# Vaccine-driven evolution of *Bordetella pertussis:*

## Changes in population structure and strain fitness

**Inge H.M. van Loo**

# Vaccine-driven evolution of *Bordetella pertussis*:
## Changes in population structure and strain fitness

## Evolutie van *Bordetella pertussis* door vaccinatie:
### Veranderingen in populatiestructuur en stam fitness

(met een samenvatting in het Nederlands)

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The road ahead is empty
It is paved with miles of the unknown
Whatever seems to be your destination
Take live the way it comes
Take live the way it is
(City to City)

*Voor mijn ouders*
*Voor Ruud*

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# 1 General Introduction

## PERTUSSIS: CLINICAL COURSE

Pertussis or whooping cough, first described in the 16th century (79), is caused by the gram-negative bacterium *Bordetella pertussis*, a strictly human pathogen that was first isolated in 1906 by Borduet and Gengou (16). In the 20th century pertussis was one of the most common childhood diseases until mass vaccination was introduced in the 1950s to 1960s. Before the introduction of vaccination almost every child contracted pertussis. The disease is most severe in neonates and children that are younger than 1 year. In The Netherlands mortality due to pertussis was 1.80/100,000 in the early 1950s (25).

After a 7-10 days incubation period the onset of the disease is characterized by symptoms of a common cold; rhinnorrhea, malaise, low grade fever and anorexia. This catarrhal stage is followed after 1–2 weeks by the paroxysmal stage, the stage, in which the typical whooping paroxysms occur. These are characterized by series of repetitive coughs followed by an inspiratory gasp. Mucus production is common and is partially responsible for the restriction of the airways. The paroxysms are frequently terminated by vomiting and exhaustion, and especially neonates might suffer from hypoxemia. After 2–4 weeks the frequency and duration of the paroxysms diminish in the convalescent stage, which lasts several weeks to months. In this period secondary complications most often occur, including mainly secondary bacterial infections of the upper and lower respiratory tract and incidentally seizures or encephalopathy. These secondary complications are often the actual cause of death (73;95).

Treatment is mainly supportive. Antibiotic treatment with erythromycin is only useful when started within three weeks after onset, because after this period the bacterium is cleared from the nasopharynx, and it is presumed that the remaining symptoms are caused by the secreted toxins. However, although erythromycin may have little effect on the clinical symptoms, it enhances the clearance of the bacterium and limits spread of the disease (99). Erythromycin prophylaxis is therefore recommended for children that are more at risk for complications of pertussis and for adults with pertussis, who may infect vulnerable children (40).

## VACCINATION AND EPIDEMIOLOGY

In the Netherlands a whole cell vaccine (WCV) was introduced in 1953. This pertussis vaccine was combined with vaccines against diphtheria, tetanus and polio (DTPP). Children were vaccinated at the age of 3, 4, 5 and 12 months. Mass vaccination very effectively reduced the number of pertussis cases and a 10-fold decrease in pertussis mortality was attained in The Netherlands in the late 1950s (25;148).

Changes in the surveillance of pertussis affected the number of cases reported. In 1976 notification of pertussis was made obligatory by law, which resulted in an increased number of notifications, while in 1988 the introduction of a stricter case definition, including clinical symptoms and laboratory confirmation, led to a decline in notifications. Further, methods for laboratory confirmation of whooping cough, such as culture, PCR and serology, varied over the years. In 1988 two-point serology and in 1997 one-point serology and positive PCR were officially introduced as diagnostics (32;97;98). As a result of the introduction of new diagnostic methods it became clear that pertussis was highly underdiagnosed in adolescents and adults. Although pertussis is most severe in children, it is now also recognized as an adult disease (17;64;122;132). For example, 13-31% of adults, who were coughing more than 1 week were shown to be infected with *B. pertussis* (122;132;160). Infected adults represent a reservoir for transmission to susceptible (unimmunized) children (10;78;160).

Despite a high vaccination coverage pertussis re-emerged in The Netherlands, with epidemic outbreaks every 3-5 years in the last two decades. In 1996 a pertussis epidemic was observed with a 5-fold increase relative to the previous three years (Fig. 1). The incidence remained high since and epidemics also occurred in 1999 and in 2001 (33;34)(personal communication, H de Melker and S de Greeff). Pertussis has also re-emerged in other countries with highly vaccinated populations, such as Canada, The United States, Australia and Poland (3;8;36;58;59). In all these countries WCVs were introduced in the 1940s to 1960s, which were very effective in reducing the pertussis incidence. However, in the 1970s negative publicity about an association between WCVs and mild and severe side effects adversely affected vaccine uptake. In The United

Kingdom the vaccine coverage dropped to 30% (86). In Sweden pertussis vaccination was discontinued in 1979, while in The Netherlands vaccine potency was decreased. As a consequence the pertussis incidence increased in these countries. This led to a recovery of vaccine coverage in The United Kingdom and an increase in vaccine potency to previous levels in The Netherlands (31;86;116).

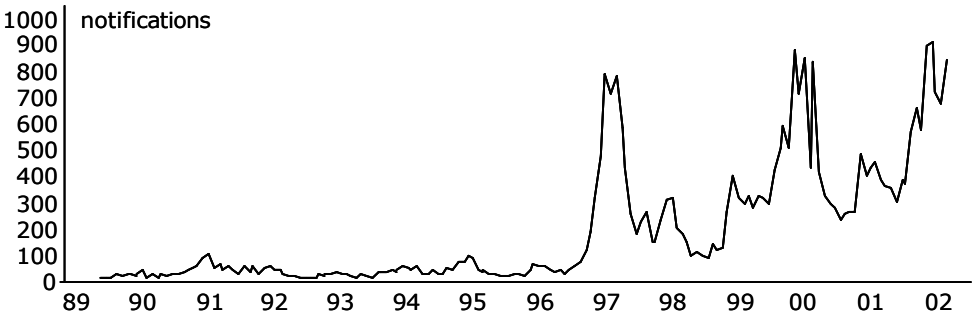


**Fig. 1.** Pertussis notifications per month in the period 1989-2001. Notifications are corrected for the first day of illness.

WCVs cause swelling and redness at the site of injection and fever (104;138). Rarely encephalitis and seizures have been reported (14;85). Although it is now clear that there is no evidence for neurological site effects of WCVs, the negative publicity about the safety of the WCVs initiated the development of acellular pertussis vaccines (ACVs)(46;55). These ACVs comprise several purified and detoxified *B. pertussis* antigens, including pertussis toxin (Ptx)(135;136), filamentous hemagglutinin (FHA)(68;123), pertactin (Prn)(21) and fimbriae (Fim2 and Fim3)(89;109) in various combinations (reviewed in (111)). These vaccines were less reactogenic and showed vaccine efficacies between 58% and 97%, dependent on the vaccine and diagnostic criteria used (reviewed in (121)). In The Netherlands the WCV from the 1950s is still used nowadays.

In The Netherlands the rise in pertussis incidence in 1996 was mainly observed in children from 1-4 and 5-9 years old (33;34). To reduce the morbidity in neonates the vaccination schedule was adapted in 1999. The DTPP vaccination schedule was changed from 3, 4, 5 and 12 months to 2, 3, 4 and 11 months.

Further, in 2001 a booster vaccination with an ACV was introduced for 4-year-old children to reduce disease burden in 5-9 year old children.

## VIRULENCE FACTORS OF *B. PERTUSSIS*

*B. pertussis* enters the airways of the host via aerosol droplets, spread by infected individuals (141). The expression of most virulence factors, necessary for colonization of susceptibles, is regulated by the BvgAS (*Bordetella* virulence genes) system, comprising the genes coding for BvgA, BvgS and BvgR (reviewed in (27;83)). This system mediates the transition between a $Bvg^+$ and $Bvg^-$ phase in reaction to environmental stimuli, such as temperature, nicotinic acid and $SO_4^{2-}$ concentration. BvgS is a transmembrane protein with a periplasmic and cytosol component, which senses the environment and is phosphorylated under $Bvg^+$ conditions. The transfer of a phosphate group from BvgS to BvgA allows the latter to activate the promoters of virulence-activated genes (*vag´*s). Further BvgR is also activated by BvgA, which downregulates the expression of virulence repressed genes (*vrg´*s)(84). The $Bvg^-$ phase, when the expression of the *vag´*s is downregulated and *vrg´*s are expressed, has been suggested to be important for survival of the animal pathogen *Bordetella bronchiseptica* outside its host (81). Since *B. pertussis* is a strictly human pathogen with no known environmental niche, the function of this system in *B. pertussis* is unknown and it might be an evolutionary remnant. However, recently a third phase, an intermediate phase ($Bvg^i$), has been identified (28;38). In the $Bvg^i$ phase some $Bvg^+$ phase genes and specific $Bvg^i$ phase genes are expressed. Although the exact function of the $Bvg^i$ phase is unknown, this phase might be important for colonization of the nasopharynx, where conditions may be intermediate between those inducing the $Bvg^-$ and $Bvg^+$ phase.

*Bordetella* virulence factors can be divided in adhesins and toxins. The first step in pathogenesis, colonization of the upper respiratory tract, requires specific adhesins, such as FHA (29;142), Prn (20), Fim2 and Fim3 (51;52;89), tracheal colonization factor (TcfA)(48), *Bordetella* resistance to killing (BrkA) (45) and Vag8 (47)(Fig. 2), while the production of toxins, i.e. Ptx (12;77;94), tracheal cytotoxin (TCT)(53) and adenylate cyclase hemolysin (Ac-Hly)(72) is important

for survival in the host and transmission to susceptibles. Kinetic studies showed indeed that adhesins are expressed first, followed by the expression of toxins (70;71). In the following paragraphs I will describe the importance of Prn, TcfA and Ptx in pathogenesis in greater detail, because these three proteins play an important role in this thesis.

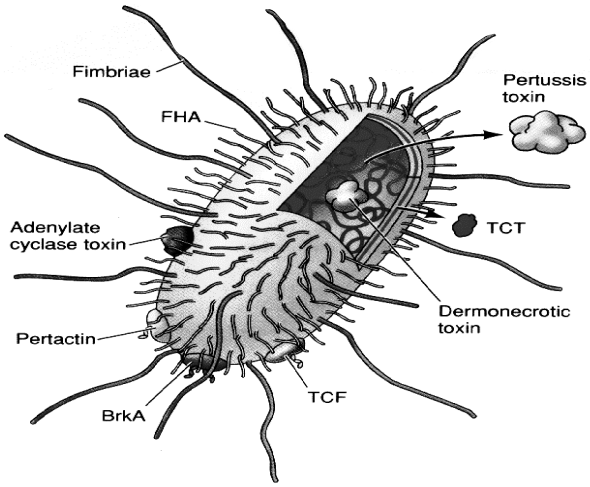


**Fig. 2.** Virulence factors of *Bordetella pertussis* (From Alison Weiss, ASM News 1997)

***Pertactin***

Prn or P.69, referring to its apparent molecular mass of 69 kDa on a SDS gel, is a protein, which belongs to the group of autotransporter proteins (62). This is a family of surface-associated proteins that direct their own export to the outer membrane, where the C-terminal end undergoes proteolytic processing.

Prn contains a RGD sequence, a tripeptide motif that binds to various mammalian adhesion proteins, such as vitronectin, vibronectin and fibrinogen (66). Via this RGD binding site Prn is thought to play a role in adherence to host receptors, although the specific receptor for Prn is unknown. Prn plays a role in attachment to CHO cells and HeLa cells (43;75). Roberts et al. showed that Prn participated in the attachment to a laryngeal epithelial cell line, while Van den Berg et al. found no difference in adhesion of a Prn-wt and Prn-ko strain to a laryngeal and bronchial epithelial cell line. (113;147). Further, Roberts et al. were not able to detect differences in colonization of mice by a Prn-wt and Prn-ko strain (113).

Several studies have shown that Prn is important for immunity against pertussis. Prn contains two repeat regions, comprising GGxxP and PQP repeats, which both

have been identified as B-cell epitopes (21;69). These repeats elicited antibody responses in humans and mice (69). Further, epidemiological studies have shown a correlation between the titer of anti-Prn antibodies and clinical protection (23;131).

***Tracheal Colonization Factor***

The recently reported virulence factor, TcfA, is an adhesion factor of 64 kDa. It also belongs to the autotransporter family and the C-terminal part has 52% sequence identity with the C terminal part of Prn. TcfA also contains a RGD motif. Deletion of TcfA impaired the ability to colonize the trachea of mice, while it did not effect the survival deeper in the lungs (48). Intranasal vaccination with an attenuated *Vibrio cholera* strain, expressing TcfA, resulted in a significant reduction in tracheal colonization, while colonization in the lungs was not affected, suggesting that antibodies to TcfA may be effective in conferring protection (22).

***Pertussis Toxin***

Pertussis toxin is the major toxin of *B. pertussis*. It is an excreted protein of 105 kDa, which consists of 5 subunits. Ptx consists of an A subunit, S1, which is the enzymatic active part and responsible for its toxicity. The B oligomer consists of the subunits S2, S3, S4 and S5 in a ratio of 1:1:2:1 and binds to receptors of eukaryotic cells and facilitates the transmembrane transport of the A subunit.

The toxic activities of Ptx are caused by the ADP ribosylating activity of the S1 subunit, which transfers ADP groups to G-proteins of eukaryotic cells (12). As a result of this, cells lose their ability to receive signals from surface receptors, which causes a general toxicity resulting in tissue specific effects. Ptx induces lymphocytosis, and hypoglyceamia in children and lymphocytosis, sensitization to histamine, enhancement of insulin secretion and induction of IL4 and IgE in animal models (93;94). Further, Ptx has been implicated in adhesion to ciliated cells and macrophages (112;142;161).

Several studies have shown that Ptx is important for immunity against pertussis. Ptx comprises several epitopes for T-cell receptors (30;120). Epidemiological studies showed that antibody responses against Ptx correlate with protection

(23;131;137). Further, the efficacy of a monocomponent pertussis toxoid ACV was 76% and decreased spread of *B. pertussis* in a population (135;136).

## MOLECULAR EPIDEMIOLOGY

A number of studies attempted to identify *B. pertussis* strains that cause epidemics in highly vaccinated populations, in order to explain why *B. pertussis* is able to persist in countries with a high vaccination coverage.

In several studies pulse field gel electrophoresis (PFGE) was used to study changes in the *B. pertussis* population. Different PFGE patterns were found associated with isolates from the prevaccination period and isolates from the 1990s in France and The United States (19;157). PFGE was useful to determine transmission patterns within communities, and in Canada and the United States two and one PFGE types, respectively, predominated in outbreaks (9;35). Further, in France, particular PFGE types were found to be linked to certain periods (87;157). There was, however, no association between PFGE type and host age, severity of infection, vaccination status and geographic distribution (11;19;157). Due to interlaboratory differences of this method it is not possible to compare the aforementioned studies to detect specific epidemic PFGE types.

Restriction fragment length polymorphism (RFLP) based on IS*1002* was used to study a Dutch *B. pertussis* population (149;150). Analysis of isolates from before and after the introduction of vaccination revealed large changes: The RFLP types from the prevaccination period disappeared and novel types emerged. Seventy one percent of the isolates from the 1994 Dutch pertussis epidemic belonged to two RFLP types, which did not predominate in the previous period 1991-1993. Further, isolates from different geographic regions showed significant differences, except 1 RFLP type that showed a worldwide distribution (151).

The changes in the population structure of *B. pertussis* may have been caused by variation in proteins that are important for immunity against pertussis. Mooi et al. studied the Dutch *B. pertussis* population by investigating polymorphism in genes coding for surface proteins (92). Vaccine strains and clinical isolates were investigated for variation in Ptx and Prn. In The Netherlands vaccine strains and

strains from the prevaccination period produced the same variants, PtxS1B, PtxS1D and Prn1. However, in the period 1960-1996 divergence between clinical isolates and vaccine strains was observed. Ninety percent of recent isolates produced the non-vaccine types PtxS1A, Prn2 and Prn3, which suggests that vaccination has shifted the competitive balance between strains, and selected for variants less affected by vaccine-induced immunity (92).

The changes in the Dutch *B. pertussis* population in the period 1996-2000, the period when the pertussis incidence was elevated, were not investigated, however. The goal of this thesis was to further investigate how *B. pertussis* evolved after the introduction of vaccination and to find an explanation for the re-emergence of *B. pertussis* in countries with a high vaccination coverage, in particular The Netherlands.

## OUTLINE OF THIS THESIS

The aim of this thesis was to explain the re-emergence of pertussis in The Netherlands and other countries with highly vaccinated populations. In **chapter 2** the abovementioned IS*1002*-based RFLP method was used to analyze strains from the period 1949-1996. In particular, isolates from the 1996 epidemic were investigated for changes in fingerprint types relative to previous years. Fluctuations in the genotypic diversity, based on the frequency and number of fingerprint types, were linked to expansion of strains that carried particular PtxS1 and Prn variants. In **chapter 3** we extended our survey of polymorphism in genes coding for surface proteins. Fifteen genes, including the genes coding for all 5 components of the current ACVs, were investigated. The polymorphic genes *ptxS1*, *ptxS3* and *tcfA* were used to define multi-locus sequence types (MLST´s), identifying 2 MLST's that could be associated with epidemics in The Netherlands. In **chapter 4** we investigated changes in the *B. pertussis* population in the first 20 years after the introduction of vaccination as they may illustrate how pathogens adapt to mass vaccination within a short time span. Isolates from the period 1949-1972, investigated for polymorphism in genes, changes in fimbrial serotype and changes in IS*1002* RFLP type, showed a decline in frequency of vaccine type variants, while a novel variant, harboring a novel PtxS1 variant and fingerprint type, increased in frequency. In **chapter 5** the effects of the variation in PtxS1 and Prn on strain fitness were investigated in a competition model in mice. The novel Prn and PtxS1 variants were less fit in the upper respiratory tract of unimmunized mice compared to the vaccine type Prn and PtxS1, while in the lungs the reverse was observed. In **chapter 6** we studied polymorphism in a number of regulatory genes. We found substantial polymorphism in the promoter region of Ptx, *ptxP*, and we investigated the effect on production of Ptx. Finally, we found that strains carrying a novel *ptxP* variant increased strain fitness in mice by increasing the ability to colonize and by causing more severe disease. In the summarizing discussion in **chapter 7** the re-emergence of *B. pertussis* in countries with a high vaccination coverage, in particular The Netherlands, is discussed within the scope of molecular epidemiology, mathematical and animal models.

# 2 Temporal trends in the population structure of *Bordetella pertussis* during 1949-1996 in a highly vaccinated population

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**ABSTRACT**

We analyzed the population structure of *B. pertussis* in The Netherlands in five successive periods, encompassing 1949-1996, by DNA typing ("fingerprinting"). In a period of 10 years following the introduction of wide-scale vaccination in 1953, a decrease in genotypic diversity (GD) was observed, suggesting clonal expansion of strains, which were adapted to vaccine-induced immunity. In subsequent periods, GD increased to prevaccination levels, probably reflecting a gradual adaptation of the *B. pertussis* population involving many lineages. In the 1990s, GD decreased again. This decrease coincided with an antigenic shift in the surface protein pertactin. No evidence was found for changes in DNA types or GD in 1996, when a large pertussis epidemic occurred. Thus gradual changes in the bacterial population previous to 1996 were probably the cause of the 1996 epidemic. Our results suggest that vaccination has selected for strains, which are adapted to a highly vaccinated population. Similar changes may have occurred in other countries, explaining the re-emergence of pertussis in vaccinated populations.

## INTRODUCTION

Despite the introduction of large-scale pertussis vaccination in 1953 (58) and a high vaccination coverage, pertussis is still an endemic disease in The Netherlands, with epidemic outbreaks occurring every 3-5 yrs. The year 1996 saw an especially severe pertussis epidemic, with an incidence, which was 5-fold higher than in previous epidemic years (33). Recent pertussis epidemics have also been observed in other countries such as Australia, Canada, Norway and The United States, notwithstanding routine vaccination (3;8;15;36). Several explanations have been put forward for the resurgence of pertussis in vaccinated populations (7;101;159). Improved surveillance, changes in case definitions and diagnostic techniques may result in an apparent increase in the pertussis incidence. However, it seems unlikely that these factors explain all cases, in which a dramatic rise in pertussis has been observed. Other factors, which may affect the incidence of pertussis include demographic changes, waning vaccine-induced immunity, changes in vaccine quality, changes in vaccine coverage, and adaptation of the *Bordetella pertussis* population to vaccine-induced immunity. In previous work, we have provided evidence for the last factor, adaptation of *B. pertussis*, by showing that the Dutch *B. pertussis* population has undergone antigenic shifts in two *B. pertussis* surface proteins, the S1 subunit of pertussis toxin and pertactin (92). Pertussis toxin and pertactin variants identical to those found in the Dutch whole cell pertussis vaccine were found in 100% of the strains isolated in the 1950s, but were gradually replaced by non-vaccine types in later years. In the 1990s, more than 90% of the strains harbored pertussis toxin and pertactin variants that were different from those produced by the vaccine strains. DNA typing (based on the insertion sequence element IS*1002*) of Dutch strains revealed little overlap in DNA types when strains from the 1950s were compared with those from the period 1978-1994, also providing evidence that shifts had occurred in the Dutch *B. pertussis* populations (151).

Here we extend our previous studies by IS*1002*-based DNA typing of a larger number of strains, especially from the critical period 1950-1978, of which only a limited number of strains were analyzed previously. Strains from the 1996 epidemic were also analyzed by DNA typing to determine whether novel strains

were involved. Finally, we investigated whether the antigenic shift observed for pertactin coincided with changes in the population structure as assessed by DNA typing. Our results show that the Dutch *B. pertussis* population has undergone major changes since the introduction of wide scale vaccination in 1953. One change occurred before 1965 and was reflected in a change in DNA types and a significant decrease in genotypic diversity. The second change occurred after 1980 and involved antigenic shifts in pertactin.

## MATERIALS AND METHODS

### *Strains*

*B. pertussis* strains were collected in the years 1949-1996. Most strains were sent to the RIVM by regional laboratories for serotyping or confirmation of identification. A potential problem for the comparison of *B. pertussis* populations from different periods, was the selection of epidemiologically related strains. Therefore, whenever possible, we selected those strains which, based on year of isolation (and if available; patient name and place of isolation) were epidemiologically unrelated. For strains isolated in 1988, and later, sufficient data were available to select epidemiologically unrelated strains. For many older strains (approximately 30%) either place of isolation or patient name was known, in addition to the date of isolation, allowing us to select strains, which differed in at least one of these characteristics. Sampling of epidemiologically related strains will tend to decrease the genotypic diversity. However, we saw the highest genotypic diversity in those periods, where lack of sufficient data about strains may have resulted in the selection of epidemiologically related strains. *B. pertussis* strains were grown on Bordet Gengou agar (DIFCO, 0048-15-7) supplemented with 1% glycerol and 15% sheep blood, or in Verwey medium (156) supplemented with 200 µg/ml cyclodextrin (Sigma) at 35$^{o}$C for 3 days.

### *IS*1002*-based DNA typing*

Some improvements of the previously described IS*1002*-based DNA typing method were implemented (151). The most important change was the use of

Field Inversion Gel Electrophoresis (FIGE), which showed a higher resolving power than the classical gel electrophoresis method used previously. FIGE resulted in the differentiation of DNA types that were previously not resolved. *B. pertussis* chromosomal DNA was purified with the Puregene DNA isolation kit (D5000A, Gentra Systems Inc, Minneapolis, USA) using the protocol for Gram-negative Bacteria as provided by the manufacturer. After purification, 1 µg of chromosomal DNA was digested for 2 hours, with 2 units of *Sma*I in a final volume of 200 µl, ethanol precipitated and dissolved in 10 µl sample buffer. Samples were applied on a 1% agarose gel (Pulse Field Certified Agarose, BioRad Laboratories, Hercules, CA, USA), and run on the FIGE mapper electrophoresis system (BioRad laboratories) for 15 hours in ½ x TBE buffer (45 mM Tris, 45 mM borate, 1 mM EDTA, pH 8.3). The following program was used: Switch time ramps 0.1-0.1 seconds, linear shape, forward voltage 180 V, reversed voltage 120 V. As MW-marker a mixture of the 1 kb-ladder (Life Technologies Inc., Rockville, Maryland, USA) and the CHEF marker (BioRad Laboratories) was used. The marker DNA was run in at least 3 lanes. The gel was transferred to Hybond N+ membrane (Amersham-Pharmacia Biotech, Uppsala, Sweden) using standard DNA blotting techniques. The 293 base pair (bp) IS*1002* probe used for hybridization was generated by PCR as follows: 5 µl of a 500 ng/µl DNA stock from strain B24 (90) was amplified with 1 unit SuperTaq (Sphaero-Q, Leiden, The Netherlands) in 1 x SuperTaq buffer, with DMSO added to a final concentration of 5%, 25 µmol of each primer, and 4 µl dNTP-mix containing 2,5 mM of each dNTP, in a final volume of 50 µl. PCR program: 1 cycle of 3 min 95$^{o}$C, 35 cycles of 15 seconds 95$^{o}$C, 30 seconds 60$^{o}$C, 60 sec 72$^{o}$C, and 1 cycle of 10 min 72$^{o}$C. PCR was performed in a Hybaid Touch-Down PCR apparatus (Hybaid Limited, Ashford, Middlesex, UK). The following primers were used: HG1 (5'-GCCGATGCGTTCCATATA-3'), HG2 (5'-AGCCCTTCTTGTTAACAGGG-3'). After PCR, the IS*1002* probe was purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany), and labeled with peroxidase according to the instructions provided with the "Enhanced Chemiluminescence Gene Detection System" (Amersham-Pharmacia Biotech, Uppsala, Sweden). Marker DNA was also labeled with peroxidase, and hybridization and detection of IS*1002*-containing DNA fragments and marker

DNA was performed according to the Amersham-Pharmacia protocol. The exposed films were scanned at 190 DPI (HP Scanjet IIcx/T, Hewlett Packard, Camas, USA). Computer-assisted analysis of the IS*1002* patterns was carried out using the GelCompar software (Windows NT version 4.1, Applied Maths, Kortrijk, Belgium). To compare IS*1002* patterns on different blots, the 5 bands of the IS*1002* type dt-29 (sizes 5050 bp, 6580 bp, 8470 bp, 11100 bp, and 15600 bp), which were shared by most DNA types, were used as internal standard to facilitate normalization. The IS*1002*-hybridizing bands in *B. pertussis* ranged in size from 2,600 to 37,800 bp. Pairwise similarities of IS*1002* DNA patterns were calculated using the Dice coefficient of similarity. Clustering of the isolates by similarity to give a dendrogram was carried out with UPGMA (unweighed pair group method with arithmetic averages).

Genotypic diversity (GD) based on DNA typing was calculated by the equation $GD= \{n/(n-1)\}(1- \sum x_i^2)$, where $x_i$ is the frequency of the ith DNA type, and n is the number of strains (100).

***Statistical analysis***

The statistical significance of the difference in DNA type frequencies between periods was calculated by cross-tabulating frequencies by period (Fig. 2, Tables 1 and 2) and applying the chi-square test. To comply with the conditions for the chi-square test (i.e. more than 80% of the cells should have an expected cell frequency $\geq 5$) the analysis was limited to the most frequent DNA types; dt-12, dt-29 and dt-35 for data in Table 1, and dt-10, dt-12, dt-29 and dt-35 for data in Table 2. For comparison of the *B. pertussis* populations in the periods 1949-1954, 1965-1972, 1976-1981 and 1991-1996, the summed DNA types of the clades A, B and C were used (Fig. 2). The statistical significance of the difference in genotypic diversity (see above for definition) was estimated as follows. By computer simulation we randomly generated 1000 cross-tabulations similar to Fig. 2, and Tables 1 and 2, under the null hypothesis of no differences in frequency of DNA types between periods (Fig. 2 and Table 2) or *prn* allele groups (Table 1) (i.e. homogeneity existed). Under that hypothesis DNA types essentially occur at random in the different periods or *prn* groups. This can be simulated by a computer, by randomly shuffling the different DNA types over the

different periods, but changing neither the total observed number of particular DNA types, nor the total number of observed DNA types per period or *prn* group (the so-called "marginal totals"). We then counted the number of times, squared differences in genotypic diversity in the simulated tables exceeded the observed squared difference, and used these counts (divided by 1000) as an estimate of the P-value of the difference in genotypic diversity.

