1998) (encoded by *vir*-activated gene-8, *vag-8*) have also shown adhesive properties. The three most widely studied adhesins FHA, Fims and Prn are discussed below (Figure 2, page 18).

FHA is suggested to act as an immunomodulatory and immunosuppressive agent, and it is considered to be the most important factor in the attachment of *B. pertussis* (reviewed in Locht 2007). It is synthesised as a 367 kDa precursor encoded by the *fhaB* gene, and secreted as a monomeric 220 kDa protein to the outer membrane and extracellular space (Coutte et al. 2003; Domenighini et al. 1990; Kimura et al. 1990; Locht et al. 1993). FHA is secreted by a T5SS/TPS pathway (Henderson et al. 2004; Hodak et al. 2006). Several different binding motifs of FHA refer to its important role in the adhesion, and the secretion of free FHA appears to contribute to the bacterial colonisation (Coutte et al. 2003). In addition, FHA has been shown to be a crucial factor in the biofilm formation of *B. bronchiseptica* (Irie et al. 2004; Irie et al. 2006) – biofilm formation of *B. pertussis* has been described (Serra et al. 2007) but the roles of virulence factors have not yet been elucidated. Besides PT, FHA is the primary component of the acellular pertussis vaccines (Mattoo and Cherry 2005; WHO 2005b).

Fims are filamentous cell surface structures used by the bacteria to adhere to one another and to the host cells. They are composed of a minor 40-kDa subunit, FimD, and a major subunit, 22-kDa Fim2 or 22,5-kDa Fim3, corresponding to the serotypes Fim2 and Fim3, respectively; serotype Fim2.3 produces both Fim2 and Fim3 fimbriae (Blom et al. 1983; Robinson et al. 1989). The subunits FimD, Fim2 and Fim3 are encoded by *fimD*, *fim2* and *fim3* genes, respectively, and play their own roles as adhesins (Geuijen et al. 1997; Geuijen et al. 1996; Willems et al. 1993). The serotype expressed by the bacteria depends on the

phase-variable expression of *fim2* and *fim3* on the level of transcription; this regulatory mechanism is called phase variation (Willems et al. 1990). In addition to *fimD*, *fim2* and *fim3*, a silent fimbrial subunit gene *fimX* appears in the *B. pertussis* genome (Pedroni et al. 1988). Besides the adhesive activities, Fims are involved in the immune interference of the host as an anti-inflammatory agent (Vandebriel et al. 2003). Despite Fim being an important virulence factor of *B. pertussis*, it is not included in most of the acellular vaccines (Jadhav and Gairola 1999).

Prn, also called as a P.69 protein, is an autotransporter 69-kDa protein located on the outer membrane of *B. pertussis* (Charles et al. 1989; Gotto et al. 1993; Junker et al. 2006). It is synthesised as a 93,5-kDa precursor, and secreted to the outer membrane by an autotransporter pathway of T5SS (Henderson et al. 2004; Junker et al. 2006; Kajava and Steven 2006). Prn has been shown to act as an adhesin (Leininger et al. 1991). Even though the mechanisms of action and the role of Prn during the infection are not yet fully understood, its role as a vaccine component is well justified: the opsonic anti-Prn antibodies were found to be essential for the antibody-mediated phagocytosis of *B. pertussis* (Hellwig et al. 2003).

2.3.4 Regulation of virulence factors

The expression of the virulence factors is mainly regulated via the *Bordetella* virulence gene (*bvg*) locus encoding a two-component gene regulatory system BvgAS and a Bvg-activated repressor protein BvgR. The two-component BvgAS system consists of a 135-kDa sensor protein BvgS and a 23-kDa activator protein BvgA, both appearing as dimers. BvgS in the inner membrane senses the environmental changes such as temperature or osmolarity and transfers the signals to the cytosolic BvgA via a complex phosphorelay (Cotter and Jones 2003). The phase variation and phenotypic modulation between the virulent (Bvg^+), the avirulent (Bvg^-) and intermediate (Bvg^i) phases of *B. pertussis* is controlled by BvgAS. (Beier and Gross 2006)

BvgAS is used by the bacteria to change the gene expression in different environmental niches according to the host and the stage of the infection. The expression levels of at least four classes of genes are dependent on the Bvg-phase enabling the temporal regulation of the virulence factors to promote the infection and to avoid the host defence. BvgAS positively regulates the expression of the virulence-activated genes (*vag*) such as PT and FHA and negatively regulates a set of virulence-repressed genes (*vrg*) whose function is unknown. The BvgAS-mediated regulation of the *vrg* genes involves the activation of expression of the 34-kDa BvgR. The *vag* encoded virulence factors are further classified as early (e.g., FHA), late (e.g., CyaA) or intermediate (BipA, see ref. Williams et al. 2005) based on their expression kinetics. (Beier and Gross 2006; Veal-Carr and Stibitz 2005)

BvgAS is autoregulated in the transcription level (Williams and Cotter 2007), and the autoregulation is suggested to function through the metabolism of cysteine (Bogdan et al. 2001). The growth phase of the bacteria and the availability of nutrients – especially glutamate – are sensed and signalled via BvgAS (Nakamura et al. 2006). Another two-component gene regulatory system, regulator of intracellular response (*ris*) locus encoding RisAS has been found; it is suggested to regulate the *vrg* genes as an activator of

expression but its role in the virulence regulation needs further investigation (Stenson et al. 2005).

BvgAS independent and virulence affecting regulation systems are used in the iron transport systems. *Bordetellae* have developed three distinct systems utilising either the native alcaligin siderophore, the enterobactin xenosiderophore or heme for retrieving iron from the environment. All three systems are controlled by different transcriptional activators and up-regulated in the presence of the optimal iron source, but the temporal and spatial functioning abilities in different hosts and regions of the respiratory tract as well as different stages of the infection are not known. As iron is necessary for bacterial growth, the ability to detect and respond to different iron sources is important for bacterial replication during the infection. (Brickman et al. 2007)

2.4 Pertussis vaccination and immunity

Pertussis is a vaccine-preventable disease, and infant immunisation programmes have been successful worldwide in reducing the morbidity and mortality of pertussis. Pertussis vaccinations were introduced in many countries already in the 1940s and 1950s. Since 1974, pertussis vaccine has been recommended by WHO. The vaccination against pertussis was estimated to avert almost 40 million cases and 607 000 deaths worldwide in 2003 (WHO 2005b).

2.4.1 Pertussis vaccines

There are two kinds of pertussis vaccines: whole-cell (wP) and acellular (aP) vaccines. The former contains suspension of inactivated *B. pertussis* bacteria, whereas the latter contains purified antigens of *B. pertussis* such as PT, FHA and Prn. Both wP and aP are usually combined with diphtheria and tetanus toxoids (DTwP or DTaP). Recently *Haemophilus influenzae* type b (Hib), hepatitis B (HepB) and inactivated poliovirus (IPV) vaccines have also been included in the combination vaccine. The standard vaccine dose is 0,5 ml administered intramuscularly in the thigh in infants or in the deltoid muscle in older children, adolescents and adults. Contraindications to pertussis vaccine are an anaphylactic reaction or other severe adverse effect after prior administration of the vaccine, unclear or progressive neurological disorder, or febrile infection. During an epidemic period, however, the febrile infection should not be considered a contraindication. Pregnancy and severe immune deficiency may be constraints, and the need of vaccinations should be valued individually. Pertussis vaccine is recommended even after suffering from the pertussis disease. Other vaccines may be administered at the same time with pertussis vaccine if injected at a different site. (KTL 2005; WHO 2005b)

The wPs are produced by many manufacturers, and the production method and the strains used for the production vary between the manufacturers. However, each lot of wP undergoes several quality tests prior to release. These include a potency assessment and toxicity testing in mice, an opacity testing to control the number of bacteria in the vaccine, and sterility control. Many wPs contain aluminium salt as an adjuvant, an agent that stimulates the immune system and increases the response to the vaccine. Thiomersal is

usually used as a preservative. Adverse reactions such as local redness and swelling, fever and agitation are frequently associated with wP (1 in 2–10 injections). More severe reactions are less common or rare. The frequency of the local reactions tends to increase with age and the number of injections, thus the wP is not recommended for adolescents and adults. (WHO 2005b)

The fear of adverse reactions and the need for adolescent and adult immunisation led to the development of aP vaccines in the 1980s (Isomura et al. 1985). The current aPs are produced as monovalent containing inactivated PT alone, bivalent containing PT and FHA, trivalent containing PT, FHA and Prn, or polyvalent containing PT, FHA, Prn and different combinations of Fim2 and Fim3. In addition to the number of antigenic components included, there are differences in the bacterial clones used for the production, the quantity of the components, the methods of purification and detoxification, adjuvants and preservatives. The frequency of adverse reactions after primary immunisation with aPs is very low or nonexistent, but the frequency and the severity of the local reactions tend to increase with each successive aP dose. Thus, aP vaccines with reduced antigen concentrations have been developed for adolescents and adults. The costs for aP development and production are considerably higher than for wP, and that is also reflected in the price per dose. Thus, wP is still the vaccine of choice in developing countries with limited resources. (WHO 2005b)

2.4.2 Vaccination schedules

WHO regularly announces the worldwide vaccination recommendations with the primary aim to protect infants from severe vaccine-preventable diseases. In the case of pertussis, WHO recommends three primary doses with either wP or aP administered at the age of 6, 10 and 14 weeks (WHO 2005b). However, the national recommendations for the schedules and the vaccines occasionally vary and change; WHO administers a monitoring system for the vaccine-preventable diseases, and distributes the national immunisation profiles from most of the countries worldwide (WHO 2007). The global summary shows that 79% of the target population – 102 million children – worldwide was vaccinated with three doses of DTaP or DTwP in 2006; the corresponding number was as low as 20% in 1980. Still, the number of unprotected children is estimated to be over 26 million.

The Global Pertussis Initiative (GPI) composed of nearly 40 experts on the pertussis field from 17 countries was established in 2001 to gather attention to pertussis problem worldwide and to evaluate and improve the immunisation strategies for pertussis control. It has recommended all countries to include the booster doses to 4–6-year-olds, adolescents and specific groups of adults. In countries where it is economically feasible, family members and close contacts of newborns as well as adults with high risk of transmitting the infection to the vulnerable infants – such as healthcare and childcare workers – should be immunised with the boosters. (Forsyth et al. 2007)

In the USA, only aP in different combinations is used and administered at the age of 2, 4 and 6 months with two booster doses at the age of 15–18 months and 4–6 years (CDC 2008b). The first dose may be given as early as the age of six weeks, and the fourth dose as early as the age of 12 months if six months have elapsed since the third dose and the child

is unlikely to return to the clinic at age 15–18 months. In pregnancy, Tdap can be given in the 2nd or 3rd trimester if needed. Recently, adult vaccination with Tdap has also been recommended for all adults younger than 65 years of age, for healthcare personnel who work in hospitals or ambulatory care settings and have direct patient contact, and for adults such as parents, grandparents, and for childcare providers who have not received a dose of Tdap and are in contact with infants younger than one year of age (CDC 2008a).

The information on the vaccination schedules in the European countries is found in the EUVAC.NET (Glismann et al. 2001), which is a surveillance community network providing national data on the occurrence and immunisation of the vaccine-preventable diseases including pertussis in 32 European countries (EUVAC.NET 2008). The first dose of the primary vaccination is given at the age of two months in 20 of the countries listed; in the rest it is given one month later. In 19 countries out of 32, the aP is used for the primary vaccination. The vaccination programmes of eleven countries in the Northern Europe are also found in EpiNorth network (EpiNorth 2008).

In Finland, the decisions on the national vaccination schedule and the vaccines included are made by the National Advisory Board of Communicable Diseases working for the Ministry of Social Affairs and Health. The advisory board is guided by the evaluations and recommendations from KTL (KTL 2007; Rapola 2007). The wP combined with D and T toxoids manufactured by Orion Oy was introduced in the schedule in 1952. The three primary doses were given at the ages of 3, 4, and 5 months. From 1952 to 1960, "Per-Dif-vaccin" and "Per-forte-vaccin" were used (personal communication, Rose-Marie Ölander, KTL). Since 1957, "Per-Dif-Tet- vaccine" was in use and the vaccination guideline from the National Board of Health from 1957 recommended two boosters at the ages of 3–4 years and 6–7 years after the three primary doses with this vaccine (Lääkintöhallitus 1957). In the guideline from 1977, the recommendations only included the primary doses administered at the ages of 3, 4, 5 and 24 months (Lääkintöhallitus 1977). A booster at the age of 3 to 6 years was recommended in a doctoral thesis (Huovila 1981), but the mobilisation of the recommendation and the extent of its usage is not reported. In the paper published in 1982, only the four doses at the ages of 3, 4, 5 and 24 months are mentioned to be included in the vaccination programme in Finland (Mertsola et al. 1982). Since the 1970s, the DTwP in use was manufactured by KTL in Finland (personal communication, Rose-Marie Ölander, KTL).

