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# EVOLUTION OF *BORDETELLA PERTUSSIS* POST VACCINATION

by

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"…we know, there are known knowns; there are things we know we know. We also know there are known unknowns; that is to say we know there are some things we do not know. But there are also unknown unknowns -- the ones we don't know we don't know."

-Donald Rumsfeld-

# ABSTRACT

TEEMU KALLONEN

**Evolution of *Bordetella pertussis* post vaccination**

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Whooping cough or pertussis is caused by the gram-negative bacterium *Bordetella pertussis*. It is a highly contiguous disease in the human respiratory tract. Characteristic of pertussis is a paroxysmal cough with whooping sound during gasps of breath after coughing episodes. It is potentially fatal to unvaccinated infants. The best approach to fight pertussis is to vaccinate. Vaccinations against pertussis have been available from the 1940s. Traditionally vaccines were whole-cell pertussis (wP) preparations as part of the combined diphtheria-tetanus-pertussis (DTP) vaccines. More recently acellular pertussis (aP) vaccines have replaced the wP vaccines in many countries. The aP vaccines are less reactogenic and can also be administered to school children and adults. There are several publications reporting variation in the *B. pertussis* virulence factors that are also aP vaccine antigens. This has occurred in the genes coding for pertussis toxin and pertactin about 15 to 30 years after the introduction of pertussis vaccines to immunisation programs. Resurgence of pertussis has also been reported in many countries with high vaccination coverage. In this study the evolution of *B. pertussis* was investigated in Finland, the United Kingdom, Poland, Serbia, China, Senegal and Kenya. These represent countries with a long history of high vaccination coverage with stable vaccines or changes in the vaccine formulation; countries which established high vaccination coverage late; and countries where vaccinations against pertussis were started late. With bacterial cytotoxicity and cytokine measurements, comparative genomic hybridisation, pulsed-field gel electrophoresis (PFGE), genotyping and serotyping it was found that changes in the vaccine composition can postpone the emergence of antigenic variants. It seems that the change in PFGE profiles and the loss of genetic material in the genome of *B. pertussis* are similar in most countries and the vaccine-induced immunity is selecting non-vaccine type strains. However, the differences in the formulation of the vaccines, the vaccination programs and in the coverage of pertussis vaccination have affected the speed and timing of these changes.

**Key words:** *Bordetella pertussis*, whooping cough, pertussis, vaccination, strains variation, CGH, PFGE, adaptation, evolution, genotyping, serotyping, cytotoxicity, cytokines

# TIIVISTELMÄ

TEEMU KALLONEN

***Bordetella pertussis* -bakteerin evoluutio hinkuyskärokotusten aloittamisen jälkeen**

Lääketieteellinen mikrobiologia ja immunologia, Lastentautioppi ja Turun biolääketieteellinen tohtoriohjelma, Turun yliopisto, Turku sekä Tartuntatautiseurannan ja -torjunnan osasto, Terveyden ja hyvinvoinnin laitos, Turku
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Hinkuyskän aiheuttaja on gramnegatiivinen bakteeri: *Bordetella pertussis*. Hinkuyskä on helposti tarttuva hengitystieinfektio ihmisellä. Hinkuyskälle ominaista on yskän puuskat joiden välissä, potilaan syvään hengittäessä, kuuluu hinkunaa. Hinkuyskä voi olla tappavan vaarallinen erityisesti rokottamattomille lapsille. Paras tapa suojata taudilta on rokottaminen. Hinkuyskä rokotteita on ollut saatavilla 1940-luvulta lähtien. Perinteisesti rokotteet olivat kokosoluvalmiste *B. pertussis* -bakteerista osana yhdistettyä difteria-tetanus-pertussis-rokotetta (DTP). Viimeaikoina soluton (aP) hinkuyskärokote on syrjäyttänyt kokosolurokotteen (wP) monissa maissa. Soluton rokote aiheuttaa vähemmän reaktioita ja sitä voidaan antaa myös kouluikäisille ja aikuisille. Lukuisissa tutkimuksissa on raportoitu muutoksia *B. pertussis* -bakteerin virulenssitekijöissä, jotka ovat myös soluttoman rokotteen antigeeneja. Muutokset ovat tapahtuneet, pertussis toksiinia ja pertaktiinia koodaavien geenien osalta, keskimäärin 15–30 vuotta hinkuyskärokotuksen rokotusohjelmaan lisäämisen jälkeen. Hinkuyskä on myös uudelleen yleistynyt monissa maissa, joissa on korkea rokotekattavuus. Tässä tutkimuksessa tutkittiin *B. pertussis* -bakteerin evoluutiota Suomessa, Isossa Britanniassa, Puolassa, Serbiassa, Kiinassa, Senegalissa ja Keniassa. Nämä edustavat maita joissa on pitkään ollut korkea rokotekattavuus ja vakiintunut rokote tai rokotetta on muutettu ajan mittaan, korkean rokotekattavuuden myöhään saavuttaneita maita ja maita joissa rokotukset aloitettiin myöhään. Bakteerin aiheuttaman sytotoksisuuden ja sytokiinierityksen mittauksilla, vertailevalla genomiikalla, pulssikenttägeelielektroforeesilla (PFGE), geno- ja serotyypityksellä havaittiin, että muutokset rokotteessa voivat viivästyttää uusien antigeenivarianttien ilmaantumista. Useimmissa maissa vaikuttaa siltä, että muutokset PFGE profiileissa ja geenien poistumiset ovat samansuuntaisia ja rokoteen antama immuniteetti on valinnut kantoja jotka poikkeavat rokotekannoista. Muutokset rokotteen sisällössä, erot rokotusohjelmissa sekä rokotekattavuudessa ovat kuitenkin vaikuttaneet muutosten nopeuteen ja ajankohtaan.

**Avainsanat:** *Bordetella pertussis*, hinkuyskä, pertussis, rokotus, kantavaihtelu, CGH, PFGE, adaptaatio, evoluutio, genotyypitys, serotyypitys, sytotoksisuus, sytokiinit

# CONTENTS

# ABBREVIATIONS

| | |
|---|---|
| ACT | adenylate cyclase toxin |
| ADP | adenosine diphophate |
| aP/ap | acellular pertussis (vaccine) |
| BB | *Bordetella bronchiseptica* |
| Bp/BP | *Bordetella pertussis* |
| bp | base-pair |
| BrkA | Bordetella resistance to killing |
| BSA | bovine serum albumin |
| BvgAS | Bordetella virulence gene activator sensor |
| CD | cluster of differentiation |
| CDC | The Centers of Disease Control and Prevention |
| cfu | colony-forming unit |
| CGH | comparative genomic hybridisation |
| CHO | Chinese hamster ovary (cell) |
| CRD | carbohydrate recognition domain |
| Cy | cyanine |
| DI | diversity index |
| DIG | digoxigenin |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| DNT | dermonecrotic toxin |
| dNTP | deoxyribonucleotide triphosphate |
| DTP | diphtheria, tetanus, pertussis |
| ECDC | European Centre for Disease Prevention and Control |
| ELISA | enzyme-linked immunosorbent assay |
| FHA | filamentous haemagglutinin |
| FIM | fimbriae |
| FRET | fluorescence resonance energy transfer |
| G protein | guanine nucleotide-binding proteins |
| GTP | guanosine triphosphate |
| Hib | *Haemophilus influenzae* type b |
| HPD | histidine phosphotransfer domain |
| IFN | interferon |
| Ig | immunoglobulin |
| IL | interleukin |
| IPV | inactivated polio virus |
| IS | insertion sequence |
| IU | international unit |
| kb | kilobase = 1000 base-pairs |
| kDa | kilodalton |
| KTL | Kansanterveys laitos, National Public Health Institute |
| LPS | lipopolysaccharide |
| MAP | mitogen-activated protein (kinase) |
| MLST | multilocus sequence typing |
| MLVA | multilocus variable number of tandem repeats |

| | |
|---|---|
| NAD | nicotinamide adenine dinucleotide |
| NF-κB | nuclear factor kappa-light-chain-enhancer of activated B cells |
| ORF | open reading frame |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PFGE | pulsed-field gel electrophoresis |
| PMA | phorbol 12-myristate 13-acetate |
| PRCB | pertussis reference culture bank |
| PRN | pertactin |
| PT | pertussis toxin |
| qPCR | quantitative real-time polymerase chain reaction |
| RD | region of difference |
| RFLP | restriction fragment length polymorphism |
| RGD | arginyl-glycyl-aspartic acid, Arg-Gly-Asp |
| RisAS | reduced intracellular survival activator sensor |
| RNA | ribonucleic acid |
| RPMI | Roswell Park Memorial Institute medium |
| RTX | repeats-in-toxin |
| SDS | sodium dodecyl sulphate |
| SNP | single nucleotide polymorphism |
| SSC | saline sodium citrate buffer |
| TCF | tracheal colonisation factor |
| TCT | tracheal cytotoxin |
| Th1/Th2 | type 1 helper T cell/type 2 helper T cell |
| THL | Terveyden ja hyvinvoinnin laitos, National Institute for Health and Welfare |
| TNF | tumor necrosis factor |
| TPS | two-partner secretion |
| vag | *vir*-activated genes |
| vrg | *vir*-repressed genes |
| WHO | World Health Organization |
| wP | whole-cell pertussis (vaccine) |

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred in the test by corresponding Roman numerals I-V. Previously unpublished data is also included.

I Eriikka Heikkinen*, Teemu Kallonen*, Lilli Saarinen, Rolf Sara, Audrey J. King, Frits R. Mooi, Juhani T. Soini, Jussi Mertsola & Qiushui He (2007) Comparative Genomics of *Bordetella pertussis* Reveals Progressive Gene Loss in Finnish Strains. PLoS ONE 2(9): e904.

II Gordana Dakic, Teemu Kallonen, Annika Elomaa, Tatjana Pljesa, Mirjana Vignjevic-Krastavcevic & Qiushui He (2010) *Bordetella pertussis* vaccine strains and circulating isolates in Serbia. Vaccine 28(5) 1188–1192

III Liu Zhang*, Yinghua Xu*, Jianhong Zhao, Teemu Kallonen, Shenghui Cui, Yungqiang Xu, Qiming Hou, Fengxiang Li, Junzhi Wang, Qiushui He & Shumin Zhang (2010) Effect of Vaccination on *Bordetella pertussis* Strains, China. Emerging Infectious Diseases, 16(11) 1695-1701

IV Teemu Kallonen, Kirsi Gröndahl-Yli-Hannuksela, Annika Elomaa, Anna Lutyńska, Norman K Fry, Jussi Mertsola & Qiushui He (2011) Differences in the genomic content of *Bordetella pertussis* isolates before and after introduction of pertussis vaccines in four European countries. Infection, Genetics and Evolution, in press, doi:10.1016/j.meegid.2011.09.012

V Teemu Kallonen, Jussi Mertsola Frits R. Mooi & Qiushui He (2011) The recently emerged *Bordetella pertussis* strains with the *ptxP3* pertussis toxin promoter allele show increased cytotoxicity towards human monocytes. (Submitted manuscript)

* Equally contributed

# 1. INTRODUCTION

Pertussis, also known as whooping cough, is mainly caused by the bacterium *Bordetella pertussis*. It is considered to be the worst-controlled vaccine-preventable disease in the world. It is especially dangerous to infants to whom the disease may be fatal. Even though vaccination programs against pertussis were initiated in most countries between the 1940s and the 1960s, pertussis is still endemic in many countries and causes epidemics. The World Health Organization (WHO) estimated that in 2008 there were 16 million cases of pertussis in the world of which 95% occurred in developing countries (WHO 2010a). According to the same estimates 195 000 children died of pertussis in 2008 (Black et al. 2010).

In recent years reports of the resurgence of pertussis have been published in many countries with long histories of vaccination against pertussis and high vaccination coverage. These countries include many European countries as well as the USA and Australia, among others (Tanaka et al. 2003; Elomaa et al. 2005; Spokes and Gilmour 2011). Reasons behind this resurgence are not entirely clear but are thought to include at least some of the following: better diagnostic methods, better awareness of the disease by medical professionals, waning of the vaccine-induced immunity and adaptation of the causative agent *Bordetella pertussis* to survive in vaccinated populations.

In Finland, vaccinations against pertussis were initiated in 1952. The vaccine composition used before 1962 remains unknown, but after that, strain 18530 was used and strain 1772 was added to the vaccine in 1976. The Finnish whole-cell pertussis (wP) vaccine remained unchanged after 1976. The primary vaccines were given at 3, 4 and 5 months of age and a booster at 20 to 24 months of age. In 2003, an acellular pertussis (aP) booster vaccine was introduced for 6-year-old children. The vaccination program was changed in 2005 when also the primary DTP vaccine (diphtheria, tetanus, pertussis) was changed to an aP vaccine (DTaP). Vaccines are given at 3, 5 and 12 months of age and booster doses are given at 4 and 14 years of age. Like Finland, most other developed countries have changed from wP vaccines to less reactogenic aP vaccines which are well tolerated and which can be administered not only to children but adolescents and adults, too (Mattoo and Cherry 2005; Jacquet et al. 2006; Zepp et al. 2011; Zhang et al. 2011).

The long history of vaccinations against pertussis has created a strong selection pressure against vaccine-type strains and, indeed, antigenic variation has been reported in *B. pertussis* virulence genes. However, it is not well known how vaccinations have affected the bacterial population; what strategies, other than antigenic variation, the bacterium has used to evade the host immune response; and how *B. pertussis* has evolved during the more than half a century during which vaccinations against it have been used.

The aims of this study were to investigate the effect of the composition of the pertussis vaccine; the time of the commencement of the vaccinations and the vaccination coverage on the *B. pertussis* population in Finland as well as three other European countries; Poland, Serbia as a part of the former Yugoslavia (later referred to as 'Serbia'), and the United Kingdom and also countries from other continents: China, Senegal and Kenya; and to elucidate, by functional tests, the reasons behind the recent changes in the bacterial population and the reasons why the recently emerged clone seems to be more virulent than old ones (Hallander et al. 2007; Mooi et al. 2009).

# 2. REVIEW OF THE LITERATURE

## 2.1. Pertussis

Pertussis, also known as whooping cough, is a disease of the human respiratory tract. It is caused by a Gram-negative bacterium of the genus Bordetella: *Bordetella pertussis*. *Bordetella parapertussis* also causes a disease with similar but often milder symptoms. The disease is spread by direct contact to excretion of the airways e.g. aerosolized droplets a pertussis patient is spreading by coughing or sneezing (KTL 2007; WHO 2010a). Pertussis is highly contagious and often the source of infection is in the household (Anderson and May 1982; Bisgard et al. 2004; Wendelboe et al. 2007; Wearing and Rohani 2009). The disease is especially dangerous and potentially fatal to infants who are too young to be vaccinated.

The pertussis disease and also the first pertussis epidemic were first described by Guillaume de Baillou in Paris, France already in 1578. He also described the clinical picture of the disease in detail. The genus of the bacterium that causes pertussis is named after Jules Bordet who with Octave Gengou first isolated and cultured the bacterium (Bordet and Gengou 1906).

### 2.1.1. Symptoms

After the initial infection, the incubation time of pertussis varies from 7 to 10 days, with a range of 4 to 21 days (Stechenberg 2008).The disease progression is typically divided into three stages: catarrhal, paroxysmal, and convalescent stages. In the first, catarrhal stage, the disease initially resembles closely a common cold with symptoms like coryza, conjunctival irritation, slight cough and possibly mild fever. The disease can therefore be easily misdiagnosed or the diagnosis may be delayed. Pertussis is most contiguous in the catarrhal stage. A prolonged paroxysmal cough, which is characteristic to pertussis, develops after one to two weeks, in the second paroxysmal stage. A whoop sound can be heard when the patient struggles for breath between coughing episodes. Other symptoms include post-tussive vomiting and subconjunctival haemorrhages. This stage can last up to six weeks. The severity of the cough and the paroxysms ease with time in the convalescent stage which can last for weeks or even moths. (Singh and Lingappan 2006; Stechenberg 2008)

Atypical symptoms (e.g. absence of whoop) can often be seen in infants as well as in adolescents and adults. Apnea is a common finding in infants. Also other frequent complications like pneumonia, pneumothorax, severe pulmonary hypertension, seizures, and encephalopathy are seen. The symptoms of adolescent and adult patients are often milder as they are usually at least partially protected by the waning immunity from previous vaccinations or infections and the disease is often misdiagnosed as common bronchitis. Other misdiagnoses include *Mycoplasma pneumoniae* infection, sinusitis, other upper respiratory tract infection, asthma, laryngitis and cystic fibrosis. (Yaari et al. 1999; Singh and Lingappan 2006; Stechenberg 2008)

Pertussis in adults is often a persistent illness associated with a cough with a mean duration of 36–54 days and lasting up to 123 days (Gilberg et al. 2002; von König et al. 2002). Pertussis in adults is also often associated with breathing difficulties, post-tussive vomiting and whooping (von König et al. 2002). Adult pertussis can also manifest itself with sweating episodes (Postels-Multani et al. 1995; De Serres et al. 2000), syncope (Jenkins and Clarke 1981) and encephalopathy (Halperin and Marrie 1991) with complications induced by severe coughing such as rib fracture, hernia, urine incontinence or back pain (Postels-Multani et al. 1995; De Serres et al. 2000). There is also a report

of carotid artery dissection (Skowronski et al. 2003) and therefore pertussis can be fatal even in adults (Mertens et al. 1999).

### 2.1.2. Pertussis case definition

There are three international recommendations for the diagnosis of pertussis. The WHO recommendation states that a clinical case is "A case diagnosed as pertussis by a physician or a person with a cough lasting at least two weeks with at least one of the following symptoms: paroxysms (i.e. fits) of coughing, inspiratory whooping, post-tussive vomiting (i.e. vomiting immediately after coughing) without other apparent cause". The laboratory diagnosis needs "isolation of *Bordetella pertussis* or detection of genomic sequences by means of the polymerase chain reaction (PCR) or positive paired serology". Pertussis cases can also be classified as clinically confirmed when you have "a case that meets the clinical case definition but is not laboratory-confirmed". A laboratory-confirmed case needs to meet the clinical case definition and be also laboratory-confirmed. (WHO 2003)

Clinical case definition of the Centers of Disease Control and Prevention (CDC, USA) is: "a cough illness lasting at least 2 weeks with one of the following: paroxysms of coughing, inspiratory "whoop," or posttussive vomiting, without other apparent cause (as reported by a health professional). This clinical case definition is appropriate for endemic or sporadic cases. In outbreak settings, a case may be defined as a cough illness lasting at least 2 weeks (as reported by a health professional)". The CDC criteria for laboratory diagnosis of pertussis are: isolation of *B. pertussis* from clinical specimen or by detection by PCR. The CDC specifically does not recommend direct fluorescent antibody testing because of low sensitivity and variable specificity or serology because of the lack of standardisation. The CDC classifies cases as "probable: meets the clinical case definition, is not laboratory confirmed, and is not epidemiologically linked to a laboratory-confirmed case" and as "confirmed: a case that is confirmed culture-positive and in which an acute cough illness of any duration is present; or a case that meets the clinical case definition and is confirmed by positive PCR; or a case that meets the clinical case definition and is epidemiologically linked directly to a case confirmed by either culture or PCR". (CDC 2010a)

The clinical case definition of the European Centre for Disease Prevention and Control (ECDC) for pertussis is "any person with a cough lasting at least two weeks and at least one of the following three: paroxysms of coughing, inspiratory "whooping", post-tussive vomiting or any person diagnosed as pertussis by a physician or apnoeic episodes in infants". A laboratory-diagnosed case has to meet "at least one of the following three: isolation of *Bordetella pertussis* from a clinical specimen, detection of *Bordetella pertussis* nucleic acid in a clinical specimen or *Bordetella pertussis* specific antibody response" The ECDC also has an epidemiological criteria for a pertussis case: "an epidemiological link by human to human transmission". The ECDC classifies cases as "A. possible case: any person meeting the clinical criteria", as "B. probable case: any person meeting the clinical criteria and with an epidemiological link" and as "C. confirmed case: any person meeting the clinical and the laboratory criteria". (EU Commission Decision 2008)

It should be kept in mind, however, that the clinical, laboratory and epidemic case definitions are separate and therefore the number of reported cases differs with different definitions. The main differences between the case definitions are related to diagnostic methods -– the WHO definition is the only one according to which a case may be diagnosed as pertussis by a physician without any other criteria, and the CDC case definition does not recommend serological diagnosis. There are also classifications of possible, probable and confirmed cases.

The multiplicity of case definitions complicates the comparison of epidemiological data from countries using different definitions. This creates a need for a unified case definition.