## RESULTS

### *IS*1002*-based DNA typing of Dutch strains isolated in 1949-1996*

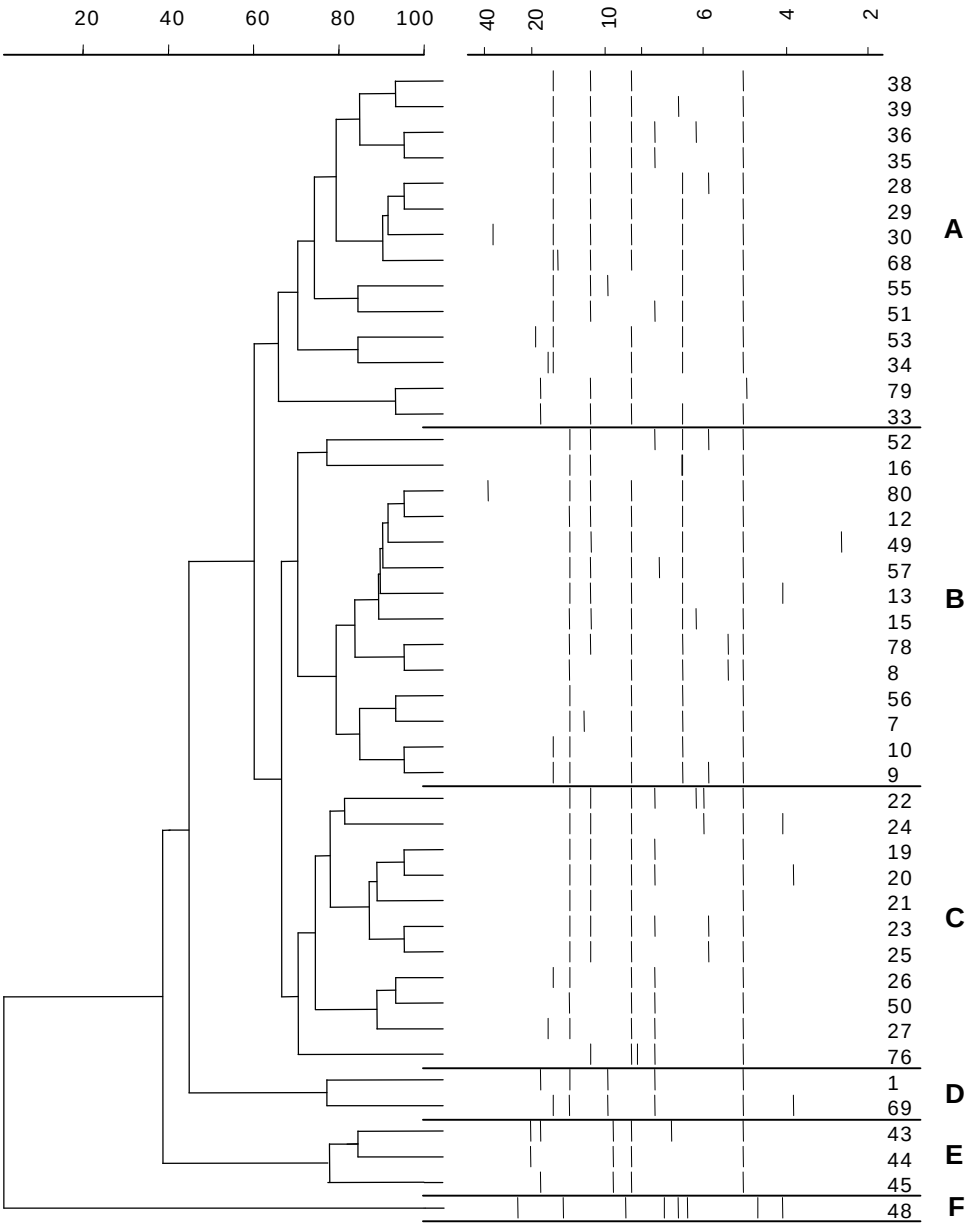
A collection of Dutch strains, collected in the period 1949-1996 and comprising 213 isolates, was investigated for IS*1002*-based restriction fragment length polymorphism (RFLP)(Fig. 1). The number of bands per DNA type varied between 4 and 8. Most DNA types (77%) contained 5 bands. Forty-five DNA types were observed, which were grouped in 6 families (A-F) or clades of related patterns with UPGMA. Clustering based on Ward or the Neighbor Joining method gave similar results (not shown).

### *Temporal trends in IS*1002*-based DNA types*

To observe temporal trends in DNA types, strains were stratified in 5 periods encompassing the years 1949-1954, 1965-1972, 1976-1981, 1982-1990 and 1991-1996, respectively (Fig. 2). Each period comprised 6 to 9 years. Since large-scale vaccination against pertussis was introduced in the Netherlands in 1953 (25), period 1949-1954 can be viewed as the prevaccination period as strains isolated in this period would have been little affected by vaccine-induced immunity.

Significant differences in DNA type frequencies were observed between the *B. pertussis* population derived from 1949-1954 and the ensuing period, 1965-1972. These two periods share only one DNA type (dt-12), which harbors only 5% of all isolates from both periods. All later successive periods shared at least 3 DNA types (i.e. dt-12, dt-29 and dt-35), that represent the majority of isolates.

In period 1965-1972 one DNA type (dt-29), which was absent from the previous period, was found to dominate, representing 61% of all isolates. Dt-29 was also found in later periods, but it decreased in frequency (to 39% in period 1991-1996).



The two Dutch vaccine strains belong to DNA types dt-19 and dt-43. Dt-19 was observed in the period 1949-1954 (frequency 8%) and in the period 1991-1996 (frequency 3%), while dt-43 was only observed in the first period (frequency 4%).

Differences between the period 1949-1954 and later periods were also evident when the distribution of strains over the different clades was compared. Strains from 1949-1954 were mainly found in the clades B and E, which contained 41% and 34% of the isolates, respectively. In later periods these clades contained between 6%-39%, and 0%-1% of the strains, respectively, while most strains (56%-82%) were found in clade A, which harbored 4% of the strains from period 1949-1954. Statistical analysis based on the distribution of DNA types over the different populations, indicated a highly significant difference ($P<0.001$) between the *B. pertussis* populations from 1949-1954 and the ensuing period, 1965-1972. In contrast, no significant differences were observed between the populations of 2 successive periods after the 1949-1954 period (Fig. 2). It may be argued that this comparison is incorrect, since the first 2 periods are separated by 10 years, while all subsequent periods are separated by 1 to 3 years. However, the periods 1965-1972 and 1982-1990 are also separated by approximately 10 years, while the *B. pertussis* populations derived from these periods do not differ significantly with respect to DNA type distributions ($P=0.17$).

The genotypic diversity of the strains isolated in the five periods was calculated (Figs. 2 and 3), and the highest (GD=0.89) and lowest (GD=0.60) diversities were observed, respectively, in period 1949-1954 and the succeeding period, 1965-1972. The difference in genotypic diversity between these two periods was significant ($P=0.016$)(Fig. 2). After period 1965-1972, the genotypic diversity increased to, respectively, 0.69, 0.88 and 0.79 in the periods 1976-1981, 1982-1990 and 1991-1996.

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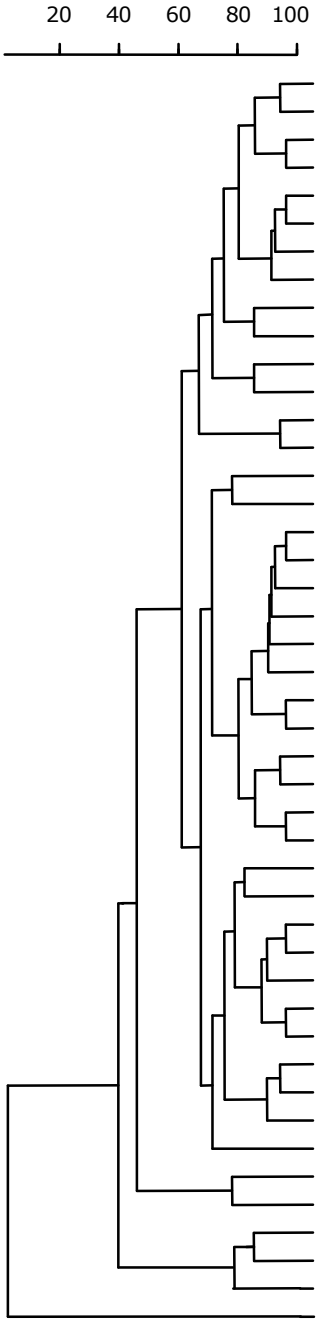
**Fig. 1.** Dendrogram of normalized DNA types found in Dutch strains. The branching points of the 6 main clades are indicated with the letters A-G. The numbers refer to the designation of the DNA types. Clustering was performed using UPGMA. The left and right bars indicate the similarity coefficient and the size of DNA fragments in kilobases, respectively.




| DT | 49-54 | 65-72 | 76-81 | 82-90 | 91-96 | clades |
|---|---|---|---|---|---|---|
| 38 | | | | | 1% (1) | A |
| 39 | | | | | 3% (3) | |
| 36 | | | | 5% (1) | | |
| 35 | | 6% (1) | 7% (2) | 14% (3) | 8% (10) | |
| 28 | | | | 5% (1) | 1% (1) | |
| 29 | | 61% (11) | 55% (16) | 32% (7) | 39% (47) | |
| 30 | | | | | 1% (1) | |
| 68 | | | 3% (1) | | | |
| 55 | | | 7% (2) | | | |
| 51 | | | | | 1% (1) | |
| 53 | | | | | 1% (1) | |
| 34 | | | 3% (1) | | 1% (1) | |
| 79 | 4% (1) | | | | | |
| 33 | | 6% (1) | 7% (2) | | | |
| 52 | | | | | 1% (1) | B |
| 16 | | | 3% (1) | | | |
| 80 | 4% (1) | | | | | |
| 12 | 4% (1) | 6% (1) | 3% (1) | 14% (3) | 22% (26) | |
| 49 | | | | | 2% (2) | |
| 57 | | | | | 1% (1) | |
| 13 | | | | 9% (2) | | |
| 15 | 29% (7) | | | | | |
| 78 | 4% (1) | | | | | |
| 8 | | | | 5% (1) | | |
| 56 | | | | | 2% (2) | |
| 7 | | | | | 1% (1) | |
| 10 | | | | | 8% (10) | |
| 9 | | | | | 2% (2) | |
| 22 | 4% (1) | | | | | C |
| 24 | 4% (1) | | | | | |
| 19 | 8% (2) | | | | 3% (3) | |
| 20 | | | | | 2% (2) | |
| 21 | | | 7% (2) | | | |
| 23 | | | | 5% (1) | | |
| 25 | | | | 5% (1) | | |
| 26 | | | | 5% (1) | | |
| 50 | | | | | 2% (2) | |
| 27 | | 22% (4) | | | | |
| 76 | | | 3% (1) | | | |
| 1 | | | | | 1% (1) | D |
| 69 | | | | 5% (1) | | |
| 43 | 4% (1) | | | | | E |
| 44 | 17% (4) | | | | | |
| 45 | 13% (3) | | | | | |
| 48 | 4% (1) | | | | 1% (1) | F |
| **N** | **24** | **18** | **29** | **22** | **120** | |
| **GD** | **0.89** | **0.60** | **0.69** | **0.88** | **0.79** | |

The difference in genotypic diversity between 1976-1981 and 1982-1990 was marginally significant (P=0.056). P-values of other, successive, periods were not significant.

Interesting differences between the five periods were also observed when the DNA types were grouped on the basis of the number of IS*1002* copies (Fig. 3). In the prevaccination period, strains with 4 and 6 copies of IS*1002* predominated (frequencies, respectively 33% and 46%). In the subsequent period (1965-1972) all strains harbored 5 IS*1002* copies, while the frequency of such strains was only 13% in the previous period. In later periods, strains with 3, 4 and 6 copies were also observed, although strains with 5 copies remained predominant.

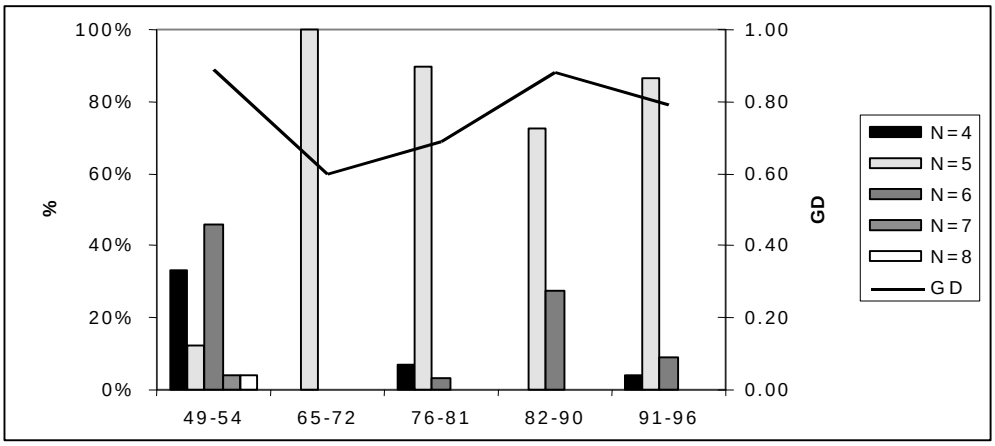



**Fig. 3.** *B. pertussis* populations characterized by IS*1002* copy numbers and genotypic diversity. Within each period the frequency distribution of strains according to their IS*1002* copy number is indicated by bars. The line shows the genotypic diversity (GD).

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**Fig. 2.** Frequency of DNA types found in the Dutch *B. pertussis* population in five successive periods. The 6 main clades (A-F) are indicated. The bar indicates the similarity coefficient. The boxes with the DNA types associated with the Dutch vaccine strains (dt-19 and dt-43) have been shaded. Between parentheses, number of isolates. Abbreviation; DT, DNA type; N, number of strains in each period; GD, genotypic diversity. P-values for differences in DNA type frequencies: 49-54 versus 65-72, P<0.001; 65-72 versus 76-81, P=0.54; 76-81 versus 82-90, P=0.089; 82-90 versus 91-96, P=0.34. P-values for differences in genotypic diversity: 49-54 versus 65-72, P=0.016; 65-72 versus 76-81, P=0.32; 76-81 versus 82-90, P=0.056; 82-90 versus 91-96, P=0.243.

### *Association between DNA types and* prn *alleles*

The association between DNA types and the 3 pertactin alleles observed in the Dutch *B. pertussis* population, *prn1*, *prn2* and *prn3*, was investigated for the period (1981-1996), in which all three types coexisted (previously, the products of the *prn1*, *prn2*, and *prn3* alleles were referred to as P.69A, P.69B and P.69C, respectively)(92). The frequency distributions of the DNA types within the 3 *prn* categories were distinct (Table 1).

For example, a high frequency (64%) of DNA type dt-29 was found in strains with *prn3*, whereas much lower frequencies were observed in strains with *prn1* and *prn2* (respectively, 25% and 32%). The frequencies of dt-12 in the 3 *prn* categories were also quite different (*prn1* 15%, *prn2* 34% and *prn3* 8%). Some DNA types were uniquely associated with a particular *prn* allele. However, with the exception of the two closely related DNA types dt-9 and dt-10 (Fig. 1), which were uniquely associated with the *prn3*, the relevance of these associations remains to be substantiated in view of the small number of strains involved. The differences in DNA type frequencies between the *prn1* and *prn3*, and *prn2* and *prn3* populations were significant (P=0.044 and P<0.001, respectively). The difference in DNA type frequencies between the *prn1* and *prn2* populations were not significant (P=0.83)(Table 1).

The genotypic diversity, calculated from the DNA type data, of the *prn1*, *prn2* and *prn3* groups were 0.91, 0.78 and 0.57, respectively (Table 1). With the exception of the difference between the *prn1* and *prn2* populations, the differences in genotypic diversity were highly significant (Table 1).

### *The 1996 epidemic*

A comparison of strains isolated from the 1996 epidemic with strains isolated in previous periods did not reveal a significant difference in population structure (Table 2). In the 3 periods analyzed, 1982-1990, 1992-1995 and 1996, three DNA types (dt-12, dt-29 and dt-35) dominated, comprising more than 60% of the isolates in each period. The genotypic diversities of the *B. pertussis* population in 1992-1995 and 1996 were identical (0.79).

**Table 1.** Association between DNA types and *prn* alleles[a].

| DNA Type | Frequency *prn* alleles[†] | | | Tot |
|---|---|---|---|---|
| | ***prn1*** | ***prn2*** | ***prn3*** | |
| 7 | 0% (0) | 2% (1) | 0% (0) | 1% (1) |
| 8 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 9 | 0% (0) | 0% (0) | 4% (2) | 1% (2) |
| 10 | 0% (0) | 0% (0) | 13% (7) | 5% (7) |
| 12 | 15% (6) | 34% (14) | 8% (4) | 18% (24) |
| 13 | 5% (2) | 0% (0) | 0% (0) | 1% (2) |
| 19 | 0% (0) | 5% (2) | 0% (0) | 1% (2) |
| 20 | 5% (2) | 0% (0) | 0% (0) | 1% (2) |
| 23 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 25 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 26 | 0% (0) | 0% (0) | 2% (1) | 1% (1) |
| 28 | 3% (1) | 2% (1) | 0% (0) | 1% (2) |
| 29 | 25% (10) | 32% (13) | 64% (34) | 43% (57) |
| 30 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 33 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 34 | 0% (0) | 0% (0) | 2% (1) | 1% (1) |
| 35 | 8% (3) | 12% (5) | 6% (3) | 8% (11) |
| 36 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 38 | 0% (0) | 2% (1) | 0% (0) | 1% (1) |
| 39 | 0% (0) | 7% (3) | 0% (0) | 2% (3) |
| 45 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 49 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 51 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 52 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 53 | 0% (0) | 2% (1) | 0% (0) | 1% (1) |
| 55 | 5% (2) | 0% (0) | 0% (0) | 1% (2) |
| 56 | 5% (2) | 0% (0) | 0% (0) | 1% (2) |
| 57 | 0% (0) | 0% (0) | 2% (1) | 1% (1) |
| 68 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| 69 | 3% (1) | 0% (0) | 0% (0) | 1% (1) |
| **Tot** | 100% (40) | 100% (41) | 100% (53) | 100% (134) |
| **GD[‡]** | 0.91 | 0.78 | 0.57 | 0.78 |

[a] Isolates from the period 1981-1996 were analyzed. P-values for differences in DNA type frequencies: *prn1* versus *prn2*, P=0.83; *prn1* versus *prn3*, P=0.044; *prn2* versus *prn3*, P=0.001. [†]Number of isolates is between parentheses. [‡]Genotypic diversity. P-values for differences in GD: *prn1* versus *prn2*, P=0.106; *prn1* versus *prn3*, P<0.001; *prn2* versus *prn3*, P=0.006.

**Table 2.** Frequency of DNA types found in the epidemic year 1996, and previous periods[a].

| DNA type | Period[†] | | |
|---|---|---|---|
| | **1982-1990** | **1992-1995** | **1996** |
| 1 | 0% (0) | 0% (0) | 1% (1) |
| 7 | 0% (0) | 2% (1) | 0% (0) |
| 8 | 5% (1) | 0% (0) | 0% (0) |
| 9 | 0% (0) | 2% (1) | 1% (1) |
| 10 | 0% (0) | 8% (4) | 9% (6) |
| 12 | 14% (3) | 23% (11) | 21% (15) |
| 13 | 9% (2) | 0% (0) | 0% (0) |
| 19 | 0% (0) | 0% (0) | 4% (3) |
| 20 | 0% (0) | 4% (2) | 0% (0) |
| 23 | 5% (1) | 0% (0) | 0% (0) |
| 25 | 5% (1) | 0% (0) | 0% (0) |
| 26 | 5% (1) | 0% (0) | 0% (0) |
| 28 | 5% (1) | 0% (0) | 1% (1) |
| 29 | 32% (7) | 40% (19) | 39% (27) |
| 30 | 0% (0) | 2% (1) | 0% (0) |
| 34 | 0% (0) | 2% (1) | 0% (0) |
| 35 | 14% (3) | 6% (3) | 10% (7) |
| 36 | 5% (1) | 0% (0) | 0% (0) |
| 38 | 0% (0) | 0% (0) | 1% (1) |
| 39 | 0% (0) | 6% (3) | 0% (0) |
| 49 | 0% (0) | 0% (0) | 3% (2) |
| 50 | 0% (0) | 0% (0) | 3% (2) |
| 51 | 0% (0) | 0% (0) | 1% (1) |
| 52 | 0% (0) | 0% (0) | 1% (1) |
| 53 | 0% (0) | 0% (0) | 1% (1) |
| 56 | 0% (0) | 4% (2) | 0% (0) |
| 57 | 0% (0) | 0% (0) | 1% (1) |
| 69 | 5% (1) | 0% (0) | 0% (0) |
| **Tot** | 100% (22) | 100% (48) | 100% (70) |
| **GD[‡]** | 0.88 | 0.79 | 0.79 |

[a] P-values for differences in DNA type frequencies: 1982-1990 versus 1992-1995, P=0.34; 1992-1995 versus 1996, P=0.92. [†]Number of isolates between parentheses. [‡]Genotypic diversity. Differences in GD were not significant (P>0.23).

## DISCUSSION

Like the use of antibiotics, vaccination can impose a strong selective force on populations of microorganisms. Nevertheless long term, population-based, studies addressing the effect of vaccination are limited. In one study, some evidence was provided for a modest increase in the incidence of invasive disease by *H. influenzae* serotype f after introduction of *H. influenzae* serotype b vaccination (144). A second study, focused on the measles virus, provided evidence that vaccination has affected the population structure of the virus, and selected for strains, which are antigenically distinct from the vaccine strain. The nucleoprotein (N) and matrix (M) genes of wild-type isolates from the prevaccination era (before 1964) were highly related to each other and to the N and M genes from the measles vaccine strain. In contrast, the N and M genes of wild type viruses isolated between 1977 and 1989 showed genetic drift, which resulted in antigenic differences with the vaccine strain (117;134). Here we extend our previous studies (92;151) on the effect of vaccination on the population structure of *B. pertussis*.

We analyzed temporal trends in the population structure of *B. pertussis* in the years 1949-1996 in The Netherlands. Strains were stratified in 5 periods encompassing the years 1949-1954, 1965-1972, 1976-1981, 1982-1990 and 1991-1996, respectively (Fig. 2). Large-scale vaccination against pertussis was introduced in the Netherlands in 1953 (25), and isolates from the earliest period analyzed, 1949-1954, probably represent a *B. pertussis* population, which has not been significantly affected by vaccination. Differences were found between the populations from the five periods, reflected in the frequency of DNA types (Fig. 2), the number of IS*1002* copies (Fig. 3), and the genotypic diversity (Figs. 2 and 3). The most pronounced differences were observed between the populations from 1949-1954 and the ensuing period, 1965-1972, and it seems likely that these changes were driven by vaccination. However, other explanations for the observed changes in the population structure of *B. pertussis*, such as random genetic drift or changes in the human population unrelated to vaccination, cannot be excluded.

The decrease in genotypic diversity (from 0.89 in 1949-1954 to 0.6 in 1965-1972, P=0.016) observed after the introduction of vaccination may have been caused by a decrease in the bacterial population size and/or by clonal expansion. Both possibilities are consistent with the effect of the introduction of vaccination, since the resultant increased herd immunity may (temporarily) reduce the circulation of the pathogen, and/or cause shifts in the relative fitness of strains. For example, strains that are antigenically distinct from the vaccine strains may be less affected by vaccine-induced immunity and expand. The observation that 61% of the isolates from 1965-1972 belonged to a single DNA type (dt-29), which was not found in the previous period, is consistent with clonal expansion. The predominance of dt-29 decreased in subsequent periods (to 39% in 1996), while the genotypic diversity of the *B. pertussis* population increased, suggesting a gradual expansion of other lineages.

In period 1965-1996 three DNA types (dt-12, dt-29 and dt-35) were predominant comprising between 60% and 73% of the isolates. Strain typing with pulsed field gel electrophoresis (PFGE) also revealed a predominance of 2-3 PFGE types in the *B. pertussis* population in Canada and England (35;133). A single PFGE type was observed during an epidemic in the US, which occurred in a small rural community (9). *B. pertussis* is thought to be a very homogenous species. For example, multi-locus enzyme electrophoresis (MLE) has revealed only 4 electrophoretic types (96;151). In contrast, IS*1002*-based DNA typing revealed 45 DNA types for 213 isolates. Thus IS*1002*-based DNA typing revealed a much faster molecular clock than MLE, presumably due to transposition of, and recombination between, the IS element. A similar observation was made for *Mycobacterium tuberculosis* (128;152).

Antigenic divergence between vaccine strains and circulating strains may be the underlying cause of the changes observed with DNA typing, and in fact such divergence has been found by us for two *B. pertussis* proteins, the S1 subunit of pertussis toxin and pertactin (92). The antigenic shifts in pertactin cannot explain the change in population structure observed between 1954 and 1965, since it occurred in the 1980s, and all strains isolated previous to 1981 produce a single pertactin type, which is identical to the type found in the Dutch pertussis vaccine. Therefore, we can discern at least two, temporally separated,

changes in the *B. pertussis* population, the first of which occurred before 1965, while the second, involving an antigenic shift in pertactin, occurred after 1980. It is not clear what adaptation caused the first shift. We have shown that the *s1* alleles found in the prevaccination era have largely been replaced by a novel type in the period 1978-1985, and this antigenic shift may explain the changes in population structure observed previous to 1965. We are currently analyzing *s1* allele frequencies in the period 1954-1965, to substantiate this possibility. However, antigenic shifts in other *B. pertussis* antigens, such as integral outer membrane proteins or lipopolysaccharide, may be the underlying cause of the changes in the bacterial population structure previous to 1965. We have not been able to associate the first change with increases in mortality or morbidity in the years 1954-1972. This may reflect the lack of adequate surveillance in this period, however. Obligatory notification of pertussis was introduced in The Netherlands in 1976.

The second change involved antigenic shifts in pertactin, a surface protein known to be protective in man and animals (54;104;114). If these shifts were due primarily to clonal expansion, one would expect this to be reflected in a decrease in genotypic diversity. The *prn1* frequency drops from 100% in the period 1949-1972 to 85%, 80% and 13% in the periods 1976-1981, 1982-1990 and 1991-1996, respectively (Table 3).

**Table 3.** Frequencies of *prn* alleles and genotypic diversity in different periods.

| Period | ***prn* frequencies** * | | | **Genotypic diversity**† |
|---|---|---|---|---|
| | ***prn1*** | ***prn2*** | ***prn3*** | |
| 1949-1954 | 100% (10) | 0% (0) | 0% (0) | 0.89 |
| 1965-1972 | 100% (10) | 0% (0) | 0% (0) | 0.60 |
| 1976-1981 | 85% (23) | 11% (3) | 4% (1) | 0.69 |
| 1982-1990 | 80% (16) | 10% (2) | 10% (2) | 0.88 |
| 1991-1996 | 13% (13) | 36% (36) | 51% (50) | 0.79 |

*Based on Mooi et al., 1998. Number of isolates is between parentheses. †Calculated from the frequency of DNA types.

Thus, the largest difference in *prn1* frequency (i.e. the largest increase in the frequencies of *prn2* and *prn3*) is found between the last two periods and this difference coincides with a decrease in genotypic diversity from 0.88 in 1982-

1990 to 0.79 in 1991-1996 (Table 3) albeit a small one, which was not statistically significant (P=0.243). In contrast the genotypic diversity increased between 1965 and 1990 (Table 3).

Subpopulations of isolates harboring distinct *prn* alleles could be distinguished on the basis of the distribution of DNA types and genotypic diversity (Table 1). The lower genotypic diversity of the *prn2* and *prn3* groups, compared to the *prn1* group (P-values, respectively, 0.106 and <0.001), is consistent with the observation that *prn1* strains represent the oldest lineage (92). The *prn3* group shows the lowest genotypic diversity, suggesting it represents a younger lineage than the *prn2* group (P=0.006). This is also suggested by the observation that *prn3* strains are geographically more restricted than *prn2* strains (unpublished data).

In 1996 there was a large and unexpected pertussis epidemic in the Netherlands (33). Such an epidemic may be caused by changes in the bacterial population, for example by the introduction (either by import or mutation) and subsequent clonal expansion of novel strains, which are less affected by vaccine-induced immunity. An epidemic may also be caused by changes in the immune status of the host population, for example due to waning vaccine-induced immunity or to a decrease in vaccine quality. A comparison of strains isolated in 1996 and previous years (Table 2) did not reveal major shifts in DNA types or changes in genotypic diversity. Thus no evidence was found for the introduction or expansion of particular strains. The antigenic divergence observed between circulating strains and vaccine strains may have gradually decreased vaccine efficacy, resulting in the crossing of a critical threshold in 1996.

This study provides evidence that the Dutch *B. pertussis* population has adapted to vaccination. Similar changes may have occurred in other countries, explaining the re-emergence of pertussis in vaccinated populations (3;8;15;36). Our results predict that WCV will protect less well against recent isolates compared to strains from the 1950s. Preliminary studies in a mouse model have confirmed this prediction (unpublished data). Most pertussis vaccines are derived from strains isolated more than 50 years ago (151). It seems plausible that such vaccines may be made more effective by using contemporary strains.

**ACKNOWLEDGMENT**

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# 3 Multi-locus sequence typing of *Bordetella pertussis* based on surface proteins: Identification of epidemic types ?

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**ABSTRACT**

Despite more than 50 years of vaccination, *Bordetella pertussis* has remained endemic in The Netherlands, causing epidemic outbreaks every 3-5 years. Strain variation may play a role in the persistence of *B. pertussis* and was studied by sequencing 15 genes coding for surface proteins, including genes for all 5 components of acellular pertussis vaccines (ACVs): pertussis toxin (Ptx), pertactin (Prn), filamentous hemagglutinin (FHA) and fimbriae (Fim2 and Fim3). A low level of allelic variation was observed, suggesting a recent evolutionary origin of *B. pertussis*. In modern isolates polymorphism was observed only in *prn*, *ptxS1*, *ptxS3* and *tcfA*. The *ptxS1*, *ptxS3* and *tcfA* genes were used to categorize isolates in Multi-locus Sequence Types (MLST's). Analysis of Dutch isolates from 1949-1999 revealed 5 MLST's, which showed a highly dynamic temporal behavior. We observed significant changes in the MLST's after the introduction of pertussis vaccination in The Netherlands. Epidemic years were found to be associated with expansion of MLST-4 or MLST-5. MLST-5 showed a remarkable expansion from 10% in 1997 to 80% in 1999. The MLST analysis was extended to a number of widely separated geographic regions: Australia, Finland, Italy, Japan and The United States. MLST-4 was found to dominate in Australia and Italy, while MLST-5 predominated in the United States. In Finland and Japan, MLST-3 and MLST-2, respectively, dominated. Thus, although each region showed distinctive MLST frequencies, in 4 of the 6 regions MLST-4 and MLST-5 were predominant. These types may represent, newly emerged, successful clones. The identification of highly successful clones may shed light on the question how *B. pertussis* is able to maintain itself in vaccinated populations.

## INTRODUCTION

Despite more than 40 years of vaccination, pertussis has remained an endemic disease, with epidemic outbreaks occurring every 3-5 years. In some vaccinated populations (e.g. in Australia, Canada, The Netherlands, The United States) the incidence of pertussis is increasing (3;8;33;34;36;58). The resilience of *Bordetella pertussis* to vaccination may be due to its highly infectious nature, its relative brief stay in the host, its ability to hamper the host immune response, shortcomings of pertussis vaccines and strain variation. Our studies on strain variation have led us to suggest that the resurgence of pertussis may be due, in part, to the adaptation of the *B. pertussis* population to vaccination (92). The aim of this work was twofold. First, to extend these studies and to find markers suitable for the analysis of *B. pertussis* populations. Second, to determine the extent of polymorphism in immunologically relevant proteins, in particular those incorporated in acellular vaccines (ACVs).