In 2003, one booster dose with commercial dtap (the letters written in the lower case indicate the lower amount of antigens in the vaccine) at 6 years of age was added to the schedule to reduce the disease burden among school-aged children. Two years later in 2005, the use of in-house produced wP was ceased with the introduction of a new vaccine and vaccination schedule. A commercial combination aP vaccine (DTaP-IPV-Hib) containing two pertussis antigens, PT and FHA, was introduced and recommended to be administered at the age of 3, 5 and 12 months. In Finland, booster vaccinations with DTaP-IPV at 4 years of age and with dtap at 14–15 years of age are included as recommended by the GPI. To complete the immunisation of the children born before 1997, the dtap booster is administered at the age of 11–13 years, as they were too old to receive the booster in 2003. As suggested by the WHO, the pertussis immunisation with the primary vaccines and

boosters is recommended in Finland even if the child had suffered from the pertussis disease prior the vaccination. (KTL 2007; Rapola 2007)

2.4.3 Differences in immunity acquired by infection and vaccination

Infection with *B. pertussis* or immunisation with wP or aP induce the production of specific antibodies within a few weeks after the exposure to the bacteria or the vaccination. The protective immunity to pertussis after natural infection or vaccination is not lifelong; the values of antibodies decrease over time until re-infection or re-immunisation induces the secondary immune response. However, the duration and the mechanism seem to differ between the immunity induced by the infection and vaccination, and further, between the wP and aP vaccination. (Wendelboe et al. 2005)

The duration of pertussis immunity has been extensively studied alongside the vaccine development. As the research data was reviewed, it was shown that the estimations for the duration of the infection-induced and vaccination-induced immunity were from 4 to 20 and 4 to 12 years, respectively, and the protective immunity after the vaccination with aP wanes faster than after vaccination with wP (Wendelboe et al. 2005). However, it seems that the protective immunity against the disease lasts longer than against the infection, and further, the re-infection – symptomatic or asymptomatic – may serve as a booster for the immunity. (Heininger 2001; Mattoo and Cherry 2005; Wendelboe et al. 2005)

The mechanisms of the immunity acquired by infection and vaccination are different. The natural infection induces IgA, IgG and IgM responses to specific antigens and the whole bacteria whereas the vaccination induces only the production of IgG and IgM antibodies. IgA has been shown to facilitate the binding, phagocytosis, and killing of *B. pertussis* and to induce cellular immune functions (Hellwig et al. 2001). Thus, the important and versatile role of IgA may explain some of the differences between the immunity after the natural infection and vaccination. Further, when immunised with aP, the antibodies are only produced against the antigens included in the vaccine. Differences in the cell-mediated immunity have also been shown. After the natural infection and wP vaccination, T-helper 1 cells (Th1) are activated, whereas T-helper 2 (Th2) biased cells or both Th1 and Th2 cells are generated after aP vaccination (Mills et al. 1999). Th cells are involved in activating and directing other immune cells to the infection site. In a simplified manner, Th1 cells are evoked by the bacterial antigens and they activate the phagocytic immune cells to kill the bacteria, whereas Th2 cells trigger the antibody production by the B cells via the production of interleukins (Wilkins 2006). (Heininger 2001; Hellwig et al. 2001; Mattoo and Cherry 2005; van den Berg et al. 2000; Wendelboe et al. 2005)

2.4.4 Herd immunity

It is well understood that in the population where an effective vaccine against any disease is used with high coverage, even the unimmunised members are less likely to encounter the disease. Due to the mass vaccination, the risk of transmission is reduced and thus the unimmunised and imcompletely immunised members as well as the vaccine failures are somewhat safe from the disease (Taranger et al. 2001). When the herd immunity increases

due to vaccination with high coverage, the circulation of the corresponding agent may cease (John and Samuel 2000).

Herd immunity is an advantage of the extensive vaccination and plays a significant role in the control and prevention of pertussis (Taranger et al. 2001). Thus, the herd immunity can be exploited on the design of vaccines and vaccination schedules even though the immunity against pertussis is not yet fully understood. However, the protective effect of herd immunity is lost if the vaccination coverage decreases due to anti-vaccine movements and parental refusal of vaccinating their children. Due to the long history of extensive and effective vaccinations, many young parents have never confronted pertussis and they may thus underestimate the value of vaccination. This again poses an unnecessary threat of pertussis to their children, to the contacts of their children, and further, to the community by the diminished herd immunity (Feikin et al. 2000; Leino and Kilpi 2005).

2.5 Pertussis epidemiology

2.5.1 Pertussis worldwide

In the prevaccine era, whooping cough was one of the main causes of death in infants and more than half of the children suffered from pertussis before beginning school (Halperin 2007). With the widespread use of pertussis vaccine, the incidences dropped dramatically. Still, pertussis is one of the leading causes of vaccine-preventable deaths in the world today. It is estimated to cause 50 million cases and 300 000 deaths every year (WHO 2003).

In the USA, pertussis caused over 200 000 cases and over 4000 deaths a year in the prevaccine era; the most recent number of cases and deaths were ~15000 (in 2006) and 27 (in 2004), respectively (Roush and Murphy 2007). In 2006, the number of reported cases in Europe was over 30 000 and almost 116 000 worldwide (WHO 2007). In the neighbouring countries of Finland, the incidence of pertussis per 100 000 inhabitants has been variable (EpiNorth 2008). The incidence observed in Norway and Estonia in 2007 was 115.2 and 30.5. These figures were even higher than what was reported in the different regions of Russia (2.3-22). The most recent figure was 7.6 in Sweden and 9.1 in Finland. However, the comparison is difficult because the reporting and diagnostic criteria vary between the countries.

The reported numbers are low compared to the above mentioned estimations by WHO; the underreporting due to underrecognition, underdiagnosis, misdiagnosis as well as poor and non-uniform surveillance systems constitute a major problem when studying the epidemiological data on pertussis. In addition, there are variations in the case definitions among countries complicating the comparisons. Despite the inconsistent practices in the reporting, however, an epidemiological commonality between the countries – despite their vaccination histories – is the endemic and periodic nature of pertussis epidemics as they follow a clear 3- to 4-year cycle (Broutin et al. 2005). Further, a shift of pertussis cases to the older age groups has been noticed in many developed countries with high vaccination coverage. (Cherry et al. 2005; Mattoo and Cherry 2005; WHO 2007)

2.5.2 Age-specific incidence

Pertussis has increased in infants, adolescents and adults during the recent years (Halperin 2007; Mattoo and Cherry 2005). At the same time, the infant deaths due to pertussis have also increased – this may be due to the transmission of the bacteria from the parents to their infants too young to be protected by the vaccination (Halperin 2007; Mattoo and Cherry 2005; Vitek et al. 2003). In a Greek study on pertussis seroprevalence, children were shown to be the main reservoir of the disease in populations with low vaccination coverage, whereas adults were the reservoir in populations with high coverage (Pavlopoulou et al. 2007). In a study with pooled data from 16 European countries, the highest but stable incidence was observed in children younger than one year old, and the incidence in adults and adolescents older than 14 year of age doubled between 1998 and 2002 (Celentano et al. 2005).

The changes in the vaccination schedules will affect the age-specific epidemiology. When the changes are mobilised, the effects will be seen rather soon among the concerning age group. Hethcote et al. used computer simulations to estimate the effects of booster vaccinations on the age-specific incidences (Hethcote et al. 2004). They suggested that the replacement of pertussis vaccination at 18 months of age with an adolescent booster would decrease the numbers of infant and adolescent cases by 30% and 25%, respectively, but increase the cases in 2–4-year-olds by 15%. In Sweden, both the overall and age-specific incidences of pertussis dropped dramatically after the introduction of aP vaccine (Gustafsson et al. 2006). However, a shift in the age-specific incidence was noticed from the 2–4-year-olds during the years without any pertussis vaccination to the 7–8-year-olds few years after the introduction of the aP referring to the waning of the immunity.

2.5.3 Pertussis in Finland

In the middle of the 19th century, estimated 2,7‰ – several thousands – of Finns died of pertussis every year. In the middle of the 20th century, the death rate had decreased to 0,01‰, or 60 deaths, but the yearly number of pertussis cases was still about 30 000. At that time, pertussis vaccination was not yet introduced in Finland, thus, the decrease was mainly due to the improved nutritional state, living conditions and healthcare. (Forsius 2005)

In 1952, wP vaccine was introduced in the national vaccination programme causing a dramatic decrease in the incidence. Since then, the vaccination coverage has remained high. The recent data showed that over 97% of the children born in 2001 obtained the DTwP vaccines by the vaccination schedule (Leino et al. 2007). However, several epidemics have occurred, and an increase in the pertussis incidence has been noticed since the 1980s (Figure 3).

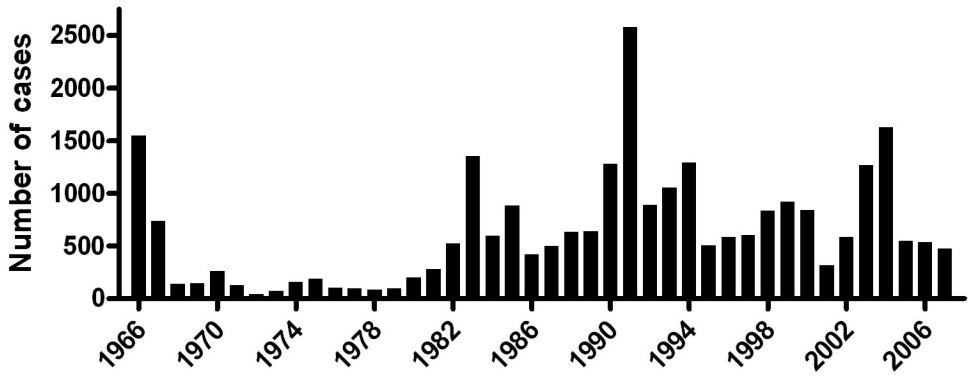

Figure 3. ***Diagnosed pertussis cases in Finland, 1966–2007. (1966–1978, Huovila, 1981; 1979–1994, Reports from Orion Oy; 1995–2007, IDR, KTL)***

Since 1995, the laboratory confirmed pertussis cases have been reported by the clinical microbiological laboratories to the Infectious Diseases Register (IDR) administered by KTL. The lowest number within the last two decades was reported in 2001 with 315 cases (Figure 3). A great proportion (39%) was diagnosed in adults (>19 years of age), 23% in school-aged children from 7 to 11 years of age and 3% in infants (<1 year of age). During the recent nationwide epidemic, the highest number in cases was 1631 in 2004 (Figure 3). Of those, one third occurred in school-aged children from 7 to 11 years of age, 26% in adults (>19 years of age) and 7% in infants (<1 year of age). Thus, the epidemic situation was most clearly seen among the school-aged children and infants whereas the proportion of adult cases decreased (Figure 4). However, a great part of the adult cases in the epidemic season is probably lost in the statistics due to the clinical diagnosis of the adults, e.g. in the families with more severe and laboratory confirmed cases of pertussis in the infants and school-aged children.

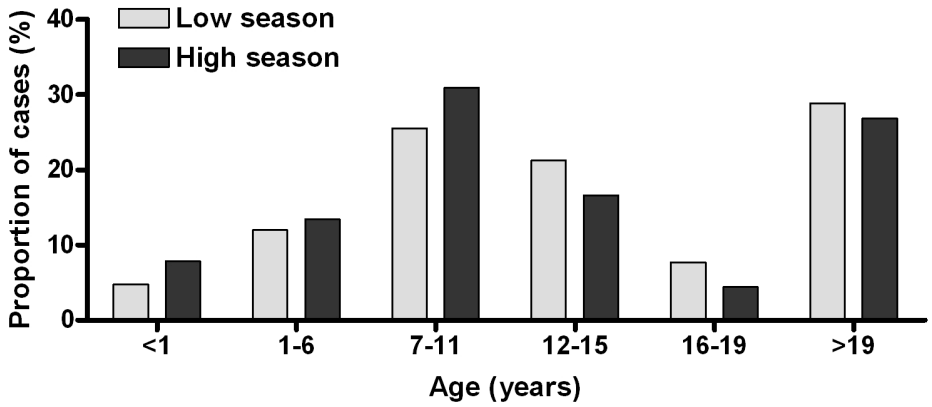

Figure 4. ***Age-specific distribution of the pertussis cases during the years of low (1996 and 2001) and high (1999 and 2004) incidence in Finland. (IDR, KTL)***

2.5.4 Disease burden of pertussis

Decisions about the national vaccination schedules and the vaccines used are always influenced by economical values. The term disease burden has been introduced to provide the basis for the calculations of the cost-effectiveness of the vaccination schedules. Disease burden is composed of both direct and indirect costs due to the disease. The direct costs include medical costs due to the hospitalisation and laboratory tests and the non-medical costs such as childcare and travel expenses. Reduced work productivity and lost working days are counted as indirect costs (Caro J et al. 2005). In 2005, WHO published a protocol to advise how the disease burden of pertussis in infants should be calculated (WHO 2005a). In addition, GPI published a thorough supplement with reviews of the studies concerning the burden of pertussis in the same year (2005).

Even though pertussis vaccinations have been widely used for decades, data on the disease burden of pertussis is rather limited. It is very hard to evaluate due to reasons similar to the epidemiological studies, but as the disease is long-lasting with serious complications and high hospitalisation rate among the infants, both the economical, physical and mental burden for the families as well as the communities is very high. Crowcroft et al. estimated that the non-fatal burden of pertussis exceeds the burden of lung cancer, and is more than double the burden of meningitis (Crowcroft et al. 2003b). In the USA, only the adolescents' pertussis was estimated to cause financial costs of $3,2 billion and further, immunisation of 10–19-year-olds would prevent 0.7–1.8 million pertussis cases and save $0.6–$1.6 billion over a decade (Purdy et al. 2004). It is also estimated that for every $1 spent on the acellular pertussis vaccine (DTaP), $24 is saved (Ehreth 2003).

2.6 Strain variation and epidemiological typing

Genomic and genetic variation occur within bacterial species shown as DNA polymorphism, loss or gain of genetic material, and antigenic changes of virulence factors leading to transcriptional and translational differences. Several methods have been developed and recommended to study the short-term evolution of *B. pertussis* and to detect the changes in the virulence factors.

2.6.1 Typing at the genome level

Restriction fragment analysis

Pulsed-field gel electrophoresis (PFGE) is based on electrophoretic discrimination of fragments obtained after digestion of the genomic DNA with a restriction enzyme (Kaufmann 1998). The bacteria are first incorporated into agarose plugs, and the plugs are treated with the restriction enzyme. For *B. pertussis*, several restriction enzymes have been tested (Lee et al. 2006), but XbaI with the recognition site at 'TCTAGA' is recommended (Advani et al. 2004; Mooi et al. 2000). The solid agarose plugs containing the bacterial DNA fragments serve as the samples that are then loaded into another agarose gel for the electrophoretic separation. When the electrophoresis is complete, the gel is stained with Ethidium Bromide. The bands are visualised and photographed under UV

illuminator. Computer-assisted analysis of the banding patterns is done to reveal the PFGE profiles of the strains. PFGE is well reproducible and has high discriminatory power, and it is widely used for analysing bacterial isolates at the inter- and intra-species levels. PFGE is used by a network that is expanding throughout the world performing standardised molecular fingerprinting of foodborne disease-causing bacteria to constitute a database of the PFGE profiles allowing the detection of disease case clusters and identification of common source outbreaks (www.cdc.gov/pulsenet/). However, PFGE profiles do not show the site where the changes have occurred and whether they are relevant for the virulence of the bacteria. In addition, the same size of bands consisting of different gene fragments may give false typing results as they are interpreted as identical. The laboratory part of PFGE is rather time-consuming and laborious, and the computer-assisted analysis of the gel images is not standardised hindering the comparison of the PFGE profiles between laboratories. To enable this kind of comparison and the correct interpretation of gel images, a reference system for *B. pertussis* PFGE has been published (Advani et al. 2004), and international reference strains for PFGE analysis are available (Mooi et al. 2000). PFGE is the most extensively used method for the genome-wide typing of *B. pertussis* isolates (Locht 2007).