### 2.1.3. Diagnosis

CULTURE

As mentioned above, there are several ways to diagnose pertussis in the laboratory. At the early stage of the disease it can be diagnosed by culturing the bacteria. Culture is especially recommended for young children when serological response may be weak (UTUlab 2009). This method should be used in the first two weeks of the disease because the sensitivity of culture decreases fast after 2 weeks of illness (Wood and McIntyre 2008). Antibiotics received by the patient may cause the culture to be negative (UTUlab 2009). Due to this and to the fact that the sensitivity of culture decreases fast, a negative culture does not necessarily mean that the patient does not have pertussis. The sample for culture should be taken from the nasopharynx through the nostril with either dacron or calcium alginate swab (Finger and von Koenig 1996; UTUlab 2009; HUSLAB 2011). If obtaining a sample through the nostril is difficult it can also be taken through the mouth with a bend swab (UTUlab 2009). The nasopharyngeal sample can also be acquired by aspiration with a suction device with a mucus trap (Finger and von Koenig 1996; Tozzi et al. 2005; Wood and McIntyre 2008). This is the recommended choice with infants (Riffelmann et al. 2005). In Finland, only nasopharyngeal swab is recommended for sampling (UTUlab 2009; HUSLAB 2011). The sampling technique affects greatly the sensitivity of culture (Finger and von Koenig 1996; Wood and McIntyre 2008). The medium of choice for culturing *B. pertussis* is the charcoal agar plate also known as Regan-Lowe medium and the Bordet-Gengou medium (Finger and von Koenig 1996).

POLYMERASE CHAIN REACTION

PCR can also be used at the early stages of the disease and its usability during the disease progression is slightly longer than with bacterial culture. PCR can show positive results even when there are no live organisms present in the patient. It has therefore greatly improved the diagnosis of pertussis (He et al. 1996). PCR is most reliable when used between one to six weeks after the start of symptoms (He et al. 1993). The most often used PCR target in pertussis diagnosis, IS*481*, is present in the genome of *B. pertussis* in more than 200 copies (Parkhill et al. 2003). This enhances the sensitivity of PCR greatly but also increases the possibility of false-positive results arising from e.g. contaminations. Strict control measures are therefore needed to ensure the reliability of PCR (Lievano et al. 2002). Problems may also arise following the fact that IS*481* is also present in the genomes of *B. holmesii* and some *B. bronchiseptica* and *B. parapertussis* strains, making the method less than perfect in specificity (Loeffelholz et al. 2000; Reischl et al. 2001; Templeton et al. 2003; Register and Sanden 2006; Bokhari et al. 2011). Nevertheless, at least in Finland and in the Netherlands this does not seem to pose a problem at the moment, as no *B. holmesii* DNA was detected in a large number of samples previously diagnosed positive for *B. pertussis* by PCR (Antila et al. 2006).

There are over 100 protocols reported for the detection of *B. pertussis* DNA by PCR (Kretsinger et al. 2006). Many of them use the previously mentioned IS*481* as their target sequence, but there have also been numerous attempts to gain better specificity by, for example, adding a second *B. pertussis*-specific PCR for confirmation of the diagnosis. The specific targets proposed are e.g. PT promoter, *B. pertussis* porin gene and the porin promoter, *rec*A and the ORF (open reading frame) BP3385 (Farrell et al. 2000; Qin et al. 2002; Antila et al. 2006; Knorr et al. 2006; Qin et al. 2007; Guthrie et al. 2008; Fry et al. 2009), although later the usability of BP3385 as an exclusively *B. pertussis*-specific target has been disproven (Register et al. 2010).

The sample for PCR is collected in the same way as the sample for culture and usually the same sample swab is used first for culture and then for PCR (UTUlab 2009; UTUlab 2010; HUSLAB 2011). Sampling with calcium alginate swabs is not recommended for PCR because of its inhibitory qualities. Dacron and rayon swabs are a better alternative (Cloud et al. 2002) and they are suitable for both PCR and culture.

SEROLOGY

At the later stages of the pertussis disease, when culture or DNA-based methods are unusable, a serological diagnosis can be performed. This means that the antibodies produced against antigens of *B. pertussis* are measured. The determination of pertussis-specific antibodies is usually done with enzyme-linked immunosorbent assay (ELISA).

Serology is useful e.g. with adolescents and adults when the patient comes late to the doctor with only prolonged cough as a symptom and culture or PCR are no longer useable (Cherry et al. 2005). Serology can be performed with one or two serum samples and with one or two cut-offs. With two-point serology, the first sample should be taken less than 2 weeks after the illness onset (acute sera) and the second 4 weeks after the acute sera measurement (convalescent sera) (Wood and McIntyre 2008). With a raise in the antibody titers in two consecutive samples the disease can be reliably verified if no vaccine has been administered in the last 12 months (Guiso et al. 2010)

Single-point serology can also be used with well defined cut-offs, but the interpretation of the results can be more difficult and therefore be less reliable. When using one cut-off the diagnosis is either positive or negative but with two cut-offs there are three possibilities: negative, low positive and positive.

In a recent paper where European pertussis reference laboratories gave recommendations on practices when performing pertussis ELISA it was stated that purified non-detoxified PT should be used as coating antigen and that there is only rationale to measure anti-PT IgG, but anti-PT IgA can be measured if a second serum sample is not available. One and two-point serology can be used with cut-offs between 50 and 120 international units (IU) per millilitre. The methods used should be validated and results should be reported in IU/ml as WHO reference standards are available (Xing et al. 2009; Riffelmann et al. 2010). Immunoblots are not recommended as the results are not quantified (Kennerknecht et al. 2011). (Guiso et al. 2010)

In Finland IgG, IgA and IgM are used in the serological diagnoses of pertussis. This is done in order to distinguish recent infection from vaccination-induced raise in the antibody levels (UTUlab 2008; HUSLAB 2010; Yhtyneet Medix laboratoriot 2011). However, the coating antigen used is soniated whole-cell bacteria.

### 2.1.4. Treatment

Antibiotic treatment of pertussis is effective when administrated at an early stage, e.g. less than 2 weeks of cough (KTL 2007). The recommended doses in Finland are for adults: azitromycin: 500 mg on the first day and 250 mg on the 2–5 following days or claritromycin: 500 mg twice a day for seven days and for children: azitromycin: age 0 to 6 months 10 mg/kg for five days; age > 6 months 10 mg/kg (max. 500 mg) on the first day and 5 mg/kg (max. 250 mg) on the following 2 to 5 days or claritromycin: age > 1 months 7.5 mg/kg (max. 500mg) twice a day for seven days. In case of macrolide allergies use sulfatrimetoprim: age > 2 months 4 mg/kg trimetoprim and 20 mg/kg sulfa twice a day for two weeks and for adults: 160 mg trimetoprim and 800 mg sulfa twice a day for two weeks (KTL 2007).

Macrolides (azitromycin, claritromycin and erythromycin) have been shown to be effective in clearing *B. pertussis* from patient airways and the resistance towards macrolides is rare in *B. pertussis*. (Bass et al. 1969; Halperin et al. 1997; Tiwari et al. 2005). The suitability of macrolides araises from the fact that they achieve high concentrations in the respiratory secretions as well as high intracellular concentrations (Hoppe 1998).

Late in disease process antibiotics are no longer effective. The long paroxysmal cough characteristic of pertussis can last for weeks and even months after the bacteria are cleared from the airways and at this point there is no point of using antibiotics. A systematic review by Altunaiji et al (2007) concluded that antibiotics effectively eliminate the bacterium from the nasopharynx and convert the patient non-infectious but do not effect the clinical course of the disease (Altunaiji et al. 2007). It also stated that prophylaxis with antibiotics had side-effects and that there is not enough evidence to recommend prophylactic treatment of pertussis contacts. In Finland, prophylactic treatment is still recommended for some cases of pertussis such as for family contacts where the diagnosis is performed early in the course of the disease (KTL 2007). The CDC also recommend that chemoprophylaxis should be considered based on the "infectiousness of patient and the intensity of the exposure, the potential consequences of severe pertussis in the contact, and possibilities for secondary exposure of persons at high risk from the contact (e.g., infants aged <12 months)" (Tiwari et al. 2005).

### 2.1.5. Epidemiology

According to WHO estimates, there were 16 million cases of pertussis worldwide in 2008 (WHO 2010a). Moreover, it was estimated that in 2008 alone, 195 000 children died of pertussis (Black et al. 2010). Most of the deaths occurred in Africa, Eastern Mediterranean and Southeast Asia (Black et al. 2010). The per case fatality rate has been predicted to be 1–3.9% in developing countries (Tan et al. 2005; WHO 2005). Whooping cough epidemics occur in most countries in cycles of 2–5 years. This was the case already in the pre-vaccine era and it is still observed today (Mattoo and Cherry 2005). The presence of the cyclic epidemics of pertussis in similar intervals as in the pre-vaccine era may indicate that the vaccines control the disease but do not control the circulation of the organism in the human population (Cherry 1996).

Be that as it may, vaccinations have greatly reduced the rate of the disease, as can be seen from figure 1. For example in the United States the incidence was reduced 150-fold (Mattoo and Cherry 2005). A similar drop in reported cases has also been observed in Finland. The incidence of pertussis in Finland decreased from 180.7 per 100 000 in the 1920s to 2.8 per 100 000 in the 1970s (Huovila 1981).

Although infants <6 months of age have the highest incidence reported (between 79.41 and 160.81 per 100 000 in 2003–2009), in recent years in the USA around half or more of the reported cases occurred in adolescents (10–19 years of age) and in adults (>20 years of age) (Hopkins et al. 2005; Jajosky et al. 2006; McNabb et al. 2007; McNabb et al. 2008; CDC 2009; CDC 2010b; CDC 2011). The same situation has also been reported in Europe. From 2002 to 2007 the highest incidence was among infants (35.5 per 100 000) but the majority of cases was in adolescents and adults (65%) (EUVAC.NET 2009). The highest incidence was also reported in infants (29.7 and 22 per 100 000) and the most cases in adolescents and adults (77.4% and 78%) in 2008 and 2009, respectively (EUVAC.NET 2010b; EUVAC.NET 2010a). Increase in the incidence in adolescents was observed. In 2009 the incidence in adolescents aged 10–14 years old (20 per 100 000) was almost as high as in infants (22 per 100 000) (EUVAC.NET 2010a). The second highest incidence was in adolescents aged 10–14 years also in 2008 (20.1 per 100 000) (EUVAC.NET 2010b).

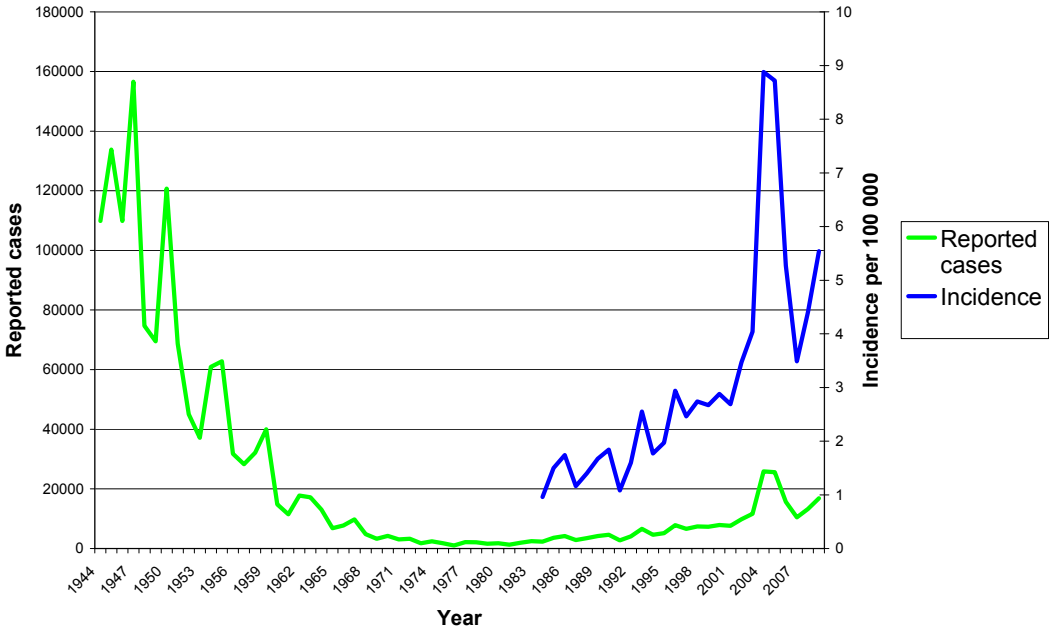


**Figure 1.** The incidence per 100 000 and the number of reported cases in the USA according to CDC (CDC 1994; CDC 1996; CDC 1997; CDC 1998; CDC 1999; CDC 2001; CDC 2002b; CDC 2003b; Groseclose et al. 2004; Hopkins et al. 2005; Jajosky et al. 2006; McNabb et al. 2007; McNabb et al. 2008; CDC 2009; CDC 2010b; CDC 2011). Vaccinations against pertussis were widely used in 1940. In 1943, the American Academy of Pediatrics approved the vaccine for routine use and in 1944 the American Medical Association recommended the use of pertussis vaccine (Shapiro-Shapin 2010).

Data from serological studies in patients with prolonged cough have shown that a round 2.6-17% of the population have anti-pertussis antibodies (Jackson et al. 2000; Cherry 2006; Aksakal et al. 2007; Dalby et al. 2010). From a healthy population with no symptoms the same figures are 6.6- 9.3% (de Melker et al. 2006; de Greeff et al. 2010; Wang and Zhu 2011). From the seroprevalence studies it was concluded that the 20 to 24-year-olds had the highest incidence of 10.8% (de Melker et al. 2006). This shows that pertussis is circulating even in vaccinated populations with a relatively high frequency.

The variations in pertussis case definitions, vaccination programs, vaccines, surveillance systems and reporting of the disease make it difficult to compare the true incidence of pertussis in different countries. Therefore seroprevalence studies with standardised methods should be undertaken to see the true burden of pertussis in different populations.

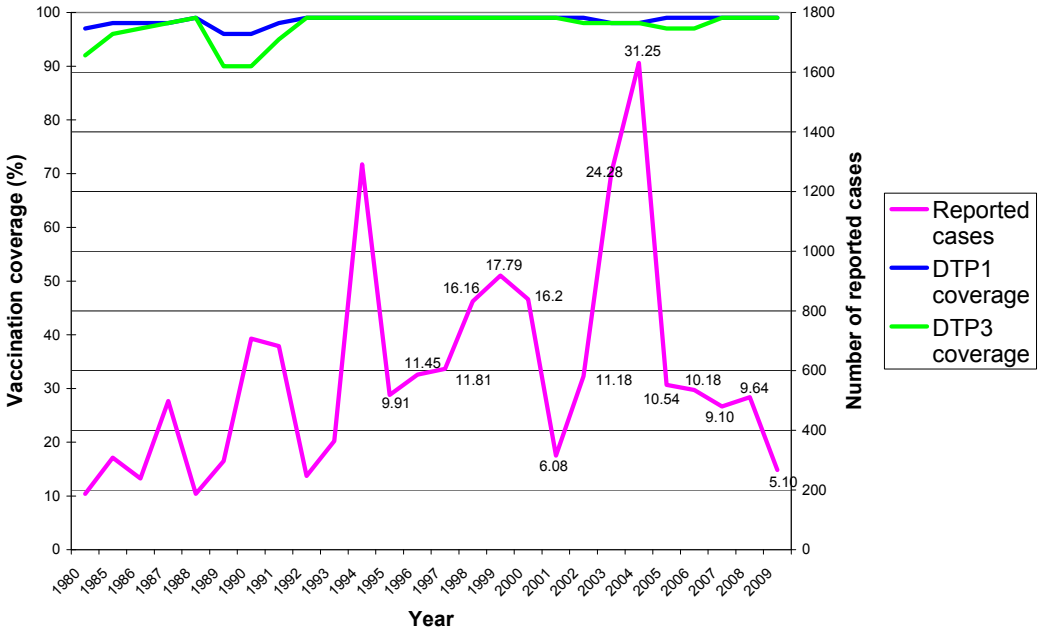


**Figure 2.** Vaccination coverage (%) and number of reported cases in Finland in 1980 and from 1985 to 2009 according to WHO and the National Infectious Disease Registry (WHO 2010b; THL 2011). Vaccination coverage is shown for both first dose of tetanus diphtheria, and pertussis vaccine (DTP1) and three doses of tetanus-diphtheria-pertussis vaccine (DTP3). Incidences of pertussis per 100 000 from 1995 onwards are written on the figure.

#### 2.1.5.1. Re-emergence of pertussis

Re-emergence of pertussis has been observed in many countries with long history of high vaccination coverage, including Argentina (Hozbor et al. 2009), Canada (Skowronski et al. 2002; Ntezayabo et al. 2003), the USA (Yih et al. 2000; CDC 2002a; CDC 2003a; Tanaka et al. 2003), Australia (McIntyre et al. 2002; Spokes and Gilmour 2011), the Netherlands (de Melker et al. 2000), Israel (Moerman et al. 2006), Spain (Crespo et al. 2011) and Finland (Elomaa et al. 2005). In the USA, for example, a clear decrease in the reported cases can be seen after the introduction of pertussis vaccine but in recent years a gradual increase in the reported cases as well as in the incidence is noticeable (figure 1). The same can also be seen in data from 1980 to 2009 from Finland (figure 2). Reasons behind the re-emergence are not entirely clear, but include at least some of the following: better knowledge and awareness of whooping cough, better diagnostic methods and more vigilant reporting of the disease, waning of vaccine-induced immunity and the adaptation of the bacteria, *B. pertussis*, to vaccine-induced immunity (He and Mertsola 2008). Other possible reasons include changes in the contact network, where age-specific contact pattern could explain the resurgence (Rohani et al. 2010), and changes in the transmission may explain in part the number of severe cases that are reported (Aguas et al. 2006).

## 2.2. *Bordetella pertussis*

Whooping cough is caused by the bacterium *Bordetella pertussis*. A similar disease, but one generally considered to cause milder symptoms, is caused by *Bordetella parapertussis*. The focus of this thesis is on *B. pertussis* and therefore *B. parapertussis* is mostly ignored in this literature review.

### 2.2.1. Characteristics of the bacterium

*B. pertussis* is a non-motile Gram-negative bacterium. It is thought to be solely a human pathogen and so far there have been no reports of its isolation from animals or the environment. Spreading of the disease happens by coughing and by nasal dripping. Therefore, the bacterium has to be able to survive for short periods of time in droplets.

Human is considered to be the sole host of *B. pertussis*, although cats, dogs and rabbits get at least minor symptoms from *B. pertussis* inoculations (Macewen 1908; Wollstein 1909). Neither monkeys nor guinea-pigs showed any symptoms after inoculation by *B. pertussis* (Wollstein 1909).

### 2.2.2. Virulence factors

*B. pertussis* produces a variety of virulence factors. These can be roughly divided into toxins and adhesins. The bacterium uses these to adhere to the airway epithelium and to alter the host's immune response. There are several dozens of factors that the bacterium produces which can be considered as virulence factors and only a selected few are gone through here in more detail.

#### 2.2.2.1. Toxins

PERTUSSIS TOXIN (PT)

The most well known toxin that *B. pertussis* produces is pertussis toxin (PT). Interestingly, it is only produced by *B. pertussis* but not by closely related species *B. parapertussis* or *B. bronchiseptica* even though both of them have almost intact *ptx* operons (Arico and Rappuoli 1987). The two close relatives that do not produce pertussis toxin have point mutations in the *ptx* promoter that prevent the expression of the genes (Arico and Rappuoli 1987; Marchitto et al. 1987; Gross et al. 1992). PT is composed of five subunits, which are coded by the genes *ptx*A to *ptx*E (Locht and Keith 1986; Nicosia et al. 1986). It is a typical AB toxin where the A part (*ptx*A) has the enzymatically active ADP-ribosyltransferase and the B part (*ptx*B to *ptx*E) has the receptor-binding function (Katada and Ui 1982; Tamura et al. 1983).

From the cytoplasm, where it is produced, PT is transported by a general export pathway, but the "liberation" out of the periplasmic space is performed by a specific transport system encoded by the *ptl* (pertussis toxin liberation) genes (Weiss et al. 1993; Farizo et al. 1996). The PT holoenzyme has to be completely assembled for it to be sufficiently transported (Pizza et al. 1990).

The A subunit only comes active after dissociation from the B subunit into the cytosol of the host cell (Kaslow and Burns 1992). In the cytosol, PT catalyses the transfer of ADP-ribose from NAD to the α-subunit of G proteins, causing their inactivation (Katada et al. 1983; Bokoch and Gilman 1984). The effects of the disruption of the G protein regulated pathways include histamine sensitisation, enhancement of insulin secretion in response to regulatory signals and suppressive as well as stimulatory immunological effects (Parfentjev and Goodline 1948; Parfentjev and Schleyer 1949; Carbonetti et al. 2004; Mattoo and Cherry 2005). PT also causes lymphocytosis, leukocytosis and decreased chemotaxis of lymphocytes, monocytes and neutrophils (Morse 1965; Morse and Bray 1969; Morse and Morse 1976; Morse 1977; Meade et al. 1984; Brito et al. 1997; Lyons 1997). The main contribution of PT to the symptoms of pertussis, according to comparative studies with naturally PT expressing *B. pertussis* and naturally PT non-expressing *B. parapertussis*, seems to be leukocytosis and lymphocytosis although the duration and the severity of the disease are longer in pertussis caused by *B. pertussis* than pertussis caused by *B. parapertussis*.

PT acts as a strong adjuvant for other antigens (Ryan et al. 1998). PT enhances the serum antibody responses to other vaccine antigens in children. It can also suppress the immune responses for example the serum antibody levels (Carbonetti et al. 2004).