Recently, Multi-Locus Sequence Typing (MLST) has been introduced as a new approach to study the molecular epidemiology of bacterial pathogens (42;127). MLST is based on the well-tested principles of Multi-Locus Enzyme Electrophoresis (MLEE), but assigns alleles at each locus directly by nucleotide sequencing, rather than indirectly from electrophoretic mobilities of their gene products in starch gels. In contrast to many current typing methods (e.g. RFLP, RAPD, etc) MLST allows unambiguous interlaboratory comparisons. Importantly, MLST has proven to be especially suitable to study longer term and global epidemiology (39;44;65).

*B. pertussis* is a very homogeneous species, and consequently finding polymorphic sites for MLST has proven to be difficult. MLEE revealed only 4 electrophoretic types (150). Consistent with this, we found very little polymorphism in *B. pertussis* genes coding for housekeeping genes (unpublished data). In view of the restricted polymorphism found in *B. pertussis*, we attempted to increase the likelihood of identifying allelic variation by analysis of genes for surface proteins, which are subject to more intensive selective pressures. In previous work we showed that the S1 subunit of pertussis toxin (PtxS1) and pertactin (Prn) were polymorphic (19;82;88;92). PtxS1 and Prn

comprise 2 of the 5 *B. pertussis* proteins of ACVs. In this work, we investigated polymorphism in the remaining *B. pertussis* proteins, which are included in ACVs: the pertussis toxin subunits S2-S5 (PtxS2-S5), filamentous hemagglutinin (FHA), and serotype 2 and 3 fimbriae (Fim2 and Fim3). Further, we analyzed variation in a number of other surface-associated proteins, some of which have been shown to be important for immunity: two integral outer membrane proteins, OmpP and OmpQ (6;76), tracheal colonization factor (TcfA)(48), *Bordetella* resistance to killing protein (BrkA)(110), Vag8 (47) and *Bordetella* intermediate phase protein (BipA)(130). The polymorphic genes were used to characterize strains from widely separated geographic areas, providing a global perspective. We identified MLST's associated with epidemics, and with strains, which have spread globally. Analysis of these strains may reveal why *B. pertussis* is able to persist, and even cause epidemics, in highly vaccinated populations.

## MATERIAL AND METHODS

### *Strains*

*B. pertussis* strains were collected from 1949 to 1999. In total 205 strains were investigated: 128 from The Netherlands, 27 from Finland, 10 from Italy, 9 from Australia, 13 from Japan and 18 from The United States. A table, detailing the origin of the strains, is available from the authors. Strains were grown on Bordet Gengou agar supplemented with 1% glycerol and 4% sheep blood at 35$^{o}$C for three days.

### *DNA Sequencing*

PCR amplification of chromosomal DNA was performed by adding 1 µl of DNA to 19 µl buffer comprising 50% HotstarTaq Master mix (Qiagen, Germany), 1 µM of each primer, 5% or 10% dimethyl sulfoxide. 5% dimethyl sulfoxide was added to the PCR mix for amplification of *fim2, fim3, fhaB, ompP, ompQ, ptxS1-ptxS5* and *tcfA*. For *bipA*, *brkA*, *prn*, and *vag8* 10% dimethyl sulfoxide was used. Amplification of genes was performed in a Hybaid Omnigene incubator, using a specific program for each gene. The PCR fragments were purified with a

Qiaquick PCR purification kit (Qiagen, Germany) and sequenced with the primers used for amplification and internal primers. The primer sequences and PCR programs can be obtained from the authors. Sequence reactions were performed with an ABI Prism Big Dye terminator reaction kit and the reactions were analyzed with a model 377 or 3700 ABI DNA Sequencer (Perkin-Elmer Applied Biosystems). For *bipA*, *brkA*, *fim2*, *fim3*, *ompP*, *ompQ*, *ptxS1*, *ptxS2*, *ptxS3*, *ptxS4*, *ptxS5*, and *vag8* the complete open reading frame was sequenced. The *fhaB* gene from 13 strains was sequenced between bases 1 and 6612. This region codes for the secreted, processed, molecule incorporated in ACVs. Subsequently, sequencing of *fhaB* was confined to the region comprising bases 2250-2750, which contained the single polymorphic locus identified. The *tcfA* gene from 5 strains was sequenced completely. Two polymorphic regions were identified between bases 1 and 945. A region comprised of bases 395-945 was sequenced for all strains. As polymorphism in the region of bases 1-395, appeared to be linked to polymorphism in region 395-945 (i.e. was non-informative), it was sequenced for 1 out of 5 Dutch strains and for all strains outside The Netherlands. Previously, 2 polymorphic regions (region 1 and 2) were identified in prn, and essentially all polymorphism was found to be restricted to region 1 (92). Region 1 was sequenced for all strains, whereas region 2 was sequenced for 20% of the strains.

## RESULTS

### *Polymorphism in* B. pertussis *genes coding for surface proteins*

We investigated polymorphism in 15 genes, coding for surface proteins (Table 1). Each gene was sequenced completely for at least 5 strains, 2 strains isolated in the 1950s and used for the Dutch WCV, and 3 epidemiologically unrelated, recent, Dutch clinical isolates. The rationale behind this approach was as follows. We presumed that vaccination has selected for strains, which are antigenically distinct from the vaccine strains and hence carry different alleles for surface proteins. Thus, by comparing the vaccine strains with recent isolates from the vaccinated population, the likelihood of finding polymorphism was enhanced. Further, we included strain 18323 in our analysis, which represents a distinct

branch in the phylogenetic tree of *B. pertussis* (96;150). Subsequently, the analysis of a subset of the 15 genes, which showed polymorphism in the Dutch *B. pertussis* population after the introduction of vaccination in 1953, was

**Table 1.** *B. pertussis* genes analyzed for polymorphism.

| Gene | allele | no of strains analyzed | Frequency (N) | GenBank Accession no |
|---|---|---|---|---|
| ***ptxS1***[1] | | 205 | | M13223 |
| | *ptxS1A* | | 80% (163) | AJ245366 |
| | *ptxS1B* | | 16% (33) | AJ245367 |
| | *ptxS1D* | | 3% (7) | AJ245368 |
| | *ptxS1E* | | 1% (2) | AJ006151 |
| ***ptxS2***[1] | | 21 | | M13223 |
| ***ptxS3***[1] | | 205 | | |
| | *ptxS3A* | | 79% (161) | M13223 |
| | *ptxS3B* | | 21% (44) | AJ420987 |
| ***ptxS4***[1] | | 22 | | M13223 |
| ***ptxS5***[1] | | 22 | | M13223 |
| ***prn***[2] | | 205 | | |
| | *prn1* | | 34% (69) | AJ011091 |
| | *prn2* | | 40% (82) | AJ011092 |
| | *prn3* | | 23% (48) | AJ011093 |
| | *prn4* | | 0.5% (1) | AJ011015 |
| | *prn5* | | 1.5% (3) | AJ011016 |
| | *prn6* | | 1% (2) | AJ132095 |
| ***fim2***[1] | | 19 | | |
| | *fim2-1* | | 84% (16) | Y00527 |
| | *fim2-2* | | 16% (3) | AJ420988 |
| ***fim3***[1] | | 12 | | X51543 |
| ***brkA***[1] | | 5 | | U12276 |
| ***fhaB***[3] | | 22 | | |
| | *fhaB-1* | | 86% (19) | M60351 J04531 X52156 |
| | *fhaB-2* | | 14% (3) | AJ420989 |
| ***ompP***[1] | | 18 | | X58488 |
| ***ompQ***[1] | | 5 | | |
| | *ompQ1* | | 17% (1) | U16266 |
| | *ompQ2* | | 83% (5) | AJ420990 |
| ***tcfA***[4] | | 205 | | |
| | *tcfA1* | | 1% (2) | U16754 |
| | *tcfA2* | | 78% (160) | AJ009785 |
| | *tcfA3* | | 20% (41) | AJ420991 |
| | *tcfA4* | | 0.5% (1) | this study |
| | *tcfA5* | | 0.5% (1) | AJ420992 |
| ***vag8***[1] | | 5 | | |
| | *vag8-1* | | 17% (1) | U90124 |
| | *vag8-2* | | 83% (5) | AJ420993 |
| ***bipA***[1] | | 5 | | |
| | *bipA1* | | 83% (5) | P. Cotter |
| | *bipA2* | | 17% (1) | this study |

extended to strains from Australia, Finland, Italy, Japan and The United States. No polymorphism was found in *brkA*, *fim3*, *ompP*, *ptxS2*, *ptxS4*, and *ptxS5*. Polymorphism was observed in *bipA*, *fhaB*, *fim2*, *ompQ*, *ptxS1*, *ptxS3*, *prn*, *tcfA* and *vag8* (Fig. 1). Data from 2 genes, *ptxS1* and *prn*, were mainly from previous studies (19;82;88;92). However, here we extended previous data by analyzing Japanese isolates and Dutch isolates from 1997-1999. In total 23 point mutations were observed in the polymorphic genes, 16 of which were non-silent. The silent mutations were found in *ptxS3* (n=1), *prn* (n=4) and *vag8* (n=2), while the non-silent mutations were found in *fim2* (n=1), *fhaB* (n=1), *ompQ* (n=1) *ptxS1* (n=4), *prn* (n=5), *tcfA* (n=3) and *vag8* (n=1). In *bipA* a variable number of repeats was found (Fig. 1). Polymorphism in *bipA*, *fim2*, *fhaB*, *ompQ* and *vag8* was restricted. Two alleles were found for each of these genes, resulting from polymorphism in 1 (*bipA*, *fim2*, *fhaB*, and *ompQ*) or 3 (*vag8*) loci (Fig. 1). Further, different alleles for these genes were only observed to co-exist in the prevaccination era in The Netherlands (*fim2*, *fhaB*), or variation was only observed relative to the atypical strain 18323 (*bipA*, *ompQ*, *vag8*). In the period 1990-1999, only *bipA1*, *fim2-1*, *fhaB1*, *ompQ2* and *vag8-2* were found in the Dutch *B. pertussis* population.

The genes *ptxS1*, *ptxS3*, *prn*, and *tcfA*, revealed allelic variation in recent isolates (i.e. from the period 1980-1999), and were selected for further analysis using isolates from widely separated geographic regions. Polymorphism in *ptxS3* and *tcfA* was not described previously. For *ptxS3*, 2 alleles were found, which differed in a single nucleotide resulting in a silent mutation (Fig. 1). Five *tcfA* alleles were found (Fig. 1). In total, 5 polymorphic loci were observed in *tcfA*, one of which consisted of a 60 bp segment flanked by 2, 15 bp, direct repeats in

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[1] Genes sequenced completely. [2] *prn* region 1 was sequenced for all strains and region 2 for 20% of the strains. [3] The *fhaB* gene from 13 strains was sequenced, between bases 1 and 6612. This is the region that codes for the secreted, processed, molecule incorporated in ACVs. For the remaining strains, the region comprising bases 2250-2750 and containing the polymorphic locus, was sequenced. [4] The *tcfA* gene from 5 strains was sequenced completely. Subsequently, the region between bases 395 and 945 was sequenced for all strains. The region between bases 1 and 394 was sequenced for 1 out of 5 Dutch strains and all strains from outside The Netherlands.

**1A**

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                1909                2995
bipA1           GGC-AAC-[NNN]360-CGG-AGC-CCC-GAG-GGC-ACC-CGC-GCC-TAT-GTG-GAC-ACC-GTG-GAC
                 G   N            R   S   P   E   G   T   R   A   Y   V   D   T   V   D

bipA2           ...-...-[XXX]360-...-...-...-...-...-...-...-...-...-...-...-...-...-...
                .   .            .   .   .   .   .   .   .   .   .   .   .   .   .   .

                    521
fim2-1          AGC-AGA-ACC
                 S   R   T
fim2-2          ...-.A.-...
                 .   K   .

                    2493
fhaB1           GAT-CAA-GCC
                 D   Q   A
fhaB2           ...-..C-...
                 .   H   .

                    1025
ompQ1           GAT-CTG-GAA
                 D   L   E
ompQ2           ...-.C.-...
                 .   P   .

                    420             692           2607
vag8-1          ACG-CGT-TTG-//-GTC-GTC-GGG-//-TCG-CGC-GGC
                 T   R   L      V   V   G      S   R   G

vag8-2          ...-..C-...-//-...-.C.-...-//-...-..T-...
                 .   R   .      .   A   .      .   R   .

                    681
ptxS3A          ATA-TGC-TGA
                 I   C
ptxS3B          ...-..T-...
                 .   C

                154        388                        451               596
tcfA1           GAG-CTG-//-GGG-GGG-GGC-ACG-CAT-GAA-//-CCG-[NNN]25-GAT-//-GCA-TCG-GGT
                 E   L      G   G   G   T   H   E      P          D      A   S   G
tcfA2           A..-...-//-...-...-...-...-...-...-//-...-[XXX]25-...-//-...-...-...
                 K   .      .   .   .   .   .   .      .          .      .   .   .
tcfA3           A..-...-//-...-...-...-...-...-...-//-...-[XXX]25-...-//-...-...-.A.
                 K   .      .   .   .   .   .   .      .          .      .   .   D
tcfA4           A..-...-//-...-...-...-...-...-...-//-...-[XXX]25-...-//-.T.-...-...
                 K   .      .   .   .   .   .   .      .          .      V   .   .
tcfA5           A..-...-//-...-...-..G-CAC-GCA-TGA
                 K   .      .   .   .   H   A
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**1B**

CTGCGCCCGGACCGGGTGGTCTGGGCCGCAATGACGAAAACTCCGAAAGTTCGCTGAATCCGGGAACTCTCGGACCTTCGCCCCGGCCGG

*tcfA1*. In the other 4 alleles, the 60 bp fragment and 1 repeat was missing, suggesting that a 75 bp deletion had occurred by recombination between the repeats. Three polymorphic sites comprised single base substitutions resulting in amino acid changes. The 5th polymorphic site comprised a homo-polymeric tract of 9 (*tcfa1-4*) or 10 G's (*tcfA5*). The additional G in *tcfA5* results in a frame shift and premature termination of translation.

### *Multi-locus sequence types*

Three genes, *ptxS1*, *ptxS3* and *tcfA*, were used to define MLST's (Table 2). Although *prn* also showed polymorphism it was not used for the MLST typing scheme, as variation in *prn* is mainly due to insertion and deletion of repeat units, a process that is expected to occur relatively frequently, and is reversible. This is in contrast to the (point) mutations observed in *ptxS1*, *ptxS3* and *tcfA*. In total 9 MLST's could be distinguished.

### *Temporal trends in MLST frequencies in The Netherlands*

The availability of an extensive, well-defined, strain collection allowed a detailed analysis of MLST frequencies in The Netherlands. To investigate temporal trends, strains were stratified in different periods: 1949-1952 (the prevaccination period), 1965-1980, 1981-1985, 1986-1990, 1991-1995, 1996-1999 (Fig. 2).
Two MLST's (MLST-1 and MLST-2) were observed in the prevaccination era comprising 45% and 55% of the population, respectively. The 2 Dutch vaccine strains each represent one of the two MLST's. MLST-1 disappeared after the

**Fig. 1.** Alleles of genes coding for *B. pertussis* surface proteins. (A). Numbers refer to the underlined residues. Numbering is relative to the start of the open reading frame. Dots indicate identity, and codons are separated by dashes. Deletions are indicated by X. In *bipA*, $[NNN]_{360}$ indicates a region of 1080 bp that is absent in *bipA2*. This region contains 4 repeats indicated by arrows. A fifth repeat, indicated by the long arrow, is found in both alleles. In *tcfA1*, $[NNN]_{25}$ indicates a region of 75 bp, which is absent from the remaining alleles. The arrow below *tcfA5* indicates an inserted G, which changes the reading frame and results in premature translational termination 2 codons downstream. (B). The 60 bp segment, flanked by 2 direct repeats (underlined), which is found in *tcfA1*. The left repeat is found in the alleles *tcfA2-5*.

introduction of vaccination, and was only observed again in the period 1991-1995, when it comprised 3% of the strains analyzed. The frequency of MLST-2 also decreased after the introduction of vaccination, dropping to 33%, 30% 10%, 3% and 4%, respectively, in the periods 1965-1980, 1981-1985, 1986-1990, 1991-1995 and 1996-1999. In the period 1965-1980, a novel MLST was found (MLST-3), initially comprising 67% of the isolates, and increasing in frequency to 70% and 90% in the periods 1980-1985 and 1986-1990, respectively. In the subsequent 2 periods, 1991-1995 and 1996-1999, the frequency of MLST-3 dropped to 19% and 30%, respectively. In the period 1991-1995, two new MLST's emerged (MLST-4 and MLST-5), which were found in frequencies of 39% and 37%, respectively, in the period 1991-1995 and 26% and 40% in the period 1996-1999.

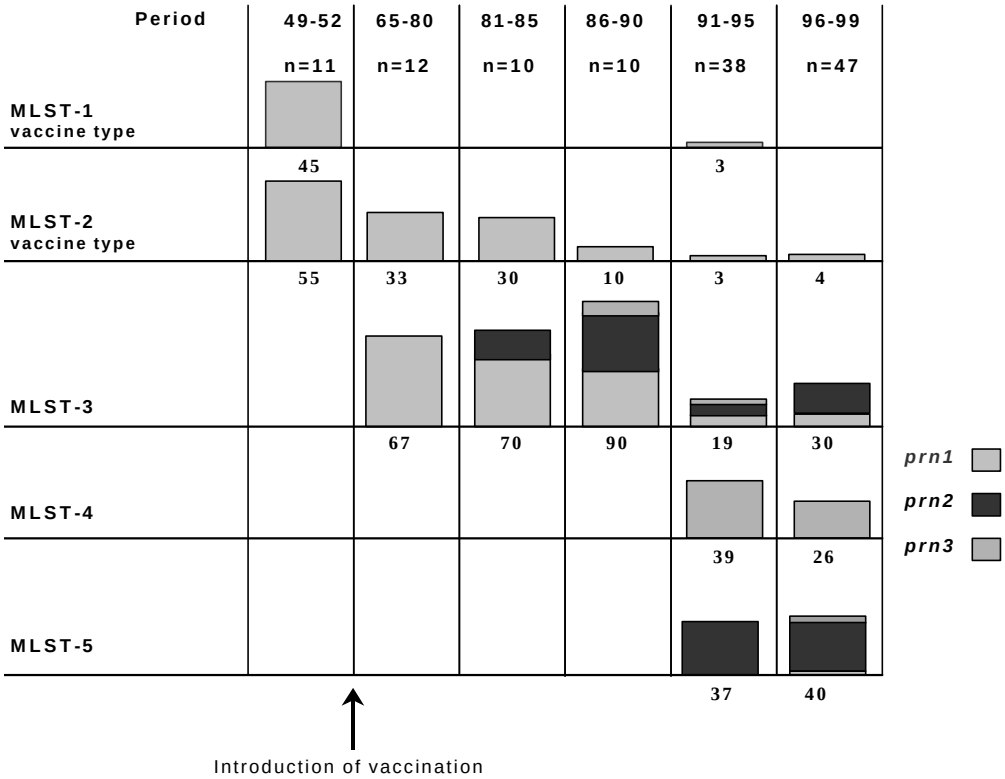


**Fig. 2.** MLST frequencies in The Netherlands in the period 1949-1999. Frequencies of MLST's within the investigated periods are indicated by numbers and bars. The distribution of *prn* alleles within a particular MLST is indicated by distinct segments.

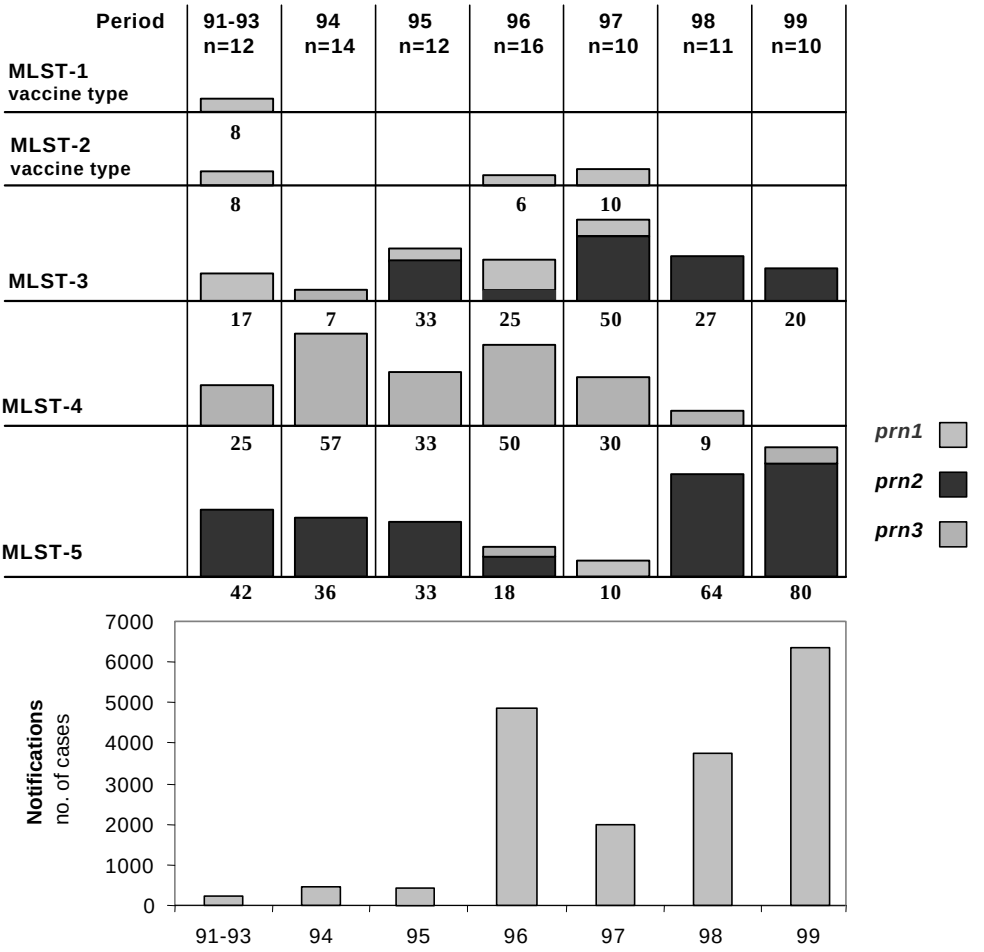


**Fig. 3.** MLST frequencies and pertussis notifications in The Netherlands in the period 1991-1999. Pertussis notifications are from the period May-April. See legend to Fig. 2 for further details.

In 1996 a severe pertussis epidemic occurred in The Netherlands, and the incidence has remained high since (33)(Fig. 3). To determine if the upsurge could be linked to particular MLST's, the period 1991-1999 was studied in more detail. Isolates were stratified in 12 month's periods: from May to April of the following year. These periods encompass the annual epidemic peak that spans the calendar year. Only a few isolates from the years 1991, 1992 and 1993 were available, and therefore they were pooled (Fig. 3). MLST-4 was observed to

dominate in the years 1994 and 1996 comprising 57% and 50% of the isolates, respectively. In the years 1991-1993, 1998 and 1999, the majority of isolates were represented by MLST-5. In the last 3 years analyzed, MLST-5 expanded in frequency from 10% to 64% and 80%.

***Global distribution of MLST's***

For a comparison of the global distribution of MLST's isolates from Australia, Finland, Italy, Japan, The Netherlands and The United States, were compared (Table 2). Isolates were stratified in 2 periods, the prevaccination period and the period 1990-1999. Strains from the prevaccination period were only available from The Netherlands and The United States. As mentioned previously, MLST-1 and MLST-2 predominated in The Netherlands in the prevaccination period (frequencies 45% and 55%, respectively). These MLST's were also observed in high frequencies in the prevaccination era in The United States, when they comprised 25% and 50% of the isolates. In The United States, an additional MLST was found (MLST-9) in this period, associated with 25% of the isolates. Although predominant in the prevaccination era, all 3 MLST's were found in low (0%-10%) frequency in the period 1990-1999 in all countries, except Japan where MLST-2 comprised 69% of the isolates. In the period 1990-1999, 8 MLST's were found in the 6 countries compared. The frequencies of the MLST's differed between countries. MLST-4 dominated in Australia and Italy with frequencies of 56% and 60%, respectively. MLST-3 dominated in Finland (frequency 89%), while MLST-5 dominated in The United States representing 60% of the isolates. MLST-4 and MLST-5 were found in approximately equal frequencies (32% and 38%) in The Netherlands in this period.

***Linkage between MLST's and* prn *alleles***

For MLST's, which were observed ≥ 3 times in a country, linkage with *prn* alleles was determined in the period 1990-1999 (Table 3). A non-random association between MLST's and *prn* alleles was observed ($P<0.0001$). MLST-4 was associated with a single *prn* allele (*prn3*). MLST-2 was linked with *prn1* (92%)

**Table 2.** Global distribution and frequencies of the MLST's in the period 1990-1999[a].

| MLST | Alleles | Country & Period | | | | | | | | Number of isolates |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Netherlands | | United States | | Australia | Finland | Italy | Japan | |
| | | prevac | 90-99 | prevac | 90-99 | 90-99 | 90-99 | 90-99 | 90-99 | |
| **MLST-1** | *ptxS1D, ptxS3A, tcfA2* | 45% | 1% | 25% | | | | | | 8 |
| **MLST-2** | *ptxS1B, ptxS3A, tcfA2* | 55% | 4% | 50% | 10% | | | | 69% | 23 |
| **MLST-3** | *ptxS1A, ptxS3A, tcfA2* | | 25% | | 10% | 22% | 89% | 40% | 15% | 54 |
| **MLST-4** | *ptxS1A, ptxS3A, tcfA3* | | 32% | | | 56% | 11% | 60% | | 41 |
| **MLST-5** | *ptxS1A, ptxS3B, tcfA2* | | 38% | | 60% | 11% | | | 15% | 42 |
| **MLST-6** | *ptxS1A, ptxS3A, tcfA4* | | | | | 11% | | | | 1 |
| **MLST-7** | *ptxS1A, ptxS3A, tcfA5* | | | | 10% | | | | | 1 |
| **MLST-8** | *ptxS1B, ptxS3B, tcfA2* | | | | 10% | | | | | 1 |
| **MLST-9** | *ptxS1E, ptxS3A, tcfA1* | | | 25% | | | | | | 2 |
| | **Number of isolates** | 11 | 85 | 8 | 10 | 9 | 27 | 10 | 13 | 173 |

[a] For The Netherlands and The United States strains from the prevaccination period have been included.

and *prn2* (8%). MLST-3 was linked with *prn1-5*, while linkage with *prn2* was most frequent (67%). Finally, MLST-5 was associated with *prn1-3*, with linkage with *prn2* (92%) being most frequent.

**Table 3.** Linkage between MLST's and *prn* alleles in the period 1990-1999[a].

| MLST | Country | *prn* alleles | | | | | Number of isolates |
|---|---|---|---|---|---|---|---|
| | | ***prn1*** | ***prn2*** | ***prn3*** | ***prn4*** | ***prn5*** | |
| **MLST-2** | Netherlands | 100% (3) | | | | | (3) |
| | Japan | 89% (8) | 11% (1) | | | | (9) |
| | **total** | **92% (11)** | **8% (1)** | | | | **(12)** |
| **MLST-3** | Netherlands | 35% (7) | 61% (13) | 4% (1) | | | (21) |
| | Finland | 4% (1) | 84% (20) | 8% (2) | 4% (1) | | (24) |
| | Italy | | | 25% (1) | | 75% (3) | (4) |
| | **total** | **16% (8)** | **67% (33)** | **8% (4)** | **2% (1)** | **6% (3)** | **(49)** |
| **MLST-4** | Netherlands | | | 100% (27) | | | (27) |
| | Finland | | | 100% (3) | | | (3) |
| | Italy | | | 100% (6) | | | (6) |
| | Australia | | | 100% (5) | | | (5) |
| | **total** | | | **100% (41)** | | | **(41)** |
| **MLST-5** | Netherlands | 3% (1) | 93% (30) | 5% (2) | | | (33) |
| | United States | | 100% (6) | | | | (6) |
| | **total** | **3% (1)** | **92% (36)** | **5% (2)** | | | **(39)** |

[a] Linkage was determined form MLST's which were identified ≥ 3 times in a country. The association between MLST and *prn* alleles was non-random ($\chi^2$-test, $P < 0.0001$). Numbers of isolates analyzed are between parentheses.

## DISCUSSION

In view of the restricted polymorphism found in *B. pertussis* house keeping genes (150)(data not shown), we attempted to increase the likelihood of identifying allelic variation by analysis of genes coding for surface proteins, which are subject to more intensive selective pressures. Our data provide the first global analysis of polymorphism in all 5 components used for ACVs. We confirmed previous observations that PtxS1 and Prn were polymorphic (19;82;88;92). Interestingly, although PtxS1 was polymorphic, the other 4

protein subunits of pertussis toxin were monomorphic. Only one, silent, mutation was detected in the gene for PtxS3. This suggests that the S1 subunit is subject to more intense (immunological) pressure. This suggests that the S1 subunit is more immunogenic compared to the other Ptx subunits, and/or that antibodies to PtxS1 affect strain fitness more severely. As to the remaining components of ACVs, a single non-silent mutation was observed in the genes for FHA and Fim2, while the gene for Fim3 was monomorphic. All Dutch isolates from 1965 or later harbored the *fhaB1* and *fim2-1*, alleles. In conclusion, of the 5 proteins included in pertussis ACVs, evidence for a mismatch between vaccine strains and clinical isolates was only found for Ptx and Prn (19;56;82;88;92). An important question is whether the observed mismatches affect the efficacy of pertussis vaccines. Using a mouse model, we have found that variation in Prn affects the efficacy of a WCV (69). One may argue that, since ACVs induce higher titers against Ptx and Prn compared to WCV, they may be less affected by the mismatch. On the other hand, protection conferred by ACVs is based on only a few proteins, while WCV induces a much broader immunity based on antigens (such as LPS), which are highly conserved. Both types of vaccines are now used, and a comparison of countries using either a WCV or an ACV, may resolve this issue in the future.