Restriction fragment length polymorphism (RFLP) goes further than PFGE as the gene fragments are blotted from the gel onto a specific membrane after the electrophoresis and then hybridised with labelled probes specific to the selected insertion sequence elements (ISEs). With this method, the presence and the number of copies of the ISEs can be determined but the discriminatory power for *B. pertussis* typing is not as high as that of PFGE. However, RFLP has been used for comparing the species of *Bordetellae* (van der Zee et al. 1996a; van der Zee et al. 1997) and as a DNA fingerprinting method alongside with PFGE (Poynten et al. 2004; van der Zee et al. 1996a).

Multilocus typing

Multilocus enzyme electrophoresis (MLEE) and multilocus sequence typing (MLST) are used for the genetic analysis of housekeeping genes. Those genes and their products are typically needed for the maintenance of the cell rather than the pathogenesis or virulence of the bacteria, and thus enable the use of neutral variation for the strain characterisation to define population structures, their relatedness and clonality (Spratt 2004). With MLEE, the variation leading to gene products with different electrophoretic mobility is detected from a bacterial protein extract run on a starch gel and stained with specific substrates yielding the electrophoretic type (ET) of an isolate. MLST is based on the nucleotide sequencing of the alleles after amplification of internal fragments of housekeeping genes. All genetic variation at the sequenced loci can be identified regardless of whether they cause translational changes. Each allele produces an allele number, and the numbers of all the loci are assigned in order to give the allelic profile and the sequence type (ST). Both MLEE and MLST show high discriminatory power while they are based on the slowly accumulating, neutral genetic variation, and are thus suitable for long-term or worldwide rather than for short-term epidemiological studies (Spratt 2004). MLST has become more popular than MLEE due to various reasons. Most importantly, the MLST results are easily comparable between laboratories, and a central www-based MLST database (http://www.mlst.net) has been established for the characterisation and comparison of the isolates of many species (Aanensen and Spratt 2005; Maiden et al. 1998). The molecular

evolution of *Bordetella* species has been studied with MLEE (Musser et al. 1986; van der Zee et al. 1997), and later with MLST with the introduction of the *Bordetella* MLST database (http://pubmlst.org/*Bordetella*/) (Diavatopoulos et al. 2005; Jolley et al. 2004).

Multilocus variable number of tandem repeat analysis (MLVA) was developed to provide a typing method with the advantages of MLST but more suitable to the short-term epidemiological studies (Lindstedt 2005). The variable number of tandem repeats (VNTRs) are DNA motifs consisting of repeated sequences of 10 to 100 basepairs, and their typing is based on PCR amplification of the known repeated sequences followed by analysis of the amplified product. While the repeated sequences themselves are usually the same between bacterial isolates of the same species, the number of times they are repeated varies. The number of repeats can be estimated by dividing the entire molecular weight of a given VNTR by the molecular weight of the repeated sequence. The most important step in the development of MLVA is the selection of the VNTR loci for the typing, and the loci should be checked for their stability and reproducibility during the long-term passaging, reculturing and retyping of the strains. MLVA methods have been developed for several bacterial species such as *Yersinia pestis* and *Escherichia coli* (reviewed in Lindstedt 2005). In the study on *Y. pestis*, the method was shown to be able to identify the likely sources of infection and to differentiate the naturally occurring cases of plague from those occurring from an intentional release of the bacteria (Lowell et al. 2005). MLVA study of *E. coli* isolates revealed a good correlation of the MLVA and PFGE profiles as the main clusters of the PFGE profiles related to the clusters noticed with MLVA (Lindstedt et al. 2003). Similar to the MLST database, a www-based database for the MLVA typing has been established (http://minisatellites.u-psud.fr/). MLVA for the typing of *B. pertussis* was developed in the Netherlands, and it showed to be a valuable new typing technique for short-term epidemiology (Schouls et al. 2004). An advantage of MLVA over PFGE is that MLVA is based on PCR amplification of known repeated sequences and does not need the bacterial culture. However, the discriminatory power for the epidemiological typing of *B. pertussis* has not been directly compared between the two methods.

Microarray methods

After the complete sequences of many bacterial pathogens have become available, microarray methods have been applied for the epidemiological typing of pathogens (Garaizar et al. 2006). In a microarray, probes of single-stranded DNA are designed to selectively capture their target sequences of DNA or transcripts of mRNA from a sample consisting either genomic DNA or cDNA (reviewed in Schoolnik 2002). The probes are printed on a solid surface such as glass slide, and the fluorescence-labelled samples are added to the array slides. After hybridisation, the spots are scanned and the data is analysed with computer software.

Comparative analysis of the genome content by comparative genomic hybridisation (CGH) array can be used for characterisation of genetic variability of bacterial populations within species and between related species (reviewed in Schoolnik 2002). In this method, the array probes are designed based on a sequenced reference strain, and the labelled sample consisting of the genomic DNA of the experimental strain is hybridised with the probes to reveal if the genes of the reference strain exist in the genome of the experimental strain.

However, the genes present in the experimental strain but not in the reference strain can not be detected. CGH was used in a study of *Bordetellae* to reveal that gene loss has had a significant role in the speciation of *B. pertussis* and *B. parapertussis* (Cummings et al. 2004). Progressive gene loss during the decades of vaccinations was noticed in a study of Finnish *B. pertussis* strains (Heikkinen et al. 2007).

Array-based gene expression profiling is used when studying the functional expression of the genomic information with genome wide experimental and computational or statistical approaches (reviewed in Dharmadi and Gonzalez 2004). The method is based on the transcriptional expression of mRNA. The functional genomics may be utilised to study bacterial response to environmental changes such as stress or heat; gene identification, genome organization, and transcriptional regulation; and genetic and metabolic engineering. The gene expression profiling was used to study the host-pathogen interaction between *B. pertussis* and human respiratory epithelial cells at the level of gene transcription (Belcher et al. 2000). Another study using the gene expression array was a comparison of a laboratory strain (Tohama I) and a recent clinical isolate revealing significant differences in their gene expression profiles, and it was shown that *B. pertussis* is able to change its expression profile noticeably after twelve passages of the clinical isolate (Brinig et al. 2006a).

2.6.2 Phenotyping and genotyping

The genotype of an organism is determined by the genes whereas the phenotype is an observed structure or function of the organism, the functional expression of the genotype. Every phenotype is coded by a genotype, but as all the genes are not expressed at the same time, the phenotype may change according to the changes in the gene expression.

Serotyping of fimbriae

The fimbrial serotypes (Fim) of *B. pertussis* are defined by the agglutination reaction with antibodies, most preferably monoclonal antibodies against the two serotypes, Fim2 or Fim3. The reaction reveals the presence or absence of the fimbriae on the surface of the bacteria thus providing the phenotypical information as a serotype. The strains reacting with Fim2, Fim3, or both antibodies are defined as Fim2, Fim3, and Fim2.3, respectively (Mooi et al. 2000). The serotypes may go through phenotypical modulation leading to the change of serotype (Willems et al. 1990). Serotyping with monoclonal antibodies for Fim2 and Fim3 has been recommended as one of the standard methods for the epidemiological typing of *B. pertussis* (Mooi et al. 2000).

Genotyping by PCR and sequencing

Genotyping has been widely used and recommended to detect the antigenic changes in the genes coding for the virulence factors of *B. pertussis*, especially for *prn* and *ptx*A (Mooi et al. 2000). As the genetic material of the sample is usually limited, the gene of interest is amplified by PCR. After that, the genotyping can be done by sequencing of the purified amplification product (De Schutter et al. 2003; van Amersfoorth et al. 2005). However, the conventional sequencing being rather laborious and time-consuming led to the development of rapid real-time PCR methods (Mäkinen et al. 2002; Mäkinen et al. 2001;

Muyldermans et al. 2004). In these methods, the PCR is designed in an allele specific manner to distinguish the different genotypes already during the amplification. As not all the genotypes can be differentiated by PCR only, the amplified products may be further separated by their sizes with gel electrophoresis.

Pyrosequencing

A modern method called pyrosequencing allows the sequencing of the template DNA simultaneously with the synthesis of the complementary strand (Ronaghi et al. 1998). This technique can be used with a special device, which detects the pyrophosphate released after the stepwise addition of the nucleotides during the DNA polymerase reaction, but the length of nucleotides that can be synthesised by pyrosequencing is short, up to 100 basepairs. Pyrosequencing can be used for DNA sequences of strong secondary structures that are difficult to sequence with conventional methods (Ronaghi et al. 1999). However, when a newly developed real-time PCR and pyrosequencing methods for the *ptx*A typing were compared, the pyrosequencing was shown to be more time-consuming and expensive than the real-time PCR method (Storm et al. 2006).

2.6.3 Definitions and nomenclature of the variants

Distinct definitions and nomenclature are established with every method, and they may be somewhat confusing as the methods are simultaneously developed in different laboratories. The definitions and nomenclature of the methods used in the original articles (I–IV) are discussed here.

Pulsed-field gel electrophoresis (PFGE)

The PFGE profiles are defined as the differences in the banding patterns of the isolates after the digestion with either XbaI or SpeI or both (Mooi et al. 2000). Recently, digestion with XbaI only has been shown to be adequate for the typing of *B. pertussis* (Advani et al. 2004). The banding patterns that differ by at least one band are defined separate, and named by the nomenclature based on the country from where the first isolates with the banding pattern is found. Thus, the PFGE profiles in Finland, Sweden and France are named BpFINR#, BpSR# and BpFR#, respectively. These PFGE profiles can also be grouped in larger clusters by the neighbour-joining clustering method with the help of computer programmes such as BioNumerics (Applied Maths, NV, Belgium). Seven major PFGE groups or clusters (I to VII) and three subgroups in PFGE group IV (IVα, IVβ and IVγ) have been determined (Hallander et al. 2007; Weber et al. 2001). However, as the French isolates (II) are only named by the clusters, they cannot be directly compared to the Swedish and Finnish isolates in other studies (I, III, IV). In addition, the same PFGE profiles may have different names in different countries, e.g. BpSR11 is the same as BpFR743. Thus, the comparison of international isolates may be complicated.

Pertactin (*prn*)

Twelve variants of the *prn* gene have been identified (*prn*1–12), and three of them (*prn*1–3) have been found to be predominant all over the world (Cassiday et al. 2000; Fry et al. 2001; He and Mertsola 2008; Mooi et al. 2000; Muyldermans et al. 2004; Poynten et al.

2004). The polymorphisms are detected as differences in the numbers of repeated sequences and point mutations in two regions of the gene, regions 1 and 2, and the three most frequent genotypes only differ in the region 1 (Mooi et al. 2000). It has been recommended that the region 1 should be sequenced for the *prn* typing, and if a novel variant is found, the *prn* gene should be completely sequenced (Mooi et al. 2000). However, the typing methods based on PCR are significantly faster than the conventional sequencing (Mäkinen et al. 2001; Muyldermans et al. 2004). The nucleotide sequences of the 12 variants are presented in Appendix I.

Pertussis toxin S1 subunit (*ptx*A)

Six variants of *ptx*A gene have been identified (Mooi et al. 2000; Poynten et al. 2004). They are distinguished by point mutations leading to changes in the amino acid synthesis of the S1 subunit. The nomenclature of *ptx*A has been fluctuating (Table 2) due to different nomenclature used in several publications (Caro V et al. 2005; Fry et al. 2001; Mooi et al. 2000; Poynten et al. 2004). The lack of *ptx*S1C is due to a sequencing error that led to the misidentification of the *ptx*S1C variant that was later withdrawn (Mooi et al. 1998). The nucleotide sequences of the four most common variants are presented in Appendix I.

Table 2. ***Nomenclature of the pertussis toxin S1 subunit variants. The names in parenthesis are not mentioned in the original publications.***

Mooi et al (2000), Poynten et al (2004)	**Fry et al (2001)**	**Caro V et al (2005)**
*ptx*S1A	*ptx*A1	*ptx*A1
*ptx*S1B	*ptx*A2	*ptx*A2
*ptx*S1D	*ptx*A3	*ptx*A4
*ptx*S1E	*ptx*A4	*ptx*A5
*ptx*S1F	(*ptx*A5)	(*ptx*A6)
*ptx*S1G	(*ptx*A6)	(*ptx*A7)

3 AIMS OF THE STUDY

Pertussis has remained endemic and even re-emerged in the countries with high coverage of pertussis vaccination such as Finland. The first purpose of this study was to examine the molecular epidemiology and the strain variation of the *B. pertussis* isolates by the means of internationally recommended typing methods and to study the effects of the change of vaccination programme on the bacterial population. The second purpose was to to evaluate the epidemiology of pertussis in Finland before and after the changes in the vaccination programme with the emphasis on the young children.

More specifically, the aims of the present study were:

1. To explore the long-term changes among the Finnish *B. pertussis* isolates during the era of whole-cell vaccination (I)

2. To compare the *B. pertussis* strains circulating in the countries with similar and different vaccination histories (II, III) and to investigate the drift of *B. pertussis* strains between Finland and Sweden (III)

3. To analyse if the change of vaccine type and vaccination schedule affected the circulating *B. pertussis* strains, the incidence of pertussis, and the need of hospitalisation among young children (IV)

4 MATERIALS AND METHODS

4.1 Bacterial strains (I–IV)

In total, 338, 455 and 61 *B. pertussis* strains were analysed at the Pertussis Reference Laboratory of KTL (Turku, Finland), the Swedish Institute for Infectious Disease Control (Solna, Sweden) and the French Pertussis National Center of Reference, Institut Pasteur (Paris, France), respectively (Table 3). The isolates were obtained from the local clinical microbiological laboratories of each country. The vaccine strains from Finland (strains 18530 and 1772) and Sweden (strain 44122) were also analysed in this study. The French vaccine strains were analysed earlier (Njamkepo et al. 2002).