Although PT is a toxin, it has been proposed to function also as an adhesin and to mediate the attachment of the bacterium to ciliated epithelial cells (Tuomanen and Weiss 1985; Carbonetti et al. 2003).

PT is an antigen included in all of the aP vaccines.

LIPOPOLYSACCARIDE (LPS)

LPS is an endotoxin and is pyrogenic, mitogenic and toxic (Ayme et al. 1980; Watanabe et al. 1990). The LPS of *B. pertussis* is quite distinct from the LPS of *B. parapertussis* and *B. bronchiseptica* as it does not have O-antigen (Peppler 1984). The LPS of *B. pertussis* consists of lipid A molecule, a branched oligosaccharide core structure and a trisaccharide (Lebbar et al. 1986; Caroff et al. 1990; Lasfargues et al. 1993).

The functions of LPS in *B. pertussis* pathogenesis are not clear. There are clear differences in LPS structure between *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* that may play a role in host specificity (Harvill et al. 2000). The O-antigen in LPS of *B. parapertussis* and *B. bronchiseptica* seems to be important for resistance to complement-mediated killing (Harvill et al. 2000). The lack of O-antigen in *B. pertussis* seems to have been compensated by resistance to serum killing mediated partly independently of BrkA (Bordetella resistance to killing) and by the structurally distinct LPS (Pishko et al. 2003; Schaeffer et al. 2004). This is interesting as BrkA is known to inhibit the bactericidal activity of the complement system (Barnes and Weiss 2001).

DERMONECROTIC TOXIN (DNT)

DNT is a single polypeptide chain of around 160 kDa that functions like a typical AB toxin with an N-terminal receptor-binding domain and a C-terminal enzymatic domain. After endocytosis DNT is cleaved by host enzymes (Matsuzawa et al. 2004). These proteolytic modifications are necessary for the cellular activity which constitutively activates intracellular Rho GTPases (Horiguchi et al. 1997). When injected intradermally to mice DNT induces localised necrotic lesions. It is lethal to mice when injected intravenously even in low doses (Iida and Okonogi 1971). DNT also induces dramatic morphological changes and stimulates DNA replication (Horiguchi et al. 1991; Horiguchi et al. 1993). The effects of DNT on *B. pertussis* pathogenesis are not entirely clear as non-functional DNT does not affect the virulence of *B. pertussis* (Weiss and Goodwin 1989).

TRACHEAL COLONISATION FACTOR (TCF)

TCF coded by the *tcfA* gene is an autotransporter expressed exclusively by *B. pertussis*. TCF is produced as a 90 kDa precursor that binds to the cell surface. It is processed to a 60 kDa functional form that is interestingly not cell surface-bound. TCF seems to have some effect on the colonisation of the trachea as TCF-deficient strain colonised mouse trachea less efficiently than wild type strain. (Finn and Stevens 1995)

TRACHEAL CYTOTOXIN (TCT)

TCT is not expressed under BvgAS (Bordetella virulence gene) control, which controls the expression of most virulence related genes in *B. pertussis* (described in section 2.2.2.3.), as it is in essence a component of the cell wall of the bacterium (Rosenthal et al. 1987; Cookson et al. 1989b). Most other bacteria recycle this component back into the cytoplasm, but due to a non-functional transporter the *Bordetella spp*. secrete it constitutively (Jacobs et al. 1995; Parkhill et al. 2003).

TCT can cause mitochondrial bloating, disruption of tight junctions, extrusion of ciliated cells and inhibition of DNA synthesis to hamster cells (Goldman et al. 1982; Cookson et al. 1989a). Experiments in human biopsies have shown that TCT causes loss of ciliated cells, cell blebbing and mitochondrial damage (Wilson et al. 1991). The loss of ciliated cells leads to e.g. disruption mucociliary clearance of the bronchi.

ADENYLATE CYCLASE TOXIN (ACT)

ACT is a bifunctional calmodulin-sensitive adenylate cyclase and haemolysin (Mattoo and Cherry 2005). It belongs to the calcium-dependent, pore-forming cytotoxins of the RTX family (Rose et al. 1995; Rhodes et al. 2001). It is strongly expressed in $Bvg^+$ phase (Mattoo and Cherry 2005). ACT is first synthesised as a protoxin of 1706 amino acids (Glaser et al. 1988; Hewlett et al. 1989). The C-terminal domain mediates the transport of the N-terminal domain with the adenylate cyclase activity to the cytoplasm of the host cell (Hewlett et al. 1989; Rogel et al. 1989; Bellalou et al. 1990). CyaC, which is transcribed from the same operon, activates ACT by fatty acid modification (Barry et al. 1991; Hackett et al. 1994; Basar et al. 2001). Actually most of the ACT is not transported into the host cells but stay on the surface of the bacterium. FHA seems to play a role in the adherence of ACT to the bacterial surface (Zaretzky et al. 2002).

ACT affects cytotoxicity and inhibits phagocytosis and ACT-deficient mutant strains are unable to cause lethal infections in mice (Weiss and Goodwin 1989; Khelef et al. 1993; Weingart et al. 2000; Weingart and Weiss 2000). ACT can suppress immune responses by inhibiting the cell surface expression of CD40 and IL-12 of the host (Skinner et al. 2004). It may also inhibit the p38 MAP kinase pathway (Skinner et al. 2004). Polymorphonuclear neutrophils seem to be the primary target of ACT (Mattoo and Cherry 2005).

ACT is not present in any of the aP vaccines currently on the market, perhaps because it has been associated with reports of the "original antigenic sin" (Mattoo and Cherry 2005). ACT is present in low amounts in wP vaccines. All *Bordetella* species that infect mammals express ACT (Mattoo and Cherry 2005).

#### 2.2.2.2. Adhesins

FILAMENTOUS HEMAGGLUTININ (FHA)

FHA is highly immunogenic and is therefore included in most acellular vaccines. It is strongly expressed in $Bvg^+$ (and $Bvg^i$) phases (Mattoo and Cherry 2005). The gene encoding for FHA is *fhaB*. It is known to be strongly regulated by the activator of transcription BvgA (Boucher et al. 1997). It is synthesised as a 367-kDa precursor and is then post-translationally modified to its 220-kDa functional form (Relman et al. 1989). FHA is transported through the outer membrane by FhaC which acts as an FHA-specific pore (Jacob-Dubuisson et al. 1999; Guedin et al. 2000). The mature form of the protein is achieved outside the outer membrane (Coutte et al. 2001; Coutte et al. 2003b). FHA and FhaC represent members of the two-partner secretion (TPS) system. Even though FHA is transported out of the cell by the TPS system some of it stays pound to the cell surface enabling it to function as an adhesin (Jacob-Dubuisson et al. 1996).

The RGD motif of FHA enables it to bind to macrophages and bronchial epithelial cells and the carbohydrate recognition domain (CRD) enables the binding to macrophages and ciliated respiratory epithelial cells. The lectin properties of FHA enable it to bind nonciliated epithelial cells (Relman et al. 1990; Menozzi et al. 1991; Prasad et al. 1993; Ishibashi et al. 1994; Ishibashi et al. 2001). The lectin-like site is required for the hemagglutinin activity of FHA (Menozzi et al. 1991). Binding of FHA activates NF-κB, which leads to leukocyte accumulation and activation at the site

of bacterial infection (Tosi et al. 1992; Bloemen et al. 1996). This activation of NF-κB may be reversed by PT (Ishibashi and Nishikawa 2002; Ishibashi and Nishikawa 2003).

Binding of FHA seems to enable the bacterium to adhere to the epithelium of the lungs and cells of the trachea. The release of FHA from the cell surface seems to enable the bacterium to spread as overly high adherence to the cell surface prevented proliferation and persistence in the lungs (Coutte et al. 2003a).

FHA is also associated with TNF-α associated apoptosis of human macrophage-like cells (Abramson et al. 2001) and suppression of IL-12 production (McGuirk et al. 2000; McGuirk and Mills 2000). Therefore it appears that in addition to functioning as an adhesin, FHA can also modulate the host's immune responses.

There are conflicting reports on the importance of FHA in the pertussis vaccine. Some studies reported that whole-cell vaccines that produced low anti-FHA antibodies were highly efficacious (Edwards et al. 1995). Others report that aP vaccines with FHA are more efficacious then those without FHA (Ad Hoc Group for the Study of Pertussis Vaccines 1988; Storsaeter et al. 1990; Storsaeter and Olin 1992; Cherry 1997). There are also reports that FHA does not play a role in protection (Cherry et al. 1998; Storsaeter et al. 1998).

PERTACTIN (PRN)

Pertactin, also known as P.69, is a 69 kDa outer membrane protein which acts as an autotransporter. The expression of PRN is regulated by the BvgA (Montaraz et al. 1985; Charles et al. 1989). The transportation of PRN from the cell is mediated by PRN itself as the name autotransporter indicates, in an energy-independent manner (Henderson and Nataro 2001). It binds to eukaryotic cells with its RDG domain. PRN consists of 16-strand parallel β-helix and is tightly associated with the bacterial surface (Emsley et al. 1994; Emsley et al. 1996).

*In vitro* studies have produced conflicting evidence about the adhesion function of PRN. Some report that PRN promotes binding to CHO cells and other cell lines (Everest et al. 1996) whereas others report that PRN-mutants do not significantly differ from the wild type in adherence to cells or in colonisation of the mouse respiratory tract (Roberts et al. 1991). However, clinical vaccine trials have shown that PRN may be a very important adhesin. Antibodies against PRN may be the most important ones for protection (Cherry et al. 1998; Storsaeter et al. 1998). Antibodies against PRN, but not against PT, FHA or FIMs are needed for efficient phagocytosis by host cells (Hellwig et al. 2003). In addition, aP including PRN performed better than similar vaccine without PRN (Gustafsson et al. 1996; Cherry 1997). The protection via PRN seems to be mediated by antibodies preventing the adhesion of the bacterium to the cell surface through PRN (Mattoo and Cherry 2005).

FIMBRIAE (FIM)

The FIM proteins comprise the *B. pertussis* serotypes Fim2, Fim3 and Fim2,3. The tip of the fimbriae is encoded by *fimD* (Willems et al. 1993; Geuijen et al. 1997). The genes (*fim2* and *fim3*) encoding the two major fimbrial subunits are not linked in any way and are separately regulated by the BvgAS system as well as homopolymeric tracts of cytosines in the promoter regions of the genes (Livey et al. 1987; Mooi et al. 1987; Willems et al. 1990). The length of these tracts is changing by slip-strand mispairing (Levinson and Gutman 1987). This contributes to the fimbrial phase variation in *B. pertussis* (Willems et al. 1990).

The major fimbrial subunits, Fim2 and Fim3, are linked to adhesion to epithelial cells and the minor subunit FimD to the adherence to monocytes (Mooi et al. 1992; Hazenbos et al. 1995). The major

subunits also bind chondroitin sulphate, heparan sulphate, and dextran sulphate with fibronectin-like regions (Geuijen et al. 1996).

Fimbriae are needed for *B. pertussis* to replicate in the nasopharynx and trachea (Mooi et al. 1992; Geuijen et al. 1997). They also play a role in the colonisation of the trachea and persistence of the infection in that site (Mattoo et al. 2000). The fimbriae are able to inhibit the killing of the bacterium by lung macrophages (Vandebriel et al. 2003). Fimbriae may also play a role in biofilm formation of *B. pertussis* (Irie et al. 2004).

Antibodies to fimbriae have been reported to contribute to protection (Miller et al. 1943; Cherry et al. 1998; Storsaeter et al. 1998). An aP containing Fim2 and Fim3 showed greater efficacy than the similar vaccine without FIMs (Olin et al. 1997).

Taken together, FIMs seem to play an important role in the adherence of the bacteria to the epithelium and macrophages as well as in protection against infection in humans.

#### 2.2.2.3. BvgAS and the regulation of virulence

A major regulator of gene expression in the Bordetella is the BvgAS two-component system with obligate multistep phosphorelay (Uhl and Miller 1994; Uhl and Miller 1996a). It is coded by the *bvgAS* locus. BvgA works as the response regulator which binds to DNA and activates the transcription of virulence-related genes and BvgS works as both sensor and response regulator component (Stibitz and Yang 1991). It can sense changes in the environment and affect the gene expression, although the environmental signals that it senses are not entirely clear. The modulation of the BvgAS regulated expression is achieved *in vitro* by high concentrations of $SO_4$ and $ClO_4$ ions, nicotinic acid, nicotinic acid analogs and low temperature (Melton and Weiss 1989; Melton and Weiss 1993).

There are three phases that can be differentiated in BvgAS modulated expression of genes which correspond to specific phenotypes in the bacterium. The phases are called $Bvg^+$-, $Bvg^-$- and $Bvg^i$-phases. In the $Bvg^+$-phase the detector, in the periplasmic domain of the BvgS, relays environmental signals to the transmitter domain in the cytoplasm, which autophosphorylates. This leads to the transfer of the phosphoryl group to the receiver domain, which can donate the phosphoryl group forward to a histidine of the histidine phosphotransfer domain (HPD) or to water. The C-terminal HPD can activate the BvgA by transfer of the phosphoryl group to it or it can transfer the phosphoryl group back to the receiver. The activation of BvgA by this method leads to the expression of the vag genes (*vir*-activated genes). BvgA binds to the promoter of vag genes and promotes their expression. These genes are mainly virulence-related genes e.g. virulence factors PT, FHA, FIM, PRN, ACT and DNT. BvgAS expression is regulated by BvgAS in an autoregulatory manner. The activation of BvgA also leads to the expression of BvgR regulator that represses the expression of vrg genes (*vir*-repressed genes) genes in the $Bvg^+$ phase. When BvgA is not active $Bvg^-$ specific genes are expressed and vag genes are silent and therefore vrg genes are expressed. $Bvg^+$-phase seems to be the virulent phase when *B. pertussis* can colonise the respiratory tract, during $Bvg^-$-phase *B. pertussis* is avirulent, but the role of this phase is not clear. The $Bvg^i$ (intermediate) phase is achieved in submodulating conditions and the bacterium shows characteristics of both $Bvg^+$- and Bvg--phases. This shows that BvgAS two-component system controls genes in more complex manner that just on or off. This is probably the case often in the natural environment of the bacterium. In the $Bvg^i$-phase the bacterium seems to be resistance to nutrient limitation and has a decreased ability to colonise the respiratory tract. It has been suggested that $Bvg^i$-phase plays a role in the respiratory transmission. (Knapp and Mekalanos 1988; Miller et al. 1992; Cotter and Miller 1994; Uhl and Miller 1994; Merkel and Stibitz 1995; Martinez de Tejada et al. 1996; Uhl and Miller 1996a; Uhl and Miller 1996b; Cotter and Miller 1997; Deora et al. 2001; Stockbauer et al. 2001; Mattoo and Cherry 2005; Williams and Cotter 2007)

The BvgAS system promotes virulence by enhancing and repressing the expression of genes temporally correctly according to environmental signals (Akerley et al. 1995).

There is also another two-component system in *B. pertussis*: the RisAS (reduced intracellular survival) system. The system is not yet well characterised, but it seems to be involved in the expression of Bvg-repressed genes as well as intracellular survival of the bacterium. (Jungnitz et al. 1998; Stenson et al. 2005)

### 2.2.3. Phylogeny and evolution of the genus Bordetellae

The genus Bordetella is classified to the Betaproteobacteria. The genus consists of nine species: *Bordetella bronchiseptica*, *B. pertussis*, *B. parapertussis*, *B. holmesii*, *B. hinzii*, *B. avium*, *B. petrii*, *B. ansorpii* and *B. trematum* (Weiss 2006; Gross et al. 2010). *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are thought to comprise the classical human Bordetellae. They are the species most often found infecting humans. Genetic analysis has shown that *B. pertussis* and *B. parapertussis* have developed quite recently from a *B. bronchiseptica*-like ancestor with a broader host range and capability to survive in the environment. Both *B. pertussis* and *B. parapertussis* cause pertussis-like symptoms in humans but *B. parapertussis* is considered to have derived more recently from the ancestral lineage (Parkhill et al. 2003; Diavatopoulos et al. 2005).

Analysis of the genomes has revealed that both *B. pertussis* and *B. parapertussis* have acquired a large number of insertion sequence (IS) elements. The proportion of IS elements of the open reading frames (ORFs) in *B. pertussis* genome is 6.8% and 2.5% of the ORFs in *B. parapertussis*. *B. pertussis* in particular has gone through major genomic rearrangement as well as genome reduction. Although *B. pertussis* shares most of its genes with either *B. parapertussis* and/or *B. bronchiseptica*, its genome size is only 4 086 186 bp compared with the 4 773 551 bp of *B. parapertussis* and 5 338 400 bp of the *B. bronchiseptica*. Probably because of the large number of IS elements the genome of *B. pertussis* has also accumulated many pseudogenes. This is because the IS elements in the genome often disrupted genes when incorporating into the genome, or when a region of the genome has relocated to the middle of a gene. (Parkhill et al. 2003) Differences between the classical human Bordetellae are summarised in table 1.

**Table 1.** Characteristics of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*. Table adapted from GeneBank, Mooi et al. (2007) and Parkhill et al. (2003).

| | ***B. pertussis*** | ***B. parapertussis*** | ***B. bronchiseptica*** |
|---|---|---|---|
| **Host range** | Humans | Humans | Mammals |
| **Disease** | Pertussis | Pertussis | Respiratory disease |
| **Reference strain** | Tohama I | 12822 | RB50 |
| **Genome size (bp)** | 4 086 189 | 4 773 551 | 5 339 179 |
| **GC content (%)** | 67.72 | 68.01 | 68.07 |
| **Coding sequences** | 3 867 | 4 467 | 5 072 |
| **Pseudogenes** | 358 | 217 | 12 |
| **Average gene size (bp)** | 978 | 987 | 978 |
| **Structural RNAs** | 61 | 65 | 66 |
| **IS*481*** | 238 | 0 | 0 |
| **IS*1001*** | 0 | 22 | 0 |
| **IS*1002*** | 6 | 90 | 0 |
| **IS*1663*** | 17 | 0 | 0 |
| **GeneBank access** | BX470248 | BX470249 | BX470250 |

## 2.3. Genetics of *B. pertussis* and strain variation

The genetics of *B. pertussis* has been studied on many levels. Great advantages in genetics have been achieved by studying different mutants, but also gene typing by SNP analysis. The SNPs in virulence factors as well as other polymorphisms and the loss of genetic material have been thought to be the main strategies for the bacterium to create variation.

Methods to study the strains variation in addition to genotyping include e.g. restriction fragment length polymorphism (RFLP) analysis (van der Zee et al. 1996; van Loo et al. 1999), multilocus sequence typing (MLST) (van Loo et al. 2002), multilocus variable number of tandem repeats (MLVA) analysis and pulsed-field gel electrophoresis (PFGE). Of the four, the two most commonly used methods today are MLVA and PFGE.

Schouls et al. published in 2004 a method for MLVA (Schouls et al. 2004) which has been later adapted also by others (Litt et al. 2009; Kurniawan et al. 2010). The most recent study utilising MLVA on *B. pertussis* further developed the method by adding two additional target sequences (Kurniawan et al. 2010). The discriminatory powers of the methods used to study *B. pertussis* have been compared (Advani et al. 2009). The results showed that PFGE has the most discriminatory power (DI (diversity index) =95) followed by MLVA (DI=76) and last MLST (DI=72). Advani et al. (2009) also reported that MLVA and MLST combined, with a DI of 91, would have almost as good a discriminatory power as PFGE alone.

In a study where a newly developed SNP analysis was compared with PFGE and MLVA in *B. pertussis* strains collected in Europe and Africa the discriminatory indices of the methods were 0.85, 0.95 and 0.83, respectively, therefore confirming the high discriminatory power of PFGE (van Gent et al. 2011a).

Although PFGE has been shown to be the most discriminatory method, it is laborious and the results are not always easily comparable between laboratories. MLVA and SNP typing are not as laborious and the nature of the methods make it easy the compare the results between laboratories and also to transfer the technique to new sites.

### 2.3.1. Genome and genomic plasticity

The first complete genome of *B: pertussis* was published in 2003 (Parkhill et al. 2003). The genome of the strain Tohama I, isolated in Japan in 1950s, was sequenced. This genome consists of 4 086 186 bp, 3 816 genes of which 358 are pseudogenes (9.4%), three rRNA operons, 51 tRNAs and 261 IS elements of which 238 were IS*481* (table 1).

Only one plasmid has been reported to be present naturally in *B. pertussis*. The role of this plasmid and the reason for its existence are not clear as it does not harbour any kind of resistance genes. It also does not seem to be widely present in the genomes of clinical strains. (Kamachi et al. 2006)

Genetic plasticity is common in bacteria. Mechanisms contributing to it include e.g. DNA rearrangements, inversion, duplication, deletion of DNA, gain of additional genetic information, integration of horizontally acquired DNA (Dobrindt and Hacker 2001).

There are some reports of genomic plasticity in *B. pertussis*. Inversions in the genome, leading to changes in the gene order, have been observed during an epidemic (Stibitz and Yang 1999). The genome of *B. pertussis* seems to be quite stable in laboratory conditions at least in relation to inversions (Brinig et al. 2006), although this may be an adaptation of a single laboratory-adapted strain as similar inversions were also reported previously with other laboratory strains (Stibitz and

Yang 1997). These facts taken together imply that the reference strains Tohama I used in the first Bordetella sequencing project (Parkhill et al. 2003) may skew data on the comparative genomics of the bronchiseptica-cluster. Therefore the rearrangements during the evolution of *B. pertussis* from the *B. bronchiseptica*-like ancestor may not have been as extent as depicted previously (Parkhill et al. 2003) or they may have been even larger. As most of the genomic research is done in reference to Tohama I, some properties of the genomes of *B. pertussis* may go unnoticed.