We also studied polymorphism in a number of surface-associated proteins, which are not found in ACVs: BipA, BrkA, TcfA, OmpP, OmpQ and Vag8. No polymorphism was detected in *brkA* and *ompP*, and with the exception of *tcfA*, polymorphism in the remaining genes was limited. Two alleles were observed for *bipA*, *ompQ* and *vag8*, only one of which was found in the period 1965-1999 in The Netherlands. The lack of polymorphism in the selected surface proteins was unexpected, as some of them have been implicated in immunity. E.g. micelles containing TcfA and Vag8 were protective in the intracerebral mouse test if low, non-protective amounts of Ptx, were added (60). OmpP is the major outer membrane protein of *B. pertussis* (6). High titers of bactericidal antibodies directed against OmpP were induced after vaccination with micelles in mice suggesting it may represent an effective vaccine component (107). The monomorphic nature of *B. pertussis* OmpP is unlikely to be due to structural constraints, as 0.4-1% divergence was observed with its *B. bronchiseptica* and

*B. parapertussis* homologues. Thus this work identifies a number of conserved proteins, which may be effective vaccine components.

Five *tcfA* alleles were identified. The *tcfA1* allele contains a 60 bp segment flanked by 2 direct repeats. The 60 bp segment and 1 repeat was not found in the *tcfA2-5* alleles, and it seems likely that it was deleted by recombination between the repeats. Since deletion of a sequence between 2 direct repeats is a more likely event than duplication of a non-repeated sequence, *tcfA1* is probably the progenitor of the *tcfA2-5* alleles. Interestingly, *tcfA1* is found in the 18323 strain, an atypical *B. pertussis* strain (13), sometimes erroneously classified more closely to *B. bronchiseptica* than to *B. pertussis*. We speculate that the 18323 strain may be closely related to the original *B. bronchiseptica* strain, from which *B. pertussis* evolved (96;150). Homologues of *tcfA* were also found in the *B. bronchiseptica* and *B. parapertussis* genomes (www.sanger.ac.uk). The *tcfA5* allele contained a frame shift mutation in a homo-polymeric G-track, suggesting that it is subject to phase variation. Homo-polymeric G- or C-tracks have been shown to be involved in fimbrial phase variation at the transcriptional level in *B. pertussis* (158).

We sequenced 27,862 bases derived from 15 gene segments. In total we found 23 point mutations and 4 insertions/deletions in 28 polymorphic loci indicating a very low degree of polymorphism in *B. pertussis*. For *Mycobacterium tuberculosis*, also a very homogeneous species, the lack of diversity has been suggested to be due to an evolutionary recent origin and worldwide spread of a clone in an episode of periodic selection (128). It is presumed that the agent of tuberculosis arose from the very closely related cattle pathogen *Mycobacterium bovis* by host specialization occurring since the domestication of this animal some 8,000-10,000 yrs ago (67). In a similar vain, the lack of allelic polymorphism in *B. pertussis* is consistent with this species evolving from a subset of *B. bronchiseptica*, which has adapted to the human host relatively recently (96;150).

The virtual absence of silent mutations in *B. pertussis* implicates that most amino acid variations observed increase strain fitness. The lack of polymorphism in *B. pertussis* surface-associated proteins in recent clinical isolates underlines the significance of the variation found in Prn, PtxS1 and TcfA. The polymorphic

loci in these proteins may interact directly with the immune system or other host targets, such as receptors used for attachment. In the period 1990-1999, most variation was observed in Prn and TcfA. Many studies have shown the important role of Prn in protective immunity (21;23;131). We have found that the Dutch whole cell vaccine is less effective against strains, which produce non-vaccine Prn types (69). Intranasal vaccination with an attenuated *Vibrio cholera* strain, expressing TcfA, resulted in a significant reduction in tracheal colonization, while colonization in the lungs was not affected, suggesting that antibodies to TcfA may be effective in conferring protection (22).

MLST has proven to be useful to study the epidemiology of bacterial pathogens (39;44;65), and we categorized *B. pertussis* isolates in MLST's, based on polymorphism in *ptxS1*, *ptxS3* and *tcfA*. Prn was not included in the MLST scheme, as variation is based on recombination within the repeated region or slipped strand mispairing, phenomena, which occur at relatively high frequency. Interestingly, the *prn* alleles were not distributed randomly over the MLST's ($P<0.0001$). Focusing on the 3 MLST's, which comprise a sufficient number of analyzed strains (i.e. 35-48) the following can be observed. MLST-3 and MLST-5 were associated with 5 and 3 *prn* alleles, respectively (Table 3). However, *prn2* predominated within these MLST's, (frequency 67% and 92%, respectively). In contrast, MLST-4 was found associated with the *prn3* allele only. Gupta et al. have shown that pathogen populations may segregate into discrete strains with non-overlapping antigenic structures, if these structures are immunodominant (57). Thus the non-random association of *prn* alleles with MLST's, may reflect strong immune selection focused on Prn and other (as yet unknown) *B. pertussis* antigens.

In accordance with our previous studies using IS*1002* based RFLP, MLST analysis revealed large changes in the bacterial population subsequent to the introduction of WCV in The Netherlands and The United States (19;151;153). MLST-1 and MLST-2, corresponding to the 2 vaccine strains, were predominant in the prevaccination period, but only detected in very low frequency in the *B. pertussis* population in subsequent periods. A plausible explanation for this phenomenon is that vaccination has shifted the competitive balance between strains, and selected for variants less affected by vaccine-induced immunity. In

1996 there was a severe epidemic of pertussis in The Netherlands, and the incidence has remained high since (33). MLST did not reveal obvious differences between the bacterial populations from 1991-1995 and 1996-1999, respectively. This may be due to lack of appropriate markers in our MLST system. Alternatively, the relationship between epidemics and bacterial strains may be complex, and involve factors unrelated to the bacterial population, such as changes in surveillance, host immunity and changes in vaccine quality. However, a more detailed analysis of the period 1991-1999 did reveal interesting trends. The twofold increase in notifications in 1994 compared to 1991-1993 was associated with an increase in frequency of MLST-4 from 25% to 57%. The 10-fold increase in notifications in 1996 compared to 1995 was associated with an increase in frequency of MSLT-4 from 33% to 50%. Also in 1998 and 1999 there was a substantial increase in notifications compared to 1997 (1,9 and 3,2 fold, respectively), associated with an expansion of MLST-5 from 10% in 1997 to 64% and 80% in 1998 and 1999, respectively. This suggests that epidemic years are associated with clonal expansion of particular strains. The expansion of MLST-5 is particularly remarkable. MLST-5 is identical to MLST-3 except for a silent mutation in *ptxS3*, and while MLST-5 increased in frequency from 10% in 1997 to 80% in 1999, the frequency of MLST-3 reduced from 50% to 20% in the same period. Since the mutation in *ptxS3* is very likely neutral, the distinctive behavior of these 2 MLST's must be due to an as yet unidentified polymorphic locus, which has a strong affect on strain fitness. We are currently trying to identify this locus. The identification of strains, which expand in epidemic years, illustrates the value of MLST analyses.

We extended our MLST analysis to include a number of widely separated geographic regions, including Australia, Finland, Italy, Japan and The United States. MLST-4 and MLST-5 were found to dominate in Australia, Italy and The United States (Table 2). Significantly, these MLST's predominated in years with the highest pertussis notifications in The Netherlands. However, in contrast to The Netherlands, where both MLST's were present in approximately equal frequencies in the period 1990-1999 (32% and 38%, respectively), only one type predominated in Australia, Italy and The United States. In Finland a third MLST (MLST-3) was found to predominate, comprising 89% of the isolates. In

Japan, MLST-2 comprised 69% of the isolates. Thus, although each region showed distinctive MLST frequencies, in 4 of the 6 regions MLST-4 and MLST-5 were predominant. Of the 4 regions, in which MLST-4 and MLST-5 predominated, 3 (Australia, The Netherlands and The Unites States) have reported an increased pertussis incidence (3;8;33;58). MLST-4 and MLST-5 may represent, newly emerged, successful clones. Further, analysis of strains belonging to MLST-4 and MLST-5 may reveal why *B. pertussis* has remained such a successful pathogen, despite intensive vaccination.

## ACKNOWLEDGMENT

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# 4 Changes in the Dutch *Bordetella pertussis* population in the first 20 years after the introduction of whole cell vaccines

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**ABSTRACT**

Despite the introduction of mass vaccination in 1953 in The Netherlands, pertussis is currently an endemic disease with regular epidemic outbreaks. Here we studied changes in the *Bordetella pertussis* population in the first 20 years after the introduction of vaccination by indexing IS*1002* fingerprint types, fimbrial serotypes and 15 genes coding for surface proteins. We compared three periods, the prevaccination period (1949-1952) and two subsequent periods 1953-1958 and 1965-1972. Except for fimbrial serotypes, there were no changes between the first two periods. Mortality decreased 5-fold and 543-fold in the periods 1953-1958 and 1965-1972, respectively, compared to the prevaccination period. The large decrease in mortality coincided with significant changes in the *B. pertussis* population with respect to the frequencies of fimbrial serotypes, fingerprint types and *ptxS1* alleles. A new fingerprint type (ft 29), associated with the novel *ptxS1* allele, *ptxS1A*, was observed in 50% of the isolates in the period 1965-1972. Of the 15 investigated genes, only *ptxS1* showed a mismatch between the vaccine strains and clinical isolates, suggesting that it played an important role in driving the observed changes. Our results suggest that, within 10-20 years after the introduction of mass vaccination, an adaptive response occurred consisting of clonal expansion of strains, which expressed a pertussis toxin variant distinct from the vaccine variants. This adaptation had very little, if any, effect on mortality, however.

## INTRODUCTION

In The Netherlands, pertussis is an endemic disease with regular epidemic outbreaks despite the fact that most children have been vaccinated against pertussis since 1953 (33;34). A remarkable increase in the pertussis incidence was observed since 1996 (33). In other countries a re-emergence of pertussis has also been observed (3;8;36;58). Further, although morbidity and mortality is highest in newly born and children, pertussis is now recognized as an important infectious disease of adults (17;58;122;132).

The *B. pertussis* population changed significantly after the introduction of vaccination in a number of countries (19;82;88;92). Antigenic divergence between clinical isolates and vaccine strains was observed, suggesting that adaptation of the *B. pertussis* population to vaccine-induced immunity may be an important factor in the resilience of *B. pertussis* against vaccination. DNA fingerprinting revealed two decreases in the genotypic diversity of the Dutch *B. pertussis* population, in the periods 1950-1972 and 1982-1996, which were associated with the emergence of novel pertussis toxin and pertactin types, respectively (153). These types were antigenically distinct from the type(s) present in the pertussis vaccine, suggesting that the novel strains were escape variants.

Changes in the *B. pertussis* population in the 1950s, the period when pertussis vaccines were introduced, are particularly interesting as they may illustrate how pathogens adapt to mass vaccination within a short time span. In the period 1949-1972, changes in frequencies of alleles for the S1 subunit of pertussis toxin were observed (92). Thus expansion of escape variants may have contributed to the observed decrease in genotypic diversity. However, only a limited number of strains and genes coding for immunologically relevant proteins were analyzed. Here we investigated changes in the *B. pertussis* population structure in this period in greater detail. Further, we looked for evidence that strain adaptation affected mortality.

## MATERIAL AND METHODS

### *Strains*

*B. pertussis* strains were collected in The Netherlands from 1949 to 1972. Most strains were sent to the RIVM by regional laboratories for serotyping or confirmation of identification. Strains were stored lyophilized or as a suspension at −70 $^{0}$C. A potential problem was the selection of epidemiologically related strains. Therefore, whenever possible, we selected those strains which, based on year or place of isolation were epidemiologically unrelated. Strains were restored by growth on Bordet Gengou agar (DIFCO, 0048-15-7) supplemented with 1% glycerol and 15% sheep blood at 35 $^{0}$C for 3 days.

### *DNA Sequencing*

PCR conditions for the amplification of *ptxS1* and *prn* were described previously (92). The amplification of the *tcfA* and *fim2* gene and the 500 bp fragment of *fhaB*, which contained the polymorphic locus was performed in 20 µl, containing 1 µl of DNA and 19 µl buffer comprising 50% HotstarTaq Master mix (Qiagen, Germany), 1 µM of each primer and 5% dimethyl sulfoxide. The *tcfA* gene from 5 strains was sequenced completely. Two polymorphic regions were identified between bases 1 and 945, which were amplified with the primers tcfAF (TTCTTGCGCGTCGTGTCTTC) and tcfAR3 (GCGGTTGCGGACCTTCAT). A region comprised of bases 395-945 was sequenced for all strains with primers tcfAR3 and tcfAF2 (TCGTCTGGCGGACATAACCC). As polymorphism in the region comprised of bases 1-395 appeared to be linked to polymorphism in region 395-945 (i.e. was non-informative), it was sequenced for 1 out of 5 Dutch strains with primer tcfAF. Primers used for amplification and sequencing of *fim2* were fim2for (GCGCCGGGCCCTGCATGCAC) and fim2rev (GGGGGGTTGGCGATT-TCCAGTTTCTC). For *fhaB* primers fha9F (ATCTCCGGCGAGGGG) and fha4450R (CGGTGTAATCGCCCTGTATC) were used for amplification and primers fha39R (GATTGGCGTGCAGCGAGTTC) and fha41R (GGCTAGCGCTGGCTCTGTCC) were used for sequencing. PCR amplification was performed by preheating the samples 15 min at 95$^{o}$C. Subsequent amplification of *tcfA* was performed by heating the samples 15 sec at 95$^{o}$C, initial annealing at 72$^{o}$C for 15 sec and

elongation for 1 min at 72°C. The annealing temperature was decreased every two cycles with 2°C until 62°C. Amplification of *fim2* was performed by heating the samples 15 sec at 95°C, initial annealing at 67°C for 15 sec and elongation for 1 min at 72°C. The annealing temperature was decreased every cycle with 1°C until 62°C. Amplification of *fhaB* was performed by heating the samples 15 sec at 95°C, initial annealing at 65°C for 15 sec and elongation for 4 min at 72°C. The annealing temperature was decreased every two cycles with 2°C until 55°C. The cycle with the final annealing temperature was repeated 25 times. Final elongation was performed for 10 min at 72°C. PCR fragments were purified with Qiaquick (Qiagen, Germany). Sequence reactions were performed with an ABI Prism Big Dye terminator reaction kit and the reactions were analyzed with a model 377 or 3700 ABI DNA Sequencer (Perkin-Elmer Applied Biosystems).

***Serotyping***

On a grease-free slide, bacterial colonies were emulsified in a drop of serum that contained Fim2 or Fim3 polyclonal antibodies, after which agglutination was determined macroscopically. To examine auto-agglutination the drop of serum was replaced by physiological saline.

***IS*1002 *DNA fingerprinting***

IS*1002*-based DNA fingerprinting was performed as described previously by digesting chromosomal DNA with *Sma*1 (153). The samples were analyzed on agarose gels (Pulse Field Certified, Biorad, USA), which were transferred to Hybond+ membrane (Amersham Pharmacia Biotech, Sweden) using standard DNA blotting techniques. A 293 bp IS*1002* probe was used for hybridization. Labeling of the probe with peroxidase and detection of hybridizing bands was performed according to the instructions of Enhanced Chemiluminescence Gene Detection System (Amersham Pharmacia Biotech, Sweden). The exposed films were scanned at 190 DPI (HP Scanjet IIcx/T, Hewlet Packard, USA). The films were analyzed using the Bionumerics software (Applied Maths, Belgium). Pairwise similarities of IS*1002* fingerprint patterns were calculated using the Dice coefficient of similarity. Clustering of the isolates was carried out with UPGMA.

Genotypic diversity (GD) was calculated with the following equation: $GD=\{n/n-1\}(1-\Sigma x_i^2)$, whereby n is the number of strains and $x_i$ is the frequency of ith fingerprint type (100).

### *Statistical analysis*

The statistical significance of the *ptxS1* frequencies and differences in fimbrial serotypes and fingerprint types between periods were calculated by applying $\chi^2$ test. To comply with the conditions for the $\chi^2$ test the analysis was performed on the summed fingerprint types of the clades. The significance of the genotypic diversity was estimated as follows. By computer simulation we randomly generated 1000 cross-tabulations similar to Fig. 3, under the null hypothesis of no differences in frequency of fingerprint types between periods or *ptxS1* allele groups. Under that hypothesis, fingerprint types essentially occur at random in the different periods or in *ptxS1* groups. We then counted the number of times the squared differences in genotypic diversity in the simulated tables exceeded the observed squared difference and used these counts (divided by 1000) as an estimate of the P value of the difference in genotypic diversity.

## RESULTS

### *Polymorphism in genes coding for surface proteins in the period 1949-1972*

Previously we investigated whether polymorphism occurred in 15 genes coding for *B. pertussis* surface proteins. In the Dutch *B. pertussis* population, polymorphism, resulting in amino acid changes, was detected only in *fim2*, *fhaB*, *prn*, *ptxS1* and *tcfA*. To determine whether changes in allele frequencies occurred in the period 1949-1972, these genes, or their polymorphic segments, were sequenced for a selection of strains. Only 1 *tcfA* allele was observed in the investigated period (n=27). Two *fim2* (*fim2-1* and *fim2-2*), 2 *fhaB* (*fhaB1* and *fhaB2*) 3 *prn* (*prn1*, *prn7* and *prn10*) and 4 *ptxS1* (*ptxS1A*, *ptxS1B*, *ptxS1D* and *ptxS1E*) alleles were observed (Fig. 1). The *prn7*, *fim2-2* and *fhaB2* alleles were found to be associated with *ptxS1D*, while *prn1*, *fim2-1* and *fhaB1* were linked to 2 other *ptxS1* alleles (*ptxS1A* and *ptxS1B*). The *ptxS1E* allele was linked to

*prn10*, *fim2-1* and *fhaB2* (n=1) and was not detected previously in the Dutch *B. pertussis* population. The *prn10* allele was not described previously, and contains an additional GGAVP repeat in region 1 compared to *prn6*. Thus, the following allelic combinations were observed: [*fim2-2*, *fhaB2*, *prn7*, *ptxS1D*](n=9), [*fim2-1*, *fhaB1*, *prn1*, *ptxS1B*](n=12), [*fim2-1*, *fhaB1*, *prn10*, *ptxS1E*](n=1) and [*fim2-1*, *fhaB1*, *prn1*, *ptxS1A*](n=8). Since each allelic combination could be unambiguously identified by sequencing *ptxS1* only, we focused on *ptxS1*. The *ptxS1* gene was sequenced for 66 strains.

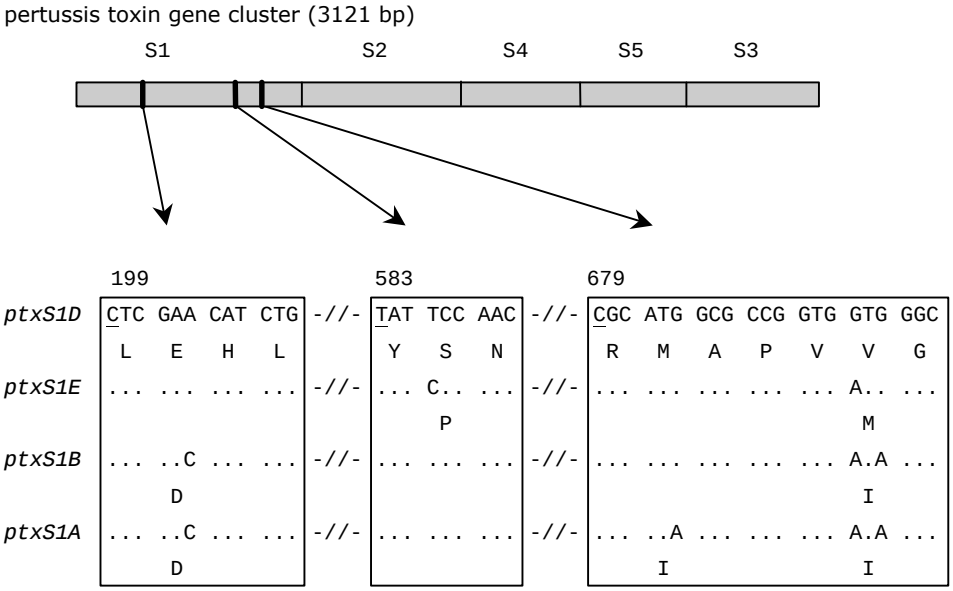



**Fig. 1.** The *ptxS1* alleles found in the Netherlands in the period 1949-1972. Dots indicate identity. The numbers indicate the position of the underlined nucleotide relative to the start of the gene.

Strains were stratified in 3 periods: 1949-1952, 1953-1958 and 1965-1972 and the frequency of *ptxS1* alleles in each period was determined (Table 1). The period 1959–1964 could not be investigated, due to lack of isolates. Large-scale vaccination in The Netherlands was started in 1953, and hence the period 1949-1952 represented the prevaccination period. In this period two *ptxS1* alleles, *ptxS1B* and *ptxS1D* were found in 58% and 42% of the isolates, respectively. In the subsequent period 1953-1958, *ptxS1B* and *ptxS1D* comprised 50% and 43%

of the isolates, respectively. The *ptxS1E* allele was found only once (7%) in the period 1953-1958. The frequencies of *ptxS1* alleles were not significantly different between the periods 1949-1952 and 1953-1958. (P=0.4). In the period 1965-1972 a shift was observed relative the previous period (1953-1958 vs. 1965-1972, P<0.0001). The frequency of *ptxS1B* decreased from 50% to 29%, while *ptxS1D* disappeared completely. Further, a new variant, *ptxS1A*, was observed in the period 1965-1972, which comprised 71% of the isolates (Table 1).

**Table 1.** Frequencies of *ptxS1* alleles and fimbrial serotypes in The Netherlands in the period 1949-1972[a].

| **allele/ serotype** | **Total** | **period** | | |
|---|---|---|---|---|
| | | **1949-1952** | **1953-1958** | **1965-1972** |
| *ptxS1A* | 30% (20) | 0% (0) | 0% (0) | 71% (20) |
| *ptxS1B* | 44% (29) | 58% (14) | 50% (7) | 29% (8) |
| *ptxS1D* | 24% (16) | 42% (10) | 43% (6) | 0% (0) |
| *ptxS1E* | 2% (1) | 0% (0) | 7% (1) | 0% (0) |
| Fim2 | 22% (13) | 29% (6) | 38% (5) | 8% (2) |
| Fim2,3 | 27% (16) | 43% (9) | 54% (7) | 0% (0) |
| Fim3 | 52% (31) | 29% (6) | 8% (1) | 92% (24) |

[a] Number of strains analyzed are shown between parentheses. The distribution of *ptxS1* alleles and fimbrial serotypes was analyzed with $\chi^2$ test. P values for the frequencies of the PtxS1 variants: 1949-1952 vs. 1953-1958, P=0.400; 1953-1958 vs. 1965-1972, P<0.0001. P values for the frequencies of the fimbrial serotypes: 1949-1952 vs. 1953-1958, P=0.426; 1953-1958 vs. 1965-1972, P<0.0001.

### *Fimbrial serotypes*

Fimbrial serotypes (Fim2, Fim2,3 or Fim3) were determined for strains, for which the *ptxS1* allele was identified (Table 1). In the period 1949-1952, Fim2, Fim2,3 and Fim3 comprised 29%, 43% and 29% of the isolates respectively. In the subsequent period (1953-1958), a decrease in Fim3 strains was observed (to 8%), while the frequencies of Fim2 and Fim2,3 increased to 38% and 54%, respectively. The frequencies of fimbrial serotypes were, however, not significantly different between the periods 1949-1952 and 1953-1958. (P=0.426). The last period (1965-1972) was quite distinct with respect to the

frequencies of fimbrial serotypes (P<0.0001, 1953-1958 vs. 1965-1972). Fim3 predominated in 92% of the isolates, while Fim2,3 was not detected.

### *IS*1002-*based DNA fingerprinting*

Strains, for which the *ptxS1* allele was identified, were also analyzed by IS*1002*-based DNA fingerprinting. Twenty-one fingerprint types were observed, which were grouped into 6 (A-F) clades of related patterns with UPMGA (Fig. 2). To observe temporal trends, strains were stratified in 3 periods: 1949-1952, 1953-1958, 1965-1972 (Fig. 3). The first 2 periods did not significantly differ in fingerprint frequencies (P=0.507). In these 2 periods most isolates were found

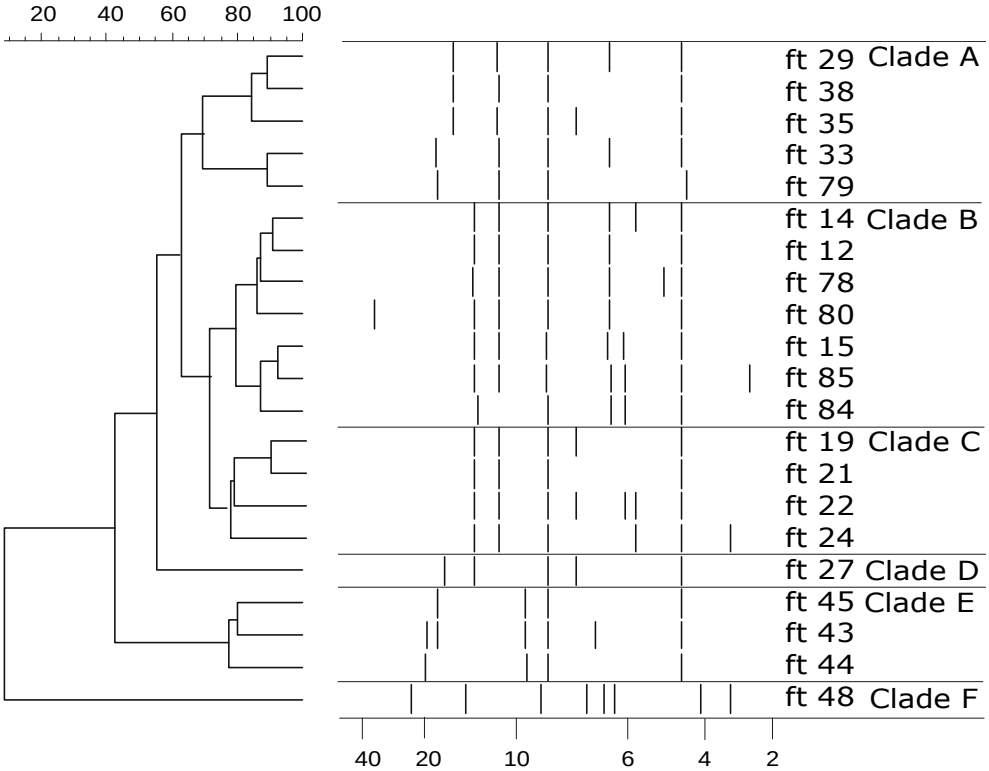
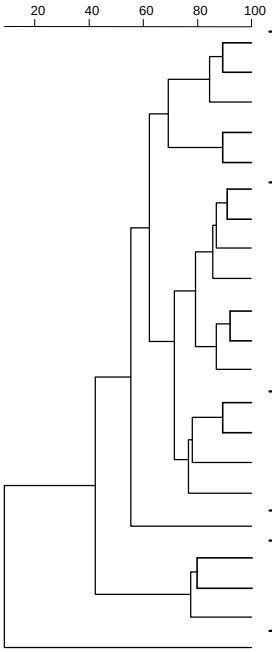



**Fig. 2.** Dendrogram of fingerprint types found in The Netherlands in the period 1949-1972. Clustering was performed using UPGMA, and 6 clades (A-F) are indicated. Upper and lower bars indicate similarity coefficient and size of fingerprint type fragments in kilobases, respectively.




| ft | | *ptxS1* | 1949-1952 | 1953-1958 | 1965-1972 |
|---|---|---|---|---|---|
| ft 29 | Clade A | *ptxS1A* | | | 50% (14) |
| ft 38 | | *ptxS1A* | | | 7% (2) |
| ft 35 | | *ptxS1A* | | | 4% (1) |
| ft 33 | | *ptxS1A* | | | 4% (1) |
| ft 79 | | *ptxS1D* | 8% (2) | | |
| ft 14 | Clade B | *ptxS1A* | | | 7% (2) |
| ft 12 | | *ptxS1B* | 4% (1) | | 11% (3) |
| ft 78 | | *ptxS1B* | 4% (1) | | |
| ft 80 | | *ptxS1B* | | 7% (1) | |
| ft 15 | | *ptxS1B* | 29% (7) | 7% (1) | |
| ft 85 | | *ptxS1B* | | 7% (1) | |
| ft 84 | | *ptxS1B* | | 14% (2) | |
| ft 19 | Clade C | *ptxS1B* | 13% (3) | 14% (2) | |
| ft 21 | | *ptxS1B* | | | 4% (1) |
| ft 22 | | *ptxS1B* | 4% (1) | | |
| ft 24 | | *ptxS1B* | 4% (1) | | |
| ft 27 | Clade D | *ptxS1B* | | | 14% (4) |
| ft 45 | | *ptxS1D* | 8% (2) | 14% (2) | |
| ft 43 | Clade E | *ptxS1D* | 4% (1) | | |
| ft 44 | | *ptxS1D* | 21% (5) | 28% (4) | |
| ft 48 | Clade F | *ptxS1E* | | 7% (1) | |
| | | **N** | 24 | 14 | 28 |
| | | **GD** | 0.87 | 0.90 | 0.73 |
| | | **Mortality** | 1.63 | 0.34 | 0.003 |

**Fig. 3.** Frequency of fingerprint types found in the Dutch *B. pertussis* population in the period 1949-1972. The period 1949-1952 represents the prevaccination period. Six clades (A-F) are indicated. Bar indicates similarity coefficient. Number of isolates is in parentheses. N is number of strains in each period. P values for differences in fingerprint type frequencies are ($\chi^2$ test): 1949-1952 vs. 1953-1958, P=0.507 and 1953-1958 vs. 1965-1972, P<0.0001. GD, genotypic diversity per period. P values for differences in GD are: 1949-1952 vs. 1953-1958, p=0.531; 1953-1958 vs. 1965-1972, p=0.004. Mortality rates are given per 100,000.

in clade B (37% and 35%, respectively) and clade E (33% and 42%, respectively). The genotypic diversity of 0.87 and 0.90 in these two periods did not differ significantly (P=0.531). Although the first 2 periods were very similar, there was a significant difference in fingerprint types and frequencies between the period 1953-1958 and the period 1965-1972 (P<0.0001). In the period 1965-1972, most (65%) fingerprint types were found in clade A, while in the 2 previous periods 9% and 0% of the fingerprint types were found in this clade. Further, in this period 50% of the strains was associated with one fingerprint

type, ft 29, a type that was not detected previously. The expansion of ft 29 strains was reflected in the genotypic diversity, which decreased to 0.73 in the period 1965-1972 (P=0.004 for 1953-1958 vs.1965-1972).