Table 3. ***B. pertussis isolates from Finland, France and Sweden analysed in the original publications (I–IV). N(studied) is the number of separate isolates as some of the isolates have been included in more than one publication. N(total) is the number of isolates collected at the Pertussis Reference Laboratory of KTL.***

Year	Finland						France	Sweden
	I	II	III	IV	N(studied)	N(total)	II	III
1953–1965	7	-	-	-	7	7	-	-
1977	10	-	-	-	10	10	-	-
1982	4	6	-	-	6	6	-	-
1991	3	-	-	-	3	6	-	-
1992	8	12	-	-	18	34	-	-
1993	8	9	-	-	15	53	3	-
1994	5	3	-	-	7	33	1	-
1995	5	-	-	-	5	14	6	-
1996	13	-	-	-	13	49	-	-
1997	5	2	-	-	6	15	8	-
1998	5	2	14	4	14	14	10	43
1999	13	16	42	10	42	42	8	137
2000	7	3	20	7	21	21	2	115
2001	2	-	3	1	3	4	-	47
2002	8	-	24	5	24	24	-	81
2003	19	-	90	25	90	93	9	32
2004	-	17	-	37	52	138	14	-
2005	-	-	-	6	6	21	-	-
2006	-	-	-	6	6	14	-	-
N(total)	122	70	193	101	348	598	61	455

4.2 Serotyping (I–IV)

Serotyping of *B. pertussis* isolates was done by slide agglutination or indirect EIA (Heikkinen et al. 2008), with monoclonal antibodies against Fim2 and Fim3 kindly provided by Dorothy Xing (National Institute for Biological Standards & Control, Hertfordshire, UK). The serotypes were indicated as Fim2, Fim3 or Fim2.3. If no agglutination was observed, the serotype was considered as untypeable. The method was described in the original publication (I).

4.3 Genotyping (I–IV)

The isolates were genotyped for *ptx*A and *prn* (Appendix I). The genotypes found among the isolates were designated based on the nomenclature described by Fry et al. (Fry et al. 2001). In II, the *ptx*A were numbered by the nomenclature of Caro et al. (Caro V et al. 2005). The isolates were genotyped with PCR and gel electrophoresis. The protocols used for the typing of the Finnish, French and Swedish isolates have been described by Mäkinen et al. (Mäkinen et al. 2002; Mäkinen et al. 2001), Weber et al. (Weber et al. 2001), and Mooi et al. (Mooi et al. 2000), respectively.

4.4 Pulsed-field gel electrophoresis (I–IV)

DNA fingerprinting of the isolates was performed by PFGE after digestion of the genomic DNA with the restriction enzyme XbaI. First, estimated 2×10^9 cfu/ml of *B. pertussis* were suspended in 1x TE buffer. Then, agarose plugs were prepared with 1:1 of the bacterial suspension and low-melting agarose (SeaPlaque® GTG ® agarose, BioWhittaker Molecular Applications). The plugs were treated with proteinase K (Proteinase K (fungal), >20 U/mg, Invitrogen) over night at +55°C, and stored in EDTA (0,5M pH8). Before the PFGE, slices of the plugs were digested with XbaI (20 000 U/ml, New England BioLabs) over night at +37°C and buffered in the running buffer (0.5x TBE buffer). The slices of the plugs were then loaded in the gel, and PFGE was completed with the running protocol of 40 hours (Advani et al. 2004) with CHEF-DR III system (BioRad Laboratories). The gel pictures were taken with GelDoc 2000 system (BioRad Laboratories) and analysed with the computer software BioNumerics (version 4.0, Applied Maths). The PFGE profiles were clustered with the unweighted pair group method using arithmetic averages (UPGMA) with 1% band tolerance and 1% optimisation settings. The protocol further described by Advani et al. (Advani et al. 2004) was used in I, III and IV. The protocol used in II has been described by Caro et al. (Caro V et al. 2005).

4.5 Pertussis before and after the change of vaccination programme (IV)

4.5.1 Study patients

The study subjects were selected from IDR. The selection criteria included patients (i) less than two years of age at the time of (ii) laboratory diagnosed pertussis (iii) from 1998 to 2006. The total number of the study subjects was 585, 490 of whose social security number was available from either the IDR or the reporting microbiological laboratory. The contact details were obtained for 465 study subjects from the Population Register Centre based on the social security numbers. However, year 1998 was excluded from the study to avoid a bias as the contact details were only available for 4 of 68 cases from that year. Thus, the study material with an envelope for a free-of-charge respondence was sent to 461 study subjects from 1999 to 2006 within all hospital districts of Finland, and the reminding letters were sent if no reply was obtained within four weeks.

With the written informed consents, the vaccination data were gathered from well-baby clinics and school nurses. In the cases of (i) guardians' information of the child being unvaccinated prior to the diagnosis, or (ii) the study subject being younger than 70 days at the time of diagnosis, no vaccination records were asked and the patients were marked as unvaccinated. The hospitalisation data were gathered from the guardians by a questionnaire to clarify if the child had been admitted to hospital due to pertussis. The collected data were combined with the diagnostic data from IDR. The strain typing data was also combined with the patient's data if the *B. pertussis* strain isolated from a patient included in the study had been sent to Pertussis Reference Laboratory after the diagnosis.

4.5.2 Linkage between the diagnostic, strain, vaccination and hospitalisation data

The written informed consents were obtained from 324 (70.3%) of the 461 study subjects. The data of both vaccination and hospitalisation were available from 319 (69.2%). The data from the different sources (Figure 5) was paired based on the social security numbers of the study subjects, and the names and the social security numbers were excised and only the study-specific identification numbers were saved for the protection of the patients' anonymities. In this study, 70 strains were isolated from the patients less than two years of age with the written informed consent available, and their typing results were included in the analysis.

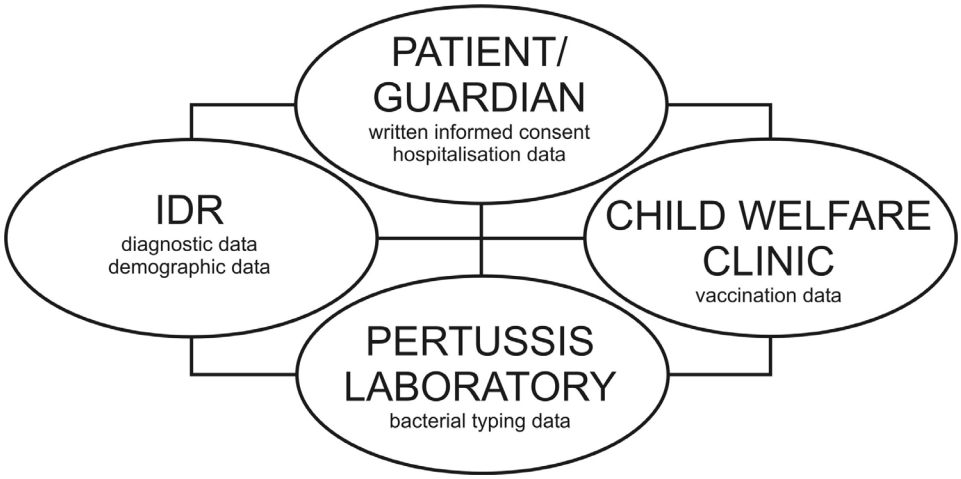

Figure 5. ***Sources and types of data acquired in the study of infantile pertussis.***

4.5.3 Ethical considerations

The research plan of IV and the documentation sent to the patients and their families was approved by the Ethical Committee of KTL (Dnro 1/2007§6) and the Ministry of Social Affairs and Health (Dnro STM/2128/2007). The data protection ombudsman was heard before contacting the patients. Written informed consents to participate in the study were obtained from the guardians before the particulars of the patients were gathered.

4.6 Statistical analysis

From the patient data, the age at diagnosis was calculated based on the date of birth and the date of sampling. The median of age at diagnosis was used for the comparison of the groups (IV). Statistical analysis using Fisher's exact test or Chi squared test, where appropriate, was performed to determine the significance of the association between the variables in the IV study (age, immunisation status, hospitalisation status, and bacterial typing data).

5 RESULTS

5.1 Serotyping (I–IV)

All three serotypes – Fim2, Fim3 and Fim2.3 – were detected among the study material. The two strains 18530 and 1772 used in the Finnish wP vaccine represent Fim3 and Fim2.3, respectively. In the Swedish wP vaccine strain, the serotypes have varied between Fim2 and Fim2.3 depending on the batches. In France, the wP vaccine consists of two strains, IM1414 and IM1416, which have been serotyped as Fim2.3 and Fim2, respectively (Njamkepo et al. 2002). Some aP vaccines used in Sweden are made of Tohama I strain, which represents Fim2. The production strains of other acellular vaccines are not known.

In Finland, the prevalent serotype during the 1980s and 1990s was Fim2, but a shift to Fim3 appeared during the last epidemic in 2003–2004 (Figure 6). In France, Fim3 was already the prevalent serotype since the beginning of 1990s. A shift from Fim2 to Fim3 also appeared during the epidemic in Sweden in the end of 1990s.

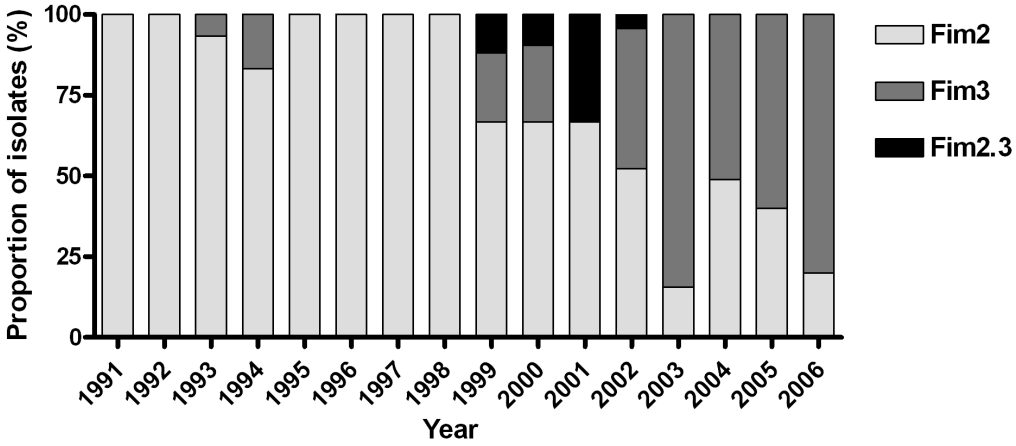

Figure 6. ***Yearly distribution of the serotypes among the Finnish B. pertussis isolates studied in the original publications.***

5.2 Genotyping (I–IV)

5.2.1 Pertussis toxin S1 subunit

The *ptx*A genotype of the Finnish vaccine strain, 18530, was *ptx*A3, and the Swedish vaccine strain was *ptx*A4. These genotypes were not found in any other strains among the studies. The French vaccine strains represent *ptx*A2 and *ptx*A3 (Njamkepo et al. 2002). The

*ptx*A2 was observed in the oldest available circulating strains, the Finnish vaccine strain 1772, and the vaccine strain Tohama I. All circulating strains since the 1970s were genotyped as *ptx*A1 (Table 4).

5.2.2 Pertactin

All vaccine strains included in this study were genotyped as *prn*1 except for the Swedish vaccine strain being *prn*10. The prevalent genotype of the circulating isolates until the 1970s was *prn*1. Since then, *prn*2 has been the most frequent *prn* genotype among the circulating strains (Table 4). In France and Sweden, *prn*2 was the predominant genotype, but *prn*1, *prn*3, *prn*7 and *prn*9 were also detected.

Table 4. ***Genotypes for pertactin (prn) and pertussis toxin S1 subunit (ptxA) found among the Finnish B. pertussis isolates studied in the original publications.***

Year	*prn* genotype (%)				*ptx*A genotype (%)	
	*prn*1	*prn*2	*prn*3	*prn*4	*ptx*A1	*ptx*A2
1953–65	100					100
1977	90	10			100	
1982		100			100	
1991	33	67			100	
1992	17	83			100	
1993	33	60	7		100	
1994		71	14	14	100	
1995		60	20	20	100	
1996	8	92			100	
1997	17	83			100	
1998		100			100	
1999	2	98			100	
2000		100			100	
2001		100			100	
2002		71	29		100	
2003		99	1		100	
2004		98	2		100	
2005		100			100	
2006		100			100	

5.3 Pulsed-field gel electrophoresis (I–IV)

The PFGE profiles of the Finnish vaccine strains 18530 and 1772 were BpFINR13 and BpSR23, respectively. The vaccine strains used for the production of the wP used in France have been analysed, but their relation to the other vaccine strains and circulating isolates has not been demonstrated (Njamkepo et al. 2002). In Sweden, the wP vaccine strain used in 1953–1979 has shown at least six different PFGE profiles from different batches of the vaccine strain (Advani et al. 2004).

5.3.1 Finnish isolates since the 1950s

In total, 42 distinct PFGE profiles were found among 295 Finnish isolates from 1991 to 2006. Two additional PFGE profiles, BpFINR1 and BpFINR14, were observed among the isolates from 1953 to 1965, but they have not been found since. Eight out of ten isolates from 1970s represented the PFGE profile of the vaccine strain 1772 (BpSR23), the other two isolates harboured BpFINR9 and BpSR46, again, profiles that have not been found since. Isolates from 1982 were all BpSR18. The dendrogram analysis of the PFGE profiles observed in two or more isolates is shown in Appendix II.

Both temporal and spatial changes were noticed among the PFGE profiles. The most frequent PFGE profile has changed periodically. The appearance and occurrence of the three PFGE profiles since 1996 in more than 20 isolates showed that the periodical change has been synchronous with the epidemic cycles of the disease (Figure 7). The spatial distribution was noticed in some PFGE profiles such as BpSR147, BpSR18 and BpFINR8 causing local outbreaks in Kisko and Salo but not significally spreading to other communities (III).

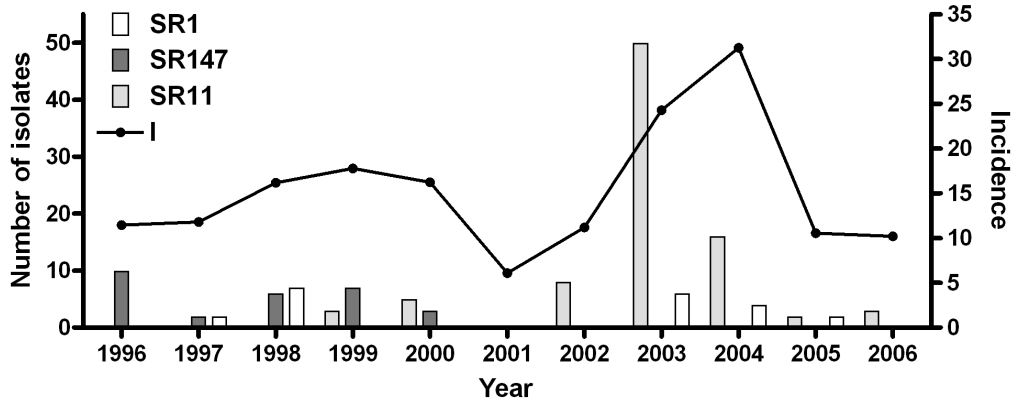

Figure 7. ***Yearly distribution of the three most frequent PFGE profiles among the Finnish B. pertussis isolates (bars), and the incidence of pertussis (number of cases per 100 000 inhabitants) in Finland (curve) (IDR).***

5.3.2 Comparison between isolates from Finland and France

The comparison between Finnish and French isolates was done in the level of PFGE clusters or groups rather than PFGE profiles. The isolates from these two countries with long-standing history of wP vaccination were found highly similar as the minimum of the overall relatedness counted from the PFGE profiles was 80%. The same group IV was predominant in both countries, but the frequencies of the subgroups IVα, IVβ and IVγ varied. In fact, IVγ that was very common in Finland in the beginning of 1990s was noticed in France a decade later. In 2000–2004, IVβ was the predominant subgroup in both countries. In addition, a completely new group VII was found among the Finnish isolates in 2004.