*B. pertussis* does not seem to have gained any genetic material, although there seems to be differences in the number of genes and genetic material in different lineages of the species (Caro et al. 2006; Bouchez et al. 2008; Caro et al. 2008; King et al. 2008).

### 2.3.2. Single nucleotide polymorphisms

Characterisation of virulence factors of *B. pertussis* has shown that many of them are polymorphic. Genotyping of these factors has been performed, from serial collections of clinical isolates, in many countries. This has shown that in almost all countries the current circulating strains are distinct from the strains used in the production on the wP vaccines. Vaccinations against pertussis were in general initialised in many countries between the 1940s and the 1960s. It is generally accepted that the changes to non-vaccine type alleles have happened after 15 to 30 years after the initiation of vaccinations (Kallonen and He 2009).

#### 2.3.2.1. Pertussis toxin subunit 1

To this day eight types of pertussis toxin subunit 1 of *ptx*A have been reported (Mooi 2010). Three different nomenclatures have been used in naming them. One of them named the first genes as S1A, S1B, S1D, S1E, S1F and S1G (Mooi et al. 2000; Poynten et al. 2004), another named them *ptx*A1, A2, A4 and A5 (Caro et al. 2005) and another named them *ptx*A1, A2, A3 and A4 (Fry et al. 2001). The four most important alleles are clearly presented in a review by He & Mertsola (2008) (He and Mertsola 2008). As the absence of *ptx*S1C in the nomenclature first mentioned is due to a sequencing error (Mooi et al. 1998; Mooi et al. 2000) and there are four alleles that are most frequently referred to (*ptx*A1-4), it is sufficient and clear to use the nomenclature of Fry et al. (2001).

All the mutations in the *ptx*A lead to a change in the amino acid sequence and are thus not silent. The mutations have also occurred in regions that are T cell epitopes (Mooi et al. 1998). There have been numerous reports of non-vaccine type *ptx*A alleles emerging 15 to 30 years after the start of vaccinations (Mooi et al. 1998; Mastrantonio et al. 1999; Mooi et al. 1999; Cassiday et al. 2000; Fry et al. 2001; Gzyl et al. 2001; Weber et al. 2001; Fiett et al. 2003; Peppler et al. 2003; Kodama et al. 2004; Poynten et al. 2004; Elomaa et al. 2005; Hallander et al. 2005; Fingermann et al. 2006; Lin et al. 2006; Borisova et al. 2007; Njamkepo et al. 2008).

Most vaccines contained *ptx*A2 and/or *ptx*A3 strains. They were gradually replaced by *ptx*A1 strains. Generally, it can be said that the change in pertussis toxin S1 has occurred before a change in pertactin, which is discussed later in section 2.3.2.3..

#### 2.3.2.2. Pertussis toxin promoter

There is great variation reported in the pertussis toxin promoter region. To this date 14 alleles have been found (Advani et al. 2009; Mooi et al. 2009; Mooi 2010). The differences of the 14 *ptx*P alleles are presented in a review by Fritz Mooi (Mooi 2010). The mutations in the promoter region occur at the sites where the activator of transcription BvgA binds (Mooi et al. 2009). The high number of variants show that it is beneficial for the bacteria to modulate the expression of PT to

suppress the host immune reactions or to gain advantage compared to other strains. The emergence of this allele has been quite recent (Mooi 2010). Variation in the PT promoter has also been linked to increased colonisation in mouse model (van Gent et al. 2011b).

#### 2.3.2.3. Pertactin

As with pertussis toxin subunit 1, also pertactin has been extensively analysed for variation. To date, 13 alleles have been reported (Mooi 2010). As mentioned above the change to non-vaccine type happened in both *ptx*A and *prn* approximately 15 to 30 years after the start of vaccinations and in general the change occurred first in *ptx*A (Mooi et al. 1998; Mastrantonio et al. 1999; Mooi et al. 1999; Cassiday et al. 2000; Fry et al. 2001; Gzyl et al. 2001; Weber et al. 2001; Fiett et al. 2003; Peppler et al. 2003; Kodama et al. 2004; Poynten et al. 2004; Elomaa et al. 2005; Hallander et al. 2005; Fingermann et al. 2006; Lin et al. 2006; Borisova et al. 2007; Njamkepo et al. 2008). Most vaccine strains were *prn*1 strains just like the circulating strains at the time of isolation of most vaccine strains. Strains representing *prn*2 have mostly replaced the previously predominant *prn*1 strains.

The variation in *prn* is focused on two immuno-dominant regions known as region 1 and region 2, although variation is found throughout the gene (Li et al. 1992). The variation in region 1 is in the number of repeats and in the sequence of the repeats of amino acids GGxxP (glysine-glysine-x-x-proline). Region 1 is close to the RGD domain which is known to be involved in binding to host receptors (Leininger et al. 1991; Leininger et al. 1992). Region 2 consists of repeats of amino acids PQP (proline-glutamine-proline). Variation in *prn* is in regions that have been identified as human B cell epitopes (Charles et al. 1991). Antibodies against this loop region may interfere with the function of pertactin and therefore the variation in this region can be of advantage to the bacterium (Emsley et al. 1996; Mooi et al. 1998).

#### 2.3.2.4. Variation in other virulence factors

Many genes coding for virulence factors of *B. pertussis* have been reported to be polymorphic (Kallonen and He 2009; Mooi 2010). The most important ones and some of the most polymorphic ones have been discussed above. Other polymorphic virulence factors are e.g. surface proteins fim2 (2 alleles), fim3 (4 alleles), tcfA (6 alleles), ptxC (2 alleles), ompQ (2 alleles), vag8 (2 alleles), bapC (2 alleles), cyaA (2 alleles) and fhaB (2 alleles) (van Loo et al. 2002; Packard et al. 2004; Kallonen and He 2009).

### 2.3.3. Insertion sequence elements

During the evolution of *B. pertussis* from a *B. bronchiseptica*-like ancestor, it has accumulated a large number of IS elements on its genome. The first report on the sequences of the genomes of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* showed that they had 261, 112 and zero IS elements, respectively (Parkhill et al. 2003). The incorporation of the elements to the genomes has disrupted the genes and therefore created pseudogenes. The number of IS elements also correlates with the number of pseudogenes in the genomes of these species as *B. pertussis* has 358 pseudogenes which corresponds to 9.4% of the genome as *B. parapertussis* has 220 pseudogenes which is 5% of the genome and *B. bronchiseptica* has 18 pseudogenes which is 0.4% of the genome.

More recent reports have confirmed that *B. pertussis* has a high number of IS elements and, in addition, the number of IS elements is actually still increasing in recent isolates (Bouchez et al. 2008).

There is at least one report of IS elements *481*a, a newly identified IS element in *B. pertussis* which is closely related to the most frequent IS element in the species (IS*481*), affecting gene expression in *B. pertussis* (Han et al. 2011). Clinical isolates express a type III effector BteA at higher levels than Japanese vaccine strains due to an insertion of IS*481*a in the vicinity of the BteA in vaccine strains. BteA of *B. bronchiseptica* has been linked to necrotic cell death as well as cytotoxicity in mammalian cells (Panina et al. 2005; Kuwae et al. 2006).

## 2.4. Vaccination and immunity

After the isolation of the causative agent of pertussis by Bordet and Gengou, it was possible to start to produce vaccines against the disease. The first vaccines produced were mostly whole-cell vaccines but there were also other kind of vaccines, used and under development, to prevent or treat pertussis, such as mixed vaccines containing also other bacteria of the upper respiratory tract, crude acellular preparations, detoxified vaccines and vaccines enriched with toxic factors (Mattoo and Cherry 2005). The first vaccines were ineffective in preventing or treating pertussis as commented upon by the American Medical Association's Council on Pharmacy and Chemistry in 1931 (Council on Pharmacy and Chemistry 1931; Shapiro-Shapin 2010)

In the 1930s and the 1940s, many trials and vaccines were tested. Very prominent work was carried out by Pearl Kendrick and Grace Eldering in Grand Rapids, Michigan, USA. They started developing and distributing their own pertussis vaccine to local physicians. By using careful control measures they tried to ensure that the vaccine was potent and safe. In 1934–1935, they conducted a vaccine trial of 1592 subjects (712 vaccinees and 880 controls) during which they recorded 89% efficacy (Kendrick and Eldering 1936). In a second trial with a slightly modified vaccine, better control measures and a larger study population the efficacy was 84.8% (Kendrick and Eldering 1939). They later conducted a trial testing alum as a vaccine preservative with good success (Kendrick 1942). After these trials the Michigan Department of Health began mass-producing the pertussis vaccine for children in 1938. By 1940, pertussis vaccine was widely distributed across the USA and already in 1943, the American Academy of Pediatrics approved the vaccine for routine use. In 1944 the American Medical Association recommended its use (Felton and Willard 1944). (Shapiro-Shapin 2010)

Eldering and Kendrick contributed to the field of pertussis research and vaccine development in many other ways as well. They developed diagnostic methods and vaccines, improved culturing methods, identified how long a child remains infectious, developed methods to measure vaccine efficacy and were, for instance, the first to identify and isolate *B. parapertussis* (Kendrick and Eldering 1934; Eldering and Kendrick 1938; Kendrick et al. 1947). They also experimented on the DTP combination vaccine that was to become the primary way of vaccinating against tetanus, diphtheria and pertussis. Kendrick also took part in a vaccine trial in the United Kingdom and consulted many European researchers in performing a vaccine trial (Bunney et al. 1965). (Shapiro-Shapin 2010)

Vaccinations against pertussis were initiated in Europe and the USA in the 1940s and 1950s. They have been very successful in diminishing the number of reported cases and incidence. It has been reported that in the USA the incidence dropped 150-fold after the start of vaccinations (Mattoo and Cherry 2005) (figure 1.)

### 2.4.1. Vaccination against pertussis

Vaccinations against pertussis are administered mostly in a series of three primary injections and additional boosters. The first dose is administered at the age of 2 to 4 months and the last from 4 to 12 months (Heininger and Cherry 2006; Zepp et al. 2011). There are significant differences in the administration of additional shots as reported by EUVAC.NET. In Europe, additional shots are given to children of 11 months to adolescents of 17 years (EUVAC.NET 2011). In France an adult booster is recommended as well as vaccination of health care workers and a cocooning strategy of vaccinating people with close contacts to newborn infants (Zepp et al. 2011).

Most European countries use acellular vaccines against pertussis. Poland is the only exception, but even Poland uses aP for the pre-school booster. Of the countries that use adult boosters, most prefer vaccines with reduced antigen amounts (dtap or dTap): Finland, Germany, Italy, Luxembourg and Sweden. Austria and France use normal aP vaccine also for adolescents. (EUVAC.NET 2011)

### 2.4.2. Immunity post infection and post vaccination

The length of the protection from an actual infection or a vaccination and the immunity arising from wP vaccine and aP vaccine can vary.

After natural infection the immunity wanes in 7–20 years and after a vaccination the time is shorter: from 4 to 12 years (Wendelboe et al. 2005). There is also evidence that aP may give shorter protection than wP (Simondon et al. 1997).

The wP vaccine raises a more Th1 type of immune response (cell-mediated immunity) and the aP vaccine gives a more broad Th1 and Th2 response (cell and antibody-mediated immunity) (Mascart et al. 2007). Natural infection, at least in infants, seems to promote Th1 response as does wP vaccine (Mascart et al. 2003). Both antibodies (Th2-mediated response) and IFN-γ (Th1-mediated response) are important for immunity against *B. pertussis* (Mills et al. 1998). Circulating antibodies may not be necessary for protection if cellular and humoral memory is still present (Mahon et al. 2000).

Macrophages and neutrophils, too, have been shown to be able to kill *B. pertussis*, but their function in eradicating the disease in unclear (Mills 2001).

### 2.4.3. Herd immunity

As the source of infection to unvaccinated infants is often a member of the family, herd immunity is vital for the protection of the unvaccinated. That is to say that the vaccination of others protects also the unvaccinated (Brisson and Edmunds 2003). There is clear evidence of herd-immunity in pertussis (Trollfors et al. 1998; Taranger et al. 2001). The results of a Swedish study show that also the unvaccinated family contacts were protected from pertussis by the vaccination (Trollfors et al. 1998). The point at which a lowered vaccine coverage starts to impair herd immunity remains unknown, but some countries have noticed that the introduction of adolescent and adult boosters is needed to increase the number of vaccinated people and therefore to protect the unvaccinated (Plans 2010).

### 2.4.4. Vaccines in Finland

Vaccinations against pertussis were initiated in 1952. For most of the 20th century, Finland used self-made wP vaccines, manufactured by the National Public Health Institute. The first vaccines

were rather poorly recorded and thus there is no data available on the strains used in their production, but from the 1962 on strain 18530 (Fim3, *prn*1, *ptx*A3), acquired from the USA, was used and from year 1976 a new strain 1772 (Fim2.3, *prn*1, *ptx*A2), acquired from the United Kingdom, was added to the vaccine (both strains in equal volumes). From the 1970s on, the vaccination program with wP vaccine was vaccination at 3, 4, 5, and 20–24 months of age. In 2003 and aP vaccine as pre-school booster at 6 years of age was added to the program (dTap, Boostrix®, GlaxoSmithKline Biologicals s.a.). In 2005, the schedule was changed to 3, 5, and 12-month-old children and the vaccine was changed to aP (DTaP-IPV-Hib, Pentavac®, Sanofi Pasteur MSD). Boosters were scheduled at 4 (DTaP-IPV, Tetravac™, Sanofi Pasteur MSD, France) and 14 years of age (dtap, Boostrix®, GlaxoSmithKline Biologicals s.a.). (Elomaa et al. 2009)

### 2.4.5. Whole cell vaccines

Whole -cell vaccines were often produced from circulating strains isolated from each country, but some countries also acquired strains for the wP from other sources (Gzyl et al. 2004; Elomaa et al. 2009; Hozbor et al. 2009; Litt et al. 2009). In general, *B. pertussis* is grown in bulk culture, harvested, concentrated by centrifugation, and suspended in a buffered saline solution (Cherry 1996). Detoxification is usually done by heat or chemical agent or a combination of those two. The vaccine components, the pertussis component, and often also diphtheria and tetanus toxoids, are absorbed onto alum (Cherry 1996).

wP vaccines are still used in many developing countries and some developed ones as well e.g. Poland and Serbia.

### 2.4.6. Acellular vaccines

As there were concerns of vaccine safety in relation to wP vaccines, the development of less reactogenic aP vaccines was begun (Miller et al. 1981; Cowan et al. 1993). The first aP vaccines were produced in Japan and the USA (Sato and Sato 1999). The aP vaccines have been shown to protect against pertussis and to be better tolerated that the wP (Zhang et al. 2011).
There are many different formulations of aP vaccines. PT is included in all the aP vaccines. In all vaccines PT has to be detoxified either by formaldehyde treatment or genetic manipulation. The chemical detoxification may impair the production of functional (protective) antibodies (Sutherland et al. 2011).

Denmark uses a self-made aP vaccine with PT as the sole component. In the current market there are vaccines with PT/FHA, PT/FHA/PRN and PT/FHA/PRN/FIM2/FIM3 vaccines. All these vaccines have been shown to be quite efficacious. Today many countries choose the cheapest vaccine available. This makes it difficult to test the effects of a certain vaccine, although many vaccines do include components from a single strain: Tohama I. Nevertheless, there is a report from Sweden stating novel *ptxP* allele *ptxP3* emerged earlier in a region with PT vaccine when compared to the rest of the country where vaccine with PT and two other components was used. The incidence of pertussis was also significantly higher in the region in which the vaccine with only PT was used (Advani et al. 2011).

# 3. AIMS OF THE STUDY

The main object of this study was to investigate the evolution of *B. pertussis*, the causative agent of whooping cough, during the 20th and the 21st century as well as to find out the possible adaptation of the bacterium to the mass vaccinations against pertussis that were initiated in most countries in the 1940s to 1960s.

We investigated clinical isolates and vaccine strains used in the production of whole-cell vaccines from several European countries including Finland. We studied strains from countries with different epidemiological situation and countries that have used different strategies when designing the composition of the whole-cell vaccine. In addition, countries outside of Europe were studied in order to complete analysis with countries where vaccinations were initiated late or vaccination coverage has been low.

Although changes in the genotypes of *B. pertussis* have been widely reported it is not clear how they affect the virulence of the bacterium. To asses this, we studied the effect of a recently emerged pertussis toxin promoter variant (*ptx*P3) on the cytotoxicity of *B. pertussis* to human monocytes.

To see the complete picture of the effects of different vaccination strategies and situations the following countries represented the different aspects that affect the evolution and adaptation *B. pertussis* (table 2).

The specific aims of this study were to investigate:

1) The evolution/adaptation of the *B. pertussis* in highly vaccinated populations

2) The effect of vaccine composition on the evolution of *B. pertussis*

3) The evolution/adaptation of *B. pertussis* in countries with low vaccination coverage

4) The evolution/adaptation of *B. pertussis* in countries with late introduction of vaccinations

5) The reasons behind the emergence of new strain types

**Table 2.** Countries representing different aspects of the study

| Country | Characteristics | Study |
|---|---|---|
| **Finland** | –High vaccination coverage | I, IV, V |
| **The United Kingdom** | –High vaccination coverage | IV |
| **Poland** | –High vaccination coverage<br>–Addition of contemporary strains to vaccine<br>–Unique PFGE profiles | IV |
| **Serbia (Former Yugoslavia)** | –Addition of contemporary strains to vaccine | II, IV |
| **China** | –High vaccination coverage achieved late | III |
| **Africa (Kenya, Senegal)** | –Vaccinations started late | (van Gent et al. 2011a) |

# 4. MATERIALS AND METHODS

## 4.1. Bacterial isolates and culture (I, II, IV, V)

The Finnish *B. pertussis* strains were obtained from the Pertussis Reference Culture Bank of the National Institute for Health and Welfare, Turku, Finland. From 2004 onwards, all the culture positive *B. pertussis* strains in Finland have been sent to the collection, as this is required by the law. International reference strains present in the collection have been acquired from Dr. Nicole Guiso, Institut Pasteur, Paris, France.

International strains in the studies comprising this thesis are from Poland, provided by Anna Lutyńska (National Institute of Public Health-National Institute of Hygiene, Warsaw, Poland); the United Kingdom, provided by Norman K. Fry (Health Protection Agency Centre for Infections, London, The United Kingdom); Serbia, provided by Tatjana Pljesa and Gordana Dakic (Torlak Institute of Immunology and Virology, Belgrade, Serbia); and from Senegal and Kenya, provided by Fritz Mooi, (RIVM, Bilthoven, the Netherlands). The strains used in the studies are summarised in table 3 and the countries of origin are shown in figure 3.

**Table 3.** The number of tested strains and their origins.

| Country | Total number of strains used | Years of isolation | Used in studies | Provider |
|---|---|---|---|---|
| **Finland** | 698 | 1953–2011 | I, IV, V | Pertussis Reference Culture Bank, National Institute for Health and Welfare, Turku, Finland |
| **Poland** | 19 | 1960–2004 | IV | Anna Lutyńska, National Institute of Public Health-National Institute of Hygiene, Warsaw, Poland |
| **Serbia (former Yugoslavia)** | 74 | 1953–2000 | II, IV | Gordana Dakic, The Institute of Virology, Vaccines and Sera Torlak, Belgrade, Serbia |
| **The United Kingdom** | 15 | 1941–2004 | IV | Norman K. Fry, HPA, London, the UK |
| **Africa (Kenya, Senegal)** | 57 | 1975–1992 | | Frits R. Mooi, RIVM, Bilthoven, the Netherlands |

**Figure 3.** Study regions. Highlighted are the countries were the study material was acquired. Countries: China, Finland, the United Kingdom, Poland and formed Yugoslavia, including Serbia, are coloured in black. Europe has been enlarged from the world map.

In addition to the strains mentioned above, the DNAs of 53 strains selected from a collection of 99 Chinese strains were tested by PFGE. The PFGE plugs were provided by Shumin Zhang (National Institute for the Control of Pharmaceutical and Biological Products, China). Selection criteria are described in detail in section 5.3. PFGE plugs made in China were analysed in the Pertussis Reference Laboratory of the National Institute for Health and Welfare, Turku, Finland.

The strains are stored at -70 °C in glycerol containing medium. The bacteria were cultured on charcoal agar plates for three days in 35 °C.

## 4.2. Serotyping (I, II, IV, V)

Serotyping was performed with monoclonal antibodies against Fim2 and Fim3 (provided by Dorothy Xing from the National Institute for Biological Standards and Controls (NIBSC), Potter Bar, the United Kingdom) either by slide agglutination test or by indirect in-house ELISA (Elomaa et al. 2005; Heikkinen et al. 2008).