***Linkage between ptxS1 alleles and fimbrial serotypes***

The association between *ptxS1* alleles and fimbrial serotypes was random in the *ptxS1D* and *ptxS1B* group (respectively, P=0,121 and P=0.501) and non-random in the *ptxS1A* group (P=0.002)(Table 2). Most notable were the high frequencies of linkage between *ptxS1D* with Fim2,3 (71%) and *ptxS1A* with Fim3 (89%).

**Table 2.** Linkage between *ptxS1* and fimbrial serotypes [a].

| **Serotype** | ***ptxS1* alleles** | | |
|---|---|---|---|
| | ***ptxS1D*** | ***ptxS1B*** | ***ptxS1A*** |
| Fim 2 | 14% (2) | 30% (8) | 11% (2) |
| Fim 2,3 | 71% (10) | 22% (6) | 0% (0) |
| Fim 3 | 14% (2) | 48% (13) | 89% (16) |

[a] P values for the distribution of fimbrial serotypes are ($\chi^2$ test): P=0.121 for *ptxS1D,* P=0.501 for *ptxS1B* and P=0.002 for *ptxS1A.*

***Linkage between* ptxS1 *alleles and fingerprint types***

There was congruence between clustering based on fingerprint types and *ptxS1* alleles (Fig. 3). In clade A, 4 out of 5 fingerprint types were associated with *ptxS1A*. The major fingerprint type in this clade, ft 29, was uniquely associated with *ptxS1A*. In clade B, 6 out of 7 fingerprint types were associated with *ptxS1B*. Clades C, D, E and F harbored only a single *ptxS1* allele, respectively, *ptxS1B*, *ptxS1B*, *ptxS1D* and *ptxS1E*.

## DISCUSSION

In 1953 large-scale pertussis vaccination was introduced in The Netherlands. Since then the WCV has been changed several times (24-26). Initially, vaccine strains were grown on solid media. However, in 1958 production was switched to liquid media and a new strain was selected (strain 509 *fim2-2*, *fhaB2*, *prn7*,

*ptxS1D*, Fim2,3), which showed a high potency when grown in liquid media (24;25). It is not clear, which strain(s) were used previous to 1958. In the early sixties a second strain was added to the vaccine (strain 134 *fim2-1*, *fhaB1*, *prn1*, *ptxS1B*, Fim3). The strains 509 and 134 are still used nowadays.

Of the 15 investigated genes, 4 were found to be polymorphic (i.e. *fim2*, *fhaB*, *prn* and *ptxS1*). Three allelic combinations were observed in the prevaccination period and the period 1953-1958: [*fim2-2*, *fhaB2*, *prn7*, *ptxS1D*], [*fim2-1*, *fhaB1*, *prn1 ptxS1B*] and [*fim2-1*, *fhaB1*, *prn10*, *ptxS1E*]. The first 2 combinations were present in the 2 vaccine strains, used since the early sixties, while the third combination of alleles was observed in 1 isolate only. In the 1960s, only one allelic combination from the prevaccination period was observed, [*prn1*, *fim2-1*, *fhaB1*, *ptxS1B*], and a novel, 4th, allelic combination was found [*prn1*, *fim2-1*, *fhaB1*, *ptxS1A*]. Thus, the only mismatch we detected in the 1960s between the vaccine strains and circulating strains was with respect to the PtxS1 subunit, suggesting that it played an important role in driving the observed changes in the *B. pertussis* population.

In the first period after the introduction of vaccination (1953-1958), no significant changes in the frequencies of fingerprint types and the alleles investigated were observed (Table 1, Fig. 3). However, changes in frequencies of fimbrial serotypes occurred. The prevaccination *B. pertussis* population was characterized by equal frequencies of Fim2 and Fim3 strains (29%), while Fim2,3 strains were found at a higher frequency (43%)(Table 1). Similar frequencies were found in other countries in the prevaccination period (18;41). In the period 1953-1958 a decrease in Fim3 strains was observed to 8%, possibly implicating that the vaccine contained a Fim3 strain in this period.

In the second period after the introduction of vaccination (1965-1972), significant changes in frequencies of *ptxS1* alleles, fingerprint types and fimbrial serotypes were observed (Table 1). The *ptxS1A* allele, not previously detected, was found in 89% of the strains, while *ptxS1B* was observed in 29% of the strains. The *ptxS1D* allele was not detected in this period. Further, fimbrial serotype 3 increased from 8% to 92%. The most notable change in fingerprint types was the emergence of ft 29, associated with 50% of the strains. The observed changes in the *B. pertussis* population in the second period after the

introduction of vaccination were much more dramatic compared to the first period. Mortality was 543-fold and 5-fold lower in the periods 1965-1972 and 1953-1958, respectively, compared to the prevaccination period (25). This suggested that vaccination was more effective in the second period and consequently had a greater effect on the *B. pertussis* population. Further, the decrease in mortality indicates that, although vaccination affected the competitive balance between strains resulting in the expansion of escape variants, the total circulation of *B. pertussis* was decreased.

Strains with *ptxS1D* disappeared before *ptxS1B* strains (Table 1). This may have been due to the fact that early vaccine strains contained the *ptxS1D* allele (as indeed strain 509 does), while the second strain, 134 (with *ptxS1B*), was added to the vaccine in the 1960s (25). It is also possible that the *ptxS1B* allele confers a higher degree of fitness on strains compared to *ptxS1D*. Consistent with this, *ptxS1D* was not detected in strains from the prevaccination era in the United Kingdom and detected in low frequencies (14%) in the United States (19;49). In the same period, *ptxS1B* was found in frequencies of 50% and 81%, respectively, in these countries. Although *ptxS1A* was not detected in the Dutch prevaccination population, we presume that it was present in low frequencies, as it was found in the prevaccination period in both the United Kingdom and the United States (frequencies 50% and 5%, respectively)(19;49). Strains with *ptxS1A* predominate in many countries with a high vaccination coverage (92). Most likely, *ptxS1A* strains were less affected by immunity induced by the vaccine strains, which harbor *ptxS1B* and *ptxS1D*. However, as yet we cannot exclude the possibility that the emergence of *ptxS1A* strains was (also) caused by other (unknown) loci, which increased strain fitness and which were linked to *ptxS1A*.

The rise to predominance of Fim3 subsequent to the introduction of vaccines with both fimbrial serotypes (1960s) was observed not only in The Netherlands, but also in other countries (13;18;41;108). It has been suggested by Preston et al. that this phenomenon is due to the fact that Fim3 is less immunogenic than Fim2 (108;108;109). Since the rise of Fim3 frequency coincides with the rise in *ptxS1A* frequency, it is also conceivable that the Fim3-*ptxS1A* combination has a higher fitness than other Fim combinations with *ptxS1A*. It is, however, unlikely

that fimbriae played a role in the expansion of particular clones. *B. pertussis* contains both fimbrial genes, which are switched on or off randomly by insertions or deletions in a homo-polymeric C-track (158). Thus strains can switch between fimbrial serotypes with relative high frequency (115).

In general, there was congruence between clustering based on fingerprint type and *ptxS1* type (Fig. 3). Two exceptions were observed, strains with *ptxS1D* and *ptxS1A* were found in clade A and B, in which *ptxS1A* and *ptxS1B* predominated, respectively. Especially the presence of *ptxS1D* in clade A was striking, as this allele was normally found in clade E, which showed a deep branching point with clade A. This observation could be explained by horizontal transfer of *ptxS1D* to a clade A strain, or chromosomal rearrangements resulting in a change in fingerprint type (Fig. 2). The *ptxS1D* allele is generally found associated with *fim2-2*, *fhaB2* and *prn7*, while *ptxS1A* was linked to *fim2-1*, *fhaB1* and *prn1*. As the *ptxS1D* strain found in clade A harbored *fim2-2*, *fhaB2* and *prn7*, it seems likely that the close relationship of this strain with *ptxS1A* strains is due to chromosomal rearrangements. Weber et al. and Cassiday et al. studied the relationship between *B. pertussis* strains with PFGE (19;157). In contrast to our observation, they did not find congruence between clustering based on PFGE and *ptxS1* type. However, they did observe an association between PFGE type and *prn* alleles. As changes in *prn* alleles occurred more recently compared to *ptxS1* (92), this may suggest that fingerprinting based on IS*1002*-based and PFGE reveal slower and faster evolutionary clocks, respectively.

The genotypic diversity as determined by DNA fingerprinting decreased significantly from 0.90 to 0.73 in the periods 1953-1958 and 1965-1972, respectively. This may have been caused by an evolutionary bottleneck and/or by clonal expansion of particular strains, possibilities that are both consistent with the introduction of a vaccine. In the period 1965-1972, 50% of the strains belonged to 1 fingerprint type (ft 29), and all of these strains contained the *ptxS1A* allele, indicating that clonal expansion had occurred. The 100-fold decrease in mortality observed after the period 1953-1958 suggested a very significant decrease in circulation of *B. pertussis*, and thus an evolutionary bottleneck. Thus the balance of evidence suggests that the introduction of vaccination resulted in an evolutionary bottleneck and clonal expansion of

strains, harboring ft 29, PtxS1A and Fim3. Strains with PtxS1A may have been introduced by import, mutation or may have existed at a low frequency before the introduction of vaccination. Although we favor the latter possibility, in all cases the competitive balance between the strains was changed, most likely due to the introduction of vaccination, resulting in a significantly higher fitness of PtxS1A strains compared to PtxS1B and PtxS1D strains.

Although the differences between the *ptxS1* alleles are small, it seems likely that they affect strain fitness (Fig. 1). All mutations in *ptxS1* are non-silent, indicating that these alleles have emerged due to positive selection. This is also suggested by the observation that one of the polymorphic sites of the PtxS1 subunit has been implicated in binding to the T-cell receptor (30;120). Hausman et al. did not find a difference in the ability of antibodies raised with ACV to neutralize pertussis toxin variants derived from *B. pertussis* and *B. bronchiseptica* (61). However, the effect of variation in PtxS1 on immunological memory and cellular immunity has not been studied. The effect of variation of PtxS1 on fitness is probably small and may be difficult to study in vitro or in animal models.

Although in The Netherlands mortality due to pertussis decreased in the 1950s and 1960s, an increase was observed in the 1990s (data not shown). We have observed potentially adaptive mutations in 3 *B. pertussis* genes since the introduction of vaccination: *ptxS1*, *tcfA* and *prn*. Mutations in *prn* have been shown to affect efficacy of a WCV in the mouse model (69). Together, these changes may act synergistically, and reduce efficacy of pertussis whole cell vaccines.

## ACKNOWLEDGEMENT

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# 5 Effect of variation in pertactin and pertussis toxin on strain fitness of *Bordetella pertussis* in mice

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**ABSTRACT**

The emergence of novel *B. pertussis* strains was identified as one of the possible causes for the resurgence of pertussis in vaccinated populations. Antigenic divergence between clinical isolates and vaccine strains was observed for several surface proteins, including pertactin (Prn) and pertussis toxin subunit 1 (PtxS1). The vaccine types Prn1, PtxS1B and PtxS1D were replaced by the novel variants Prn2, Prn3 and PtxS1A. In this study we investigated the role of Prn in colonizing ability and the effect of variation in Prn and PtxS1 on strain fitness. For this purpose, we developed an in vivo competition mouse model, in which mice were co-infected with two isogenic strains. One strain served as reference (vaccine type) and the other as test strain (novel variant). We determined relative fitness (RF) by comparing the colonizing ability of the test strain with the reference strain. We first investigated the effect of a pertactin knock-out (Prn-ko) mutation on fitness and demonstrate here for the first time that a Prn-ko mutation reduced RF compared to the wild type strain. Next we studied the natural variation in Prn and PtxS1. We observed that in the upper respiratory tract of mice the RF of strains, carrying Prn2, Prn3 and/or PtxS1A, was reduced compared to the reference strain, carrying Prn1 and PtxS1B. In the lungs, however, the reverse was observed; the RF of the Prn2 and Prn3 strains (in combination with PtxS1A) was increased compared to the reference strain. We assume that fitness in the nasopharynx and trachea is most relevant for the human situation, since *B. pertussis* primarily is an infection of the upper respiratory tract. Thus our data suggest that strains with Prn2, Prn3 and/or PtxS1A (carrying non-vaccine type protein variants that predominate in the current *B. pertussis* population), are less fit compared to Prn1, PtxS1B strains (carrying vaccine type protein variants predominant in the prevaccination era). Vaccination may have changed the competitive balance between strains; due to immune pressure, the fitness of strains with Prn2, Prn3 and PtxS1A increased relative to strains with Prn1 and PtxS1B.

## INTRODUCTION

Whole cell vaccines against pertussis, introduced in many countries in the 1940s and 1950s, have been very successful in controlling this disease. However, in the 1990s pertussis re-emerged in a number of countries with vaccinated populations such as Australia, Canada, The Netherlands, and The United States (3;8;33;34;36;58;59). Further, although morbidity is highest in newly born and children, pertussis is now recognized as an infectious disease in adults (17;58;122;132).

The emergence of novel *B. pertussis* strains was identified as one of the possible causes for the resurgence of pertussis (91). Antigenic divergence between clinical isolates and vaccine strains was observed for several surface proteins, including pertactin (Prn), tracheal colonization factor (TcfA) and pertussis toxin subunit 1 (PtxS1)(19;59;82;88;92;157). Several studies have provided evidence that Prn and Ptx play an important role in immunity against pertussis. Sero-epidemiological studies have shown a correlation between the titer of anti-Prn antibodies and clinical protection (23;131). Vaccination with a monocomponent pertussis toxoid vaccine-induced significant protection for at least 2 years and decreased the spread of *B. pertussis* in a population (135;136). Further, the polymorphic sites of Prn and PtxS1 comprise B- and T-cell epitopes (21;30;69;120). Finally, King et al. have shown that variation in Prn affects vaccine efficacy in a mouse model (69).

In The Netherlands 3 Prn and 4 PtxS1 types were identified Prn1, Prn2 and Prn3, and PtxS1A, PtxS1B, PtxS1D and PtxS1E. The vaccine types Prn1, PtxS1B and PtxS1D were replaced by the novel variants Prn2, Prn3 and PtxS1A in the 1990s (92). The *prn2* and *prn3* alleles have not been found in strains collected in the prevaccination era in a number of countries, while the *ptxS1A* allele was not observed, or observed in low frequencies, in most countries, with the notable exception of the United Kingdom, where *ptxS1A* was found in 50% of the prevaccination strains (19;49;88;92;115). We hypothesized that the fitness of Prn2, Prn3 and PtxS1A strains was lower in unimmunized populations compared to strains with Prn1, PtxS1B and Ptxs1D. Introduction of vaccination may have allowed the emergence of novel Prn and PtxS1 variants, because the fitness of

the vaccine type strains decreased, while that of non-vaccine type strains increased, because they were less affected by vaccine-induced immunity.
In this study we show for the first time that Prn is important for colonization of the mice respiratory tract. Further, we investigated the effect of variation in Prn and PtxS1 on the ability of *B. pertussis* to colonize mice. For this purpose, we developed an in vivo competition mouse model, in which it was possible to detect small differences in colonization efficiency. Further, since clinical isolates may differ at many loci, we constructed isogenic strains, which differed only in the Prn or PtxS1 type expressed, and an antibiotic resistance marker.

## MATERIAL AND METHODS

### *Construction of strains*

Strains and plasmids used in this study are shown in Table 1 and Table 2. Isogenic derivatives of the Tohama strain B213 were constructed by allelic exchange. First, two derivatives were constructed by inserting a kanamycin (*kan*) or gentamycin (*gen*) resistance gene in *fim3*, a fimbrial gene, which is not expressed in the Tohama strain (89). To this purpose, the *kan* and *gen* genes were inserted into a copy of *fim3* contained in pKAS32 (125). The resulting plasmid, pKH7-27 or pKH8-62, was transferred to *E. coli* SM10 (136), which allowed subsequent transfer to *B. pertussis* by conjugation. The plasmid pKAS32 is not able to replicate in *B. pertussis*, and selection for kanamycin or gentamycin resulted in the isolation of *B. pertussis* clones, in which pKH7-27 or pKH8-62 was inserted into the *B. pertussis* chromosome by homologous recombination. Plating on media with kanamycin and streptomycin, or gentamycin and streptomycin, respectively, resulted in excision of pKH7-27 or pKH8-62, while the *kan* or *gen* gene, respectively, was retained. The insertion was checked by PCR and DNA sequencing and 2 strains, B1584 (*fim3::kan*) and B1585 (*fim3::gen*), were selected for further modification. The Tohama strain carried the *prn1* allele (69) and Tohama derivatives containing *prn2* or *prn3* alleles were constructed using B1585 (*prn1*, *fim3::gen*) as parental strain by exchange of the polymorphic region (region 1) as follows: Primers PrnM13*Xba*1 (GCTCTAGATGTAAAACGACGGCCAGTGGGCG-GGCAGCGGGG) and PrnM13*EcoR*1

**Table 1.** Strains used in this study.

| Strain number | Derived from | Antibiotic resistance | Genotype | Reference |
|---|---|---|---|---|
| B213 | *B.pertussis* Tohama | $Nal^R$, $Strep^R$ | *prn1, ptxS1B* | |
| B1584 | B213 | $Nal^R$, $Strep^R$, $Kan^R$ | *prn1, ptxS1B, fim3::kan* | this study |
| B1585 | B213 | $Nal^R$, $Strep^R$, $Gen^R$ | *prn1, ptxS1B, fim3::gen* | this study |
| B1586 | B1585 | $Nal^R$, $Strep^R$, $Gen^R$ | *prn2, ptxS1B, fim3::gen* | this study |
| B1587 | B1585 | $Nal^R$, $Strep^R$, $Gen^R$ | *prn3, ptxS1B, fim3::gen* | this study |
| B1684 | B1587 | $Nal^R$, $Strep^R$, $Gen^R$ | *prn3, ptxS1A, fim3::gen* | this study |
| B1814 | B1586 | $Nal^R$, $Strep^R$, $Gen^R$ | *prn2, ptxS1A, fim3::gen* | this study |
| B1686 | B213 | $Nal^R$, $Strep^R$, $Kan^R$ | *prn::kan, ptxS1B* | this study |
| MC4100?pir | *E. coli* | | | Skorpuski et al. |
| JM109 | *E. coli* | | | Promega, USA |
| SM10 | *E. coli* | $Kan^R$ | | Simon et al. |

**Table 2.** Plasmids used in this study.

| Plasmid | Derived from | Antibiotic resistance | ***Genotype*** | Reference |
|---|---|---|---|---|
| pKAS46 | | $Strep^S$, $Amp^R$, $Kan^R$ | *bla,kan, rpsL, oriT* | Scorupski et al. |
| pSS1129 | | $Strep^S$, $Amp^R$, $Gen^R$ | *bla, rpsL, oriT,gen* | Sitbitz et al. |
| pKH7-27 | pKAS32 | $Strep^S$, $Amp^R$, $Kan^R$ | *fim3::kan* | this study |
| pKH8-62 | pKAS32 | $Strep^S$, $Amp^R$, $Kan^R$ | *fim3::gen* | this study |
| pKH8-69 | pKAS46 | $Strep^S$, $Amp^R$, $Kan^R$ | *prn2* | this study |
| pKL-1.1 | pKAS46 | $Strep^S$, $Amp^R$, $Kan^R$ | *prn3* | this study |
| pIvL4-1.1 | pKAS46 | $Strep^S$, $Amp^R$, $Gen^R$ | *ptxS1A* | this study |
| pSR2-1 | pSS1129 | $Strep^S$, $Amp^R$, $Gen^R$ | *prn* PCR fragment | this study |
| pSR2-1.1 | pSS1129 | $Strep^S$, $Amp^R$, $Gen^R$, $Kan^R$ | *prn::kan* | this study |

(GGAATTCCAGGAAACAGCTATGACCCCAGCTCC-GGCGCCTCG) were used to obtain PCR products with region 1 from *prn2* and *prn3*. The PCR fragments (307 and 292 bases in size, respectively) were cloned into pKAS46, resulting in pKH8-69 or pKL-1.1. Subsequently, the procedure was similar to the insertion of the *kan* and *gen* genes, as described above, except that it was not possible to select for the insert. Therefore, strains, in which region 1 of *prn2* or *prn3* was inserted, were identified by DNA sequencing. Two strains were selected for further experiments, B1586 (*prn2, fim3::gen*) and B1587 (*prn3, fim3::gen*). The Tohama strain harbors the *ptxS1B* allele, and the same procedure as described for *prn* was used to construct Tohama-derivatives with *ptxS1A*. A PCR product of

the variable region of *ptxS1* was amplified using the primers S1AF-*Xba*1 (GCTCTAGAGCTTCC-AACGCTCGCTACGTCA) and S1AR2-*Xba*1 (GGTCTAGAA-CGAATACGCGATGCTTTCGTAG)(240 basses in size) and was cloned into pKAS46, resulting in the plasmid pIvL4-1.1. The latter plasmid was used for allelic exchange. Strains containing *ptxS1A* were identified by sequencing of the complete gene. Two strains were selected, B1684 and B1814, which were *ptxS1A* containing derivatives of B1586 and B1587, respectively. To construct a Prn knockout mutant (Prn-ko), a PCR product of *prn* (469 bases in size) was amplified using primers Prn*Xba1*F (GCTCTAGAGCCTG-GCATCCAATGAACATGT) and Prn*EcoR1*R (GGAATTCCTGTTCGCCGGCCACATAG), and cloned into pSS1129 (129), resulting in pSR2.1. Subsequently, the *kan* gene was cloned into the *Rsr*II site located in the *prn* PCR fragment (pos 184 relative to atg of *prn*), resulting in pSR2-1.1. The latter plasmid was used to introduce the *kan* gene into *prn1* of strain B213. Correct insertion was checked by sequencing and one strain, B1686 was selected for further studies. Expression of Prn and Ptx in all clones was confirmed by immunoblotting.

***Mouse infection model***

Strains were stored at –80$^{o}$C and were recovered by growth on Bordet Gengou agar (DIFCO, 0048-15-7), supplemented with 1% glycerol, 15% sheep blood and 30 µg/ml streptomycin, at 35$^{o}$C for 24 hrs. Subsequently, a number of colonies were transferred to a fresh plate without streptomycin, and incubated at 35$^{o}$C for 24 hrs, after which bacteria were harvested in Verwey medium (156). Optical densities of bacterial suspensions were measured at 600 nm and adjusted to approximately $10^{10}$ bacteria/ml. Finally, the bacterial suspensions were flash-frozen in ethanol/dry ice in small aliquots in Verwey medium with 15% glycerol and stored at –80$^{o}$C. The viability of the frozen cell suspensions was determined twice prior to infection by plating each strain on 4 plates. For infection, cells were thawed at RT and mice were infected with $2*10^{7}$ CFU of a single strain, or with $1*10^{7}$ CFU of each strain, when a mixture of 2 strains was used.

The mixture comprised a reference strain and a test strain, which were marked with either a kanamycin or gentamycin resistance gene. The ratio of the 2

strains was verified by plating a sample of the inoculum on BG plates supplemented with kanamycin or gentamycin. The ratio of the two strains in the inoculum varied between 0.7 and 1.2. After recovery of reference and test strains from mice, the number of CFUs were adjusted according to the ratio in the inoculum. Plating efficiency for BG plates supplemented with kanamycin or gentamycin were identical.

For infection, BALB/c mice (Harlan, OlHsd) 4-6 weeks old, were lightly anaesthetized with ether and 20 µl of the inoculum was placed on top of each nostril and allowed to be inhaled. After 3 days mice were sacrificed by intraperitoneal injection of Nembutal$^{R}$ (Sanofi/Algrin). The nasopharynx was sampled by flossing the nostrils with a suture, followed by introducing Verwey medium from the internal side of the animal, and collecting the first 5 droplets from the nostrils. The trachea and lungs were removed. The trachea was vortexed in 500 µl Verwey medium with 5 glass pearls for 30 sec at RT. The lungs were homogenized in 900 µl Verwey medium for 10 sec at 20,000 rpm with a blender (pro scientific, pro200) at RT. For the determination of CFU appropriate dilutions were plated on BG plates supplemented with kanamycin or gentamycin, and the number of CFUs was determined after 3 days of incubation at 35$^{o}$C. After co-infection, the relative fitness (RF) of the test strain and the reference strain was calculated as follows for each mouse: RF= log CFU test strain – log CFU reference strain. To determine RF of strains inoculated in separate infections, the average CFU of 8 mice infected with the reference strain, or the test strain, was determined, and used to calculate the RF as described above. Per strain, or strain pair, 8 mice were infected. All data are based on at least 3 independent experiments, except for the data from the nasopharynx, which were based on 1 or 2 experiment(s).

***Statistical analysis***

Means and SEM were calculated of the $^{10}$log CFU. Differences in log CFU between strains, co-infected in one mouse, were assessed by the paired Student's t-test and differences between strains infected in separate mice and RF of strain pairs were assessed by unpaired Student's t-test. Significance was accepted at the $P<0.05$ level, and all tests were two-tailed.

## RESULTS

### *A mouse model to compare strain fitness*

The aim of this study was to determine the effect of variation in the *B. pertussis* surface proteins Prn and Ptx on strain fitness. Differences in the amino acid sequences of Prn and Ptx variants, however, are small (Fig. 1) and we anticipated that they would have a minor effect on strain fitness, requiring a highly reproducible animal model. For this purpose we developed a competition model, in which mice were co-infected with two isogenic strains. One of the 2 strains served as internal reference, while the other was the test strain. The reference strain contained *prn1* and *ptxS1B*, the vaccine variants, while the test strain varied in *prn* and/or *ptxS1* allele. The advantage of using an internal reference is, that variation in absolute counts due to variation in dosage, the efficiency of inhalation of the inoculum and the size (or fraction) of the organ excised, is corrected for. Further, infection was performed with frozen cell suspensions, which retained their viability for extended periods of time, allowing reproducible dosage of the number of viable bacteria. With this approach the ratio of the two strains in the inoculum varied between 0.7 and 1.2.

After co-infection the 2 strains were recultured from the mouse nasopharynx, trachea and lungs, and their differential ability to persist was calculated as follows: relative fitness (RF) = log CFU test strain – log CFU reference strain. As the same reference strain was used within an experiment, the obtained value allowed comparison of the fitnesses of different test strains. Further, as the test strains were isogenic, differences in fitness of test strains could be attributed to distinct alleles. Co-infection required that the 2 strains were marked with distinct antibiotic resistance genes (*kan* and *gen*), and we first investigated, whether the 2 genes affected strain fitness differently.

### *The effect of antibiotic resistance genes on fitness of* B. pertussis *in the mouse model*

To investigate the effect of the antibiotic resistance genes, *kan* or *gen*, on strain fitness, mice were infected with a mixture of 2 strains B1584 (*kan*) and B1585 (*gen*), which were isogenic, except for the antibiotic resistance gene inserted

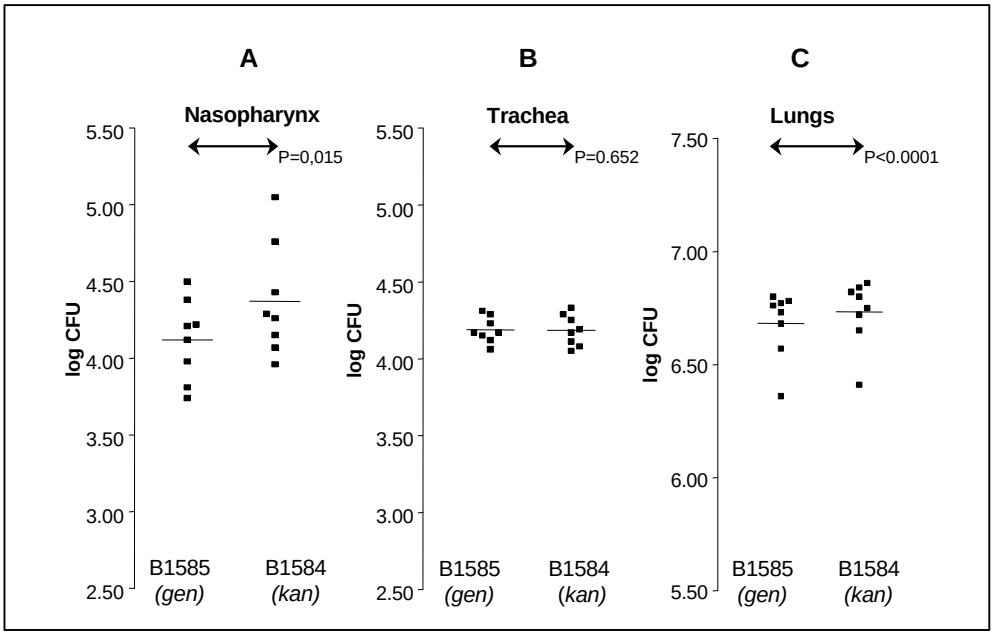
into a silent fimbrial gene (Table 1). Three days after infection the number of CFUs of both strains was determined in the nasopharynx, trachea and lungs (Fig. 2A, B and C).

```
        256
        |
Prn1    TIRRGDAPA-GGAVP-GGAVP-GG AV P-GGFGP-GGFGP- xxxxx -VL
Prn2    TIRRGDAPA-GGAVP-GGAVP-GG AV P-GGFGP-GGFGP- GGFGP -VL
Prn3    TIRRGDAPA-GGAVP-GGAVP-GG FG P-GGFGP-GGFGP- xxxxx -VL

            226
            |
PtxS1B      V R R M P
```

**Fig. 1.** Pertactin (Prn) and pertussis toxin S1 (PtxS1) variants used in this study. Differences in amino acid sequence are blocked. In the Prn sequence, the RGD sequence involved in attachment to host receptors has been underlined and the 5 amino acid repeats are separated by dashes. Gaps in the sequence are indicated by x. Prn and PtxS1 comprise 911/916 and 270 amino acids, respectively. Numbering of amino acids is relative to the N-terminus.