5.3.3 Paimio outbreaks

All the 31 isolates from Paimio from 1992 to 2004 were analysed to study molecular epidemiology of *B. pertussis* during and between local epidemics. Although all the isolates had *ptx*A1 and *prn*2 genotypes, the analysis of the PFGE groups showed a change of the prevalent groups relative to the nationwide epidemics (Figure 8). Appearance of Fim3 strains was also evident during the study period. This indicates that the *B. pertussis* population is not sustainable within the community between the epidemics but the bacterial population within a community is as dynamic as within the country.

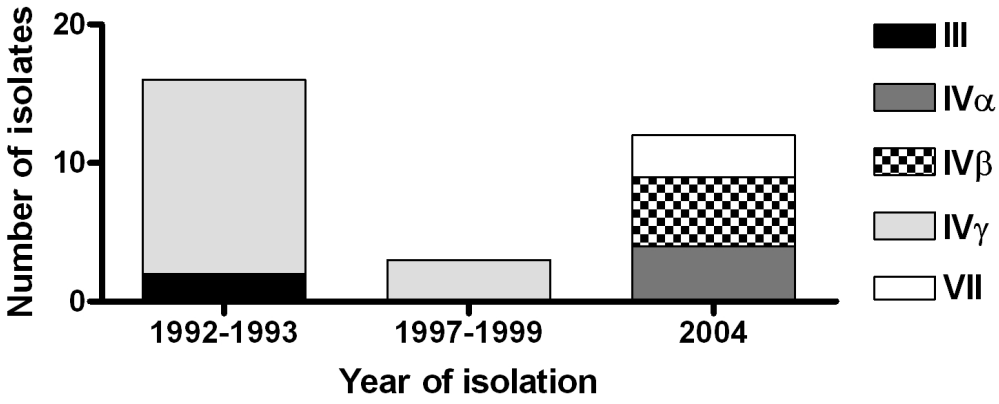

Figure 8. ***B. pertussis isolates from Paimio from 1992 to 2004 grouped by PFGE clusters and epidemic cycles.***

5.3.4 Comparison between isolates from Finland and Sweden

The PFGE profiles of the isolates from the two countries with different vaccination history showed a minimum of the overall relatedness of 70%. The heterogeneity of the PFGE profiles in Finland was similar to that in Sweden as the percentage of heterogeneity was 14,5% in both countries (28 PFGE profiles/193 Finnish isolates; 66 PFGE profiles/455 Swedish isolates). Of these profiles, only 15 were found in both countries leaving 13 and 51 PFGE profiles unique among Finnish and Swedish isolates, respectively. The shared PFGE profiles were mainly detected first in Sweden and a few years later in Finland (Table 5). The shared PFGE profiles (except BpSR23, the PFGE profile of the vaccine strain 1772) were grouped in the same cluster relative to the group IV in the French comparison study.

Table 5. ***Ten of the shared PFGE profiles between Finland and Sweden, the first years of detection in both countries, and the number of representative isolates included in III.***

PFGE profile	First year of detection		No. of isolates from 1998 to 2003	
	Sweden	Finland	Sweden	Finland
BpSR18	1972	1982	1	16
BpSR1	1986	1991	18	13
BpSR7	1987	1995	3	14
BpSR10	1994	1999	28	3
BpSR16	1994	2001	38	10
BpSR33	1994	1998	2	3
BpSR11	1997	1999	167	66
BpSR12	1997	2000	9	18
BpSR5	1997	1999	28	5
BpSR147	1998	1996	2	15

Most of the PFGE profiles in both countries were seen in a limited number of isolates as only 6 and 8 PFGE profiles were represented in >10 Finnish and Swedish isolates, respectively. The most frequent PFGE profile in both countries was BpSR11, which was first detected in Sweden in 1997, and became predominant during the epidemic in 1999–2000 (Figure 9). In Finland, BpSR11 was first found in 1999, and it became predominant during the nationwide epidemic in 2003–2004 (Figure 9). This PFGE profile was found in France already in 1994 (named FR743, Caro V et al. 2005), and by the French gouping it belongs to group IVβ.

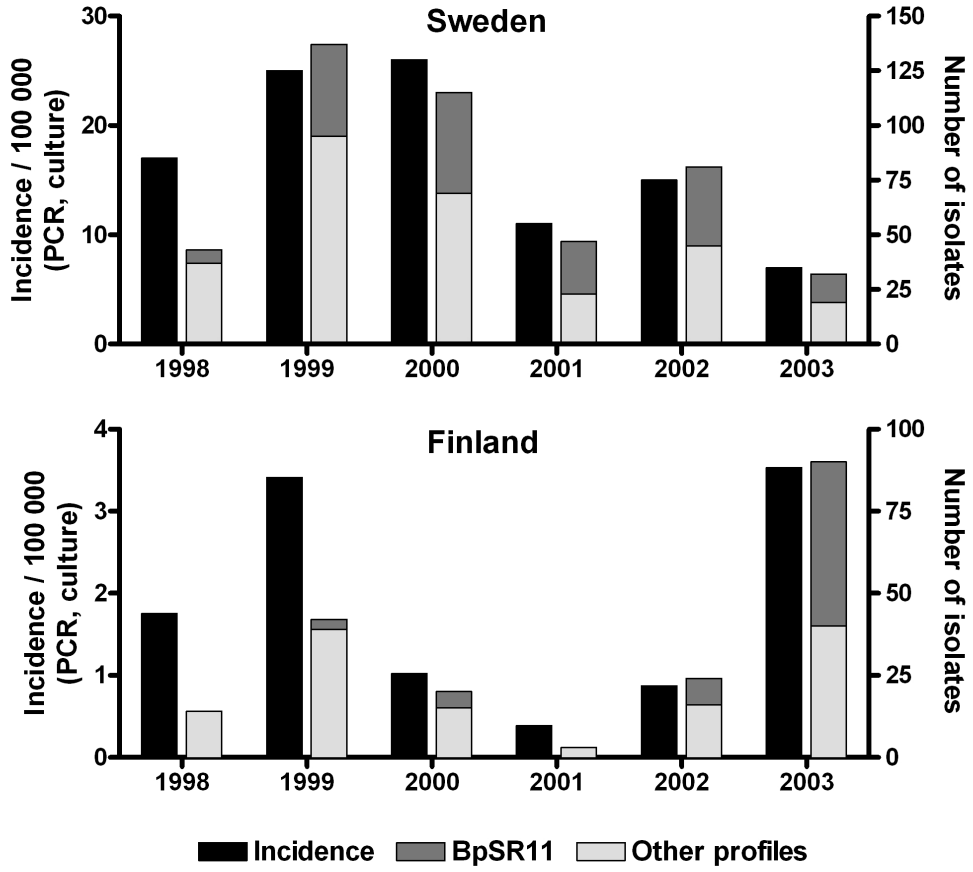

Figure 9. ***Pertussis incidence in relation to BpSR11 appearance in Finland and Sweden. In the incidence figures, only PCR and culture confirmed cases were included to enable the comparison between the countries, as the serology is not used for the diagnosis in Sweden.***

5.3.5 Isolates from children <2 years of age

From 1999 to 2006, *B. pertussis* strains were available from 97 children younger than two years of age. Of those, 70 isolates representing 20 different PFGE profiles were included in the study after the written informed consents in study IV were obtained. Again, BpSR11 was clearly the most frequent PFGE profile with 22 isolates followed by BpSR20 (8 isolates) and BpSR1 (6 isolates). In this study, BpSR20 was only observed in infants younger than five months of age whereas BpSR11 and BpSR1 did not show any age-specificity. Overall, the *B. pertussis* population isolated from the infants was shown to be highly similar to the population isolated from other age groups (Figure 10) except that the percentage of heterogeneity was higher (28,6% in infants vs. 14,5% in >2-year-olds).

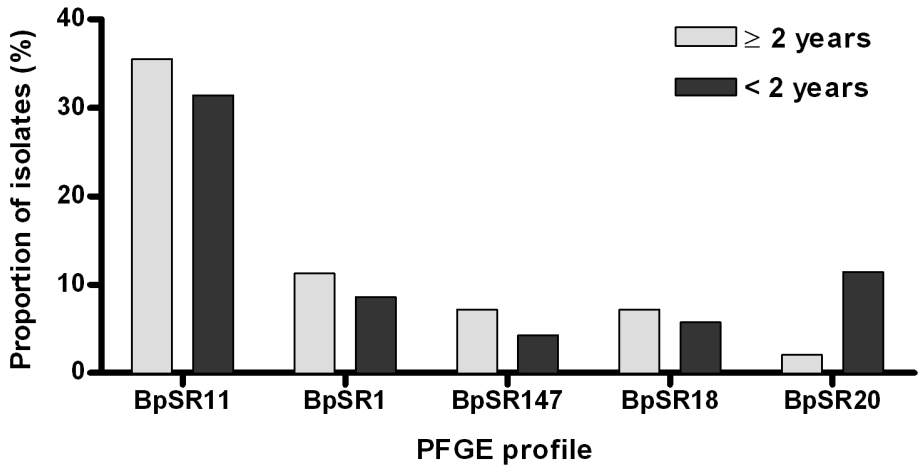

Figure 10. ***The age-group specific proportions of the most frequent PFGE profiles among the Finnish B. pertussis isolates among infants (<2 years of age) and other age groups.***

5.3.6 Association between serotypes, genotypes and PFGE profiles

There seems to be an association between certain PFGE profiles, Fim serotypes and *prn* genotypes. The increase of Fim3 strains during the epidemic in 2003–2004 was in relation to the increase of BpSR11 isolates representing Fim3. The most frequent PFGE profiles and their correlations with *prn* and Fim are presented in table 6. Correlation with *ptx*A genotype is not relevant in practice, as all the circulating isolates represent the same genotype, *ptx*A1.

Table 6. ***The most frequent PFGE profiles, their prn genotypes and Fim serotypes.***

PFGE profile	*prn* genotype (%)		Serotype (%)		
	*prn*2	*prn*4	Fim2	Fim3	Fim2.3
SR11	100	0	1	95	3
SR1	97	3	93	7	0
SR147	100	0	100	0	0
SR18	100	0	94	0	6
SR20	100	0	100	0	0

5.4 Pertussis before and after the change of vaccination programme (IV)

5.4.1 Vaccination data (IV)

The study subjects were divided into three groups based on their vaccination status: (1) unimmunised children three months of age or younger, (2) age-appropriately immunised, and (3) children with delayed immunisations (Figure 11). The immunised groups were further subgrouped by the vaccine type administered (DTwP or DTaP-IPV-Hib), and the number of vaccine doses administered before the diagnosis was determined. The vaccination data is summarised in table 7.

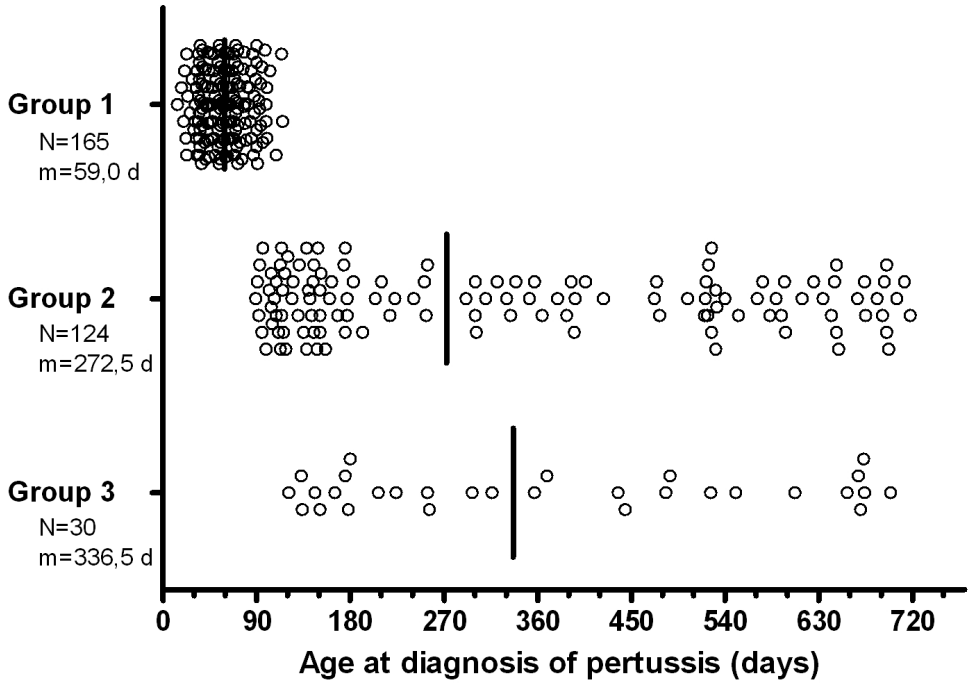

Figure 11. ***Study subjects divided according to their vaccination status. Group 1, unimmunised, ≤3 months of age; group 2, age-appropriately immunised; group 3, immunisation delayed. The vertical lines show the median of age within the groups.***

Table 7. ***Vaccination status and number of vaccine doses administered prior to the diagnosis in the children (<2 years of age) with the vaccination and hospitalisation data available.***

Immunisation status	Number of cases	Number of vaccine doses			
		1	2	3	4
1: Unimmunised	165	-	-	-	-
2: Age-appropriately immunised	124	38	17	65	4 #
wP	103	26	10	63	4 #
aP	21	12	7	2	-
3: Immunisation delayed	30	2	3	12	-
non	13	-	-	-	-
wP	13	-	3	10	-
aP	4	2	-	2	-

intermediate phase immunisation schedule (primary immunisation with wP at 3, 4, 5 months of age, fourth dose with aP at 1-year-old)

5.4.2 Hospitalisation data (IV)

The need of hospitalisation due to pertussis was determined as an overnight stay at the hospital. The duration of the stay or the severity of the illness were not systemically declared by the hospitals. However, the data obtained from the parental questionnaire showed extensive variation in the duration of the hospital stay from only one night to nearly two months. As expected, most of the children in group 1 were hospitalised. The numbers of the hospitalised cases divided by their vaccination status are presented in table 8.