## 4.3. SNP detection (I, II, IV, V)

SNP detection or genotyping of different *ptx*A and *prn* variants was performed with Light Cycler qPCR (Makinen et al. 2001; Makinen et al. 2002). The protocol of *ptx*A genotyping was based on

PCR, FRET probe melting curve analysis. The detection of *prn* variants was also based on PCR, FRET probe melting curve analysis as well as agarose gel electrophoresis.

The genotyping for *ptxP* was performed in most cases by allele-specific LightCycler qPCR. The method has been described in the manuscript V. The melting temperature of the PCR products derived from the strains with *ptxP3* was about 84 $^{o}$C. There was no specific amplification from the strains with *ptxP1*. Therefore, the two alleles can easily be differentiated by this method (figure 4).

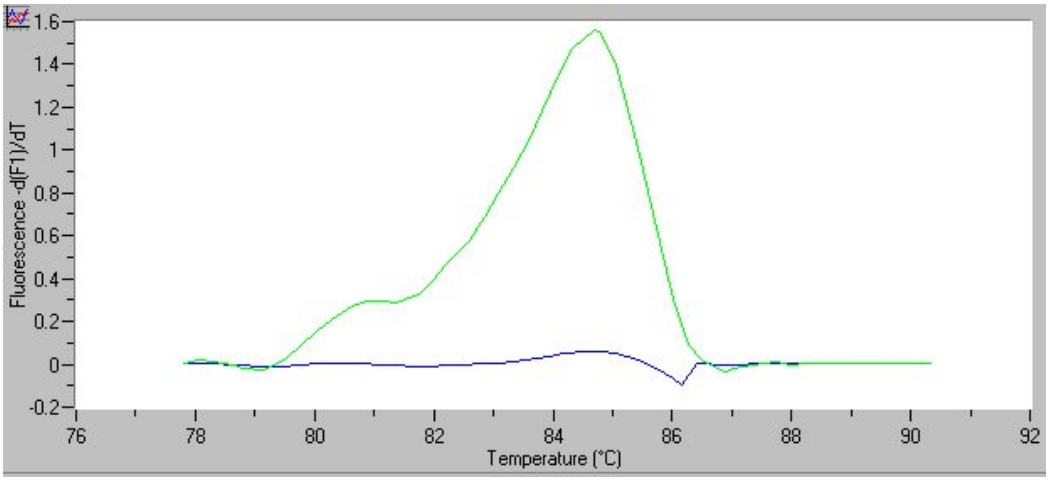


**Figure 4.** The identification of *ptxP3* strains. The green line presents a *ptxP3* strain and the blue line a *ptxP1*strain. The area under the curve and specific melting temperature of PCR products are used to distinguish the two alleles.

## 4.4. Pulsed-field gel electrophoresis (I, II, III, IV)

### 4.4.1. Sample preparation and gel electrophoresis

The method for PFGE used in this study has been published in 2004 (Advani et al. 2004). Bacterial cultures were performed as described before in manuscript I. Bacterial cells were harvested from charcoal agar plates and suspensed in PBS, and the bacterial concentration was adjusted to 2 x $10^9$ cfu/ml. Mixture of 150 µl of low melting agarose (SeaPlaque® GTG ® agarose, BioWhittaker Molecular Applications) and bacterial suspension was pipetted to plug moulds. The bacteria in the agarose plugs were treated with proteinase K (Proteinase K (fungal), >20 U/mg, Invitrogen) to destroy the cell wall. The plugs can be stored after the proteinase K treatment or taken straight to DNA digestion with *Xba*I (20 000 U/ml, New England BioLabs). Digested samples were run on 1% PFGE certified agarose (Biorad Laboratories) gels for 40 hours with the parameters described previously by Advani et al. 2004. The gel electrophoresis was performed in CHEF-DR III system (Biorad Laboratories)

### 4.4.2. Analysis

The digital picture of the gel was taken with GelDoc 2000 system (BioRad Laboratories). Analysis of the PFGE gel pictures was performed with Bionumerics version 4.01 (Applied Maths). The

unweighted pair group method using arithmetic averages (UPGMA) with 1% band tolerance and 1% optimization settings was used as the clustering method.

The results from the PFGE analysis have been stored to a single database and can therefore be used in subsequent analyses. The database contains data from 790 strains analysed in our laboratory. Some of the strains have also been analysed elsewhere. Therefore, it is also possible to compare results from different laboratories.

In the field of *B. pertussis* research, two kinds of nomenclature are used in when assigning strains to different PFGE profiles. In these studies we used the parameters and nomenclature first reported by Advani et al, (2004) , but the more broad classification in to PFGE groups introduced by Caro et al, (2005) was also included in the analysis (Caro et al. 2005).

## 4.5. Microarray based comparative genomic hybridisation (I, IV)

### 4.5.1. Array design

The microarray was designed according to the published genome of Tohama I (Parkhill et al. 2003). Each gene was presented by one 70-mer probe. The probe library covered 3 582 DNA sequences (94%) of the 3 816 ORFs present in Tohama I. The probes were acquired as an Array-Ready Oligo Set™ from Operon Biotechnologies GmbH and spotted onto UltraGaps™ coated slides (Corning Life Sciences) in the Finnish DNA Microarray Centre, Turku Centre for Biotechnology, Turku, Finland.

Twenty negative control probes with no match to *B. pertussis* strains Tohama I were spotted in 8 separate spots to control non-specific binding. Water was spotted to 244 spots to control the contamination of printing tips and the non-specific binding of DNA to slide surface. To control the intra-array variation, 12 "housekeeping genes" of *B. pertussis* were spotted 10 times on one array.

### 4.5.2. DNA isolation, labelling and hybridisation

Genomic DNA was isolated from approximately 2 x $10^9$ cfus of bacteria with GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich Inc.) and digested with *Sma*I (10 U/µg DNA, New England Biolabs) as instructed by the manual of the BioPrime®Array CGH Genomic Labeling System (Invitrogen Life Technologies). Isolated and digested DNA was run on agarose gel (1.5%) to asses the quality.

4 µg of digested DNA was used in the labelling reactions. DNA from the strain Tohama I, the strain according to which the microarray was designed, was used as reference material and labelled with Cy3 (Amersham Biosciences UK Limited). An equal amount of DNA from a testing strain was used as an experimental sample and labelled with Cy5 (Amersham Biosciences UK Limited). The labelling was performed according to the protocol of BioPrime®Array CGH Genomic Labeling System (Invitrogen Life Technologies). The purification module included in the kit was used for DNA purification. After purification, the DNAs of the reference strain and the testing strain were combined and the concentration and quality of the labelled DNA was measured with ND-1000 Spectrophotometer (NanoDrop Technologies). The reagents and their volumes are presented in table 4.

Before hybridisation the slides were cross-linked with UV-light (90 mJ cm-2) and washed for 1 minute in 0.1% of SDS and 2 minutes in water, after which an incubation at 50°C for 30 minutes in a solution containing 1% BSA fraction V, 2 x SSC and 0.1% SDS to block nonspecific binding, was performed. The slides were further washed in 2 x SSC for 3 minutes and then in 0.2 x SSC for 3 minutes.

The hybridisation reactions were performed in hybridisation chambers under LifterSlip™ coverslip (Erie Scientific Company) at 65°C for 16 hours. After the hybridisation the slides were washed at 65°C for 10 minutes with 1xSSC and 0.1% of SDS. Subsequent washes were performed at room temperature for 10 min with 0.5xSSC and 0.01% SDS, and for 3 minutes and 1 minute with 0.1xSSC. Slides were dried in slide centrifuge and scanned with ScanArray® 5000 (PerkinElmer).

**Table 4.** The reagents included in the hybridisation solution and their volumes

| Solution | Volume | Information |
|---|---|---|
| **Labelled DNA** | 15.7 µl | |
| **Yeast tRNA** | 2.5 µl | 10 mg/ml, Invitrogen Life Technologies |
| **20xSSC** | 4.3 µl | 1xSSC was 8.8 g/l NaCl and 4.4 g/l trisodiumcitrate |
| **10% SDS** | 0.8 µl | |
| **10xBlocking Solution** | 1.7 µl | DIG-Wash and Block Buffer set (Roche) |
| **Molecular grade water** | fill to 25 µl | |

### 4.5.3. Analysis

Quantitation was performed using ScanAlyze program (Michael B. Eisen, University of California at Berkeley). The numerical values obtained from ScanAlyze were analysed with R/Biocondutor (Ihaka and Gentleman 1996; Gentleman et al. 2004; R Development Core Team 2008).

Initially the analysis included a crude analysis of the spot intensities (I). By constructing an MA-plot, mean of intensity (A) was plotted against the log transformed intensity ratio (M) of each spot. This enabled us to classify the spot (genes) as absent or present. From the plot it was then easy to spot the genes that were absent as most of the spots centered close to zero and the absent genes fell into a separate cluster with low M-values. This group could be defined by an M-value <-2. This method could also be used to detect duplications as genes with duplications or other multiplications were easily identifiable as they had higher M-values that the majority of genes. An example of an MA-blot is presented in figure 5.

Later on, a more refined method was utilised in the analysis of the CGH results (IV). Microarray data analysis was performed with R/Bioconductor (Ihaka and Gentleman 1996; Gentleman et al. 2004; R Development Core Team 2008). Print-tip-loess normalisation was performed with default parameters and the gains/losses were filtered with Limma package (Smyth 2005).

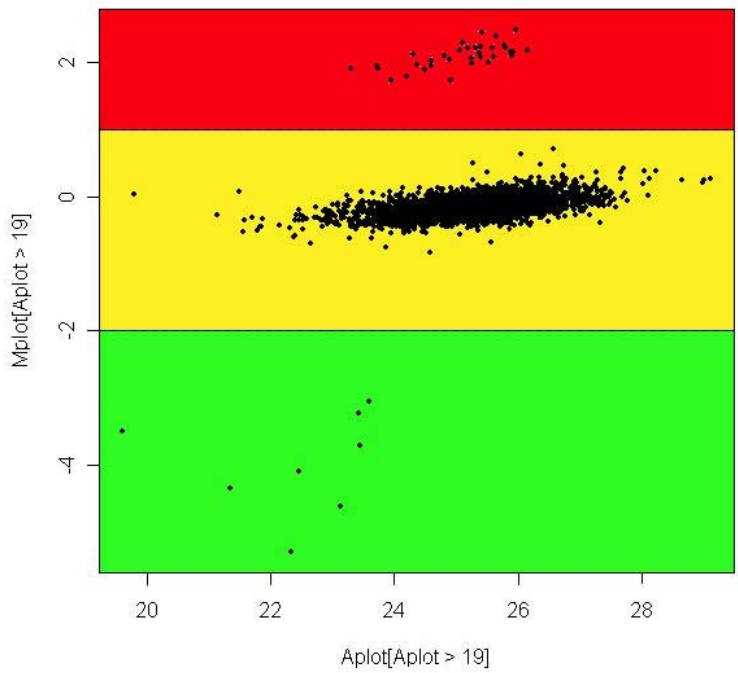


**Figure 5.** MA-blot used to classify genes as present, absent and duplicated. In the figure there are three areas indicated by red, yellow and green colours. The yellow indicates the genes that are present in the tested strain and the reference strain. Red indicates genes that are present in higher copy numbers e.g. in duplications in the tested strain than the reference strain. Green presents genes that are lost in the tested strain.

### 4.5.4. PCR for confirmation of the CGH results and detection of absent genes

All the CGH results of the study I were confirmed by PCR and the same protocols (see below) were later used to detect the possible absence of the corresponding region from the tested strains. The primers were designed with the Primer3 program (Rozen and Skaletsky 2000). The PCR primers were designed so that the PCR reaction would extend over the boundary of the absent region. This enabled us to detect the loss of genetic regions but also to detect any location changes of the region in relation to the genome of the reference strain Tohama I.

In the PCR amplifications, 5–50 ng of purified DNA was used as template. The PCR reaction also included 200 µM of each dNTP (Promega), 20 pmol of each primer (Sigma Aldrich), 5% DMSO (Merck), 1xBuffer II, 1.5–3 mM of MgCl2, 0.625–2.5 U of AmpliTaq Gold® DNA polymerase (Applied Biosystems). The reaction volume was adjusted to 50 ml with Molecular Biology grade water (Eppendorf). PCR was run in MJ Research PTC 200 Thermal Cycler (Global Medical Instrumentation) with the following protocol: initial denaturation step at 95°C for 8 min followed by 30–38 cycles of 30 sec at 94°C, 30 sec to 1 min at 60–62°C and 1 to 3 min at 72°C, and with a final elongation of 5 min at 72°C. The correct lengths of the PCR products were confirmed on agarose gel electrophoresis.

The presence or absence of genes not found in Tohama I but later reported to be present in *B. bronchiseptica* and *B. parapertussis* and in many European *B. pertussis* strains were also investigated with PCR (Brinig et al. 2006; Caro et al. 2008). The primers were designed according to the results of Brinig et al (2006). The presence of the gene BP1225 was further studied with PCR as well as the presence of the lost regions from recent European strains. The primers were designed

to amplify a short region (~250–350 bp in size) in the middle of the genes at the ends of the regions of difference. In some regions of difference, also genes in the middle of the region were tested. PCR reactions were performed as described above.

## 4.6. Cytotoxicity detection and cytokine measurements (V)

### 4.6.1. Cytotoxicity

Bacterial cells stored in -70°C were plated and cultured as described earlier. After three days the bacteria were plated again and cultured for another three days.

The culture method for human U937 cells has been previously published (Ekman et al. 2002). The method used in this study was otherwise identical except for the fact that we used 1% FCS according to the manual of the cytotoxicity detection kit (Cytotoxicity Detection Kit$^{PLUS}$ (LDH), Roche Diagnostics GmbH) to reduce the background from lactate dehydrogenase (LDH) present in FCS. In short, human monocytic U937 cells were harvested and stimulated to differentiate to a macrophage-like phenotype by incubating them with phorbol myristate acetate (PMA) for 24 hours. After the stimulation the old RPMI-medium was discarded and replaced with fresh medium with suspensed *B. pertussis* bacteria. The eukaryotic cell amount was optimised as suggested by the kit manufacturer. The number of cells which gave the largest difference in LDH release between untreated and lysed cells was $10^5$ cells. The multiplicity of infection was also optimized and eukaryotic cells to bacteria ratio of 1:5 was used in the experiments. The experiments were performed in triplicate in 96 well plates. The U937 cells were incubated with *B. pertussis* cells for 16h after which the cytotoxicity was measured according to the manufacturer's recommendations. The cytotoxicity percentage was calculated with the following formula: (experimental value – low control)/(high control – low control)*100. Low control = cells with no treatment, high control = lysed cells.

The Mann-Whitney *U* test was used to compare the cytotoxicity of *ptx*P1 and *ptx*P3 strains. Kruskal-Wallis test was used to compare the differences in cytotoxicity between different groups of genotypes: *ptx*P3 group (*prn*2/*ptx*A1/*ptx*P3), consisting of all the *ptx*P3 strains; main *ptx*P1 group, consisting of (*prn*2/*ptx*A1/*ptx*P1) strains and the rest of *ptx*P1 strains (*prn*1/*ptx*A2/*ptx*P1, *prn*1/*ptx*A1/*ptx*P1 or *prn*4/*ptx*A1/*ptx*P1). A p-value $< 0.05$ was considered significant.

### 4.6.2. Cytokines

Production of cytokines by the monocytic cells treated with bacteria was measured by collecting culture supernatants at 6 and 16 hour time points. Experiments were performed twice and all treatments were done in triplicate. Cytokines were measured only from U937 cells treated with selected six *B. pertussis* strains, representing three *ptx*P1 strains (Tohama I, PRCB163 and 223) and three *ptx*P3 strains (PRCB382, 406 and 677). Positive control was not included as we only investigated the difference between different strain types. We measured the concentrations of pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 with Quantikine Immunoassay Human TNF-α and IL-10 (R&D Systems Inc.).

The U937 cell line was selected for the experiments since it has been used to study the interaction of host cells with *B.* pertussis as well as other bacteria (Abramson et al. 2001; Boldrick et al. 2002; Ekman et al. 2002). U937 cells are known to produce the selected cytokines (Ekman et al. 2002). TNF-α is a characteristic proinflammatory cytokine and a marker for inflammation. IL-10 is a characteristic anti-inflammatory cytokine. Both of these cytokines are measured in many studies investigating the interaction between the host and bacteria.

# 5. RESULTS

## 5.1. Strain variation of Finnish *B. pertussis* strains post vaccination (I, V)

Strains analysed in Finland have clearly become different after the start of vaccination program against pertussis in the 1952. The serotypes of *B. pertussis* isolates have changed from a mixed population of Fim2, Fim2,3 and Fim3 to a population expressing almost exclusively Fim3, in recent years. At one point there was, however, a period of Fim2 predominance. The predominance of Fim2 in a highly vaccinated population is uncommon. This may be due to the fact that only roughly 30% of vaccinees produces antibodies against Fim2 even after the addition of a Fim2,3 strain to the vaccine in 1976. Of the vaccinees, around 75% produced antibodies against Fim3 (Huovila 1982).

A new variant of pertussis toxin promoter has been described recently (Mooi et al. 2009). The allele *ptx*P3 has replaced the previously predominating allele *ptx*P1 in the Finnish *B. pertussis* population.

Pertactin and pertussis toxin have experienced a similar shift as serotypes. In the period after the initiation of vaccinations, most of the strains were *prn*1/*ptx*A2 but for many years all strains have been *ptx*A1 and most of the strains are *prn*2. After 1977, all isolates have been *ptx*A1. The shift in serotypes and genotypes of *prn* and *ptx*P are presented in figure 6.

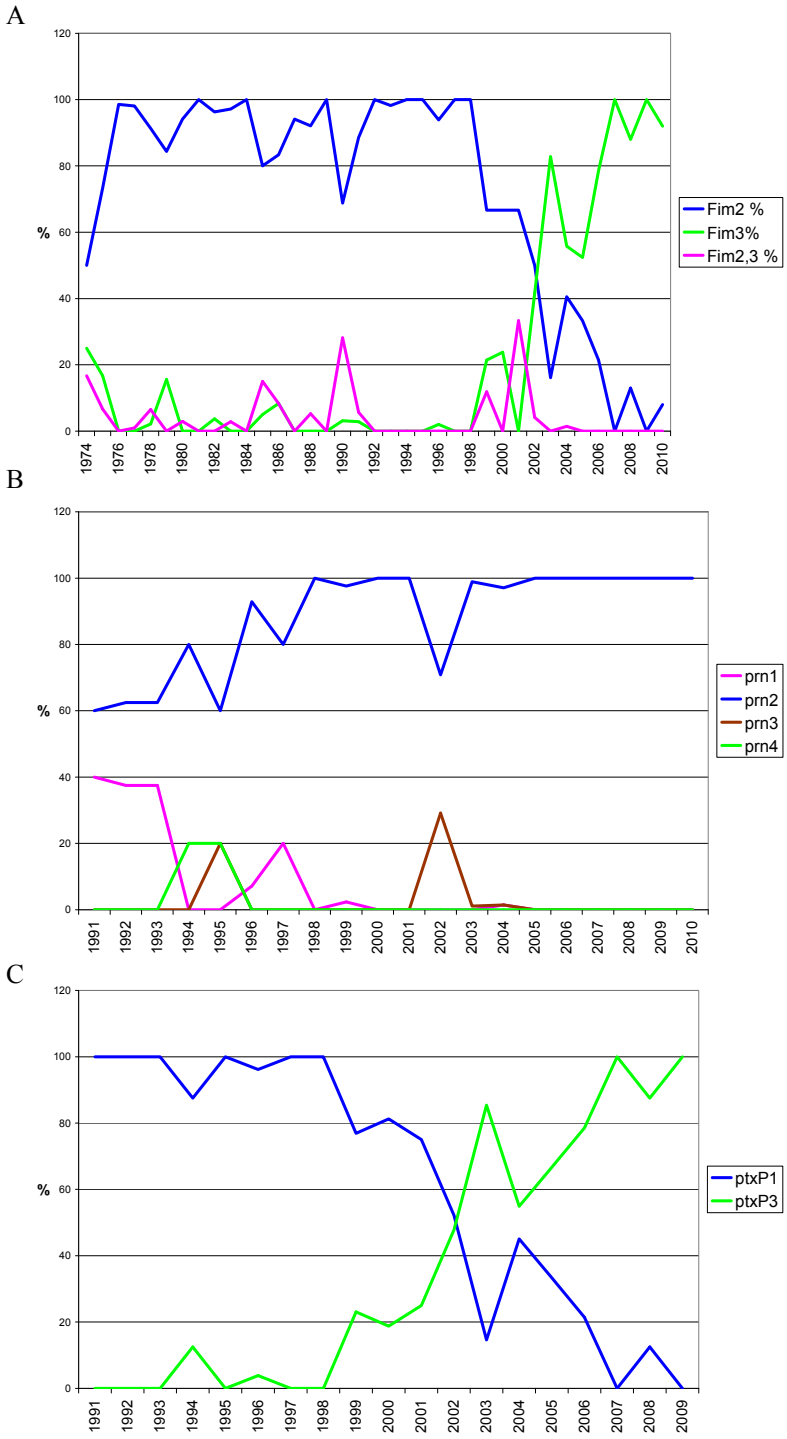


**Figure 6.** The annual proportions of strains presenting different serotypes (A) and alleles for *prn* (B) and *ptx*P (C). The pertussis vaccinations were initiated in Finland in 1952.