We found that in the nasopharynx and the lungs the *kan* strain colonized significantly better than the *gen* strain (P=0.015 and P<0.0001, respectively). The small difference in CFUs observed in the lung (i.e. 7%), was highly significant, illustrating the sensitivity of the approach. No significant difference was found in the trachea (P=0.652)(Fig. 2). The RF of the 2 strains used to co-infect is shown in Fig. 2D. Any significant deviation from zero indicates that *gen* and *kan* affect fitness differently. The RF value was negative in the nasopharynx, indicating that insertion of *gen* decreased fitness relative to *kan*, but was very close to zero in the trachea and lungs, indicating little or no differential effect.
To correct for the differential effect of *kan* and *gen*, changes in RF due to polymorphism in *prn* and *ptxS1* were compared to a reference pair comprised of strains differing only in the resistance genes.



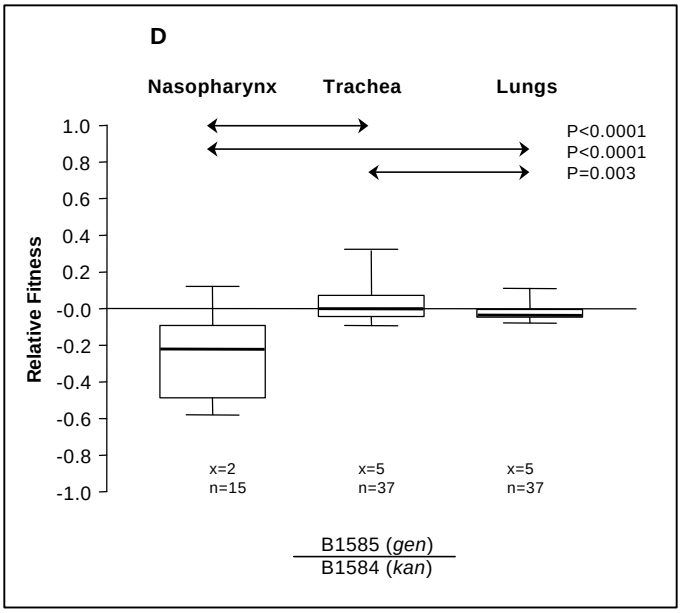
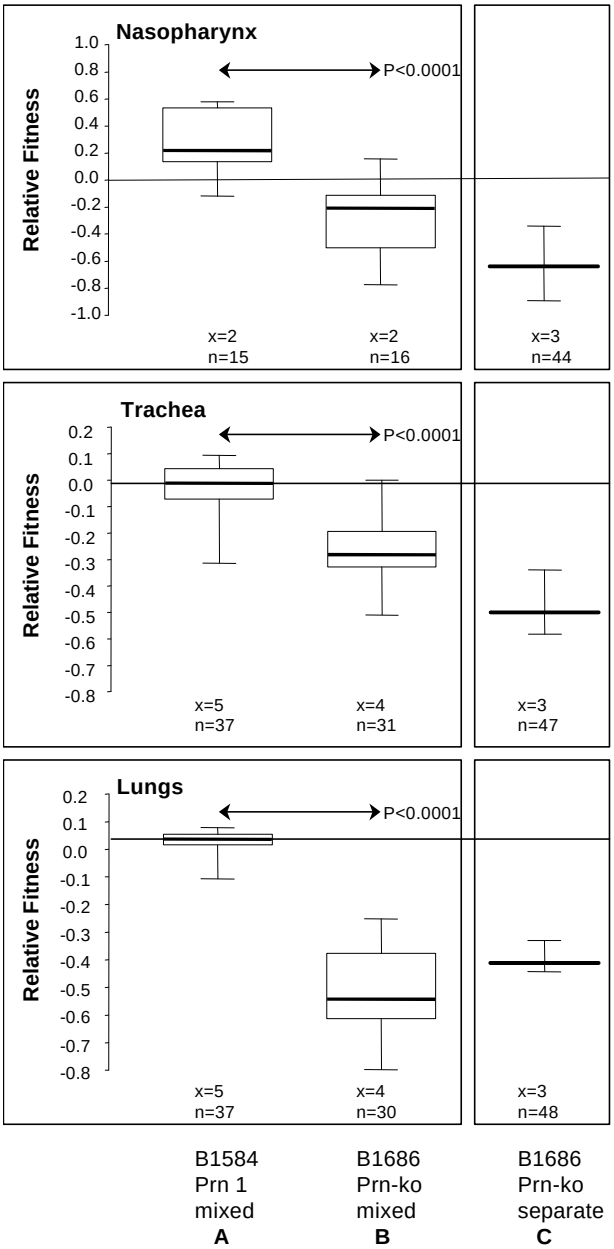




**Fig. 2.** Effect of *kan* and *gen* genes on strain fitness in the mouse. Mice were infected with a suspension containing an equal number of CFUs of strain B1584 (*kan*) and strain B1585 (*gen*). After 3 days, the number of CFUs in nasopharynx (A), trachea (B) and lungs (C) was determined. In Fig. 2A-C a representative result of 1 experiment is shown. Relative Fitness (D) was calculated as described in the text. In Fig. 2D data from all experiments were pooled. The number of experiments (x) and the total number of mice (n) are indicated. Box plots encompass 50% of the data points (25th to 75th percentile), the thick line indicates the median, whereas whiskers show the range of all data. P values are indicated.

### *The effect of inactivation of the* prn *gene on fitness of* B. pertussis *in the mouse model*




We next determined the effect of a pertactin knock-out (Prn-ko) mutation on strain fitness. Mice were co-infected with the strain pair B1686 (*prn::kan*) and B1585 (*prn1, gen*) and RF of this pair was compared with the reference pair, comprising B1584 (*prn1, kan*) and B1585 (*prn1, gen*).

**Fig. 3.** Effect of a pertactin knock-out mutation on strain fitness in the mouse. Mice were co-infected with a mixture of 2 strains: the internal reference strain B1585 (*prn1*, *gen*) in combination with the following test strains: **A.** B1584 (*prn1*, *kan*), **B.** B1686 (*prn::kan*). The horizontal line indicates the median of the RF value of B1584. In **C** mice were infected separately with B1585 (*prn1, gen*) and B1686 (*prn::kan).* Relevant characteristics of test strains are shown below the graphs. For the co-infections the RF was calculated per mouse and for the separate infections per experiment. Significant P-values are indicated. See legend to Fig. 2 for further details.

The RF of the Prn-ko strain (B1686) was significantly reduced in nasopharynx, trachea and lungs compared to the reference (B1584), which harbored the wild type *prn1* gene (Fig. 3; P<0.0001 for all 3 anatomical sites). The largest decrease in RF caused by the Prn-ko mutation compared to the reference pair was found in the lungs (lungs vs. nasopharynx and lungs vs. trachea P<0.0001). No difference in RF was found between the nasopharynx and the trachea (trachea vs. nasopharynx P=0.555). Thus, although Prn was important for persistence at all 3 anatomical sites, its role was more important in the lungs.

Our observation that a Prn-ko mutation affected RF was not consistent with results published by Roberts et al, who did not find any effect of a Prn-ko mutation on persistence in the mouse trachea and lungs (113). Because Roberts et al. used a model, in which mice were infected with single strains, we also infected mice separately with B1585 (*prn1, gen*) and B1686 (*prn::kan*), respectively. We confirmed the results of the co-infection: compared to the *prn1* strain, the Prn-ko strain was less well able to colonize nasopharynx, trachea and lungs (Fig. 3). Differences in CFUs of the Prn1 and Prn-ko strain in nasopharynx, trachea and lungs were significant (respectively, P=0.036, P<0.0001 and P<0.0001). To compensate for the differential effect of the antibiotic resistance genes, the separate infections were also performed with 2 kanamycin resistant strains: B1584 (*prn1, kan*) and B1686 (*prn::kan*). The results were similar to the separate infections of B1585 (*prn1, gen*) and B1686 (*prn::kan*)(results not shown).

### ***The effect of natural variation in pertactin on fitness of* B. pertussis *in the mouse model***

We next investigated whether strains, which carried distinct, naturally occurring,

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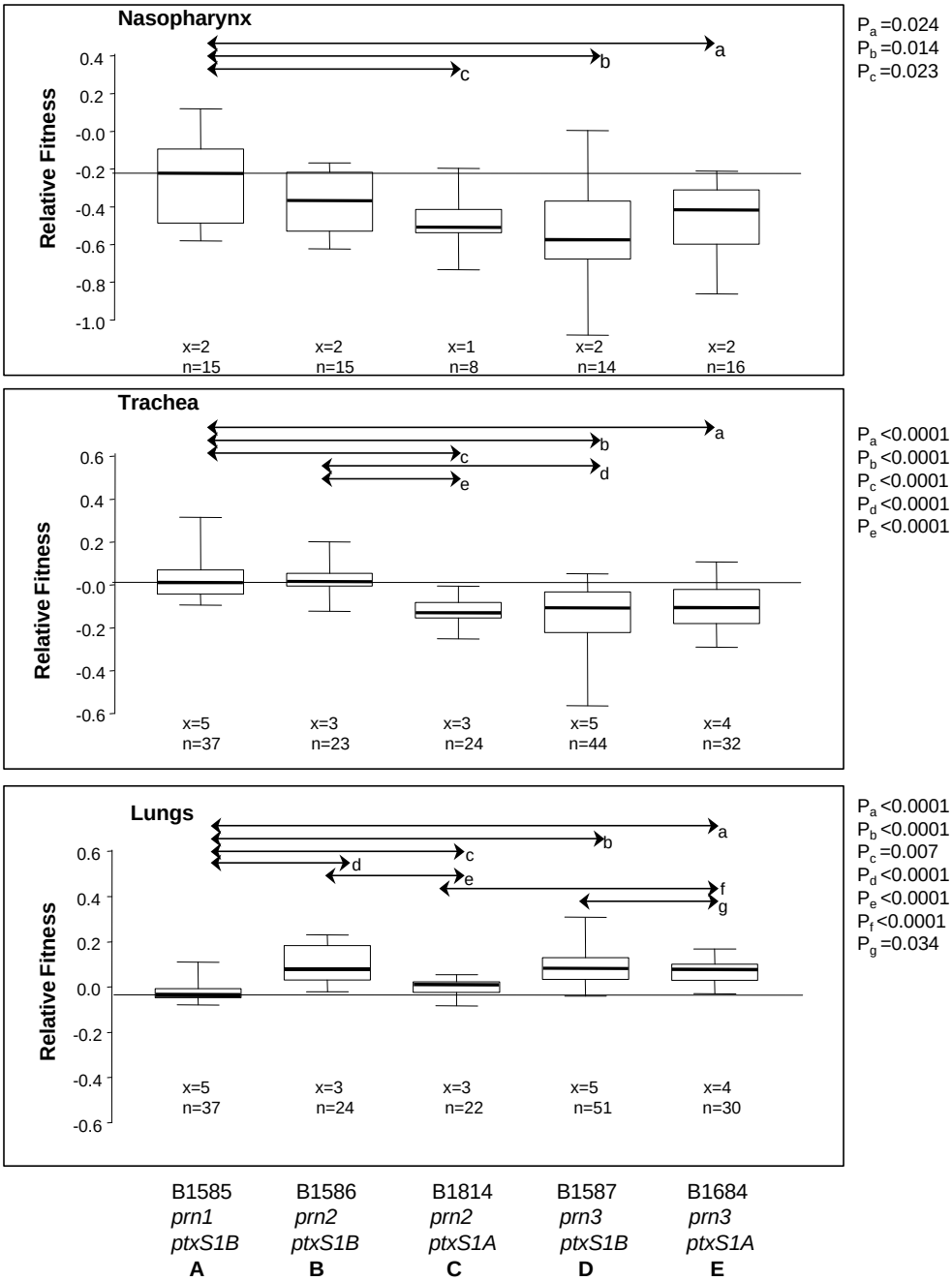
**Fig. 4.** Effect of variation in Prn and PtxS1 on strain fitness in the mouse. Mice were co-infected with a mixture of 2 strains: the internal reference strain B1584 (*prn1*, *ptxS1B*, *kan*) in combination with the following test strains: **A.** B1585 (*prn1*, *ptxS1B*, *gen*), **B.** B1586 (*prn2*, *ptxS1B*, *gen*), **C.** B1814 (*prn2*, *ptxS1A*, *gen*), **D.** B1587 (*prn3*, *ptxS1B*, *gen*) and **E.** B1684 (*prn3, ptxS1A, gen*). Relevant characteristics of test strains are indicated below the graphs. The horizontal line indicates the median of the RF value of B1585. See legend to Fig. 2 for further details.


Nasopharynx
Relative Fitness
0.4
0.2
-0.0
-0.2
-0.4
-0.6
-0.8
-1.0
a
b
c
x=2 n=15
x=2 n=15
x=1 n=8
x=2 n=14
x=2 n=16
Pa =0.024
Pb =0.014
Pc =0.023
Trachea
Relative Fitness
0.6
0.4
0.2
-0.0
-0.2
-0.4
-0.6
a
b
c
d
e
x=5 n=37
x=3 n=23
x=3 n=24
x=5 n=44
x=4 n=32
Pa <0.0001
Pb <0.0001
Pc <0.0001
Pd <0.0001
Pe <0.0001
Lungs
Relative Fitness
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
a
b
c
d
e
f
g
x=5 n=37
x=3 n=24
x=3 n=22
x=5 n=51
x=4 n=30
Pa <0.0001
Pb <0.0001
Pc =0.007
Pd <0.0001
Pe <0.0001
Pf <0.0001
Pg =0.034
B1585 prn1 ptxS1B A
B1586 prn2 ptxS1B B
B1814 prn2 ptxS1A C
B1587 prn3 ptxS1B D
B1684 prn3 ptxS1A E

*prn* alleles, showed different fitnesses in the mouse model. Mice were co-infected with the reference strain B1584 (*prn1, kan*) in combination with B1586 (*prn2, gen*) and B1587 (*prn3*, *gen*), respectively. The RFs of these strain pairs were determined as described above and compared with the reference pair B1585 (*prn1, gen*) and B1584 (*prn1, kan*) to compensate for the effect of the antibiotic resistance gene on fitness.

In the nasopharynx the *prn2* allele (B1586) did not significantly affect RF, whereas the *prn3* allele (B1587) reduced RF compared to the *prn1* allele (B1585)(Fig. 4; P=0.128 and P=0.014, respectively). The RF of the *prn3* strain (B1587) was reduced compared to the *prn2* strain (B1586), although this difference was not significant (P=0.120). Thus the results obtained with the 3 strain pairs suggested that *prn1* conferred the highest fitness in the nasopharynx, while the *prn2* allele did not significantly affect RF and *prn3* conferred the lowest fitness relative to *prn1*, i.e. *prn1* = *prn2* > *prn3*.

In the trachea, the RF of the *prn2* strain (B1586) was not affected compared to the *prn1* strain (1585) (P=0.809). However, as observed for the nasopharynx, the RF of the *prn3* strain (B1587) was reduced compared to the *prn1* (B1585) and *prn2* (B1586) strains (P<0.0001, in both cases). Based on these results the following RF order can be derived for the trachea: *prn1* = *prn2* > *prn3*.

In the lungs RF of both the *prn2* (B1586) and *prn3* (B1587) strain increased compared to the *prn1* strain (B1585)(P<0.0001 in both cases). No significant difference was observed between the *prn2* and *prn3* alleles with respect to their effect on RF in the lungs (P=0.930). Thus the fitness order in the lungs was *prn2* = *prn3* > *prn1*.

***The effect of natural variation in pertussis toxin on fitness of* B. pertussis *in the mouse model***

The effect of the *ptxS1* alleles on RF was studied by constructing strains with different combinations of *prn* and *ptxS1* alleles (Table 1). The *ptxS1A* and *ptxS1B* alleles were tested in a *prn2* and *prn3* background. Mice were co-infected with the reference strain B1584 (*prn1, ptxS1B, kan*) in combination with B1814 (*prn2, ptxS1A, gen*) and B1684 (*prn3, ptxS1A, gen*), respectively.

In the nasopharynx, exchange of the *ptxS1B* allele by *ptxS1A* did not significantly affect RF of strains with a *prn2* background (Fig. 4; compare B1586 with B1814, P=0.238). However, the combination of *ptxS1A* and *prn2* (B1814) conferred a significantly lower RF compared to the combination *ptxS1B* and *prn1* (B1585)(P=0.023), while a significant effect of *prn2* only (B1586) was not observed compared to *prn1* (B1585). In a *prn3* background no effect of the exchange of the *ptxS1B* allele by *ptxS1A* was observed (Fig. 4; compare B1587 with B1684, P=0.372). The combination of *ptxS1A* with *prn3* conferred a significantly lower RF compared to *ptxS1B* and *prn1* (Fig. 4; compare B1585 with B1684, P=0.024).

In the trachea similar effects were observed for replacement of *ptxS1B* by *ptxS1A*, i.e. a decrease in RF in a *prn2* background and no effect on RF in a *prn3* background, respectively (Fig. 4; compare B1586 with B1814 (P<0.0001) and B1587 with B1684 (P=0.128)). The allele combinations *ptxS1A-prn2* (B1814) and *ptxS1A-prn3* (B1684) reduced RF compared to the allele combination *ptxS1B-prn1* (B1585)(P<0.0001 for both).

In the lungs replacement of *ptxS1B* by *ptxS1A* resulted in a significant decrease in RF in both a *prn2* and *prn3* background (compare B1586 with B1814, P<0.0001 and B1587 and B1684, P=0.034). However, strains with *ptxS1A* in combination with *prn2* or *prn3* (respectively, B1814 and B1684) conferred a significantly higher RF in the lungs compared to the strain with *ptxS1B* and *prn1* (B1585)(P=0.007 and P<0.0001, respectively).

## DISCUSSION

The aim of this work was to explain the emergence of novel Prn and Ptx variants after the introduction of vaccination, a phenomenon observed in geographically separated regions (19;59;82;88;92;157). Our approach was to study the effect of different *prn* and *ptxS1* alleles on relative fitness (RF) in a mouse model. Relative fitness was used in a restricted sense, as the ability to colonize mice relative to a reference strain. Because of the small differences in the amino acid sequences of the Prn and PtxS1 variants (Fig. 1), we expected small effects on strain fitness, requiring a highly reproducible animal model. This was attained by

infecting mice with a suspension that contained two strains in equal numbers of CFUs, a reference strain and a test strain. The 2 strains were marked with different antibiotic resistance genes, allowing discrimination on selective plates. Further, the strains were isogenic, except for the *prn* or *ptxS1* alleles. The isogenic strains were (assumed to be) identical, except for the changes introduced deliberately. However, unlinked mutations may have been introduced, which may affect RF. We are currently investigating this possibility by reconstructing the parental genotype by back mutation, which should restore the original fitness. The sensitivity of this approach is illustrated by the finding that very small differences in CFUs recovered from mice (e.g. 7%), were found to be highly significant. Further, in our model we were able to observe significant effects of single amino acid substitutions on RF at 3 anatomical sites, nasopharynx, trachea and lungs. We assume that fitness in the nasopharynx and trachea is most relevant for the human situation, since *B. pertussis* primarily is an infection of the upper respiratory tract. However, in severe cases *B. pertussis* may colonize the lungs (126) and/or secondary complications as pneumonia might occur.

The antibiotic genes affected RF differently in the nasopharynx and lungs of mice, but not in the trachea, a phenomenon we cannot as yet explain. We compensated for this effect by relating all changes in RF to a strain pair differing only in the antibiotic gene inserted.

To our knowledge we demonstrate here for the first time a role for Prn in fitness of *B. pertussis* in an animal model. We found that the RF of a pertactin knock-out (Prn-ko) strain was decreased in nasopharynx, trachea and lungs of mice (Fig. 2). Roberts et al. did not observe an effect of a Prn-ko mutation in mouse trachea and lungs, using a model, in which mice were infected with a single strain (113). We also infected mice with the Prn-ko and wild type strain separately and observed results similarly to the mixed infections. The discrepancy between the work of Roberts et al. and our work might be due to differences in the method of infection (aerosol vs. intranasal) or differences in strains used to construct a Prn-ko (Wellcome vs. Tohama).

Although the reduction in RF was highly significant at all 3 anatomical sites, the largest decrease was observed in the lungs. This suggests that, at least in the mouse model, Prn plays an important role in colonizing the lung.

We observed that small changes in Prn (i.e. substitution of 2, or the insertion of 5 amino acids, (Fig. 1) affected RF. Interestingly, this effect was dependent on the anatomical site. E.g. while Prn1 strains were fitter in the nasopharynx and trachea compared to Prn3 strains, the reverse was observed in the lungs. This suggests that Prn may have different functions or recognize different receptors in the upper and lower respiratory tract. The variable region of Prn is located close to the RGD sequence involved in attachment to host receptors (43;74;75), and it is likely that variation affects this process.

The effect of *prn* alleles on strain fitness in trachea and nasopharynx showed interesting parallels with epidemiological data. We observed that the RF conferred by *prn* alleles in the mouse upper respiratory tract was highest for *prn1* and *prn2* and lowest for *prn3*. Prn1 was found in higher frequencies in non-vaccinated children compared to those that were vaccinated (92). Further, Prn1 strains were predominant in the prevaccination era (92). Both observations suggest that Prn1 strains are more fit than Prn2 and Prn3 strains in unvaccinated populations. After the introduction of vaccines based on Prn1 strains, Prn2 and Prn3 emerged and largely replaced Prn1. The frequencies of Prn1, Prn2 and Prn3 in vaccinated populations might reflect the level of Prn1 antibodies induced by vaccines. In vaccinated populations, Prn2 generally occurs in higher frequencies compared to Prn3 (19;59;82;88;92;157). This suggests that Prn2 confers a fitness, intermediate between Prn1 and Prn3. The observation that Prn3 confers a lower RF in the mouse upper respiratory tract suggest that Prn3 may be found in populations, in which vaccines are used, which induce high levels of Prn antibodies. Indeed, after the introduction of ACVs, which induce higher levels of Prn antibodies than WCV (37), in Sweden the frequency of Prn3 strains rose from 2% to 20% in 5 years (Hans Hallander, personal communication). In contrast, in Japan where ACVs are used that induce low amounts of Prn antibodies, Prn1 predominates, while Prn2 is found in low frequencies and Prn3 has not yet been detected (56;154). Thus vaccination may have changed the competitive balance between strains; due to immune

pressure, the fitness of Prn2 and Prn3 strains increased relative to Prn1 strains, after the introduction of Prn1 containing vaccines.

In the lungs the fitness of the Prn2 and Prn3 variants increased relative to Prn1. The significance of this observation for the biology of *B. pertussis* is not clear. However, as *B. pertussis* is found in the lungs of patients in severe cases (126), it might indicate that Prn2 and Prn3 strains are potentially more virulent than Prn1 strains.

Most vaccine strains harbor the *ptxS1B* and *ptxS1D* alleles, which were predominant in the prevaccination *B. pertussis* population (19;88;92). However, after the introduction of vaccination *ptxS1A* emerged and largely replaced the 2 vaccine types. Nowadays, most strains harbor *ptxS1A* in combination with *prn2* and *prn3*. Therefore, we studied the effect of replacement of *ptxS1B* with *ptxS1A*. This replacement decreased RF in a *prn2* background in the nasopharynx, trachea and lungs (only significant in trachea and lungs). In a *prn3* background replacement of *ptxS1B* with *ptxS1A* resulted in a decrease in RF in the lungs, while no significant effects were observed in nasopharynx and trachea. The *prn2*, *ptxS1A* and *prn3*, *ptxS1A* strains (harboring the allele combinations that predominate in the current *B. pertussis* population), were less fit compared to *prn1*, *ptxS1B* strain (representative of strains from the prevaccination era) in the nasopharynx and trachea. In the lung the reverse was observed. As discussed above, vaccination may have shifted the competitive balance by decreasing the fitness of strains harboring alleles that are present in vaccine strains (i.e. *prn1* and *ptxS1B*). The observation that the effect of exchange of *ptxS1* alleles was dependent on the *prn* allele may suggest that Prn and Ptx cooperate.

The frequency of strains is a function of their fitness in immune (vaccinated or naturally infected) and non-immune individuals. Introduction of vaccination has resulted in a shift in competitive balance between *B. pertussis* strains. In the case of *B. pertussis* changes in the population structure were observed 10 to 30 years after the introduction of vaccination. Thus, the consequences of altering levels of pathogen competition by vaccination may generally become obvious only on time scales longer than those of clinical trials. It is, therefore, important to develop mathematical models (63;145) or animal models, as presented here,

in which these processes can be studies in a shorter time frame. We are currently using this model to compare fitness of Prn and Ptx variants in immunized mice to gain insight in shifts in fitness due to vaccination.

## ACKNOWLEDGMENT

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# 6 Polymorphism in the promoter region of pertussis toxin: Has vaccination selected for more virulent strains?

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**ABSTRACT**

Although vaccination against pertussis in The Netherlands was very effective during 45 years, the disease has shown a remarkable comeback in the 1990s. Several studies showed that the *B. pertussis* population changed after the introduction of vaccination. Polymorphism in genes coding for surface proteins was observed, and temporal trends revealed antigenic divergence between vaccine strains and clinical isolates. In this study we extended our analysis of the *B. pertussis* population to genes that are involved in the regulation of virulence genes. No polymorphism was observed in the FHA promoter region, and only limited polymorphism in the *bvgAS* genes and the promoter regions of *bvgAS* and pertactin. However, a fair degree of polymorphism was observed in the promoter region of pertussis toxin (Ptx), *ptxP*. Eight alleles were observed in 6 different countries. In The Netherlands 2 alleles were observed in recent clinical isolates. One of these, *ptxP3*, emerged in 1993 and increased in frequency from 13% in 1996 to 85% in 2000, while the other, *ptxP1* decreased in frequency. Comparison of Ptx production of *ptxP1* and *ptxP3* strains showed that *ptxP3* strains produced more Ptx. Mice experiments showed that a *ptxP3* strain induced higher leucocytosis and was better able to colonize compared to a *ptxP1* strain, suggesting that *ptxP3* strains cause more severe disease compared to *ptxP1* strains. Strains, which cause more severe clinical symptoms, may show a higher transmission rate. We propose that vaccination may have selected for more virulent *B. pertussis* strains because of their improved transmissibility.

## INTRODUCTION

Although vaccination against pertussis in The Netherlands was very effective during 45 years, *B. pertussis* is currently an endemic pathogen. Further, as in many other countries, a remarkable increase in the pertussis incidence was observed in The Netherlands in the 1990s, despite the fact that most children have been vaccinated (3;8;33;34;36;58). Several studies showed that the *B. pertussis* population changed after the introduction of vaccination. Antigenic divergence between vaccine strains and clinical isolates was observed. In the 1990s, 90% of the clinical isolates contained novel, non-vaccine type, pertussis toxin and pertactin variants (19;56;59;82;88;92;157). Further, multi-locus sequence typing (MLST) revealed two MLST´s that were associated with epidemics. One of these MLST´s, MLST-5, which expanded remarkably in the years 1997 to 1999, was identical to a MLST, MLST-3, that declined in frequency in the same period, except for 1 silent mutation (154). Since it is very likely that this mutation is neutral, the distinctive behavior of MLST-3 and MLST-5 must be due to an as yet unidentified polymorphic locus, which has a strong affect on strain fitness.

In this study, we extended our studies on polymorphism to genes that are involved in the regulation of the virulence genes of *B. pertussis*. The expression of the virulence genes in *Bordetella* is regulated by the *bvgAS* system (reviewed in (83)). This system mediates transition between the virulent $Bvg^+$ phase and the avirulent $Bvg^-$ phase in response to particular environmental stimuli. In the $Bvg^+$ phase, BvgS, the transmembrane sensory protein, becomes phosphorylated and a phosphate group is transferred to BvgA, the DNA binding protein. BvgA subsequently activates promoters of virulence genes (e.g. genes coding for filamentous hemagglutinin (FHA), pertactin (Prn) and pertussis toxin (Ptx)), while in the $Bvg^-$ phase the expression of these genes is down regulated. The sensing and regulation by this system might be affected by polymorphism in BvgS and/or BvgA, resulting in altered expression of virulence genes. Further, polymorphism in promoter regions of the virulence genes of *B. pertussis* might affect binding of BvgA and RNA polymerase, which in its turn may affect expression of virulence genes.

Here, we analyzed polymorphism in *bvgA* and *bvgS*, and in the promoter regions of *bvgAS* (*bvgP*), FHA (*fhaP*), Prn (*prnP*) and Ptx (*ptxP*)(4;5;70;77). We observed significant polymorphism in the promoter region of Ptx (*ptxP*) and subsequently investigated its effect on expression of Ptx and on strain fitness in mice. Finally, we showed that a particular promoter variant is linked to MLST-5, which has expanded dramatically in recent years, explaining the distinct behaviors of MLST-3 and MLST-5.

## MATERIAL AND METHODS

### *Bacterial strains*

*B. pertussis* strains were collected from 1949 to 2000. In total 493 isolates were included: 390 from The Netherlands, 10 from Australia, 49 from Finland, 11 from Italy, 16 from Japan and 17 from The United States. The isolates were grown on Bordet Gengou agar (BG) supplemented with 1% glycerol and 4% sheep blood at 35$^{o}$C for three days.