Table 8. ***The need of hospitalisation grouped by the vaccination status.***

Immunisation status	Number of cases	Hospitalised	
		Yes	No
1: Unimmunised	165	145	20
2: Age-appropriately immunised	124	60	64
wP	103	48	55
aP	21	12	9
3: Immunisation delayed	30	11	19
non	13	6	7
wP	13	4	9
aP	4	1	3

5.4.3 Bacterial typing, vaccination and hospitalisation data combined

When data of the typing, vaccination and hospitalisation were combined, it was noticed that 64.2% of the *B. pertussis* strains were isolated from the unvaccinated children in group 1. Thus, the hospitalisation rate among the patients with bacterial isolates available was high, as expected (Table 9).

Table 9. ***Combined data of PFGE typing, vaccination and hospitalisation from the cases with all the data available.***

Immunisation status	Number of cases	PFGE profile (N, isolates)			
		BpSR1	BpSR11	BpSR20	other
1: Unimmunised	45	4	16	6	19
Hospitalised	41	2	16	5	18
Non-hospitalised	4	2		1	1
2: Age-appropriately immunised	16	2	5	2	7
Hospitalised	8	1	1	2	4
Non-hospitalised	8	1	4		3
3: Immunisation delayed	9		1		8
Hospitalised	5		1		4
Non-hospitalised	4				4

As shown in table 9 and figure 12, there was no clear correlation between the hospitalisation or vaccination status and the isolates representing the most frequent PFGE profiles. Most of the BpSR20 and BpSR11 isolates were from the unimmunised and hospitalised infants (Table 9). The vaccinated children with BpSR20 isolates were all hospitalised unlike children with BpSR11 (Table 9). This may, however, be due to the young age of the patients as all the BpSR20 isolates were from children <5 month of age. The medians of age among the children with BsSR20, BpSR11, BpSR1 and other profiles were 84 (36–138), 72 (32–504), 74.5 (14–697) and 85.5 (22–625) days, respectively.

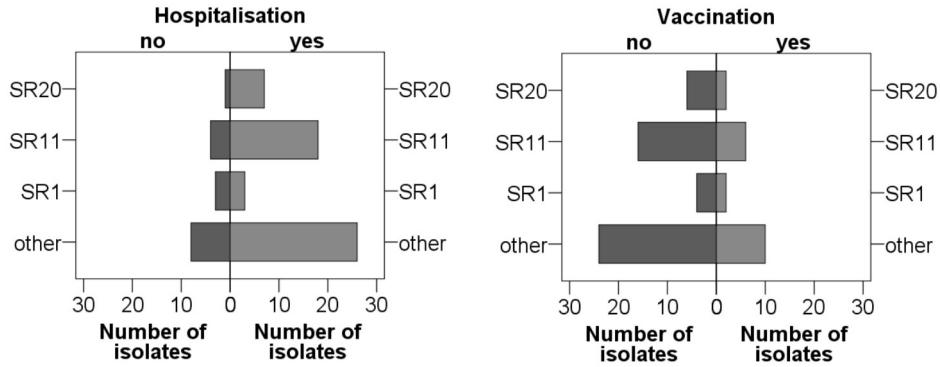

Figure 12. ***PFGE profiles and the distributions of the isolates by the hospitalisation (no/yes) and vaccination (no/yes) status.***

When the data of vaccination and hospitalisation were combined with the serotypes of the isolates, significant but contradictory correlation was found in groups 1 and 2 (Table 10). The unimmunised infants (group 1) who were infected with Fim3 strains were more likely to be hospitalised than the ones infected with Fim2 strains. However, in group 2, Fim2 strains were more likely than Fim3 strains to cause severe pertussis with the need of hospitalisation. When the hospitalised and non-hospitalised cases were pooled together despite the immunisation status, 57.4% of the isolates among the hospitalised cases were Fim3, whereas the corresponding proportion among the non-hospitalised cases were 40.0% (Table 10).

Table 10. ***Combined serotyping, vaccination and hospitalisation data from the cases with all the data available.***

Immunisation status	Number of cases	Serotype (N, isolates)		P value
		Fim2	Fim3	
1: Unimmunised	45	19	26	
Hospitalised	41	15	26	
Non-hospitalised	4	4		0.026
2: Age-appropriately immunised	15[#]	7	8	
Hospitalised	8	6	2	
Non-hospitalised	7[#]	1	6	0.041
3: Immunisation delayed	9	6	3	
Hospitalised	5	2	3	
Non-hospitalised	4	4		0.167
All together	69	32	37	
Hospitalised	54	23	31	
Non-hospitalised	15	9	6	0.257

[#] one untypeable isolate was excluded from this table

5.4.4 Diagnostics and geographical data (IV)

D i a g n o s t i c s

The methods used for the laboratory confirmed diagnosis in Finland are PCR, culture and serology. In the study of pertussis among children <2 years of age (IV), 73% of the cases were diagnosed by PCR, 14% by serology, and 12% by culture (Figure 13). Based on the strain collection of the Pertussis Reference Laboratory, 70 isolates were obtained from the 319 cases. However, only 39 (55.7%) were reported in IDR as diagnosed by culture. As both PCR and culture are often used in parallel, the confirmation method may be marked as PCR only, because the results are produced faster than by culture.

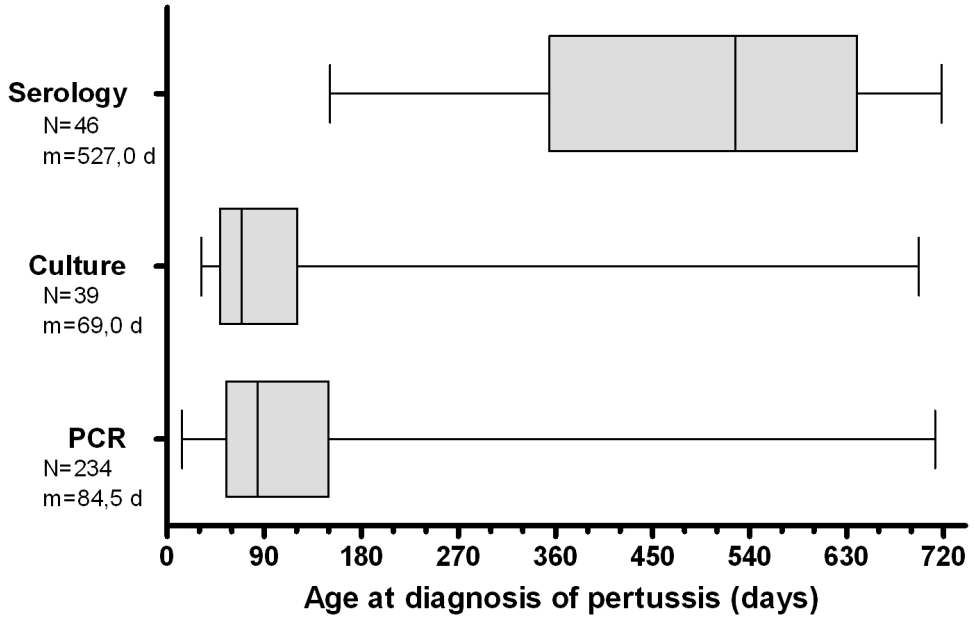

Figure 13. ***Diagnostic methods used in the children (<2 years of age). N, number of cases; m, median age of the patients.***

When comparing the proportions of the diagnostic methods used, the numbers of cases confirmed by PCR or culture were combined and compared to those of serology. The proportions of the diagnostic methods used in the children younger than two years of age differ significantly from those of older patients (Table 11). From 1999 to 2006, a total of 6876 pertussis cases were reported to IDR. Of those, only 18% were diagnosed by PCR or culture (personal communication, Jan-Erik Löflund, KTL) whereas the proportion was 86% in this study.

Table 11. ***Diagnostic method used in the pertussis cases reported to IDR.***

Diagnostic method	**Age (years)**		**P value**
	<2	**≥2**	
Diagnosed cases	517	6359	
PCR and/or culture	439	823	
Serology	78	5536	0.0001

Geographical distribution of the cases

All of the 22 hospital districts of Finland were represented in the diagnosed pertussis cases included in the study. One third of the cases were from the Hospital District of Helsinki and Uusimaa, followed by the Hospital District of Southwest Finland, the Northern Ostrobothnia Hospital District, and the Pirkanmaa Hospital District (Table 12). According to the population figures, these are the largest hospital districts in Finland, and in fact, 30% of the Finnish children (<7 years) live within the Hospital District of Helsinki and Uusimaa (Kunnat.net 2008). Thus, the 319 cases included in the study represented well the situation of the entire country (Table 12).

Table 12. ***Hospital Districts (HD) of Finland with >200 000 inhabitants (Kunnat.net 2008), and the distribution of the pertussis cases studied in IV.***

Hospital District	**Abbreviation**	**Inhabitants / total population[#] (%)**	**Cases within HD / total number of cases* (%)**
Helsinki and Uusimaa	Hus	28	32
Pirkanmaa	PSHP	9	7
Southwest Finland	VSSHP	9	13
Northern Ostrobothnia	PPSHP	7	11
Central Finland	KSSHP	5	5
Northern Savo	PSSHP	5	6
Satakunta	SatSHP	4	4
Päijät-Häme	PHSHP	4	4

[#] 5 276 955 inhabitants in 2006; * 319 cases included in the study IV

6 DISCUSSION

6.1 Strain variation and clinical outcome of pertussis disease

Pertussis infection is a complex interaction between bacterial virulence factors and the immune system of the human host. The ability to adapt to the hostile environment within the host is fundamental to the survival and replication of the bacteria. Bacteria use different mechnisms to avoid or weaken the immune reaction of the host, one of which is the antigenic variation of the virulence factors.

The relationship between the antigenic variation and clinical outcome of pertussis disease has been studied by PFGE analysis of *B. pertussis* isolates. In Sweden, when a large number of strains isolated from 1997 to 2004 were studied, the strains with PFGE profile BpSR11 were shown to correlate with longer hospital stay than other PFGE profiles (Advani et al. 2007). However, correlation was not found between the PFGE profiles and the disease severity in the studies performed in the USA and Germany (Bisgard et al. 2001; Heininger et al. 2004a). The results represented in IV did not show significant correlation with the need of hospitalisation and the isolates with BpSR11. However, the duration of the hospital stay was not analysed. The discrepancy between the results in these studies might be explained by the number of strains analysed or the collection periods of strains. As also suggested by Heininger et al., PFGE might not be suitable for the comparison of the virulence of different *B. pertussis* strains, as the analysis is not based on any specific virulence factors but the whole genome (Heininger et al. 2004a).

In Belgium, genotyping of *prn* and *ptx*A was done in addition to the PFGE analysis, and the results showed that identical strains could cause both symptomatic and asymptomatic pertussis (De Schutter et al. 2003). The role of Fims in the disease severity has been studied with contradictory results from the UK and Sweden. In the UK, serotype Fim2 was connected to a more severe disease than Fim3 (Van Buynder et al. 1999). In Sweden, however, no correlation was noticed with the serotype and severity of pertussis (Advani et al. 2007). In our studies, Fim3 was correlated with the recent epidemic in Finland but not with the need of hospitalisation (III, IV).

6.2 Relation of national vaccination history to strain variation

Pertussis vaccination was introduced already in the 1940s and 1950s in many countries worldwide including Finland in 1952. Antigenic variation has been connected to the pertussis re-emergence in some countries with a long history of pertussis vaccination. Whether the antigenic variation is vaccine-driven or not, is under debate among the researchers.

In the Netherlands, the RFLP analysis already in 1996 showed that the recently isolated *B. pertussis* strains varied from the vaccine strains and the strains isolated in the pre-vaccination era (van der Zee et al. 1996b). Similar results have since been obtained with

other methods such as PFGE and the genotyping of *prn* and *ptx*A in many countries including Argentina, France, Italy, the Netherlands, Poland, Taiwan, and the USA (Cassiday et al. 2000; Fingermann et al. 2006; Gzyl et al. 2001; Lin et al. 2006; Mastrantonio et al. 1999; Mooi et al. 1998; Van Loo and Mooi 2002; Weber et al. 2001). In Finland, the circulating strains have also been shown to be different in their *ptx*A and *prn* genotypes (Mooi et al. 1999) and PFGE profiles (I). Despite different vaccination schedules and vaccine coverages, common to all these countries is that wP vaccine has been produced from strains harbouring *prn*1 and *ptx*A2 or/and *ptx*A3. The circulating strains in these countries harbour the non-vaccine types such as *ptx*A1 and *prn*2, *prn*3 or *prn*4. Thus, the changes in *ptx*A and *prn* genotypes have been proposed to be the consequences of the vaccine-driven evolution of the bacteria (Mooi et al. 1998).

In Australia and the UK, the wP vaccines have been produced from other type of strains. The Australian wP vaccine is produced from *ptx*A1 and *prn*1 type of strains (Poynten et al. 2004). In the UK, the wP vaccine is produced from strains harbouring *ptx*A1 and *ptx*A2 and *prn*1 (Fry et al. 2001). Interestingly, the *ptx*A type of the circulating strains in both Australia and the UK is still *ptx*A1, but the other vaccine strain used in the UK, *ptx*A2, seems to have vanished. In the case of *prn*, the non-vaccine types such as *prn*2 and *prn*3 have increased in both countries. These findings impugn the inducible effect of vaccination on the antigenic variation at least in the case of *ptx*A.

Japan has been unique in pertussis prevention as the aP vaccines were introduced already in 1981. The production strain of these vaccines was Tohama I representing *ptx*A2 and *prn*1. Even though the alleles included in the aP are included in the wPs used in the Netherlands and Finland, the *B. pertussis* population seems to have developed in another direction in Japan as half of the studied strains from 1991 to 2007 still represented the vaccine types *ptx*A2 and *prn*1 (Han et al. 2008). It should be kept in mind that in many countries the strains with non-vaccine types *ptx*A and *prn* appeared about 20 to 30 years after the the vaccination was started. In addition, the situation in Japan is complicated since different vaccine formulations have been used within the country during the last decades.