The strains from Finland have also evolved in regards to the genome content. We used CGH to test Finnish *B. pertussis* strains isolated during the over 50 years (1953–2004) when vaccination against pertussis was in the vaccination program in this country. Even the oldest strains differed from the reference strain Tohama I, a strain that was used in the design of the microarray and as the reference material in the hybridisations. The oldest Finnish strains (from 1950s and 1960s) had lost a region in the genome covering seven genes (BP1135-BP1141, RD5) and 8.6 kb. The Finnish strains from 1970s to 1996 had lost the same region as the oldest ones as well as a new region covering 24 genes (BP0910A-BP0934, RD3) and 24kb. The strains isolated from 1999 to 2004 had lost the same regions as older strains but again had lost an additional genomic region covering 18 genes (BP1948-BP1966, RD10) and 22.7 kb. These were the most commonly found regions of difference when comparing the reference strains Tohama I and the Finnish clinical isolates. The positions of the lost regions can be seen in figure 7. In addition to those regions, one strain isolated in 1996 had lost a region of 16 genes (BP2088-BP2103) and 17.7 kb and one strain isolated in 1965 had a duplication in its genome of more than 100 genes.

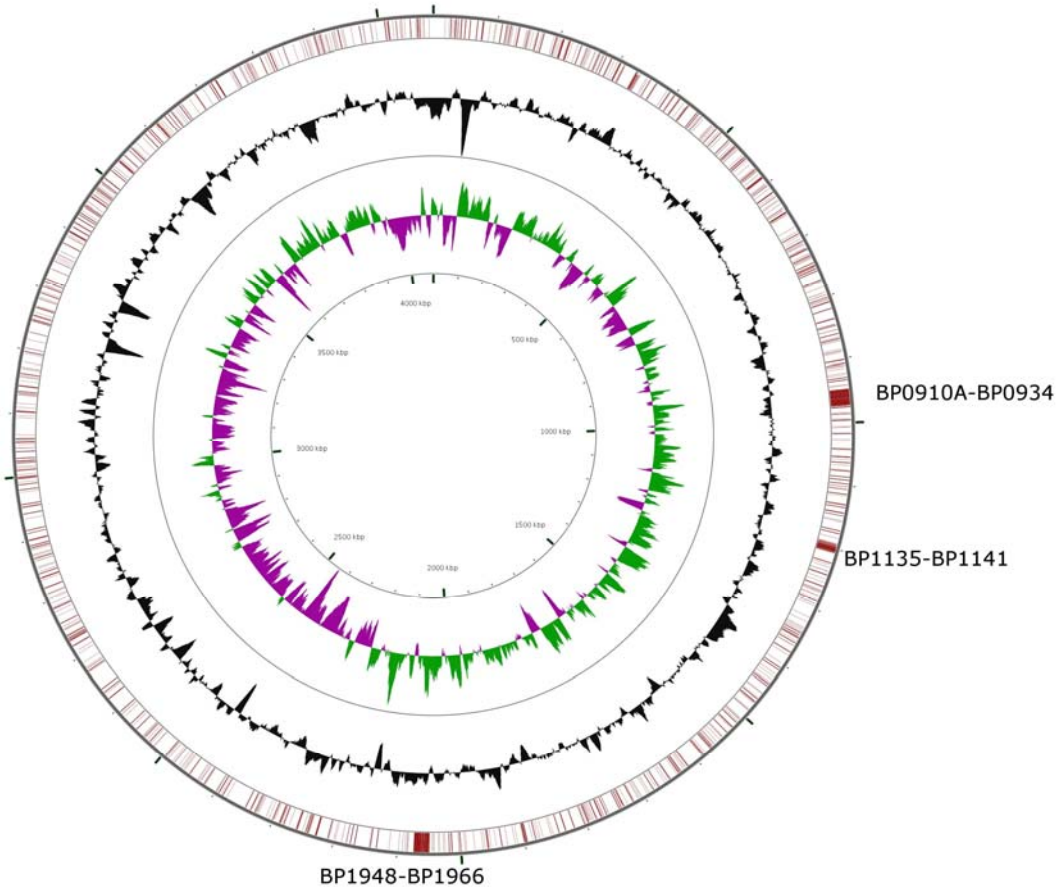


**Figure 7.** Most commonly lost regions of genes in Finnish *B. pertussis* strains are presented on the genome of Tohama I. The innermost circle shows the genome position, the next circle the GC skew, the next the GC content and the outermost circle depicts the positions of the lost regions of genes. The regions are marked by gene codes BP0910A-BP0934, BP1135-BP1141 and BP1948-BP1966. Figure made by CGView Server V 1.0 (2007) (Grant and Stothard 2008).

Proportionally more lost genes were reported as being pseudogenes than can be thought to be lost on the basis of the genome of the reference strain Tohama I which has 9.4% of pseudogenes in its genome and of the 65 lost genes identified in the tested strains 16.9% were pseudogenes (n=11). The functions of the lost genes were mostly in same proportions as they are present in the genome of Tohama I, except for the genes involved in energy production and conversion and inorganic ion transport and metabolism, especially iron transport and storage.

Genes in the duplicated region were involved in cell motility, intracellular trafficking and secretion, cell wall/membrane biogenesis, and posttranslational modification, protein turnover and chaperones. Many genes were related to flagella even though the flagella operon is thought to be inactive in *B. pertussis*. The results from the study on Finnish strains are deposited to the Gene Expression Omnibus (GEO) and can be accessed with the number GSE8092

We confirmed that digestion of DNA did not affect the results as there was a restriction site (CCCGGG) of *Sma*I in 55 (1.5%) oligonucleotides. Of these, 48 were in the present genes, five in the duplicated genes, and two in the absent genes. Therefore, restriction sites on oligonucleotides did not affect the identification of absent or duplicated genes in the microarray. Treatment of the testing material did not influence the results, but the digested DNA was used for all experiments to avoid the possible effects of the length and the conformation of long chromosomal DNA fragments on labelling.

All lost regions were flanked by IS*481* insertion sequence elements. This shows that the loss of genes is mediated by these IS elements in *B. pertussis*, probably by homologous recombination.

The changes of PFGE profiles have been extensively reported in the Finnish *B. pertussis* population. In the studies comprising this thesis, we found that the PFGE results correlate with the CGH results as the strains with different genomic content clustered together.

The Finnish *B. pertussis* population has not experienced notable changes. The previously very common PFGE type, BpSR11, which has spread across Europe, was missing at some point, but then it reappeared. The detected percentages of BpSR11 from 2004 to 2010 were 55%, 42%, 44%, 43%, 0%, 13%, 17% and 8%, respectively. The fact that BpSR11 was not detected may also be explained by the low number of strains received and tested. Profile BpFINR32 predominated for a few years and profile BpSR3 has also continued to be detected in the bacterial population in high percentages.

## 5.2. Strain variation of European *B. pertussis* strains pre and post vaccination (II, IV)

For the purposes of this study, we analysed 74 strains from Serbia with serotyping, genotyping of the *ptx*A and *prn* genes and with PFGE. In addition, we analysed clinical isolates and vaccine strains from Poland (n=14, isolated in 1960–1968), the United Kingdom (n=10, isolated in 1941–1963) and Serbia (n=10, isolated in 1953–1984) with serotyping, genotyping of the genes *ptx*A and *prn,* PFGE and CGH. To understand the changes in the bacterial population after the pertussis vaccination programs were initiated, we also analysed recent strains from Poland (n=5), the United Kingdom (n=5) and Serbia (n=4) with PFGE and PCR designed to detect lost regions. We also included in the analysis previously published and analysed Finnish strains, 5 old (1953–1965) and 8 recent (1996–2004).

SERBIA (FOCUS ON GENOTYPING AND PFGE)

We concluded that the Serbian isolates were distinct from isolates from many other European countries. In the pre-vaccine era the serotypes Fim2,3 and Fim2 predominated. In the period between 1957 and 1984 all the serotypes were present, contrary to many other countries with high vaccination coverage where Fim3 is the predominant serotype. In the most recent strains, isolated between 1985 and 2000, Fim2 had become the predominant serotype but Fim3 was also detected.

In the isolates from the pre-vaccine era in this country, we only detected *ptx*A2 type of strains. During 1957–1984 and 1985–2000 both genotypes, *ptx*A1and *ptx*A2, were found but *ptx*A1 was found more frequently with percentages of 66% and 65%, respectively. A similar change in *ptx*A genotypes has been observed in many countries but usually the *ptx*A1 is clearly the predominant with close to 100% of isolates.

In the pre-vaccine era no variation in the *prn* gene was detected. The only allele detected was *prn*1. This is also the most common *prn* type in the pre-vaccine era in other European countries. Between 1957 and 1984, *prn* types *prn*1, *prn*2, *prn*3 and *prn*11 were identified, but only *prn*1 in high frequency. The frequencies for *prn*1, *prn*2, *prn*3 and *prn*11 were 91%, 2%, 4% and 2%, respectively. From 1985 to 2000 *prn*1 was still the most frequently found, but the other *prn* types 2, 3 and 11 were also highly represented with 47%, 12%, 12%, 29%, respectively. The percentages of *prn*2, *prn*3 and *prn*11 are unusually high. The data on serotypes and genotypes of *ptx*A and *prn* are presented in figure 8.

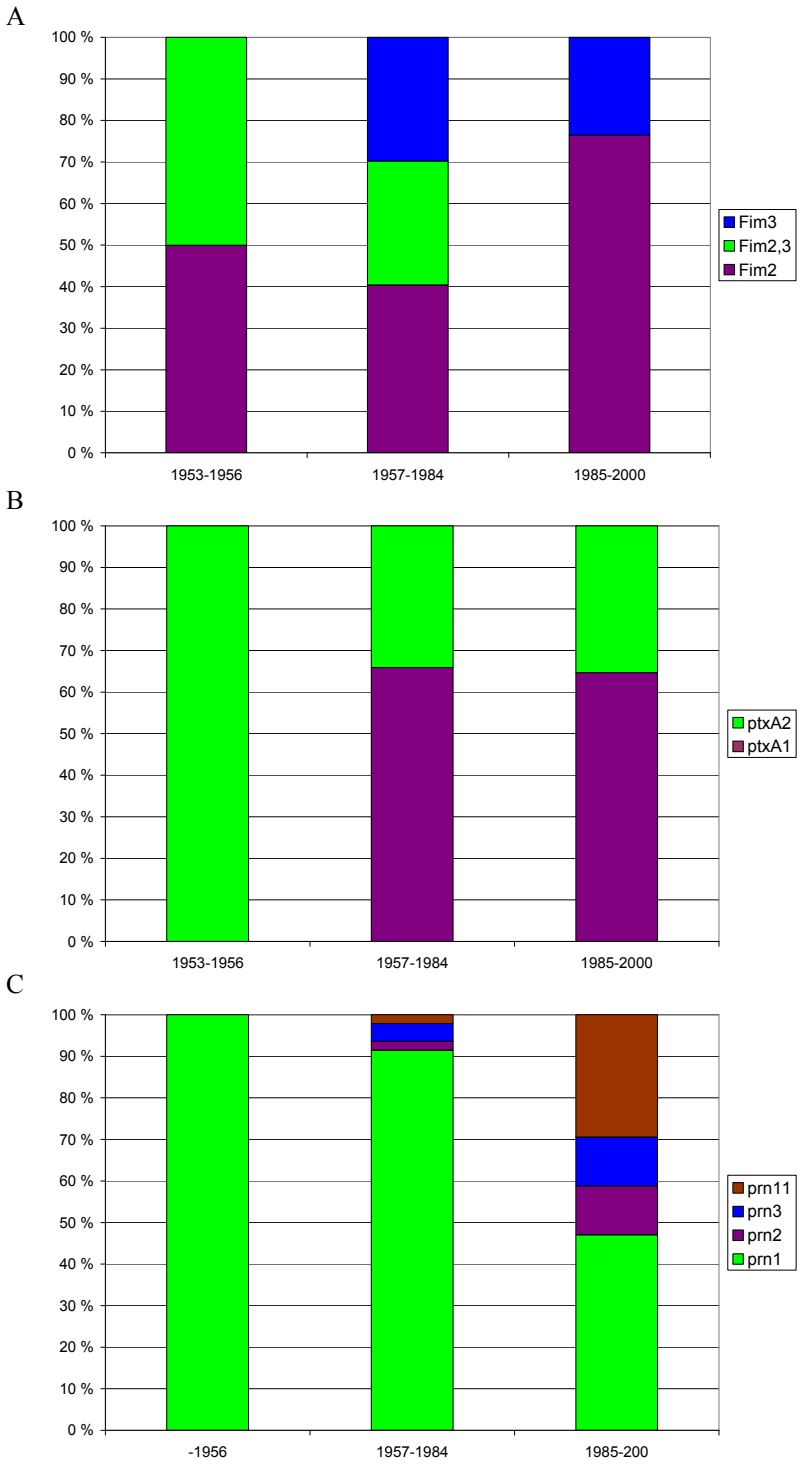


**Figure 8.** Frequencies of the serotypes (A), *ptx*A alleles (B) and *prn* alleles (C) in Serbia during three time periods.

From the 60 strains from Serbia tested with PFGE, 22 distinct profiles were identified. 43% of the strains presented PFGE profiles only found in Serbian isolates. Changes in the profiles were observed during different time periods. The most predominant PFGE profiles from the time periods 1953–1956, 1957–1984 and 1985–2000 were BpFINR1 (62.5%); BpSR23 (40%); and BpSBR6 and BpSR23 (23.5%), respectively. Old profiles, which were first identified in the 1950s, were replaced later by new profiles except for BpSR23 which was found throughout the study period. The PFGE analysis was able to identify three clades to which the strains could be assigned. Of the strains, 5% resembled the reference strains Tohama I. These were three old isolates from 1953 and 1957. All strains with *prn*11 belonged to the same clade. The strains presenting *prn*2 and *prn*3 belonged to another clade.

FINLAND, POLAND, THE UNITED KINGDOM, SERBIA (FOCUS ON CGH)

There is some overlap in the material of the previous study, focused on genotyping and PFGE analysis of isolates from Serbia, and this study, focused on the genome content of *B. pertussis* from four European countries. This is mainly in the old isolates and vaccine strains from Serbia.

All serotypes were present in the old isolates and vaccine strains from these four countries. In all the countries except for Poland, the most recent strains were mostly Fim3. In Poland they were Fim2.

In the old strains from Finland and Serbia, only *ptx*A2 was found. In Poland and the United Kingdom, both *ptx*A1 and *ptx*A2 were present. The recent strains from Finland and the United Kingdom were exclusively *ptx*A1 but in Serbia one of the four recent strains analysed was *ptx*A2.

All the old isolates and vaccine strains were *prn*1 except for one old isolate from Poland, isolated in 1962, and one vaccine strain from Serbia, isolated in 1984, which were *prn*2. The recent isolates from Finland and the United Kingdom were all *prn*2 but the recent strains from Serbia were 50% (n=2) *prn*2, 25% (n=1) *prn*3 and 25% (n=1) *prn*11.

Most of the recent isolates from Finland and the United Kingdom were *ptx*P3 but all of the recent isolates from Poland and half of the recent isolates from Serbia were *ptx*P1.

The CGH results show that the old clinical isolates and vaccine strains from Poland, Serbia and the United Kingdom are similar to old isolates from Finland. They have lost the same regions as the Finnish strains from 1950s–1960s or 1970s–1996 (RD3 and RD5). Contrary to Finland where only one region was lost in the oldest strains and the second RD was lost in the 1970s, in other European countries RDs 3 and 5 were both lost in the oldest strains. The region (BP1948-BP1966) that was lost in the most recent Finnish strains from 1999 to 2004 was not lost in any of the old isolates or vaccine strains from Europe. However, this region was lost in all of the recent strains from the United Kingdom and half of the recent strains from Serbia when tested by PCR. This region was not found to be lost in any of the recent strains from Poland.

Interestingly a single gene (BP1225, RD29) that was lost in most of the old isolates was not lost in any of the recent strains tested. The Sanger Institute's GeneDB (old.genedb.org) classifies the gene as "putative Ecf-type RNA polymerase sigma factor".

Three strains, the same ones that also by PFGE resembled Tohama I, were found to have almost identical genomic contents to Tohama I.

The results of the CGH experiments as well as PCR detections are shown in table 5.

**Table 5.** The typing results of vaccine strains and clinical isolates from four European countries tested also by CGH. +=present, -=absent, +=duplication detected. Vaccine strains are in white background, clinical isolates in coloured background.

| Country | Strain | Isolation Year | Serotype | *prn* | *ptxA1* | BP0910A -BP0934 | BP1135 -BP1141 | BP1225 | BP1948 -BP1966 | Duplication |
|---|---|---|---|---|---|---|---|---|---|---|
| **Finland** | 1772 | | 2,3 | 1 | 2 | - | - | - | + | |
| | 18530 | | 3 | 1 | D | + | + | + | + | |
| | KKK22 | 1953 | 3 | 1 | 2 | + | - | - | + | |
| | KKK1277 | 1964 | 2,3 | 1 | 2 | + | - | - | + | |
| | KKK1330 | 1965 | 2 | 1 | 2 | + | - | - | + | + |
| **Poland** | 186/65 | 1965 | 2.3 | 1 | 2 | + | - | - | + | |
| | 629/65 | 1965 | 2.3 | 1 | 2 | + | - | - | + | |
| | 606/77 | 1977 | 3 | 1 | 2 | + | - | - | + | |
| | 3/60 | 1960 | 2 | 1 | 2 | + | - | - | + | |
| | 7/60 | 1960 | 2 | 1 | 1 | - | - | + | + | |
| | 12/60 | 1960 | 2,3 | 1 | 2 | + | - | - | + | |
| | 21/60 | 1960 | 2 | 1 | 1 | - | - | + | + | |
| | 6/62 | 1962 | 2 | 2 | 1 | - | - | + | + | |
| | 1326/62 | 1962 | 2 | 1 | 2 | + | - | - | + | + |
| | A/63 | 1963 | 2 | 1 | 1 | - | - | + | + | |
| | 7/68 | 1968 | 2.3 | 1 | 2 | + | - | - | + | + |
| | 9/68 | 1968 | 3 | 1 | 1 | - | - | + | + | |
| **Serbia** | 1772/57 | 1957 | 2,3 | 1 | 2 | - | - | - | + | |
| | 2047/57 | 1957 | 2,3 | 1 | 2 | + | - | - | + | + |
| | 23/81 | 1981 | 3 | 1 | 1 | - | - | + | + | |
| | 8/84 | 1984 | 2 | 2 | 1 | - | - | + | + | |
| | 22/53 | 1953 | 2 | 1 | 2 | + | + | + | + | |
| | 124/54 | 1954 | 2 | 1 | 2 | + | - | - | + | + |
| | 162/54 | 1954 | 2,3 | 1 | 2 | + | - | - | + | + |
| | 1496/57 | 1957 | 2 | 1 | 2 | + | + | + | + | |
| | 1828/57 | 1957 | 2,3 | 1 | 2 | + | + | + | + | |
| | 2154/58 | 1958 | 2,3 | 1 | 2 | - | - | - | + | |
| **UK** | CN2992 | 1949 | 2.3 | 1 | 2 | - | - | + | + | |
| | CN3099 | 1950 | 2 | 1 | 1 | - | - | + | + | |
| | CN5476 | 1963 | 3 | 1 | 1 | - | - | + | + | |
| | CN0128 | 1941 | 2 | 1 | 2 | - | - | + | + | |
| | CN0137 | 1941 | 2.3 | 1 | 2 | + | - | + | + | + |
| | CN0142 | 1942 | 2 | 1 | 1 | - | - | + | + | |
| | CN0909 | 1944 | 3 | 1 | 2 | + | - | + | + | |

The results from Poland, Serbia and the United Kingdom have been deposited to the Gene Expression Omnibus (GEO) and can be accessed with the number GSE29579.

The presence of genes not found in the genome of Tohama I was detected with PCR. All tested European strains had genes that are not present in the genome of Tohama I. There were a total of four regions of genes reported to be present in the genomes of clinical isolates that are not found in Tohama I. All the tested strains had all four regions which are not found in the genome of Tohama I except the Finnish vaccine strains 18530 and the three old strains from Serbia (22/53, 1496/57 and 1828/57) that resembled Tohama I, in other ways, too. The Finnish vaccine strain 18530 did not have one side of the region BB0916-BB0921, but it did have all the rest of the regions not present in Tohama I. The three strains from Serbia had all other regions that are not present in the genome of Tohama I except region BB0916-BB0921.

## 5.3. *B. pertussis* strains from other continents (III)

CHINA

A total of 99 strains from China were tested, of which 96 were clinical isolates and three vaccine strains. Of the isolates, 25 were from 1953–1958, 52 from 1963–1985, and 19 from 1997–2005. These periods represent the pre-vaccine period prior to the introduction of wP vaccines in 1960 the period of wP vaccine; and the period after aP vaccine was added to the vaccination program in 1995. The clinical isolates were isolated from different parts of China: 26 from Beijing, 5 from Gansu, 8 from Fujian, 4 from Hebei, 3 from Hubei, 4 from Jilin, 18 from Shanghai and 5 from Sichuan. For 23 clinical isolates the place of isolation was not known.