### *Construction of isogenic strains*

Strain B1584, a streptomycin, kanamycin and nalidixin resistant derivative of Tohama, which harbors *ptxP1*, was used to construct a *ptxP3* isogenic derivative. For this, a 414 bp large PCR fragment, containing *ptxP3*, was amplified with the primers ptxP*BamH*1F and ptxP*EcoR*1R and cloned into pSS1129 (129)(Table 1). The resulting plasmid p407ptxP3, was transformed into JM109 cells (Promega, USA) for amplification and subsequently transferred to *E. coli* SM10 (124), which allowed subsequent transfer to *B. pertussis* by conjugation. The plasmid pSS1129 is not able to replicate in *B. pertussis*, and selection for gentamycin resulted in the isolation of *B. pertussis* clones, in which p407ptxP was inserted into the *B. pertussis* chromosome by homologous recombination. Plating on media with streptomycin resulted in excision of p407ptxP, resulting in strains with *ptxP1* or *ptxP3*. Strains with the *ptxP3* allele were identified by sequencing, and one clone, B1880, was selected.

**Table 1.** Primers for amplification and sequencing and PCR programs[a].

| Primer name | Primer sequence (5′ to 3′) | Position | Accession | PCR program |
|---|---|---|---|---|
| ptxP*BamH1*F | GGAATTCGCTGCAGTCCAAGGCGGTCGTC | -416 to -394 | M13223 | 15 min 95 °C<br>15 sec 95 °C > 30 sec 74 °C - 64 °C > 60 sec 72 °C<br>decrement of annealing temp. with 2 °C per 2 cycles. Final annealing temp.<br>30 cycles; 10 min 72 °C |
| ptxP*EcoR1*R | AGGATCCCGTCTTCCCCTCTGCGTTTTGATG | -26 to -3 | M13223 | |
| *bvgAF* | GACGCATCAATGACATTACGAG | -71 to -50 | M25401 | 15 min 95 °C<br>15 sec 95 °C > 30 sec 72 °C - 62 °C > 60 sec 72 °C<br>decrement of annealing temp. with 2 °C per 2 cycles. Final annealing temp. 25 cycles; 10 min 72 °C. |
| *bvgAR* | CCATGCCAATAGCGCCTGA | 867 to 885 | M25401 | |
| *bvgSF* | GGTGGAACTGATAGACCTCGCC | 588 to 609 | M25401 | 15 min 95 °C<br>15 sec 95 °C > 30 sec 72 °C - 62 °C > 60 sec 72 °C<br>decrement of annealing temp. with 2 °C per 2 cycles. Final annealing temp.<br>25 cycles; 10 min 72 °C. |
| *bvgSF2* | AGGCTACGCTGCATTACTTCC | 1207 to 1227 | M25401 | |
| *bvgSF3* | GTGGGCGTCGACGATGTATTGAT | 3111 to 3133 | M25401 | |
| *bvgSM2R* | GGTCTTGCCCAGTACCGCAT | 2497 to 2510 | M25401 | |
| *bvgSM1F* | TGACGCCTACCTCGACACCTT | 2462 to 2482 | M25401 | |
| *bvgSR* | GTAAGGTCCGCGCAAGCAG | 4384 to 4402 | M25401 | |
| *bvgAS-PF = fhaBF* | GTGTTTCCGACGGTGCAA | - 513 to -495 | M25401/ X52948 | 15 min 95 °C<br>15 sec 95 °C > 30 sec 59 °C > 60 sec 72 °C 30 cycles<br>10 min 72 °C |
| *bvgAS-PR = fhaBR* | TTGAGCCCGTCAATGCCGTTA | 101 to 121 | M25401 | |
| *prn-PF* | TGTGCTTCGGGGTCCTCAA | -393 to -375 | AJ006152 / www.sanger.ac.uk | 15 min 95 °C<br>15 sec 95 °C > 30 sec 59 °C > 60 sec 72 °C 30 cycles<br>10 min 72 °C |
| *prn-PR* | CAGTCCATCGTCAAGACCGGT | 115 to 135 | AJ006152 | |
| *ptx-PF* | AATCGTCCTGCTCAACCGCC | - 446 to -427 | M13223 | 15 min 95 °C<br>15 sec 95 °C > 30 sec 70 °C - 60 °C > 60 sec 72 °C<br>decrement of annealing temp. with 2 °C per 2 cycles. Final annealing temp.<br>25 cycles; 10 min 72 °C |
| *ptx-PR* | GGTATACGGTGGCGGGAGGA | 200 to 225 | M13223 | |

[a] Position of primers are relative to the A of the initiation codon. For *bvgS*, the primer positions are relative to the A of the initiation codon of *bvgA.*

***Sequencing***

PCR amplification of chromosomal DNA was performed in 20 µl, containing 1 µl of DNA and 19 µl buffer comprising 50% HotstarTaq Master mix (Qiagen, Germany), 1 µM of each primer and 5% dimethyl sulfoxide as previously described (154). The primers used for amplification and sequencing of *bvgA, bvgS, bvgP, fhaP, prnP* and *ptxP* and the specific PCR conditions are shown in Table 1. Allele designation was based on the complete gene sequence, except for *bvgS*. The gene for *bvgS* was sequenced completely for 6 strains, while for the remaining 16 strains allele designation was based on the sequence of the polymorphic region (comprising bases 1-400). Sequence reactions were performed with an ABI Prism Big Dye terminator reaction kit and the reactions were analyzed with a model 377 or 3700 ABI DNA Sequencer (Perkin-Elmer Applied Biosystems).

***Western Blotting***

Cell-free lysates were prepared from bacterial cultures that were incubated for 48 hrs on BG agar or 24 hrs in Thijs medium (139). Cell suspensions were adjusted to $4*10^9$ cells (OD at 600 nm) or to 1 mg/ml protein (Pierce BCA-kit, Pierce, USA), and 5 µl was added to each slot. Samples were analyzed by electrophoresis using SDS-10% polyacrylamide gels. Electrophoretic transfer of proteins to Hybond-P+ membranes (Amersham Pharmacia biotech, USA) was accomplished by semi-dry blotting according to manufacturer's instructions. After transfer of the proteins, the membranes were incubated overnight at $4^oC$ or 1 h at RT in phosphate saline buffer (PBS) containing 0.5% non-fat dry milk, 0.1% BSA and 0.1% tween 20. Subsequently, the membranes were incubated with monoclonal antibody Pem9 (α-PtxS1), P1 (α-PtxS1) or SU4 (α-PtxS4) diluted 1:1000 for 1h at RT. Membranes were washed 3 times for 5 min with PBS containing 0.1% tween 20 and subsequently incubated with alkaline phosphate (AP) labeled antimouse IgG for 1 h at RT. Membranes were developed with AP substrate after washing twice for 10 min with PBS containing 0.1% tween 20. After 5 min membranes were scanned with a fluor imager (Fluor Imager 595, Molecular Dynamics, USA). Quantification of the amount of PtxS1

was performed by calculating the area of a signal peak. PtxS1 expression by *ptxP1* and *ptxP3* strains was determined in 5 independent experiments.

***Mouse model***

Strains were flash-freezed and stored at –80°C, as previously described (155). The viability of the cell suspensions was determined prior to infection (155). For infection, BALB/c mice (Harlan, OlHsd) 4-5 weeks old, were lightly anaesthetized with ether and 20 µl of the inoculum was placed on top of each nostril and allowed to be inhaled. Five to eight mice per group were infected with either $4*10^5$ or $2*10^7$ CFUs of strain B1584 (*ptxP1*) or B1880 (*ptxP3*). Non-infected mice served as controls. After 7 days mice were sacrificed by intraperitoneal injection of Nembutal$^R$ (Sanofi/Algrin). Blood was collected by eye extraction in 16 µl $K_3$EDTA (Venoject, Belgium). Leucocyte counts were determined on a cell counter (Beckman Coulter, USA). For determination of CFUs, trachea and lungs were removed. The trachea was vortexed in 500 µl Verweij medium with 5 glass pearls for 30 sec at RT. The lungs were homogenized in 900 µl Verweij medium for 10 sec at 20,000 rpm with a blender (pro scientific, pro200) at RT. Finally, CFU appropriate dilutions were plated on BG plates supplemented with kanamycin, and the number of CFUs was determined after 3 days of incubation at 35°C. This experiment was performed 3 times.

***Statistical analysis***

Means and SEM were calculated of the leucocyte counts and the $^{10}$log CFU. Differences in expression of PtxS1, leucocyte counts and colonization were assessed by unpaired Student's t-test. Significance was accepted at the $p<0,05$ level, and all tests were two-tailed.

**RESULTS**

***Polymorphism in regulatory genes***

We investigated polymorphism in 6 genes (Table 2). Each gene was sequenced completely for at least 6 strains, the two strains that are used for the Dutch WCV and 3 epidemiologically unrelated, recent, Dutch clinical isolates. Further,

**Table 2.** *B. pertussis* genes analyzed for polymorphism[a].

| gene | allele | Number of strains | frequency of alleles | Accession number |
|---|---|---|---|---|
| *bvgA* | | 6 | | M25401 |
| *bvgS* | | 22 | | |
| | *bvgS1* | | 95% (21) | M25401 |
| | *bvgS2* | | 5% (1) | this study |
| *bvgAS-P* | | 6 | | |
| | *bvgP1* | | 83% (5) | M25401 |
| | *bvg-P2* | | 17% (1) | this study |
| *fhaP* | | 6 | | |
| *prnP* | | 6 | | |
| | *prnP1* | | 83% (5) | AJ006158 |
| | *prnP2* | | 17% (1) | this study |
| *ptxP* | | 493 | | |
| | *ptxP1* | | 63% (310) | M13223 |
| | *ptxP2* | | 2% (12) | this study |
| | *ptxP3* | | 33% (163) | this study |
| | *ptxP4* | | 0.6% (3) | this study |
| | *ptxP5* | | 0.2% (1) | this study |
| | *ptxP6* | | 0.2% (1) | this study |
| | *ptxP7* | | 0.2% (1) | this study |
| | *ptxP8* | | 0.2% (1) | this study |

[a] Allelic designation was based on the complete gene sequence, except for *bvgS*. The *bvgS* gene was sequenced completely for 6 strains, while for the remaining 16 strains the allelic designation was based on sequencing of the polymorphic region, comprising bases 1-400.

we included strain 18323 in our analysis, which represents a distinct divergent branch in the phylogenetic tree of *B. pertussis* (96;150). Subsequently, the analysis of one gene, *ptxP*, which showed substantial polymorphism in the Dutch *B. pertussis* population, was extended to strains from Australia, Finland, Italy, Japan and The United States.

No polymorphism was observed in *bvgA* and *fhaP*. For *bvgP* and the upstream region of *prn*, polymorphism was only observed relative to strain 18323 (Fig. 1). Strain 18323 contained one base substitution relative to all other strains analyzed. The promoter region of *bvgAS* (*bvgP*) contains 4 promoters, $P_{1-4}$, and the polymorphic site was found near the –35 box of $P_2$ (118;119).

The polymorphic site in *prnP* was located 38 bases downstream the start site for transcription and 113 bases upstream of the initiation codon of *prn* (70). We

```
            364
bvgS1  GGG CAG ATC GAC CTC
        G   Q   I   D   L
bvgS2  ... ... .C. ... ...
        .   .   T   .   .

             -41    -35box     -10box of P2
bvgP1  CCAGCGCAGGAATTCAGA-//-TAGGAT
bvgP2  .......G..........-//-......

       -35 box   -10 box    +1            +38       +151
prnP1  TTGCTTA-//-GAGAAT-//-CTG-//-GACAAATACCGTGC-//-ATG
prnP2  .......-//-......-//-...-//-........G.....-//-...
```

**Fig. 1.** Alleles of *bvgS*, and the promoter regions for *bvgAS* (*bvgP*) and *prn* (*prnP*), respectively (70; 118;119). Dots indicate identity. Numbers refer to the underlined base. Numbering of *bvgS* is relative to the A of the initiation codon, while numbering of the promoter sequences is relative to start of transcription. For *prnP* the initiation codon has been indicated.

observed two alleles for *bvgS* in Dutch *B. pertussis* strains one of which, *bvgS2*, was observed in one clinical isolate only (n= 22) (Fig. 1). Polymorphism in the abovementioned genes was not analyzed further, because of the low degree of variation observed.

For *ptxP*, in total 8 alleles were found: *ptxP1-8* (Fig. 2). Compared to *ptxP1*, the alleles *ptxP2, ptxP3, ptxP5, ptxP6 ptxP7* and *ptxP8* each contained 1 polymorphic site, while *ptxP4* harbored 8 polymorphic sites. The polymorphic sites of *ptxP2*, *ptxP5, ptxP6* and *ptxP7* were found near or at the BvgA binding site (Fig. 2). The polymorphic sites of *ptxP3* and *ptxP8* were found near or at the RNA polymerase binding site, while the polymorphic sites in *ptxP4* were found in the whole promoter region (77;80).

### *Temporal trends in frequencies of* ptxP *alleles in The Netherlands*

The availability of an extensive, well-defined, strain collection allowed a detailed analysis of temporal trends in the frequencies of the *ptxP* alleles in The Netherlands. Analysis of 390 Dutch isolates revealed the presence of the alleles *ptxP1*, *ptxP2*, *ptxP3*, *ptxP4*, *ptxP6* and *ptxP7* (Fig. 2). To study temporal trends,

```
-170      -160      -150      -140      -130      -120      -110      -100       -90       -80
  |         |         |         |         |         |         |         |         |         |
AACGCGCATGCGTGCAGATTCGTCGTACAAAACCCTCGATTCTTCCGTACATCCCGCTACTGCAATCCAACACGGCATGAACGCTCCTTCGGCGCAAAGT   ptxP1

......T.......................................................................................   ptxP2
.......................................................................................................   ptxP3
......T........G...G.....................A.................................G.A.....................   ptxP4
.......................................................A.......................................   ptxP5
.......................................................T.......................................   ptxP6
...............................................G.......................................   ptxP7
.......................................................................................................   ptxP8

 -70       -60       -50       -40       -30       -20       -10       +1        +10       +20
  |         |         |         |         |         |         |         |         |         |
CGCGCGATGGTACCGGTCACCGTCCGGACCGTGCTGACCCCCCTGCCATGGTGTGATCCGTAAAATAGGCACCATCAAAACGCAGAGGGGAAGACGGGATG   ptxP1
                                                                          *---------------->
.......................................................................................................   ptxP2
........A..............................................................................................   ptxP3
.................................................................C.G.......................   ptxP4
.......................................................................................................   ptxP5
.......................................................................................................   ptxP6
.......................................................................................................   ptxP7
....................T..................................................................................   ptxP8
```

**Fig. 2.** DNA sequence of the *ptxP* alleles. Bases are numbered relative to the start of transcription, which is marked by an asteriks. Bases –156 to – 117 (box) comprise the BvgA binding site. The bases –60 to –41 comprise the RNA binding site. The shaded area and arrows indicate inverted or direct repeats. Bases –41 to –35 and –12 to –6 (underlined) are the –35 and –10 boxes (77;80).

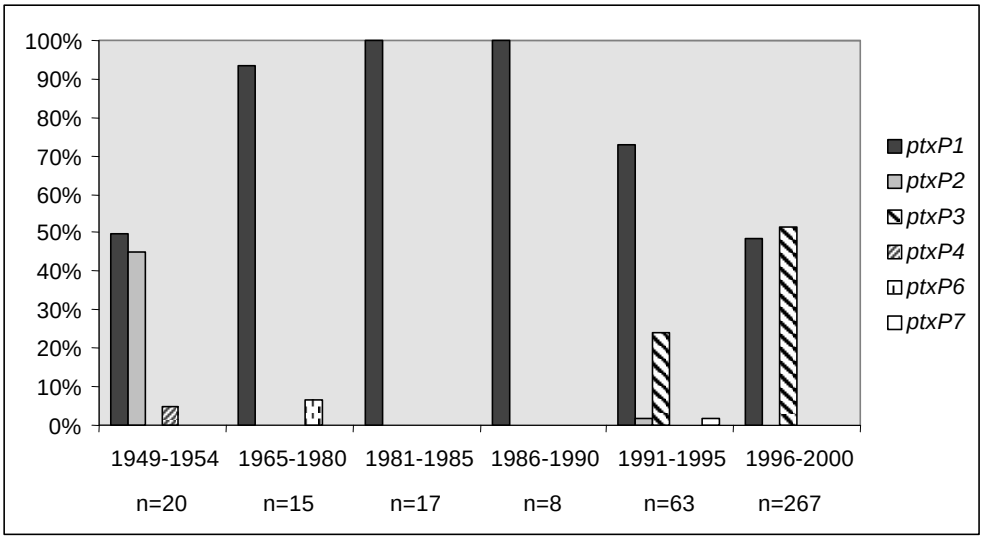


**Fig. 3.** Temporal trends in frequencies of *ptxP* alleles in The Netherlands in the period 1949-2000. N indicates number of isolates.

strains were stratified in the periods 1949-1954, 1965-1980, 1981-1985, 1986-1990, 1991-1995 and 1996-2000 (Fig. 3). The alleles *ptxP1* and *ptxP2* were present in the 2 strains used to produce the Dutch WCV and were found in, respectively, 50% and 45% of the strains from the period 1949-1954. One isolate from this period contained *ptxP4* (5%). In the period 1965-1980 2 alleles were observed, *ptxP1* and *ptxP6*, with frequencies of 93% and 7%, respectively. In the 2 subsequent periods, 1981-1985 and 1986-1990, only *ptxP1* was observed. In the period 1991-1995 *ptxP1*, *ptxP3*, *ptxP2* and *ptxP7* were found at frequencies of 73%, 24%, 2% and 2%, respectively. In the period 1996-2000 the frequency of *ptxP3* increased to 52%, while *ptxP1* was found in 48% of the isolates.

In 1996 a severe pertussis epidemic occurred in The Netherlands and the incidence has remained high since (33;34). Therefore the period 1990-2000 was studied in more detail by stratifying strains in smaller periods (Fig. 4). Data from the years 1990-1992 were pooled, because of the low numbers of isolates available. In the period 1990-1992 *ptxP1*, *ptxP2* and *ptxP7* were found with frequencies of 88%, 6%, and 6%, respectively. The *ptxP3* allele was first

observed in 1993 and found in 21% of the isolates. The *ptxP3* allele increased in frequency to 41% in 1995, followed by a drop to 13% in 1996. In the subsequent years a remarkable expansion of *ptxP3* strains was observed. The *ptxP3* allele was found in 33% of the strains in 1997 and strains with *ptxP3* expanded to frequencies of 56%, 67% and 85% in 1998, 1999 and 2000, respectively.

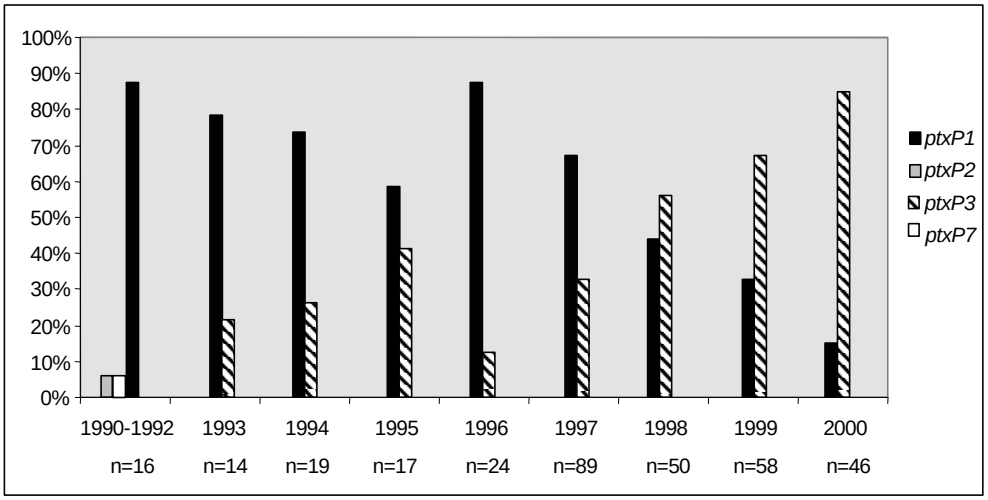


**Fig. 4.** Temporal trends in frequencies of *ptxP* alleles in The Netherlands in the period 1990-2000. Data from the years 1990-1992 were pooled, due to low number of isolates. N indicates number of isolates.

### *Global distribution of* **ptxP** *alleles*

For a comparison of the global distribution of *ptxP* alleles, isolates from the period 1990-2000 from Australia, Finland, Italy, Japan, The Netherlands and The United States, were compared (Table 3). From The Netherlands and The United States, isolates from the prevaccination period were also included. In this period, the alleles *ptxP1*, *ptxP2* and *ptxP4* were found at frequencies of 43%, 29% and 29% in The United States, compared to 50%, 45% and 5% in The Netherlands. In the period 1990-2000, the allele *ptxP1* was found in all countries investigated. However, it was observed in much higher frequencies (81%-98%) in Australia, Finland, Italy and Japan, compared to The Netherlands and the United States, where it was found at frequencies of 53% and 30%, respectively.

**Table 3.** Global distribution and frequencies of *ptxP* alleles in the period 1990-2000[a].

| ***ptxP* allele** | **Country & period** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Netherlands** | | **United States** | | **Australia** | **Finland** | **Italy** | **Japan** |
| | **Prevacc** | **1990-2000** | **Prevacc** | **1990-2000** | **1990-2000** | **1990-2000** | **1990-2000** | **1990-2000** |
| *ptxP1* | 50% (10) | 53% (178) | 43% (3) | 30% (3) | 90% (9) | 98% (48) | 91% (10) | 81% (13) |
| *ptxP2* | 45% (9) | 0.003% (1) | 29% (2) | | | | | |
| *ptxP3* | | 46% (153) | | 70% (7) | 10% (1) | 2% (1) | | 13% (2) |
| *ptxP4* | 5% (1) | | 29% (2) | | | | | |
| *ptxP5* | | | | | | | 9% (1) | |
| *ptxP7* | | 0.003% (1) | | | | | | |
| *ptxP8* | | | | | | | | 6% (1) |

[a] From The Netherlands and The United States isolates from the prevaccination period have been included. Number of isolates is in parenthesis.

In this period *ptxP3* was observed in all countries, except Italy. High frequencies of this allele were only observed in The Netherlands and the United States (46% and 70%, respectively). Finally, the alleles *ptxP5*, *ptxP7* and *ptxP8* were found in single isolates in Italy, The Netherlands and Japan, respectively.

### *Effect of polymorphism in* ptxP *on expression of PtxS1*

In order to determine the effect of the polymorphism in *ptxP* on expression of Ptx cell-free extracts of clinical isolates that contain *ptxP1*, *ptxP2* or *ptxP3* (the *ptxP* alleles that were observed relatively frequently) were examined by Western blot analysis (Fig. 5).

The membranes were incubated with a monoclonal antibody directed against PtxS1, Pem9 and PtxS1 expression was quantified by densitometry. For *ptxP1*, *ptxP2* and *ptxP3*, 3 clinical isolates were examined. The average signal of the *ptxP1* isolates was adjusted at 100%. The *ptxP2* isolates produced 61% PtxS1 relative to *ptxP1* strains (P=0.104), while *ptxP3* isolates produced 208% PtxS1 relative to *ptxP1* strains (P=0.002). This experiment was repeated 5 times and PtxS1 expression of *ptxP3* relative to *ptxP1* strains varied between 130% to 208%.

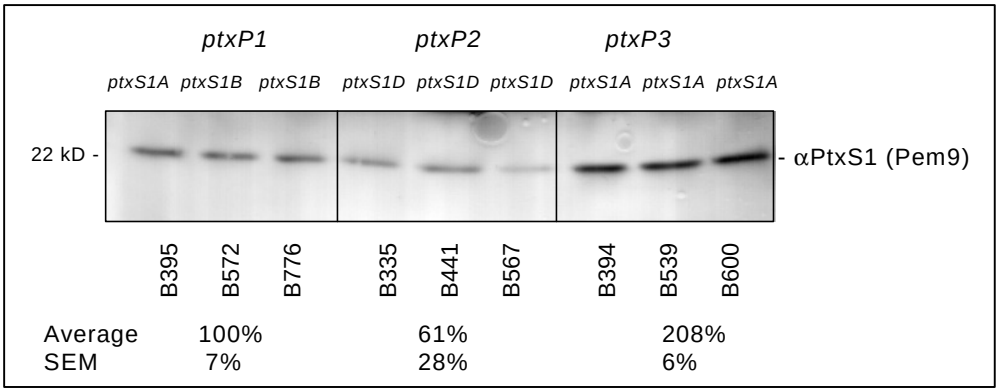


**Fig. 5.** The effect of polymorphism in *ptxP* on expression of pertussis toxin. Equal amounts of strains were analyzed by Western blotting using a monoclonal antibody against PtxS1. Expression of PtxS1 was quantified by densitometry, and the average PtxS1 expression of *ptxP1* strains was adjusted to 100%. Average of PtxS1 expression of *ptxP2* and *ptxP3* strains relative to *ptxP1* strains is shown. SEM of the *ptxP1*, *ptxP2* and *ptxP3* is shown. The *ptxP* and *ptxS1* alleles harbored by the strains are indicated above the lanes.

Monoclonal antibody Pem9 bound to PtxS1, the subunit of Ptx that contains several polymorphic sites (92). The 3 *ptxP1* strains expressed either PtxS1A or PtxS1B, while the *ptxP2* and *ptxP3* strains expressed PtxS1D and PtxS1A, respectively (Fig. 5). Therefore, it was possible that the Western blot results were affected by differential binding of Pem9 to PtxS1. To investigate this, Western blots were also performed with another monoclonal antibody against PtxS1 (designated P1) and a monoclonal antibody directed against PtxS4, which is monomorphic (designated SU4). For P1 and SU4 similar results as for Pem9 were found (data not shown).

***Effect of variation in* ptxP *on colonization and leucocytosis***

Mice were infected with the isogenic strains, B1584 (*ptxP1*) or B1880 (*ptxP3*) with either $5*10^4$ or $2*10^7$ CFU. The ability of these strains to colonize trachea and lungs and cause leucocytosis was determined 7 days after infection.

At the low dose, B1880 (*ptxP3*) colonized the trachea and lungs of mice significantly better compared to B1584 (*ptxP1*)(p=0.002 and p=0.005, respectively)(Fig. 6). At the high dose, no significant difference in colonization was observed (Fig. 6).

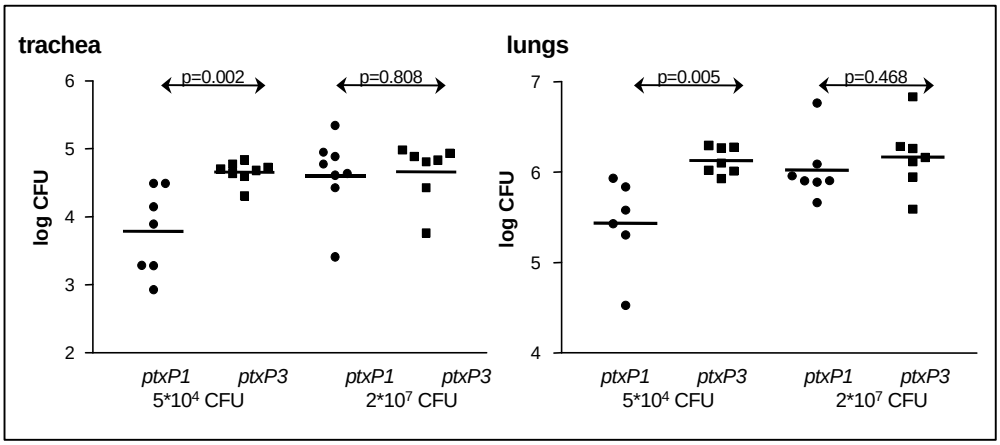


**Fig. 6.** Effect of polymorphism in *ptxP* on colonization of mice. Mice were infected with $5*10^4$ or $2*10^7$ CFUs of B1584 (*ptxP1*) and B1880 (*ptxP3*) and colonization of trachea and lungs was determined 7 days after infection. The line indicates the median. P-values are shown. This experiment was performed three times and a representative result is shown.

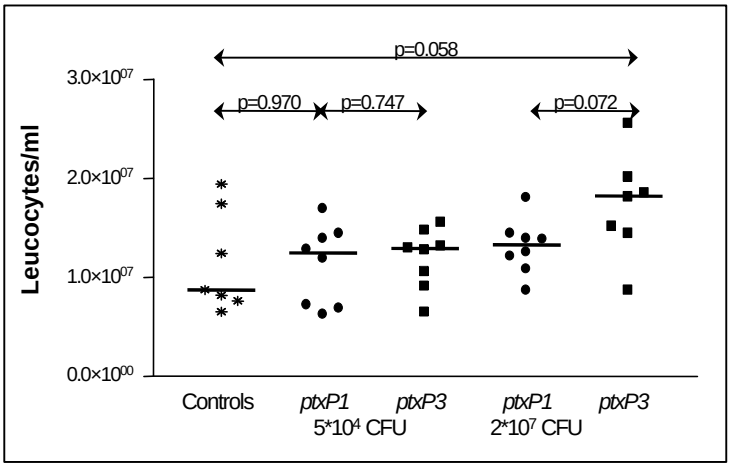


**Fig. 7.** Effect of polymorphism in *ptxP* on leucocyte counts in mice. Mice were infected with $5*10^4$ or $2*10^7$ CFUs of B1584 (*ptxP1*) and B1880 (*ptxP3*). Leucocyte counts were determined 7 days after infection. The line indicates the median. P-values are shown. This experiment was performed three times and a representative result was shown.