In Sweden, the wP vaccination with *ptx*A4 and *prn*10 type of strain was included in the national vaccination programme from 1953 to 1979 followed by a seventeen-year period without pertussis vaccination (Hallander et al. 2005a). In 1996, an aP vaccine produced from *ptx*A2 and *prn*1 type of strain was introduced. In Sweden, the *ptx*A1 was already prevalent during the wP period and *prn*2 became prevalent during the years without vaccination (Hallander et al. 2005a). This indicates that the vaccine-driven changes might have already started before 1979 as shown in I and III, or the strains might have originated in other countries where the vaccine-driven changes had already occurred, or the changes in *ptx*A and *prn* might not be directly vaccine-driven. When the Finnish and Swedish *B. pertussis* isolates were compared with PFGE, the populations were found to be rather similar despite the different vaccination history (III). This may be due to the host population with waning immunity in both countries. It is possible that the bacterial evolution has mainly occurred among the adolescent or adult population with waning immunity. Thus, the bacterial evolution might have happened distinct from the well-immunised infant and children population indicating that the changes were driven by waning immunity rather than the vaccination.

The Fims have shown temporal changes in the prevalence of Fim2 and Fim3 serotypes during decades of vaccination. Generally, *B. pertussis* Fim2 strains predominate in unvaccinated populations, whereas Fim3 strains are often isolated in vaccinated populations. Despite extensive vaccinations in Finland, however, Fim2 strains were the most common serotype from 1970s to 1990s. The exact reasons for the dominance of serotype Fim2 strains in Finland are not known. Emergence of Fim3 strains started in 1999 and coincided with nationwide epidemics (I–III). Interestingly, most of the aP vaccines do not contain Fims, and in Sweden, the Fim3 emerged only after the introduction of aP vaccination with vaccines not containing Fims (Hallander et al. 2005a). Thus, the role of Fims remains unclear even though the temporal differences seem to be related to the epidemic cycles of the disease (I–III).

6.3 Strain variation and vaccine efficacy

The hypothesis of the vaccine-driven evolution of *B. pertussis* has also been studied from the viewpoint of vaccine efficacy. In the case of Prn, the antigenic changes have mainly been found among region 1 (Appendix I). Region 1 consists of repeats and is located adjacent to an RGD motif that has been implicated in adherence (Leininger et al. 1991). In a Dutch study, the *prn* region 1 was found to harbour the protective epitope of Prn, and furthermore, the changes in this region were found to affect the efficacy of the wP vaccine (King et al. 2001). Similar results were observed in Poland, where the current wP was shown to be less efficient in clearing the *B. pertussis* strains harbouring the non-vaccine *ptx*A/*prn* than the vaccine type of strains from the lungs of the immunised mice (Gzyl et al. 2004). The human serological study performed in Finland consolidates these findings, as the infection caused by *prn*2 strains did not induce *prn*1 antibodies (He et al. 2003). In a more recent study, an Argentinean group showed that the vaccine produced from a currently circulating strain provides better protection against the currently circulating strains than the vaccine produced from the Tohama I strain (Bottero et al. 2007). Thus, the effects of the strain variation on the efficacy of pertussis vaccines is a matter of concern especially since the aP vaccines are usually produced from the strains harbouring the non-circulating *ptx*A/*prn* alleles. Even though the bacterial evolution may not be vaccine-driven, the development of new vaccines should be driven by the efficacy-affecting changes noticed among the circulating strains.

6.4 Dynamics of *B. pertussis* populations

As discussed above, the population structure of *B. pertussis* seems to be rather similar worldwide with some temporal and geographical variation and differences in the frequencies of different types of strains (Broutin et al. 2005; Caro V et al. 2005; Cassiday et al. 2000; van Amersfoorth et al. 2005). The dynamics of the *B. pertussis* population can be also studied with PFGE as it has higher discriminatory power than genotyping or serotyping.

The PFGE analysis is well suitable for tracking the transmission of the bacteria (Bisgard et al. 2001), which was also noticed as the emergence of local epidemics, e.g. in Kisko, 1998

and in Salo, 1999 and 2002–2003 (III). However, some strains seem to be more capable of spreading or adapting more effectively to the host population with waning immunity as profiles such as BpSR11 cause nationwide epidemics (I, III). In a broader scale, the PFGE analysis of the circulating isolates revealed that the sequential pertussis epidemics in Sweden (1999) and Finland (2003–2004) were caused by clonal expansion of certain strains harbouring the PFGE profile BpSR11, first noticed in France in 1994 (III). However, it is not known whether this strain was originated in France or drifted there from other countries such as Spain or Portugal where the incidence of pertussis was higher and the vaccination coverages were relatively low. In addition, the strain variation of *B. pertussis* in other neighbouring countries of Finland such as Norway, Estonia and Russia has not been widely studied, and thus, the drift of the strains from these countries to Finland cannot be excluded.

Further analysis of the BpSR11 strains with oligonucleotide-based microarray showed that these strains along with some other recent strains, representing Fim3 and *prn*2, had lost a locus of 18 genes present in older isolates and vaccine strains (Heikkinen et al. 2007). In Finland, the strains with BpSR11 were not detected in 2007 (unpublished data). It remains to be shown if the strains with BpSR11 have disappeared in Finland and other countries. One reason for the potential disappearance might be that the high prevalence of BpSR11 strains during the epidemic induced efficient protection against the type of strains and thus dramatically limited the transmission. Another reason might be the change of vaccine from wP to aP after the epidemic. However, the latter hypothesis does not seem probable as the BpSR11 strains appeared in Sweden only after the aP was introduced (Hallander et al. 2005b).

Our results from the PFGE analysis of the Finnish *B. pertussis* isolates also confirmed the earlier studies from Belgium and Taiwan that the *B. pertussis* strains circulating among infants, adolescents and adults are not age-specific (De Schutter et al. 2003; Lee et al. 2003). These findings validate the epidemiological studies stating that the disease of the infants is often transmitted from adolescent or adult contacts (Long et al. 1990; Mertsola et al. 1983; Schellekens et al. 2005). A recent study on the role of social contacts to the spreading of infectious diseases showed that during disease outbreaks, contact tracing in home, school, workplace and leisure settings would reveal over 80% of the contacts (Mossong et al. 2008). Most likely, this would also be the case during pertussis outbreaks.

6.5 Transmission of *B. pertussis*

The role of vaccination in the disease transmission has been widely studied (Broutin et al. 2005; Gandon and Day 2007; Restif and Grenfell 2007). Even though pertussis vaccinations have been in use for decades, the dynamics of pertussis disease is still not fully understood. It has been shown that the recently immunised children in contact with clinical pertussis may be infected, as the colonised bacteria can be found by PCR, but without developing symptomatic pertussis disease (Águas et al. 2006; Cherry 2005; He et al. 1994; Long et al. 1990). It is known that the pertussis vaccination protects better from

the typical disease than from the infection, thus, affecting the disease severity and transmission rather than the susceptibility of the infection.

Águas et al. introduced a well-arguable mathematical model for the pertussis disease transmission, where they also considered the above-mentioned facts of pertussis immunity (Águas et al. 2006). They concluded that the increase in pertussis incidence and a shift to older age groups result from the reduced transmission of the disease leading to the decrease of natural boosting after mild infections. Furthermore, due to the waned immunity of the older age groups, the reduced transmission has induced an increase of severe and thus reported cases of pertussis. The validity of this model is worth testing, and addresses the importance of maintaining the protective immunity among adolescents and adults after the primary vaccinations to diminish the disease transmission to infants too young to be vaccinated (Wendelboe et al. 2007). In IV, the source of infection was within the family in 25 cases out of the 29 cases where the parents addressed the potential source of infection for the child <2 years of age. Other sources besides the siblings or parents were the grandparents, cousins and family friends. One case might have been infected by the healthcare personnel of the maternity ward, as the child was symptomatic already at the age of eight days, diagnosed at the age of 14 days, and all visitors and family members were tested negative.

Thus, adolescents and adults with waning immunity act as a reservoir of *B. pertussis*. As they travel between home, school, work and even countries, they may effectively transmit the bacteria widely around their environment. It is possible that *B. pertussis* has adapted to the host population with waning immunity to maintain the bacterial reservoir in this population. As the strains infecting all age groups originate from the same reservoir, the comparison of *B. pertussis* strains from unvaccinated and vaccinated persons does not reveal dramatic differences between the bacterial strains. The analysis of the strains by PFGE together with the RFLP analysis by Mastrantonio et al. consolidate this hypothesis as no significant changes were noticed among the strains between age groups (Figure 10) and vaccination status (Mastrantonio et al. 1999). Thus, the effect of the strain variation on the transmission of *B. pertussis* might occur through the reduced vaccine efficacy due to the antigenic changes leading to the increased transmission and incidence rather than the development of more virulent strains.

6.6 Pertussis after introducing acellular vaccine

Acellular vaccines have been introduced in many developed countries, and the effects of the change from wP to aP have been studied in the USA, Canada and Austria (Bettinger et al. 2007; Bisgard et al. 2005; Rendi-Wagner et al. 2006). In the study of Bisgard et al., three doses of any DTaP or DTwP vaccine was found highly protective among the children aged from 6 to 59 months, but the disease characteristics such as severity or the need of hospitalisation were not discussed and compared between the DTwP and DTaP recipients (Bisgard et al. 2005). The hospitalised cases <16 years of age and diagnosed with pertussis during the vaccine transition period were studied in Austria (Rendi-Wagner et al. 2006). They noticed that during the aP era, even 19% of the hospitalised cases were fully vaccinated compared to 2% during the wP era. Surprisingly high proportion of the

hospitalised cases was noticed among the children aged between 6 and 14 years, probably related to the high proportion of unvaccinated cases (63% of all with known vaccination status). Recently, regular boosters with ten-year intervals have been recommended for adolescents and adults in Austria (Rendi-Wagner et al. 2006). After the introduction of aP in Canada, significant improvement in the protection was noticed as a decreased incidence among children <5 years of age and decreased need of hospitalisation among the infants aged between 4 to 59 months (Bettinger et al. 2007). As stated, the improvements were mainly due to the better effectiveness of the aP compared to the wP previously used in Canada.

In IV, the effects of the vaccine change were examined among all the diagnosed cases of pertussis in children <2 years of age. Due to the low season of pertussis, we only observed a limited number of cases during the aP era. However, it seems that the children with pertussis who had been age-appropriately vaccinated with aP were more often hospitalised than those vaccinated with wP (57% vs. 47%, P value 0.344). This may be due to the changed schedule as the wP was administered at 3, 4, 5 months and the aP at 3, 5, and 12 months. The old schedule most likely protected the children already at the age of 4–5 months whereas the new schedule provides stronger protection a couple of months later. The youngest ones are more vulnerable to the severe disease and need hospitalisation, and thus, the difference is probably more related to the vaccination schedule than the vaccine itself. However, as the aim of the pertussis vaccination is to provide protection for the youngest ones, the finding indicates that the third dose in this Scandinavian schedule is rather late when given at the age of one year.

6.7 Vaccination schedule and age-specific incidence

The design of the national vaccination programme includes the selection of the vaccine and the schedule. The decisions are based on both economical and scientific evaluations, and they have far-reaching effects in both individual and community level. Potential vaccination strategies to prevent pertussis were recently reviewed by GPI, an international group of pertussis specialists (Forsyth et al. 2005). These strategies include vaccination of different target groups such as new mothers, healthcare workers or adolescents. They concluded that current vaccination programmes are not sufficient. The GPI recommended adolescent boosters with aP or TdaP vaccine as the Td vaccine is already recommended to adolescents in many countries. In Finland, the adolescent booster to 14–15-year-olds was included in the national vaccination programme in 2005. Additional strategies would be e.g. the vaccination of the healthcare and childcare workers and the cocoon strategy, i.e. vaccination of the household members of newborns (Forsyth et al. 2007).

In Australia, a preschool booster to 4–5-year-olds was introduced in 1994 and its effects on the age-specific incidences was studied (Torvaldsen and McIntyre 2003). It was observed that the booster clearly reduced the age-specific incidences among the age-groups eligible to the booster vaccinations. Still, the preschool booster did not significantly decrease the notification rate of the infants. Similar results were obtained in Denmark in a retrospective long-term study of the children born in 1977–2001 (Hviid et al. 2006). It was, however, estimated that the preschool booster at 5 years of age would prevent 18% of the

hospitalisations due to pertussis among children <2 years of age even though the reduced transmission in the community was not considered. In the developed countries, many of the preschool children attend daycare, where infections are transmitted between the children and the childcare workers. Thus, the role of the reduced transmission in this age group might be more considerable to the overall burden of pertussis.

Computer simulations have also been used to estimate the effects of different vaccination strategies to the age-specific incidences. In Australia, the pertussis vaccine dose given at the age of 18 months was replaced with an adolescent booster dose to the 15–17-year-olds in 2003, and the potential long-term effects were estimated (Hethcote et al. 2004). After the change, the pertussis vaccines were given at 2, 4 and 6 months, 4 years and 15–17 years. Compared to the earlier schedule, the change was estimated to decrease the pertussis cases in adolescents and 0–23-months-olds for 25% and 30%, respectively. However, the removal of the dose at 18 months of age increased the cases among the 2–4-year-olds as could be expected. The overall effect of the change still remained positive without any increase in the costs of the vaccination programme (Hethcote et al. 2004).

In Finland, the pertussis vaccination programme was changed twice during the last few years. First, a preschool booster at 6 years of age was introduced in 2003. The effect of the preschool booster was noticed right away during the pertussis epidemic in 2004. Among the 4–14-year-olds reported to the IDR, the 6- and 7-year-olds were the only age groups with lower incidence in 2004 than in 2003. In 2005, the whole vaccination schedule was changed with concomitant change of the vaccine type from wP to aP. When the vaccination schedule was changed in 2005, the children who were too old for the booster introduced in 2003 were vaccinated at the age of 11–13 years. After that, a dramatic decrease in the number of cases among 12-year-olds was noticed in 2006 (IV). Thus, the booster vaccination has a major effect on the disease burden at least among the boostered age group. The more wide-ranging effects on the other age groups due to the reduced disease transmission from adolescents remain to be seen.