The results obtained from PFGE performed in China were validated and compared to the PFGE data bank in the Pertussis Reference laboratory of the National Institute for Health and Welfare, Turku, Finland. At least one strain was selected for each profile. If only two strains had identical profiles, both were retested in Finland. When multiple strains belonged to the same profile, several strains isolated in different years and isolated at different places were tested. Altogether, 53 strains were retested in Finland. Only the results from the PFGE analysis have been included here.

The vaccine strains represented two PFGE profiles. P3s10 and CS were BpCHR6 and 18530 was BpFINR13 as the Finnish vaccine strains 18530. The 96 clinical isolates represented 27 profiles out of which 4 had earlier been also identified in Europe (BpFINR9, BpSR127, BpSR23, and BpSR11). The profile BpCHR6 was present in vaccine strains and clinical isolates.

A dendrogram analysis was performed on the 27 PFGE profiles identified from Chinese strains. Eight international PFGE reference strains were also included in the analysis to assign the profiles into PFGE groups, where possible. Interestingly many Chinese strains fell into groups previously populated only by very few isolates. For example, three profiles from Chinese strains belonged to group I, previously represented only by the international reference strains 18323 and one clinical isolate (Njamkepo et al. 2008), eight profiles belonged to group II, previously represented only by the international reference strains Tohama I and three strains from Serbia (II). PFGE profiles identical to Tohama I were actually found among Chinese strains. Three profiles of clinical isolates and the Chinese vaccine strain 18530 were grouped together with Finnish vaccine strain 18530 (previously the only strain in an unnamed group). The strains belonging to groups I and II were old strains mostly isolated in the 1950s and 1960s. The more recently isolated strains belong to groups III, IV and VII or could not be assigned to a PFGE group. Group III has previously been populated mostly by old European isolates and group IV has been predominantly populated by recent isolates.

SENEGAL AND KENYA (AFRICA)

African strains from Senegal and Kenya (n=58) were analysed with PFGE. The results form a part of a larger study in which a novel SNP detection system for the typing of *B. pertussis* strains was published (van Gent et al. 2011a). The PFGE results from the strains isolated in Africa show that all of the strains belong to the PFGE group III or could not be assigned to a previously named PFGE group (figure 9). Group III can be described as the PFGE group where most of the old European isolates fall. Previously, when strains from Senegal were analysed with PFGE, the conclusion was that all the strains belonged to the PFGE group III (Njamkepo et al. 2008).

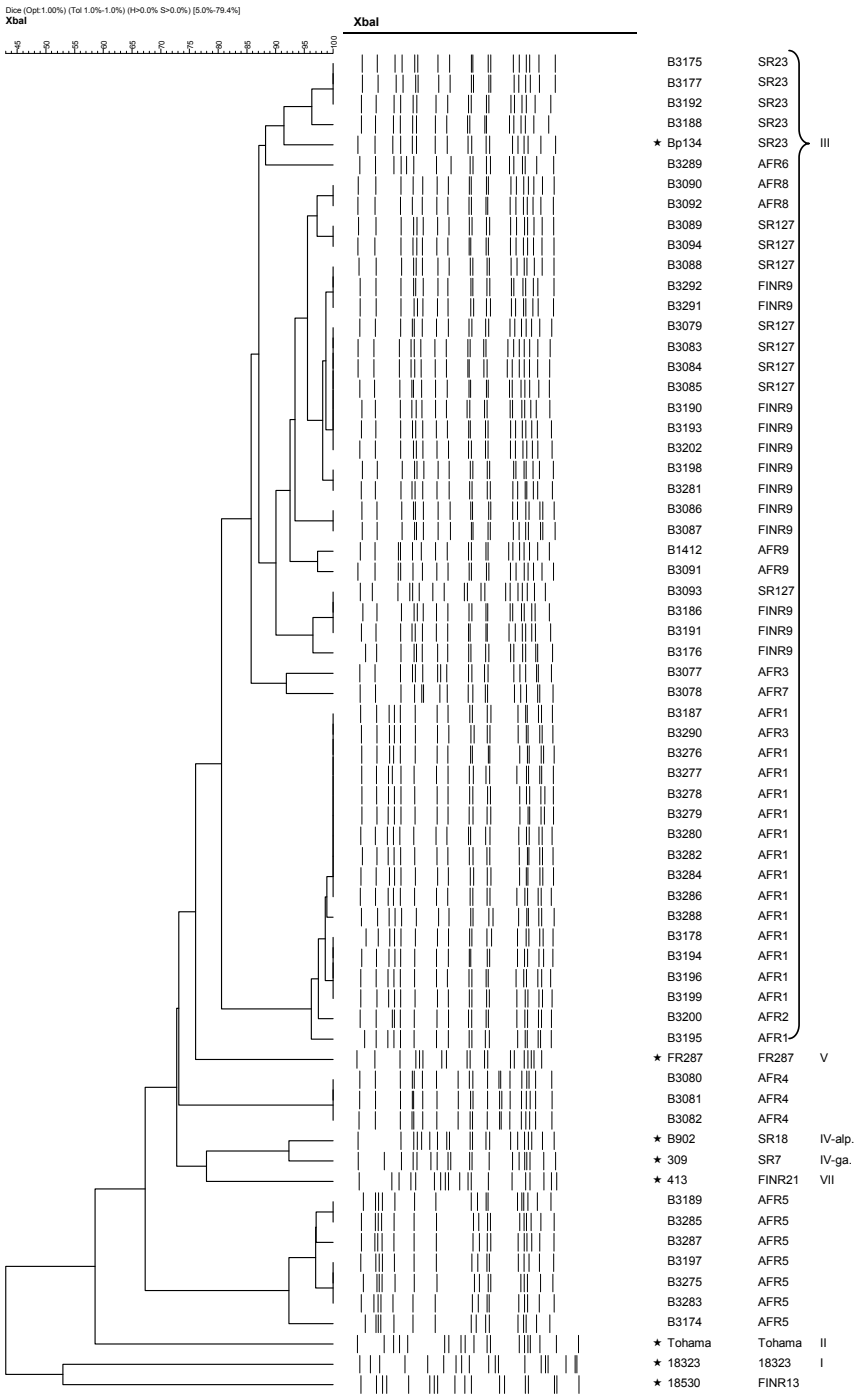


**Figure 9.** PFGE analysis of the African strains. In addition to African strains eight international PFGE reference strains have been included, marked with stars.

## 5.4. Effect of strain variation on cytotoxicity (V)

For the purposes of this study, we tested the cytotoxicity of 45 clinical *B. pertussis* isolates from Finland representing *ptx*P1 (n=20) and *ptx*P3 (n=25) strains as well as international reference strains Tohama I and 18323, PT-deficient mutant BP32 (also called as BP3311, which was constructed on BP370 background) (Stibitz et al. 1986; Black and Falkow 1987) and heat treated PRCB406 and Tohama I. Tohama I and PRCB406 were included in every experiment. The two strains were tested altogether eight times.

The averages of cytotoxicity percentages for the strains Tohama I and PRCB406 were 11.5% (SD=7.0) and 52.0% (SD=6.3), respectively. The PT-deficient mutant B32 had a cytotoxicity of 21%, the heat-killed Tohama I 8.3% and heat-killed PRCB406 25.3%. The international reference strain 18323 was found to be non-cytotoxic. The average cytotoxicity of *ptx*P1 strains was 49% (median 53.20%) and the cytotoxicity of *ptx*P3 strains was 61% (median 61.94). The difference between these two types of strains was statistically significant (p=0.021) (figure 10). There was also a statistical difference (p=0.013) in the cytotoxicity between the three groups based on different genotypes (*ptxP3* group, main *ptxP1* group and the rest of *ptxP1* strains). The statistically significant difference, according to the Kruskal–Wallis test, was between the *ptxP3* group and the main *ptxP1* group.

No other marker e.g. *ptx*A or *prn* type or PFGE prolife could be shown to have a statistically significant effect on the cytotoxicity of *B. pertussis* strains.

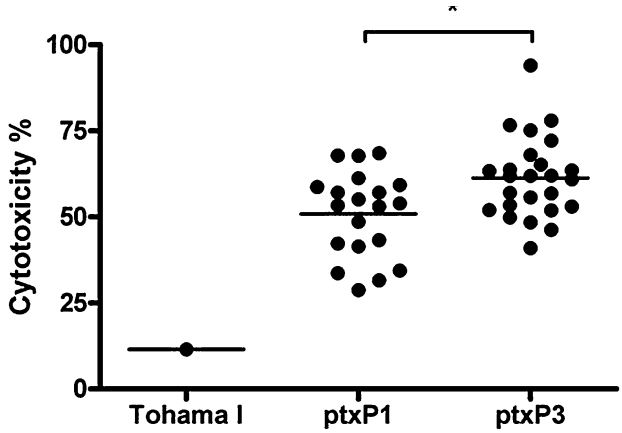


**Figure 10.** The cytotoxicity of Tohama I, *ptx*P1 strains and *ptx*P3 strains.

## 5.5. Effect of strain variation on cytokine secretion of human macrophages (V)

We measured TNF-α and IL-10 levels at two different time points (6h and 16h) from three *ptx*P1 strains (Tohama I, PRCB163 and 223) and three *ptx*3 strains (PRCB382, 406 and 677). Concentration of TNF-α was 105 and 167 pg/ml at 6 and 16 h in cultured cells without bacteria (figure 11 A & B). Only marginal differences in TNF-α concentrations were observed in cultured cells with bacteria at 6 and 16 hour time points (figure 11 A and B). The production of IL-10 was observed at 6 and 16 hour time points to be higher in cultured cells with bacteria than in those

without bacteria (figure 11 C & D). Also the concentration of IL-10 was higher in cells incubated with the six clinical isolates (range: 195 - 247 pg/ml) at 6 hour time point than with Tohama I (60 pg/ml) (figure 11 C). It seems that the *ptx*P allele did not influence the production of cytokines.

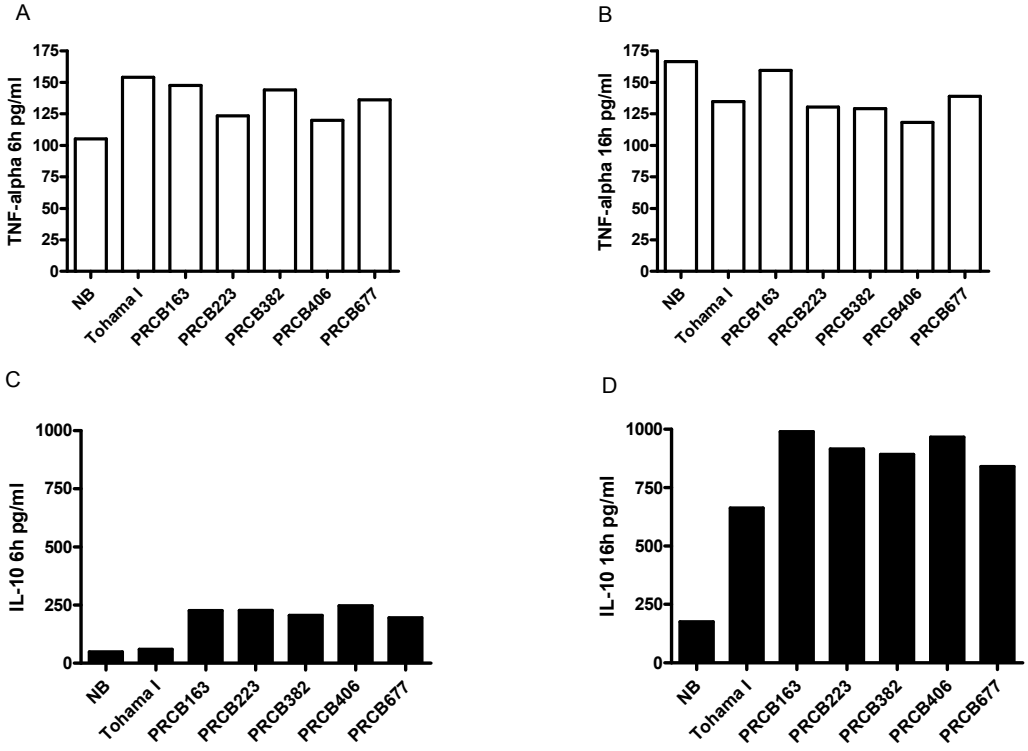


**Figure 11.** Concentration of TNF-α in the supernatants of U937 cells incubated with bacteria or with no bacteria (NB) at 6 hour time point (A). The concentration of TNF-α in the supernatants of U937 cells incubated with bacteria or with no bacteria (NB) at 16 hour time point (B). The concentration of IL-10 in the supernatants of U937 cells incubated with bacteria or with no bacteria (NB) at 6 hour time point (C). The concentration of IL-10 in the supernatants of U937 cells incubated with bacteria or with no bacteria (NB) at 16 hour time point (D).

# 6. DISCUSSION

## 6.1. Genomics

Genomics, the study of genomic content, has become a commonly used method for studying bacteria. Genomics has been used to identify specific differences between strains and species that are responsible for virulence and host adaptation as well as to study the human microbiota. The acquired knowledge will have impact on diagnosis, vaccine development, forensics and epidemiological investigations in the coming years. It will also shed light to the evolution of bacteria and their close relationship with humans (Relman 2011). For the purposes of this study, we have used comparative genomic hybridisation to analyse genomic contents of *B. pertussis* strains of several countries during different time periods. These countries have used different vaccination strategies against pertussis, therefore providing a useful basis to evaluate the effect of vaccinations on the bacterial population. We have observed differences as well as similarities in the genomic contents of the *B. pertussis* strains of different countries.

### 6.1.1. Genome reduction

The results of this study as well as other studies have clearly demonstrated that genome reduction occurs in *B. pertussis* and that the genomes of European *B. pertussis* isolates have lost genes in the last 50 to 60 years after the initiation of vaccination programs (Brinig et al. 2006; Caro et al. 2006; King et al. 2008; King et al. 2010). The loss of genetic material seems to be mediated by homologous recombination of the numerous IS elements in the genome of *B. pertussis*. There are no known virulence factors found in the lost genes. The unproportionally over-presented absent genes were pseudogenes and genes involved in iron acquisition and iron storage.

The loss of pseudogenes can be easily explained by the advantage of loosing genetic material that has no function. In theory, the reduction of genome size may shorten the generation time of the bacterium because the synthesis of DNA will take less time.

The loss of several genes involved in iron acquisition and iron storage can be explained by differences in host species. The bacterium can survive in its current host, the human, without these genes if they were important for the ancestral bacterium's survival in other host species or the environment. These genes can therefore still be found in *B. bronchiseptica* and also in *B. parapertussis* (Parkhill et al. 2003).

### 6.1.2. Gene acquisition

The limitation of the CGH methods used in this study prevented the acquisition of any information about the gain of new genetic material in the *B. pertussis* isolates tested. However, there are still several recent publications that shed light to this question. Bases on these studies, it can be deducted that *B. pertussis* has not gained any new genes (Brinig et al. 2006; Bouchez et al. 2008; Caro et al. 2008; Bouchez et al. 2009; Bart et al. 2010; King et al. 2010). The report that the recent European and American strains (Brinig et al. 2006) have genes that are not present in the reference strain Tohama I seem to indicate that Tohama I, and the clinical isolates investigated in these studies, are from different lineages of *B. pertussis* that have differentiated from an ancestral lineage several

decades ago and therefore seem to have differences in the genome content. The genotype of Tohama I isolated in Japan is not common in Europe.

In addition the strains from the region where Tohama I was isolated are not as well studied in this regard as the ones from Europe, for instance. Therefore we were not able to conclude whether the genes that are present in recent European strains are important for the bacterium to survive (in Europe) or if they increase the fitness of these strains or if they are present in the genome just by chance. The fact that there are no pseudogenes in these regions argues against any claims of them being unimportant. The ongoing sequencing project of strains collected in different continents including Japan will hopefully provide answers to this question (Parkhill, J., personal communication)

### 6.1.3. Genome rearrangement

Genome rearrangements during the evolution of *B. pertussis* seem clear. The major genomic rearrangements found in the analysis between *B. pertussis* strains Tohama I, *B. bronchiseptica* strain RD50 and *B. parapertussis* strain 12822 indicate that they have played some role in the evolution of these species or they reflect the evolution of the species (Parkhill et al. 2003).

There is a lack of evidence supporting the claim that genome rearrangements would play a role in the adaptation of the bacteria in recent times. One report states that there are no major rearrangements during laboratory cultures (Brinig et al. 2006). The laboratory-adapted reference strain Tohama I did not present any rearrangement in 224 passages and clinical isolate passages 12 times gave similar results (Brinig et al. 2006). Furthermore, the same study concluded that some rearrangements have occurred during the evolution of the recent strains from a Tohama I-like old strain. However, this might not be the case as in the genome of Tohama I the rearrangements may have occurred during its adaptation to laboratory conditions and the current genome structure may not reflect very closely the genome of the first clinical isolate from 1954.

Reports on genomic plasticity, however, would indicate that there are rearrangements even during a single epidemic, meaning that they are not a rare event (Stibitz and Yang 1997; Stibitz and Yang 1999). Furthermore, strains with different genotypes were isolated in the same outbreak and even in the same patients, although the possibility that the patients were infected by different strains can not be ruled out (Makinen et al. 2005; Cassiday et al. 2008) Rearrangements of the genome could quickly change the gene expression profile of the strains and therefore produce great amounts of material for natural selection (Brinig et al. 2006).

### 6.1.4. Insertion sequence elements

The genetic content of the *B. pertussis* strains seems to be relatively stable in their respective lineages as gene loss is quite rare. Then again, there are reports stating that the number of IS elements is increasing in the circulating strains (Bouchez et al. 2008). This would, with high probability, increase the frequency of genome rearrangements as they seem to be mostly mediated by homologous recombination of IS elements. The increase in the number of IS elements would also increase the instability of the genome as well as change the gene expression levels. The genetic plasticity provided by frequent recombination events has been shown to enable bacteria to adapt quickly to clinical interventions (Croucher et al.; Snitkin et al. 2011). A high number of IS elements in the genome of *B. pertussis* may also facilitate similar adaptations. IS elements have been associated with increased genetic diversity, which is linked to new adaptations in other bacteria (Mancilla et al. 2011)

Similar to *B. pertussis*, IS elements and their expansion have, through genome reduction and rearrangements, contributed to the evolution of *Yersinia pestis* from its ancestral species (Gu et al. 2007). Insertion of IS elements has also been shown to enable the bacterium to escape host immunity (Cornelius et al. 2009).

All of these examples provide evidence that the IS elements play important roles in the evolution and adaptation of different bacteria.

## 6.2. Evolution of *B. pertussis* in Finland post vaccination

In Finland distinct changes have been detected in the *B. pertussis* population after the addition of pertussis vaccine to immunisation programs. A shift has been observed in the serotype from a mixed population through Fim2-dominated population to a Fim3-predominated population. Fim3 is also most common in other countries with high vaccination coverage (Hallander et al. 2005; Litt et al. 2009).

The *ptx*A alleles have changed from *ptx*A2 to *ptx*A1. Pertactin allele *prn*2 is the predominant one in Finland. This is true for most countries that have had high vaccination coverage for a long period of time. Different PFGE profiles predominate in different times. The most frequently found in recent years are BpSR11, BpFINR32 and BpSR3.

In this study we found that the Finnish *B. pertussis* strains have progressively lost regions of genes. The oldest Finnish strains were already different from the reference strains Tohama I and became even more different during the 50 years following the introduction of pertussis vaccine via progressive gene loss. Only one genome type was found at one time. Moreover, the old Finnish isolate had a region of duplication in the genome. As the loss of genes and the clustering into clades in PFGE analysis go hand-in-hand, it is possible that the loss of genetic material occurs simultaneously with the rearrangements since most regions of difference are too small to be detected by PFGE.

The number of the tested strains, the methods used and the main finding of each study are summarized in Table 6.