6.8 Diagnostic dilemma of pertussis

The clinical presentation of pertussis varies exceedingly depending on several factors such as age and immunisation status (reviewed in Tozzi et al. 2005). The wide spectrum of the symptoms together with limited diagnostic methods complicates the diagnosis of pertussis. The best specificity and sensitivity is achieved when the methods are used in parallel, and when the tests are done as soon as possible after the symptoms appear (Tozzi et al. 2005).

Culture is the oldest but still useful method for the diagnosis during the first weeks of the disease. However, as the bacteria is cleared by the host immune system some weeks after the symptoms appear, the method cannot be used in the later stages of the illness. PCR has become the method of choice, because it can also detect the fragments of the bacteria in addition to the living bacteria, it is faster than culture, and it is very specific. However, culture is still recommended, as it is the only method to provide the actual bacterial strains for the public health purposes (Mooi et al. 2000; Wolf and Daley 2007). Both culture and PCR are suitable during the early stage of the disease, but serological tests are needed when the symptoms have lasted for several weeks. The serological diagnosis would generally

need two samplings from the patient, which are acute sera and later another sample, to show the increase in the antibody titers. If paired sera is not available and a single sera shows high antibody titers, the diagnosis may be considered as confirmed in adolescents and adults, who have not been immunised against pertussis since childhood. Thus, serology is more useful for the confirmation of the diagnosis in adults and adolescents than for the rapid diagnosis in infants (Muller et al. 1997). However, the boosters given to the adolescents and adults complicate the serological diagnosis as the antibody responses to the natural infection and vaccination are very hard or even impossible to distinguish.

New diagnostic methods and approaches are thus needed to improve the diagnosis of pertussis especially in young infants who need a timely diagnosis to get decent treatment before the disease threatens their lives. A model exploiting PCR, culture and serological samples from both mother and infant was developed by Grimprel et al., but the real use of the model might be limited due to the unavailability of the maternal prepartum sera (Grimprel et al. 1997). Recently, the usefulness of the basic laboratory tests for the prediction of pertussis in infants was studied (Guinto-Ocampo et al. 2008). Correlation of fifteen variables from patients tested positive for pertussis was analysed, and the absolute lymphocyte counts were found the best predictor of the infant's pertussis before confirmation with other diagnostic methods. However, this approach needs more evaluation and validation before it can be officially recommended.

As pertussis is often coexistent with other respiratory pathogens and may appear without typical and recognisable symptoms, the differential diagnostics to either confirm or exclude pertussis is important. However, physicians are often unaware of pertussis, and thus the delay in the suspicion and testing for pertussis may restrain the diagnosis for weeks. Especially for young infants, the delay in the diagnosis causes anxiety. This was reported already by Johnston et al. in 1985 (Johnston et al. 1985), and the feedback from many parents of the children included in IV showed a similar effect still during the last decade in Finland. As the medical knowledge and the diagnostic methods have improved significantly since the Johnston report (Johnston et al. 1985), the reason might be the physicians' unawareness of the disease among the vaccinated population. Thus, the healthcare workers and physicians should be reminded of the possibility of pertussis despite the vaccination history of the patient especially during an epidemic season.

6.9 Presence and future of pertussis prevention

Finland is in the same situation as many other developed countries with an increasing trend of pertussis especially among adults and adolescents (Celentano et al. 2005). The recent addition of the adolescent booster in Finland will most likely have a reducing effect on the disease burden of pertussis at least among adolescents (IV). What remains to be seen is if the booster has any effect on the incidence among the youngest infants. The effects of this concomitant change of the vaccination schedule and the vaccine from wP to aP will be seen when the next epidemic occurs likely in a few years. Until now, the two-component aP (containing PT and FHA) used in Finland does not seem to be worse than the wP used for the preceding decades (IV) even though the one- and two-component pertussis vaccines have been shown to be less efficacious than the vaccines with three or more

components (Jefferson et al. 2003). However, the disease pressure has been low during the study period (IV). This, together with herd immunity as a consequence of the decades of vaccination with efficient vaccine and high coverage, may have restrained the effects of the change of the vaccine. Finland is, to our knowledge, the only country where the two-component aP vaccine is recommended as most countries have selected the vaccines containing Prn and/or Fim2/3 in addition to PT and FHA. The experience in Sweden (Ad Hoc Group 1988; Tindberg et al. 1999) and the lower price of this two-component vaccine led Finland to take the vaccine into the national vaccination programme in 2005. However, the two-component aP will be replaced by a three-component aP in 2009. As of adult vaccination, the dT booster recommended for the adults in ten-year intervals may be replaced by dtap (Halperin et al. 2006), but for now, the dtap vaccine will not be offered for free and needs a prescription from a physician.

In a global scale, two matters are of concern. The first is the pertussis situation in the developing countries where the case fatality rates in infants are estimated to be as high as 4% (Crowcroft et al. 2003a). The developing countries may not have facilities and economical possibilities to execute the extensive vaccination programmes, and the use of new vaccines may be prohibited due to the high costs (Singh and Lingappan 2006). In many African countries, the vaccination coverage is less than 50% (WHO 2007). Thus, political decisions towards universal vaccination of the infants should be executed (Music 2005).

Another matter of concern in the field of vaccine-preventable diseases is the anti-vaccine movement in the developed countries. In the case of tetanus, the vaccine refusal only puts the unvaccinated individual at risk, whereas in the case of pertussis, the anti-vaccine movement may pose a notable threat even to the vaccinated individuals in the community. Recently, the anti-vaccine movement often provoked by the representatives of the natural or alternative medicine has strengthened in the USA raising the discussion on the individual and community risks of the refusal of vaccination (Feikin et al. 2000; Leino and Kilpi 2005). For the meantime, the anti-vaccine movement is not a significant menace to the community in Finland. Only 13 out of 154 children older than 4 months of age with diagnosed pertussis were not vaccinated at all and three of the cases were unvaccinated due to the parental refusal (IV). In addition, the official statements of the national and the Scandinavian society of the homeopaths are not against vaccinations and they do not encourage the parents to leave their children out from the national vaccination programme (personal communication, Marja-Terttu Pakkanen, the president of the Scandinavian society of the homeopaths). As the successful vaccination programme has significantly reduced the incidence of pertussis, many of the adolescents and adults have never witnessed a child suffering from severe pertussis. Thus, the parents refusing to vaccinate their children should be informed about the consequences of not vaccinating at both individual (put the child at risk of severe but avoidable disease) and community level (put the contacts of the child at risk).

The only beneficial matter that originated from the anti-vaccine movements in the 1970s was the development of the aP vaccines (Sato and Sato 1999). The vaccine industry together with the researchers worldwide is continuously exploring new vaccine components and vaccination approaches. New acellular outer membrane vaccines might

provide a wider protection than the traditional aP vaccines and the mechanisms of protection might be different because the epitopes are presented to the host in a more native conformation (Roberts et al. 2008). The development of improved wP vaccines is also under investigation, because the production costs of the wP vaccines are significantly lower than those of the aP vaccines, the antibody responses to the wP vaccination are more similar to the natural infection than the responses to the aP vaccination, and the immunity seems to last longer after wP than after aP vaccination (Bisgard et al. 2005; Heininger 2001; Wendelboe et al. 2005). Promising results were obtained from the study by Geurtsen et al., who noticed in a mouse study that the wP vaccines supplemented with non-toxic lipopolysaccharide analogs were more efficacious and induced less adverse effects than the traditional wP vaccines (Geurtsen et al. 2008). They have also shown that a similar approach may be used to improve the efficacy of the aP vaccines (Geurtsen et al. 2007).

Recent research has suggested that a birth dose of aP vaccine at the age of 2 to 5 days will result in earlier immune responses than the conventional vaccination schedule starting at the age of 2 months (Knuf et al. 2008). Maternal vaccination during pregnancy has also proved to be safe and efficacious for the protection of newborns (Mooi and de Greeff 2007). However, both of these approaches may not obtain unreserved support from the parents despite the recommendations and justification from the scientific community. More attractive might be the newly developed intranasal vaccines with the genetically attenuated *B. pertussis* strains that would enable immunisation of the infants without injections already very early in life (Mielcarek et al. 2006).

New vaccination strategies might enable better prevention of pertussis (Forsyth et al. 2007). Availability of less reactogenic aP vaccines has enabled booster vaccinations for adults and adolescents already recommended in many countries. The cocoon strategy, i.e. the vaccination of household members of newborns, has also been recommended, as the universal adult vaccination is not yet achievable in most countries. For the improved prevention, the vaccine coverage among adults and adolescents should be increased to the level of infant vaccinations. It should also be reminded that the primary vaccines and boosters should be given even if the child, adolescent or adult had suffered from the pertussis disease prior the vaccination. The achievements gained from the decades of extensive pertussis vaccinations will be lost without the maintenance and improvement of the current vaccination programmes.

SUMMARY AND CONCLUSIONS

The aims of this thesis were (1) to examine the antigenic changes in *B. pertussis* population during the era of vaccination in Finland, and (2) to compare the bacterial populations between the countries with similar and different vaccination history. The strains were analysed using the molecular methods recommended for the epidemiological typing of the *B. pertussis* isolates. In addition, (3) the potential effects caused by the change of the vaccination programme in Finland were studied especially among the children <2 years of age.

1. The results showed the continuous evolution of the *B. pertussis* population in Finland since the beginning of the nationwide pertussis vaccinations in the 1950s. The antigenic variation of PT and Prn was observed, and the *ptx*A and *prn* alleles among the circulating isolates differ from the vaccine strains. The three serotypes, Fim2, Fim3 and Fim2.3 were all detected during the study period, but changes in the frequencies were noticed. The PFGE analysis of the isolates collected since 1950s showed that *B. pertussis* is dynamic and continuously evolving. Most recently, the significant increase of strains with Fim3 serotype was associated with the PFGE profile BpSR11. These strains were responsible for the epidemics first in Sweden and then in Finland.

2. Although the comparison of the *B. pertussis* isolates from Finland, France and Sweden did not refer to significant differences that would be resulting from the different vaccination histories, temporal and spatial differences among the PFGE profiles were observed. The circulating strains in these three countries differed from their vaccine strains in respect to *ptx*A and *prn*. The strains with marked antigenic and genomic changes such as *ptx*A1/*prn*2/Fim3/BpSR11 seem to have adapted to the host population with waning immunity in order to maintain the bacterial reservoir among adolescents and adults. The advantages acquired by the adaptation might relate to the better metabolism, increased fitness and colonisation, or better adaptation to the hosts with partial immunity.

3. The *B. pertussis* population isolated from the infants showed to be highly similar to the population isolated from other age groups. This may be because the strains infecting all age groups originate from the same reservoir among adolescents and adults. The antigenic variation does not seem to play a major role in pertussis epidemiology within a few years after vaccination. There was no clear link between the hospitalisation or vaccination status and certain *B. pertussis* isolates. However, attention should be paid to the infants too young to be vaccinated according to the current schedule, as 52% of the pertussis cases reported in children <2 years of age were diagnosed among the unvaccinated infants ≤3

months of age. The hospitalisation rate among this group was high (88%). Until now, the two-component aP used for the primary vaccinations in Finland seems to provide adequate protection against pertussis disease. However, the disease pressure has been low probably due to the epidemic cycle of pertussis. The real efficacy of the new vaccination programme remains to be seen during the next years.

In addition to the studies on the molecular evolution of *B. pertussis*, future research should be aimed at the development of better strategies or vaccines or both to prevent pertussis especially in infancy. Improved and rapid diagnostics as well as continuous follow-up of the circulating strains is also important. Despite the successful worldwide vaccinations that led to the dramatic decrease in the mortality and morbidity in the 1940s–1950s, today pertussis is not even near to eradication in any parts of the world.

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APPENDIX I

Pertactin

	102	260	266							291	337	532	589	853
Prn1	S	RGD	GGAVP	GGAVP	GGAVP	GGFGP	GGFGP	-----	-----	VLD	S	L	PQP	H
Prn2	.	...	GGAVP	GGAVP	GGFGP	GGFGP	GGFGP	GGFGP	-----	VLD	.	.	...	.
Prn3	.	...	GGAVP	GGAVP	GGFGP	GGFGP	GGFGP	-----	-----	VLD	.	.	...	.
Prn4	.	...	GGAVP	GGAVP	GGFGP	GGFGP	-----	-----	-----	VLD	.	.	...	.
Prn5	.	...	GGAVP	GGFGP	GGFGP	GGFGP	-----	-----	-----	VLD	.	.	...	.
Prn6	F	...	GGGVP	GGAVP	GGAVP	GGFGP	GGFGP	-----	-----	VLD	F	R	---	R
Prn7	.	...	GGAVP	GGAVP	GGAVP	GGFGP	GGFGP	-----	-----	VLD	.	R	...	.
Prn8	.	...	GGAVP	GGAVP	GGFGP	GGFGP	-----	-----	-----	VLD	.	R	...	.
Prn9	.	...	GGAVP	GGAVP	GGFGP	GGFGP	GGFGP	GGFGP	GGFGP	VLD	.	.	...	.
Prn10	F	...	GGGVP	GGAVP	GGAVP	GGAVP	GGFGP	GGFGP	-----	VLD	F	R	---	R
Prn11	.	...	GGAVP	GGAVP	GGAVP	GGAVP	GGFGP	GGFGP	-----	VLD	.	.	...	.
Prn12	.	...	GGAVP	GGAVP	GGAVP	GGAVP	GGFGP	GGFGP	-----	VLD	S	L	...	H

Pertussis toxin S1 subunit

	196						580						679						
ptxA2	GTG	CTC	GAC	CAT	CTG	-//-	GAG	TAT	TCC	AAC	GCT	-//-	CGC	ATG	GCG	CCG	GTG	ATA	GGC
	V	L	D	H	L		E	Y	S	N	A		R	M	A	P	V	I	G
ptxA1	...	...	...	...	...	-//-	...	...	...	...	...	-//-	...	..A	...	...	...	...	...
														I					
ptxA3	...	...	..A	...	...	-//-	...	...	...	...	...	-//-	...	...	...	...	...	G.G	...
			E															V	
ptxA4	...	...	..A	...	...	-//-	...	...	C..	...	...	-//-	...	...	...	...	...	..G	...
			E						P									M	

APPENDIX II

PFGE profiles observed in more than one Finnish *B. pertussis* isolate.

The most frequent profiles are marked with ★, the vaccine strains of the Finnish DTwP with ⊙, and the international reference strains with ⚑. The columns present the identification code of the isolate, PFGE profile and the first year of detection in Finland.