**Table 6.** Summary of the strains, methods and the main findings of the conducted studies.

| Study | Country | Methods used | No of strains tested by each method | Main results | Section |
|---|---|---|---|---|---|
| I | **Finland** | Serotyping<br>Genotyping<br>PFGE<br>CGH | 22<br>22<br>22<br>22 | In Finland where pertussis vaccination was introduced in 1952 and the vaccination coverage has been high, progressive gene loss was observed. Bacterium may use gane loss as a strategy to survive in vaccinated population. | 5.1. & 6.2. |
| II | **Serbia** | Serotyping<br>Genotyping<br>PFGE | 74<br>74<br>60 | In Serbia where vaccination was introduced in 1957 and vaccination coverage has been high, but contemporary strains were added to the vaccine in 1985, the *B. pertussis* population is different from other countries with a long history of vaccination. | 5.2. & 6.3. |
| III | **China** | PFGE | 53 | The late achievement of high vaccination coverage in China has affected the bacterial population | 5.3. & 6.4. |
| IV | **Four European countries** | | | | |
| | Finland | Serotyping<br>Genotyping<br>PFGE<br>CGH/PCR<br>Sequencing | 13<br>13<br>13<br>13<br>13 | At least three clusters of *B. pertussis* circulated in Europe in the pre-vaccine era. The genomes of European isolates were distinct from the genome of the international reference strain Tohama I. Progressive gene loss occurs in European *B. pertussis* population with time. Difference in frequency of the lost genes was observed among the countries. In contrast to many European countreis, the novel pertussis toxin promoter allele *ptxP3* was not found in Poland. | 5.1., 5.2., 6.2. & 6.3. |
| | Poland | Serotyping<br>Genotyping<br>PFGE<br>CGH/PCR<br>Sequencing | 19<br>19<br>19<br>17<br>11 | | |
| | Serbia | Serotyping<br>Genotyping<br>PFGE<br>CGH/PCR<br>Sequencing | 14<br>14<br>14<br>14<br>10 | | |
| | The UK | Serotyping<br>Genotyping<br>PFGE<br>CGH/PCR<br>Sequencing | 15<br>15<br>15<br>12<br>8 | | |
| V | **Finland** | Genotyping<br>Cytotoxicity<br>Cytokines | 511<br>48<br>6 | The *ptxP3* strains have replased the previously predominant *ptxP1* strains. The *ptxP3* strains proved to be more cytotoxic towards human monocytic cells than *ptxP1* strains. This may reflect the increased virulence of *ptxP3* strains. | 5.4., 5.5. & 6.4. |
| | **Senegal & Kenya** | PFGE | 57 | In Senegal and Kenya where pertussis vaccination was introduced late the *B. pertussis* strains resemble old isolates from Europe. | 5.3. & 6.4. |

### 6.2.1. Effect of vaccine strains on the evolution of Finnish strains

The circulating strains in Finland are different from the vaccine strains 18530 and 1772 as well as the strain Tohama I which is used in the production of the current aP vaccines. This is evident in virulence factors PT, PRN and perhaps also in serotype. The genetic content is also different in recent strains. Therefore there seems to be vaccine-driven selection of strains different from the vaccine strains at least with regard to the virulence factors mentioned. The situation in other countries has been referred in section 2.3.2.1. and 2.3.2.3..

The effect of the gene content of the genomes of vaccine strains on the circulating isolates is difficult to show incontrovertibly, because of the extensive changes in the vaccine compositions. Vaccinations still seem to drive gene loss, and thus the genome of vaccine strains should have an effect on it.

It is possible that the lost genes are unimportant for the bacterium and were lost purely by chance. However, this is unlikely as the same genes have been lost also in circulating isolates in other countries (Brinig et al. 2006; Caro et al. 2006; King et al. 2008).

As seems to be the case with the loss PRN, an immune response raised against a gene product may drive the loss of a gene (Bouchez et al. 2009). It is possible that there were genes that raised

immune responses in the lost regions but which were nevertheless relatively unimportant for the survival of the bacterium. When these genes were lost the strain gained an advantage over other strains because it could evade part of the immune response.

The wP preparations were treated before adding to the vaccines to reduce their toxicity. This has been shown to have an effect of the antigen epitopes (Sutherland et al. 2011). It is possible that the treatment during the preparation of vaccines, e.g. detoxification changed some of the proteins coded by the lost genes, which resulted in strong immune responses raised against them. Therefore it was advantageous for the bacterium to lose those genes. From the results of this study it is difficult to draw definitive conclusion about the importance of single lost genes. This would need more detailed genetic studies.

## 6.3. Evolution of *B. pertussis* in Europe post vaccination

In other European countries (Poland, Serbia and the United Kingdom) in addition to Finland, the circulating strains are different from the vaccine strains. The old isolates resembled the vaccine strains rather significantly. However the vaccine strains are mostly circulating strains from these countries and therefore similarity can be expected.

Most countries with a long history of vaccination with high coverage have nowadays circulating strains with the following characteristics: Fim3/*prn2*/*ptxA1* (Hallander et al. 2007). The Serbian strains studied were different from other countries in regard to the virulence factors, as in the most recent isolates from Serbia there were both alleles of *ptx*A1 and *ptx*A2 present at the same time and in the same isolates, a large proportion was other than *prn*2. This is different from most countries in Europe. Especially *prn*1 and *prn*11 are very rare in recent isolates in such high proportions (Hallander et al. 2007).

Clustering three old isolates from Serbia together with Tohama I is interesting. In Finland and other countries studies (excluding data acquired from China during the studies included in this thesis) have not identified such strains. It can not be ruled out that this could be a result of the limited number of tested strains. These strains also resembled Tohama I in genetic content since they had almost all the same genes as Tohama I. All other European strains had lost some regions of genes.

Recent strains from the United Kingdom resembled the ones from Finland, since they had lost the same genes as Finnish isolates after 1996. The Polish recent strains from 2004 resembled the Finnish strains from 1977 to 1996 and had lost only two regions of genes. Serbian recent isolates from 2000 had lost either three regions of genes as the recent Finnish and British strains, but half had lost only one region as Finnish strains before 1977. All recent strains had gene BP1225 which was lost in high proportion of old isolates.

The studies included in this thesis and others show that Tohama I is not a good representative of the clinical European isolates (Caro et al. 2008).

The number of tested strains, the methods used and the main finding of each study are summarized in table 6.

### 6.3.1. Effect of vaccine strains on the evolution of European strains

With regards to virulence factors, in other European countries investigated, there was similar shift from vaccine type strains to nonvaccine type strains as in Finland. The fact that Serbia introduced contemporary strains with *ptx*A1/*prn*2 strain into their vaccine, may have had an effect on the *B.*

*pertussis* population, as it has not been previously reported that in recent strains, from 1985 to 2000, both *ptx*A1 and *ptx*A2 are present in almost identical proportions and that there are four *prn* alleles present at the same time in relatively high proportions. Especially the presence of *prn*11, which has been previously been reported only in low numbers from Australia, is an interesting finding (Poynten et al. 2004; Hallander et al. 2007). In this study, a relatively large number of isolates collected before pertussis vaccinations were included in the immunisation programs. The results of genotyping and PFGE analysis indicate that strains circulating before the introduction of vaccination were heterogeneous, suggesting an effect of vaccination on the *B. pertussis* population.

It is difficult to assess, from the other European countries than Finland, if vaccine strains have affected the genome content of circulating isolates as, no clear progressive gene loss is seen in the old strains, because strains that have lost RD5 and strains that have lost RDs 3 and 5, are present at the same time. This was not the case in Finnish strains. Clearly these two genotypes were present in Europe at the same time and from our material it is not clear what their genetic relationship is.

Because of the fact, that in Europe two genotypes were present at the same time, it is possible that the Finnish strains did not lose genes, but only the arrival of new strains from continental Europe or the British Isles at different time were observed and new strains quickly took over from the old strains. This has been seen in reference to PFGE profiles when comparing Sweden and Finland. PFGE profiles causing epidemics in Sweden come to Finland later and caused epidemics (Elomaa et al. 2007).

There were significant differences in the genomic contents of recent strains. The strains from the United Kingdom resembled the one from Finland isolated at the same time, perhaps because the vaccine strains are similar and the wP vaccines have remained stable for a long time. The wP vaccine from the United Kingdom had already at the beginning of vaccination program against pertussis *ptx*A1 allele, but this does not seem to have affected the bacterial population. One explanation may be that strains with *ptx*A1 are less immunogenic than strains with *ptx*A2 and are therefore still present in the bacterial population.

Recent strains from Poland represented the genome content as Finnish strains from the 1977 to 1996. This type of strains later disappeared from Finland. The continual addition of contemporary strains to the vaccine formulation has already been reported to have affected the PFGE profiles of Polish strains and the genomic content may only reflect the overall difference of the Polish strains from other European countries (Gzyl et al. 2004; Hallander et al. 2007).

In Serbia, the addition of contemporary strains to the wP vaccine in 1985, has clearly affected the virulence factors of the circulating strains. It may have also affected the bacterial population in a way of preserving the old type of strains as the selection pressure from the vaccine has been as strong to the old and new strains and that is why the genetic content of the most recent strains from 2000 represent the most recent type having lost three regions of genes and an old type having lost only one.

The observation that many old strains had lost the gene BP1225 (putative Ecf-type RNA polymerase sigma factor), may be a result of vaccination in general. As this gene is present in all recent strains tested, it may indicate that it is important for the survival of *B. pertussis* in highly vaccinated population. However, this hypothesis needs more detailed investigations.

## 6.4. Effect of the initiation of pertussis vaccination programs and vaccination coverage on the evolution of *B. pertussis* strains

From the results from China and African countries: Kenya and Senegal, we can see that late achievement of high vaccination coverage and the late introduction of pertussis vaccine have an effect on the *B. pertussis* population. The late achievement of high vaccination coverage has postponed the emergence of non-vaccine type strains in China. This is also clearly shown in analysis of PFGE among the current circulating isolates. The same results can be seen from the isolates from Africa in the 1990s. As a results of late introduction of vaccines, all the strains resembles old European PFGE profiles found before 1970s and PFGE group III found before 1990s.

It is interesting that even when comparing remote places like Europe and China the evolution of *B. pertussis* seems to go into the same direction. The predominant PFGE profile from Europe, BpSR11 (Hallander et al. 2007), was also observed in a recent strains from China. Either this represents a situation that evolution is generally driven by vaccines into the same direction or in this time of frequent travel, successful strain types from e.g. Europe arrive in China and became established in the circulating *B. pertussis* population. Because to date there is no good analysis of the origin of for example BpSR11 type strains, it can not be said which of the hypothesis is true, if either. With the completion of the global SNP analysis of *B. pertussis* in the coming years, this question may be answered.

## 6.5. Strain variation

There have been numerous reports of strain variation with respect to *ptx*A and *prn* in the recent years (Mooi et al. 1998; Mastrantonio et al. 1999; Mooi et al. 1999; Cassiday et al. 2000; Fry et al. 2001; Gzyl et al. 2001; Weber et al. 2001; Fiett et al. 2003; Peppler et al. 2003; Kodama et al. 2004; Poynten et al. 2004; Elomaa et al. 2005; Hallander et al. 2005; Fingermann et al. 2006; Lin et al. 2006; Borisova et al. 2007; Njamkepo et al. 2008). The most recent and one of the most interesting is the identification of *ptx*P alleles and the finding that *ptx*P3 strains have become predominant in several countries. They also correspond largely to PFGE profile BpSR11 and strains related to it. These strains also produce more PT than *ptx*P1 strains, that predominated previously, and are more virulent (Mooi et al. 2009; Advani et al. 2011).

### 6.5.1. Cytotoxicity and cytokine expression in different genetic backgrounds

When genotypes of *ptx*A, *prn, ptxP* and PFGE profiles were analyzed, the only genetic marker that was associated to elevated cytotoxicity was *ptx*P3. The observed cytotoxicity of *B. pertussis* strains was not only caused by PT, as PT- mutant was also cytotoxic. Live bacteria were more cytotoxic that heat-killed, even though the heat-killed bacteria did not lose cytotoxicity entirely after the treatment. Interestingly the laboratory (adapted) strains were low in cytotoxicity (Tohama I) or non-cytotoxic (18323). It seems that during the adaptation to laboratory conditions the strains have become less cytotoxic and perhaps less virulent. The increased cytotoxicity and the reported increased virulence fit well together, but it is still not clear if the change in the pertussis toxin promoter is the (only) cause of the elevated virulence or if other changes in the genome also play a role. For example genome rearrangements may have happened in the *ptx*P3 strains causing differences in gene expression, which might explain these findings. This will become clear with the sequencing and the completion of a physical map of *ptx*P1 and *ptx*P3 strains. The currently available map of Tohama I (*ptx*P1) is not sufficient to answer these questions, since it has been

shown extensively that Tohama I is not a good representative of the species. Therefore a physical map(s) of both (recent) clinical *ptx*P1 and *ptx*P3 strains are needed.

Different genetic markers did not seem to affect to production of TNF-α of IL-10. The relatively high production of TNF-α by the cells not treated with bacteria may be explained by the late time points of sample collection and the previous stimulation with PMA. This may have induced a stress response that led to the production of TNF-α.

## 6.6. Vaccinations and *B. pertussis* strains without expression of vaccine antigens

The several different vaccines used against pertussis seem to function well despite strain and antigenic variation. In general, infants are well protected by the vaccines even though local epidemics occur regularly. However, problems may arise if the recent developments in France and Japan are found to happen also elsewhere as *B. pertussis* strains not expressing vaccine antigens emerged in these countries (Bouchez et al. 2009; Stefanelli et al. 2009; Otsuka et al. 2010). The loss of PRN has been reported in France, Italy and Japan and the frequency of strains not expressing PRN seems to be increasing. The loss of PT has only been reported in France.

## 6.7. Evolution and adaptation of *B. pertussis* in the future

The developments from France, Italy and Japan, that is the loss of aP antigens (Bouchez et al. 2009; Stefanelli et al. 2009; Otsuka et al. 2010), can become a common problem when acellular vaccines have been longer in use in high coverage around Europe. The countries, where this phenomenon has to this date been reported, are some of the countries with the longest use of aP vaccines and high coverage of adolescent and adult boosters. The loss of aP vaccine components will have an impact on the vaccine efficacy and new and better vaccines have to be developed. If the evolutionary pressure from the acellular vaccines is strong enough it might be possible that most, if not all, of the vaccine component are lost from the bacterium.

The loss of PT may be explained by the fact that *B. parapertussis* does induce somewhat similar symptoms and similar disease as *B. pertussis*, but does not express PT. Therefore it can be assumed that *B. pertussis* could also cause an infection without PT. Pertactin probably is important for the bacterium, but it also raises strong immune response, as pertactin antibodies have a protective effect (Cherry et al. 1998; Storsaeter et al. 1998). As FHA is present in all three species, it may be too important to be lost. We will see if this is the case. It might be possible that the selection pressure for Fim2 and Fim3 would drive their loss. The bacterium may survive and cause infection even without Fims, as there are untypeable strains with regards to serotype. These strains express very little Fims on their surface *in vitro*. The *in vivo* regulation of serotype is still not entirely clear (Heikkinen et al. 2008) and the untypeable strains *in vitro* may express Fims *in vivo.*

To detect the future changes in *B. pertussis* it is important to maintain long-term monitoring of *B. pertussis* strains in order to evaluate the effectiveness of vaccines!

## 6.8. Future of pertussis vaccines

As mentioned the loss of aP vaccine components indicates that there is a need for improved vaccines against pertussis. Optimally these vaccines would induce broad long-term immunity against *B. pertussis* and perhaps closely related species as well.

According to the results of the studies included in this thesis it can be recommended that currently circulating strain(s) be used in the production of the vaccines. This should ensure the best possible immune response against currently circulating strains. It would also seem that this makes the selection pressure diverge and therefore slow down the adaptation of the bacterium. To ensure that the old type of strains do not reappear it may be considered to include old strains in the vaccines as well.

The loss of PRN and PT in some countries would suggest that the so-called deep immunity induced by the aP vaccines may not be beneficial. A more broad immunity, produced by more whole-cell like vaccine, should also protect against the disease, but it may prevent the loss of virulence factors. A live attenuated vaccine may be considered. The attenuated *B. pertussis* strains BPZE1 administered intranasally has been studied intensively and is in phase I clinical trial in Sweden (Mielcarek et al. 2006; Feunou et al. 2008; Ho et al. 2008; Skerry et al. 2009; Feunou et al. 2010; Kavanagh et al. 2010; Mielcarek et al. 2010; Fedele et al. 2011; Li et al. 2011). It may be a future model for inducing protection against pertussis.

There are also other alternatives for vaccine components than the ones in the current aPs. In a recent report attenuated *B. bronchiseptica* strain, not expressing any of the components of the current aPs nor ACT of BipA, elicited cross protection also against *B. pertussis* (Sukumar et al. 2010). The core genes (genes in all *B. pertussis* isolates) identified by the comparative genomic analyses may also be good source to search for vaccine candidates.

It may also be required to improve the vaccination schedules to protect the very young infants from pertussis. Regular boosters for the adolescents and adults are needed. The decennial Td vaccine should be replaced by the dTap vaccine. Maternal vaccination has been proposed as well as cocooning strategy to protect newborn infants (Kretsinger et al. 2006; Mooi and de Greeff 2007; Gall et al. 2011). In the cocooning strategy the closest family members of the newborn are pre-emptively vaccinated to prevent the transmission of pertussis from the parent of grandparents to the infant. Medical professionals in close contacts with infants should also be vaccinated to prevent them from functioning as the source of infection to the infants.

# 7. SUMMARY AND CONCLUSIONS

Pertussis continues to cause considerable health problems despite extensive vaccinations. In recent years resurgence of pertussis has been reported in many countries and antigenic variation between vaccine strains and circulating isolates has been observed. The aims of this thesis project were (1) to investigate the evolution of the *B. pertussis* population in Finland, a high vaccination coverage country, after the start of vaccinations, (2) to investigate the effect of different compositions of vaccines on the evolution of *B. pertussis* population in four European countries, (3 and 4) to understand the effect of late introduction of vaccines and the late achievement of high vaccination coverage to the *B. pertussis* population in China and Africa represented by Kenya and Senegal, and (5) to use functional tests to study the reasons for the expansion of *ptxP3* strains, a recently identified pertussis toxin promoter variant, and the reasons behind their increased virulence.

1) The results of the studies reveal that in Finland in addition to antigenic variation and the emergence of *ptx*P3 strains, progressive gene loss has occurred in the circulating strains after the start of the vaccination programs against pertussis. The bacterium may use the loss of genes as a strategy to survive in a vaccinated population.

2) The composition of vaccines has clearly had an effect on the circulating isolates in Poland and Serbia. The addition of new contemporary strains to the vaccines has postponed the emergence of strains/variants predominating in other countries. Also the addition of different antigenic variants to the vaccine has delayed the expansion of such strains, contributing to the heterogeneity of the bacterial population. Furthermore, the identified genomic contents and the PFGE profiles reflect the other differences in the genome of *B. pertussis* strains. Gene BP1225 may be important for the bacterium to survive in vaccinated populations.

3 & 4) The late introduction of vaccines in Africa and the late achievement of high vaccination coverage in China have had an effect on the *B. pertussis* population. The PFGE profiles of the strains in these countries resemble the old European isolates. However, in China the PFGE profiles that clonally expanded in Europe (most importantly BpSR11) can already be identified in some of the most recent isolates.

5) It was shown that the recently emerged strains carrying pertussis toxin promoter variant *ptx*P3 presented higher cytotoxicity towards human macrophages than the previously predominant *ptx*P1 strains. This observation can be considered as a sign of increased virulence and it may in part elucidate the reasons behind the expansion of *ptx*P3 strains.

To conclude, the adaptation of *B. pertussis* in response to vaccination was observed in the studies comprising this thesis. The evolution of *B. pertussis* after the start of vaccinations in different countries occurs towards non-vaccine type strains, although the speed and timing of changes in *B. pertussis* populations differ according to e.g. the differences between vaccines, vaccination schedules, time of the start of pertussis vaccination, and the features of the host population. The bacterium may use gene loss as a strategy to survive in a vaccinated population. The emergence of strains with BpSR11 is found in almost all of the studied bacterial populations and it marks the success of this particular strain type. The findings also stress the importance of monitoring the *B. pertussis* strains. Further studies are needed to elaborate the mechanisms behind the success of the new strain type.

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# APPENDIX

Appendix I

Amino acid sequence of pertactin alleles

```
          102 130   260  266                                                 337 404 532 590 853
          |   |     |    |                                                   |   |   |   |   |
prn1      S   V     RGD  GGAVP GGAVP GGAVP GGFPG GGFPG ----- ----- VLD  S   S   L   PQP H
prn2      .   .     RGD  GGAVP GGAVP GGFPG GGFPG GGFPG GGFPG ----- VLD  .   .   .   ... .
prn3      .   .     RGD  GGAVP GGAVP GGFPG GGFPG GGFPG ----- ----- VLD  .   .   .   ... .
prn4      .   .     RGD  GGAVP GGAVP GGFPG GGFPG ----- ----- ----- VLD  .   .   .   ... .
prn5      .   .     RGD  GGAVP GGFPG GGFPG GGFPG ----- ----- ----- VLD  .   .   .   ... .
prn6      F   .     RGD  GGGVP GGAVP GGAVP GGFPG GGFPG ----- ----- VLD  F   .   R   --- R
prn7      .   .     RGD  GGAVP GGAVP GGAVP GGFPG GGFPG ----- ----- VLD  .   .   R   ... .
prn8      .   .     RGD  GGAVP GGAVP GGFPG GGFPG ----- ----- ----- VLD  .   .   R   ... .
prn9      .   .     RGD  GGAVP GGAVP GGFPG GGFPG GGFPG GGFPG GGFPG VLD  .   .   .   ... .
prn10     F   .     RGD  GGGVP GGAVP GGAVP GGAVP GGFPG GGFPG ----- VLD  F   .   R   --- R
prn11     .   .     RGD  GGAVP GGAVP GGAVP GGAVP GGFPG GGFPG ----- VLD  .   .   .   ... .
prn12     .   .     RGD  GGAVP GGAVP GGAVP GGFPG GGFPG GGFPG ----- VLD  .   .   .   ... .
prn13     .   .     RGD  GGAVP GGAVP GGFPG ----- ----- ----- ----- VLD  .   .   .   ... .
```

Modified according to (Mooi 2010). Dots and dashes indicate identical and absent amino acids, respectively. Numbering is relative to the N-terminal methionine of Prn1