At the low dose, leucocyte counts of B1584 (*ptxP1*) and B1880 (*ptxP3*) infected mice were not different (p=0.747) (Fig. 7). Although leucocyte counts of mice that were infected with the low dose of B1584 (*ptxP1*) or B1880 (*ptxP3*) were higher than those of control mice, the difference was not significant (p=0.970). At the high dose, B1880 (*ptxP3*) infected mice showed higher leucocyte counts compared to B1584 (*ptxP1*) infected mice (p=0.072) (Fig. 7). Interestingly, with the high dose B1880 (*ptxP3*) infected mice showed symptoms of discomfort: they were less alert, ate less and had an unkempt appearance. In contrast, mice that were infected with the same dosage of B1584 (*ptxP1*) did not show these clinical signs.

## DISCUSSION

To address the hypothesis that adaptation of *B. pertussis* to vaccination might be a cause for the re-emergence of pertussis, we previously studied polymorphism in genes coding for surface proteins (3;8;33;34;36;58). We observed variation in a limited number of surface proteins and divergence between vaccine strains and modern clinical isolates (19;56;59;82;88;92;157). In this study we extended our analysis of polymorphism in *B. pertussis* to genes that are involved in regulation of expression of virulence factors.

In *B. pertussis* the expression of virulence genes is regulated by the BvgAS system, which showed only limited polymorphism, however. Polymorphism was observed in *bvgS* that occurred as 2 alleles in Dutch *B. pertussis* strains. The mutation in *bvgS* was non-silent and was located in the environmental sensing domain (143). The $Bvg^-$ phase has been suggested to be important for survival of the animal pathogen *Bordetella bronchiseptica* outside its host (81). Since *B. pertussis* is a strictly human pathogen with no known environmental niche, the function of the $Bvg^-$ phase in *B. pertussis* is unknown and it might be an evolutionary remnant. Although the role of the $Bvg^-$ phase for *B. pertussis* is not clear, an intermediate phase, $Bvg^i$ phase, has recently been identified that might be important for colonization of the nasopharynx, where conditions may be intermediate between those inducing the $Bvg^-$ and $Bvg^+$ phase (28;38). Since there was no expansion of strains with *bvgS2*, it is less likely that this allele is beneficial for the bacterium. Alternatively, this allele might be linked to other; as yet unknown, polymorphic sites that affect strain fitness negatively.

No polymorphism was observed in the FHA promoter region. Polymorphism in *bvgP* and *prnP* was observed relative to strain 18323, only. The upstream region of *bvgAS* contains 4 promoters ($P_{1-4}$) and the polymorphic site was located near the −35 box of $P_2$ (118;119). Thus it is conceivable that variation in this site affects transcription of *bvgAS*. The polymorphic site in the promoter region of *prn* was located 38 bases downstream the start site for transcription and 113 bases upstream of the initiation codon (70). Thus it is unlikely that the polymorphism affects transcription or translation.

A remarkable degree of polymorphism was observed in the promoter region of Ptx. In contrast to Nygren et al., who did not observe variation in *ptxP* in Swedish strains (n=11), we observed 8 different *ptxP* alleles in 6 countries (103). Three *ptxP* alleles, *ptxP1*, *ptxP2* and *ptxP3*, were observed with relative high frequencies. In The Netherlands and The United States *ptxP1*, *ptxP2* and *ptxP4* were observed in the prevaccination period (Table 3). In recent clinical isolates, two alleles *ptxP1* and *ptxP3* were frequently observed. Strains with *ptxP3* occurred in high frequencies in The Netherlands and The United States (respectively, 46% and 70% in the period 1990-2000), two countries that have reported an increased pertussis incidence (8;33;34;58). However, in Australia, a

country that also showed an increased pertussis incidence, *ptxP3* strains were found with a frequency of 10% only, while *ptxP1* dominated (3).

Previously, we identified two multi-locus sequence types (MLST's) that were associated with countries that experienced an increased pertussis incidence; MLST-4 and MLST-5 (154). MLST-5 expanded remarkably in the years 1997-1999 in The Netherlands and predominated in The United States in the period 1990-2000 (154). MLST-5, however, differed only in a neutral mutation in *ptxS3* from MLST-3, which declined in frequency in the same period. We found that *ptxP3* was linked to *ptxS3B*, harbored by MLST-5 strains, in all isolates tested. In contrast, MLST-3, harboring *ptxS3A*, was linked to *ptxP1*. We propose that the distinctive behavior of MLST-3 and MLST-5 is caused by the linkage to *ptxP1* and *ptxP3*, respectively (see below).

The polymorphic locus of *ptxP3* was located near the RNA polymerase binding site (77;80) and affected the expression of PtxS1. We found that *ptxP3* isolates produced (130% to 208%) more PtxS1 compared to *ptxP1* isolates. Since the amount of PtxS1 reflects the amount of Ptx produced (102), *ptxP3* strains produce more Ptx. Ptx is an ADP ribosyltransferase, which blocks the GTPase regulated pathways, resulting in tissue specific effects, such as hypoglyceamia and leucocytosis in humans and leucocytosis, hyperinsulinaemia, IL4 and IgE secretion in mice (93;106;140). We hypothesized that strains, which express more Ptx, cause more severe pertussis.

To address this hypothesis, we used a mouse model. Mice were intranasally infected with equal amounts of isogenic strains carrying *ptxP1* or *ptxP3*, and virulence was determined by measuring leucocytosis and colonization. We found more pronounced leucocytosis in mice that were infected with the *ptxP3* strain. In human infants severity of pertussis infection was correlated with extreme leucocytosis. Pierce et al. found significant higher leucocytosis in children, who did not survive a *B. pertussis* infection, compared to children, who survived the infection (105). Thus, these data indicate that *ptxP3* strains may cause more severe disease relative to *ptxP1* strains. Further, we found that the colonization ability of the *ptxP3* strain was increased relative to the *ptxP1* strain. These data are consistent with those of Kinnear et al, who challenged mice with inocula that contained various amounts of purified Ptx and found that colonization increased

with the amount of Ptx added (71). These data suggest that pertussis toxin is advantageous for survival in the host.

In the UK severity of pertussis disease has been associated with the expression of particular serotypes. Clinical isolates that expressed fimbrial serotype 2 (st2), which frequency increased in the period 1995-1997, were associated with more severe pertussis (146). It would be interesting to investigate if the British strains also harbor *ptxP3*. However, in The Netherlands *ptxP3* was found in 152 st3 strains and only in 2 st2 strains.

Epidemiological data also support the observations that *ptxP3* strains cause more serious disease. An increase in number of deaths due to pertussis was observed since 1996. In the period 1996-1999 8 deaths due to pertussis were observed, compared to 0, 1, 0, 0 and 2 deaths in the periods 1976-1979, 1980-1983, 1984-1987, 1988-1991 and 1992-1995 (Fig. 8).

Calculation of lethality (number of deaths / number of hospital admissions) also showed

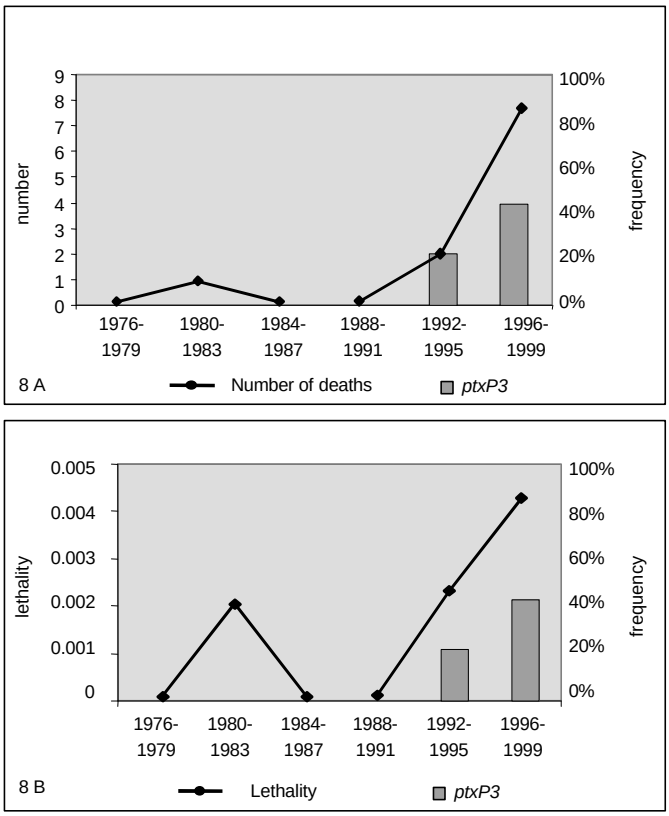


**Fig. 8.** Deaths due to pertussis and *ptxP3* frequency in the period 1976-1999. The bars show the *ptxP3* frequency. (A) The line shows the cumulative number of deaths per period of 4 years. (B) The line shows pertussis lethality (number of deaths due to pertussis / hospital admissions) per period of 4 years.

an increase from 0.0000, 0.0022, 0,0000, 0.0000 and 0.0024 in the periods 1976-1979, 1980-1983, 1984-1987, 1988-1991 and 1992-1995 to 0.0046 in the period 1996-1999 (Fig. 8). Eighty-eight percent of the deaths due to pertussis are observed in children < 3 months, the unimmunized group. Gandon et al. showed in a mathematical model that some type of vaccines may select for more virulent strains (50). Expansion of such strains result in an increased mortality in naive individuals. More severe clinical symptoms, especially coughing, are beneficial for transmission of *B. pertussis*. Further, transmission may increase due to the better colonization ability of *ptxP3* strains. We propose that increased Ptx production may increase transmission by enhancing colonization and clinical symptoms like coughing. The enhanced clinical symptoms may affect mortality.

The novel *ptxP3* allele may affect vaccine efficacy of WCVs and ACVs in several ways. First, increased Ptx production may accelerate the onset of clinical symptoms and hence the transmission to the next host, leaving the (primed) immune system less time to react. Second, higher titers of (pre-)existing antibodies may be required to neutralize Ptx. In this regard, the higher levels of Ptx antibodies induced by ACVs compared to WCVs might be beneficial (37). Ptx antibodies play an important role in immunity against clinical symptoms and inhibition of transmission. A recent report of Taranger et al. observed a decline in spread of *B. pertussis* in a population that recently has been vaccinated with a monocomponent pertussis toxoid ACV (135). Further, Alonso et al. suggested that the presence of (inactive) Ptx on the surface of the bacterium is a direct target for the immune system of the host (1), suggesting that *ptxP3* strains would be more immunogenic in immunized individuals and thus less well able to survive. We are currently investigating the effect of polymorphism in *ptxP* on immunity.

*Bordetella pertussis* is one of the most contagious pathogens. We propose that vaccination has reduced the transmission rate of *B. pertussis*, and selected for strains with increased transmission rates. As transmission is coupled to clinical symptoms, such strains are more virulent. Here, we showed that fifty years after the introduction of vaccination the current circulating strain may have adapted to vaccination by increasing its transmissibility.

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# 7 Summarizing Discussion

Whooping cough or pertussis is a highly contagious disease of the respiratory tract, caused by *Bordetella pertussis*. In the prevaccination period almost every child contracted pertussis. The clinical course is most severe in neonates and infants and most hospitalized cases are children < 2-3 months of age, the age group that is too young to be vaccinated. Although viewed as a childhood disease, pertussis is now recognized as an important infectious disease in adults (17;58;122;132), who represent a reservoir for transmission to susceptible (unimmunized) children (10;78;160).
In The Netherlands mass vaccination against *B. pertussis* was introduced in 1953 and was very effective in reducing the pertussis incidence and mortality (25). Although vaccination was initially very effective, the pertussis incidence increased remarkably after 1996 in The Netherlands. Epidemics have been reported in 1996, 1999 and 2001, while in the intermittent years the incidence did not return to levels observed previous to 1996 (33;34). Increased pertussis incidences were also observed in other countries with a high vaccination coverage, such as Australia, Canada, Poland and The United States (3;8;36;58;59). The goal of this thesis was to find an explanation for the re-emergence of *B. pertussis* in countries with highly vaccinated populations, in particular The Netherlands.

The re-emergence of pertussis might be explained by changes in surveillance and diagnostic criteria, changes in vaccine quality or a decrease in vaccine coverage. However, for The Netherlands these possibilities have been excluded (33). Waning vaccine-induced immunity and strain adaptations are other possible explanations. Indeed, we observed that the introduction of vaccination affected the *B. pertussis* population significantly (chapter 2, 3 and 4). Notable changes were observed in the population structure of Dutch *B. pertussis* strains in 2 periods: the 1960s, a decade after the introduction of vaccination and in the 1990s, the period, when pertussis re-emerged in The Netherlands (chapters 2 and 4). IS*1002*-based fingerprint typing showed a change in fingerprint types a decade after the introduction of vaccination. Fingerprint types from the prevaccination period disappeared, while novel fingerprint types emerged. In particular the frequency of one fingerprint type, ft 29, increased remarkably

from 0% in the 1950s (the prevaccination period) to 50% in the 1960s. These changes were reflected in the genotypic diversity, which was 0.9 in the prevaccination period and decreased to 0.6 in the 1960s (chapters 2 and 4)(Fig. 1).

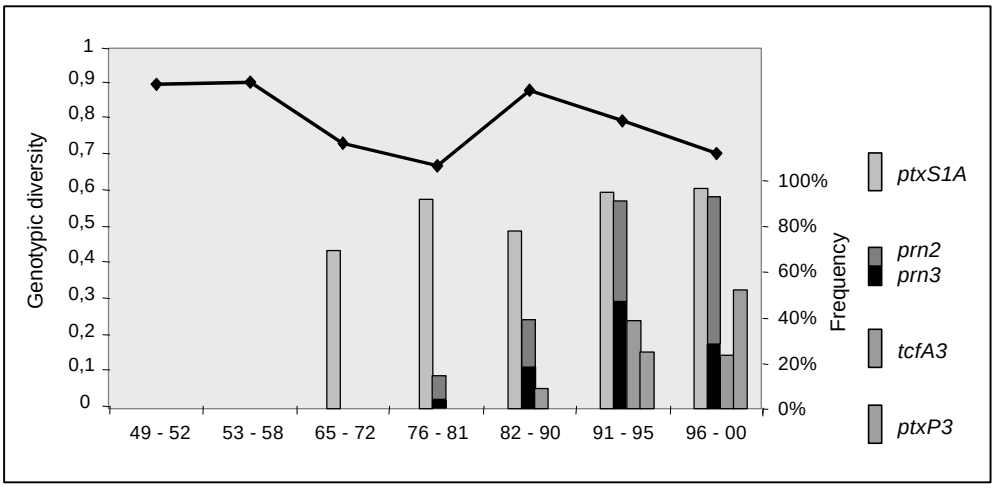


**Fig. 1.** Correlation between genotypic diversity and the emergence of novel alleles of *ptxS1*, *prn*, *tcfA* and *ptxP*. The line and bars represent the genotypic diversity and novel alleles, respectively. The novel alleles are distinct from those found in the vaccine strains.

We hypothesized that antigenic divergence between vaccine strains and circulating strains may be the underlying cause of the changes observed with IS*1002* fingerprint typing. To address this hypothesis, we studied polymorphism in genes coding for surface proteins (chapter 3 and 4). In the 1960s the only mismatch we detected between the vaccine strains and circulating strains was with respect to PtxS1. The observed decrease in genotypic diversity in this period was found to be associated with expansion of strains with the *ptxS1A* allele, which was distinct from the alleles found in the vaccine strains (*ptxS1B* and *ptxS1D*)(Fig. 1). Although we observed divergence between vaccine strains and clinical isolates, suggesting that vaccination selected for strains that were less affected by vaccine-induced immunity, vaccination remained very effective, since mortality decreased 543-fold in the late 1960s compared to the prevaccination period (25).

The second period showing changes in the population structure was observed in the 1990s. In this period we observed divergence between vaccine strains and clinical isolates in the surface associated proteins pertactin (Prn) and tracheal colonization factor (TcfA)(chapter 3)(92). For TcfA, the vaccine type, TcfA2, was replaced by the novel variant TcfA3, while for Prn, the vaccine types Prn1 and Prn7, were replaced by Prn2 and Prn3. Polymorphism was not restricted to genes coding for surface proteins, but was also observed in regulatory genes, such as the promoter region for pertussis toxin, *ptxP* (chapter 6). The alleles *ptxP1* and *ptxP2*, found in the vaccine strains, were replaced by *ptxP3* in the 1990s (Fig. 2).

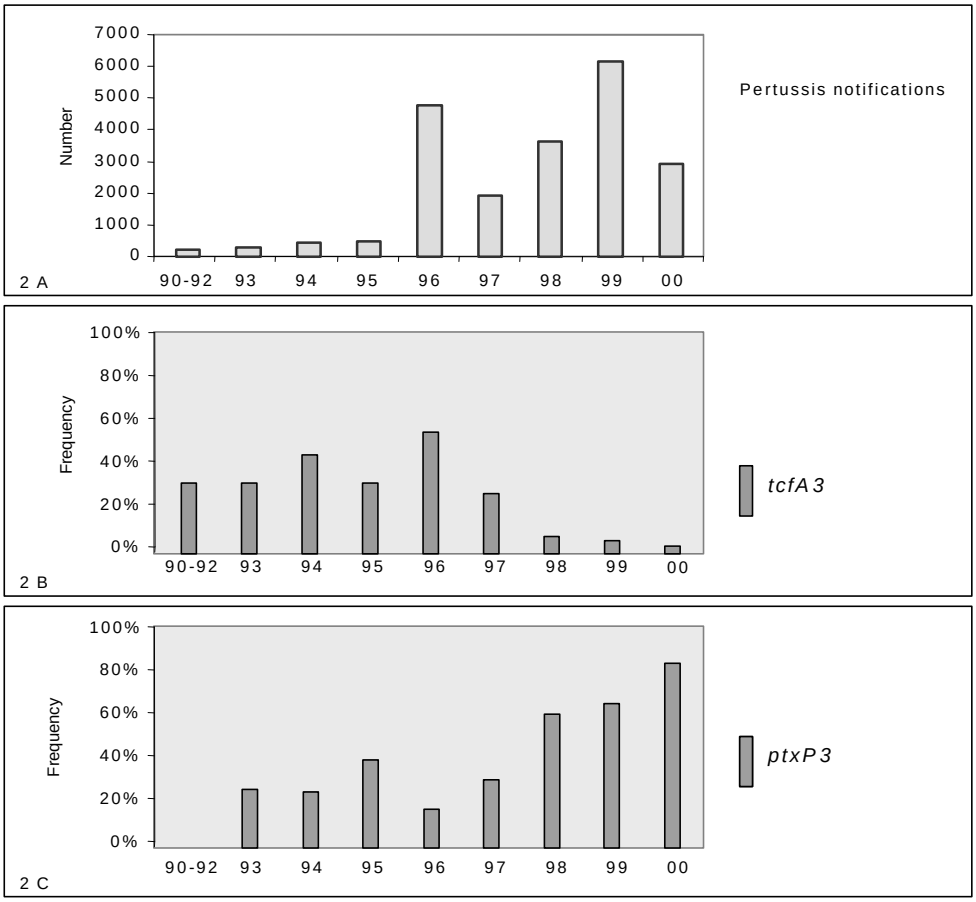


**Fig. 2.** Pertussis notifications (A) and the frequencies of novel alleles for *tcfA* and *ptxP* (B, C).

In The Netherlands the pertussis incidence increased after 1996 (Fig. 2). However, comparison of the periods before and after 1996 did not reveal significant differences in fingerprint types and frequencies of alleles (chapter 2 and 3).

A detailed analysis of the period 1990-2000 did reveal interesting trends in frequencies of the polymorphic proteins TcfA and Prn (Fig. 2). The twofold increase in notifications in 1994 compared to 1993 and the 10-fold increase in notifications in 1996 compared to 1995 was associated with an increase in frequency of strains harboring the non-vaccine type variant TcfA3. Thus, the observed increase in notifications may have been caused by a change in the *B. pertussis* population. This assumption is consistent with results from mathematical modeling performed by Boven et al., which suggested that the most likely cause for the 1996 epidemic was a change in the *B. pertussis* population (145). After 1996 strains with the *tcfA3* allele decreased in frequency. This may have been caused by the naturally induced immunity against TcfA3 strains.

In 1998 and 1999 there was a substantial increase in notifications compared to 1997 (1,9 and 3,2 fold, respectively). This increase was associated with expansion of strains harboring the *ptxP3* allele, again suggesting that epidemic years are associated with clonal expansion of particular strains (chapter 6).

The number of deaths due to pertussis has increased since 1996. In the period 1996-1999, 8 deaths due to pertussis were observed compared to 0, 1, 0, 0 and 2 deaths, respectively, in the periods 1976-1979, 1980-1983, 1984-1987, 1988-1991 and 1992-1995 (Fig. 3). As 88% of the deaths were children < 3 months, this suggests an increased number of deaths in unimmunized individuals. Gandon et al. showed in a mathematical model that some types of vaccines may select for more virulent strains (50). Expansion of such strains caused higher mortality rates in unimmunized individuals.

Calculation of lethality (number of deaths / number of hospital admissions) also showed an increase from 0.0000, 0.0022, 0.0000, 0.0000 and 0.0024 in the periods 1976-1979, 1980-1983, 1984-1987, 1988-1991 and 1992-1995, respectively, to 0.0046 in the period 1996-1999 (Fig. 3). The increase in lethality coincided with an increase in the frequency of strains with *ptxP3*,

suggesting that *ptxP3* strains may cause more severe disease due to increased Ptx production (chapter 6). Animal studies showed that leucocyte counts of mice infected with a *ptxP3* strain were significantly higher compared to those of *ptxP1* infected mice (chapter 6). In human infants the level of leucocytosis was correlated with severity of infection and lethality (105). More severe clinical symptoms, especially coughing, are beneficial for transmission of *B. pertussis*. Further, transmission may increase due to the better colonization ability of *ptxP3* strains (chapter 6). We propose that increased Ptx production may be beneficial for transmission by enhancing clinical symptoms and colonization. Both factors may affect mortality.

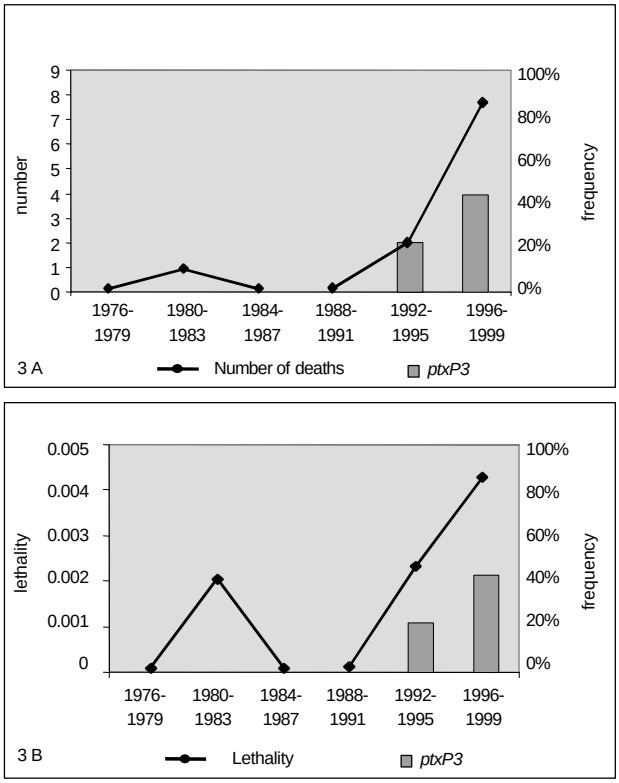


**Fig. 3.** Deaths due to pertussis and *ptxP3* frequency in the period 1976-1999. The bars show the *ptxP3* frequency. (A) The line shows the cumulative number of deaths per period of 4 years. (B) The line shows pertussis lethality (number of deaths due to pertussis / hospital admissions) per period of 4 years.

We observed a non-random association between *prn* alleles and alleles of other polymorphic genes; e.g. *tcfA3* was linked with *prn3* and *ptxP3* with *prn2* (chapter 3). This was unexpected, as variation in Prn is based on recombination within the repeated region or slipped strand mispairing, phenomena, which occur at relatively high frequency and are reversible. Gupta et al. have shown that pathogen populations may segregate into discrete strains with non-

overlapping antigenic structures, if these structures are immunodominant (57). The non-random distribution of *prn* alleles may reflect strong immune selection focused on Prn, TcfA and other, as yet unknown, *B. pertussis* antigens.

Several observations underline the importance of Prn, TcfA and Ptx in immunity. Sero-epidemiological studies have shown a correlation between the titer of anti-Prn antibodies and clinical protection (23;131;131). Vaccination with a monocomponent pertussis toxoid vaccine-induced significant protection for at least 2 years and decreased the spread of *B. pertussis* in a population (135;135;136). Intranasal vaccination of mice with an attenuated *Vibrio Cholerae* strain expressing TcfA resulted in significant reduction of colonization of the trachea (22). The fact that the polymorphic sites in Ptx and Prn comprise B- and T-cell epitopes indicate that these sites are immunologically relevant (21;30;69;120). Finally, variation in Prn was shown to affect the efficacy of a whole cell pertussis vaccine in a mouse model (69). Protection was best against Prn1, the vaccine type Prn, and lower against Prn2 and Prn3. We observed that strains carrying non-vaccine types Prn and PtxS1 are less well able to colonize naive mice compared to those carrying vaccine types (chapter 5), explaining the absence of the non-vaccine types in the prevaccination period (92). These data indicate that vaccination has shifted the competitive balance between strains, resulting in expansion of strains that are less fit in unimmunized individuals.

The next question to address is how to control pathogens with polymorphic antigens, such as *B. pertussis*. Mathematical modeling emphasizes the importance of developing vaccines against a set of conserved antigens, even if these are less immunogenic (2). We identified a number of conserved antigens, such as BrkA, FHA, Fimbriae, OmpP, OmpQ and Vag8, which may be included in future vaccines (chapter 3). Because of the importance in immunity against *B. pertussis* the polymorphic proteins TcfA and Prn may be potential vaccine components, especially when the immune response is directed to conserved epitopes to avoid the emergence of escape variants.

The data presented in this thesis provide an explanation for the re-emergence of pertussis and a better understanding of how pathogens evolve after the introduction of vaccines. We show that vaccination may result in the emergence of more virulent strains. This knowledge may contribute to the development of better pertussis vaccines.

# References

1. **Alonso S**, Pethe K, Mielcarek N, Raze D, Locht C. Role of ADP-Ribosyltransferase activity of pertussis toxin in toxin-adhesin redundancy with filamentous hemagglutinin during *Bordetella pertussis* Infection. *Infect. Immun.* 2001;69(10):6038-43.
2. **Anderson RM**, Donnelly CA, Gupta S. Vaccine design, evaluation, and community-based use for antigenically variable infectious agents. *Lancet* 1997;350(9089):1466-70.
3. **Andrews R**, Herceg A, Roberts C. Pertussis notifications in Australia, 1991 to 1997. *Commun. Dis. Intell.* 1997;21(11):145-8.
4. **Arico B**, Miller JF, Roy C, Stibitz S, Monack D, Falkow S, Gross R, Rappuoli R. Sequences required for expression of *Bordetella pertussis* virulence factors share homology with prokaryotic signal transduction proteins. *Proc. Natl. Acad. Sci. U.S.A.* 1989;86(17):6671-5.
5. **Arico B**, Scarlato V, Monack DM, Falkow S, Rappuoli R. Structural and genetic analysis of the *bvg* locus in *Bordetella* species. *Mol. Microbiol.* 1991;5(10):2481-91.
6. **Armstrong SK**, Parr TR Jr, Parker CD, Hancock RE. *Bordetella pertussis* major outer membrane porin protein forms small, anion-selective channels in lipid bilayer membranes. *J. Bacteriol.* 1986 Apr;166(1):212-6.
7. **Bass JW**, Stephenson SR. The return of pertussis. *Pediatr. Infect. Dis. J.* 1987;6(2):141-4.
8. **Bass JW**, Wittler RR. Return of epidemic pertussis in the United States. *Pediatr. Infect. Dis. J*. 1994;13(5):343-5.
9. **Beall B**, Cassiday PK, Sanden GN. Analysis of *Bordetella pertussis* isolates from an epidemic by pulsed-field gel electrophoresis. *J. Clin. Microbiol.* 1995;33(12):3083-6.
10. **Beiter A**, Lewis K, Pineda EF, Cherry JD. Unrecognized maternal peripartum pertussis with subsequent fatal neonatal pertussis. *Obstet. Gynecol.* 1993;82(4 Pt 2 Suppl):691-3.
11. **Bisgard KM**, Christie CD, Reising SF, Sanden GN, Cassiday PK, Gomersall C, Wattigney WA, Roberts NE, Strebel PM. Molecular epidemiology of *Bordetella pertussis* by pulsed-field gel glectrophoresis profile: Cincinnati, 1989-1996. *J. Infect. Dis.* 2001;183(9):1360-7.
12. **Black WJ**, Munoz JJ, Peacock MG, Schad PA, Cowell JL, Burchall JJ, Lim M, Kent A, Steinman L, Falkow S. ADP-ribosyltransferase activity of pertussis toxin and immunomodulation by *Bordetella pertussis*. *Science* 1988;240(4852):656-9.
13. **Blaskett AC**, Gulasekharam J, Fulton LC. The occurrence of *Bordetella pertussis* serotypes in Australia, 1950- 1970. *Med. J. Aust.* 1971 Apr;1(15):781-4.
14. **Blumberg DA**, Lewis K, Mink CM, Christenson PD, Chatfield P, Cherry JD. Severe reactions associated with diphtheria-tetanus-pertussis vaccine: detailed study of

children with seizures, hypotonic-hyporesponsive episodes, high fevers, and persistent crying. *Pediatrics* 1993;91(6):1158-65.

15. **Blystad H**, Bäckman-Breidvick G, Eide Anestad KA. Meldingssystem for Smittsiomme Sykdommer. (MSIS)-Rapport, OSLO, *MSIS* 1997 week 43.
16. **Borduet**, Gengou. Le micorobe de la coqueluche. *Ann. Inst. Pasteur.* 1906;20:731-41.
17. **Brennan M**, Strebel P, George H, Yih WK, Tachdjian R, Lett SM, Cassiday P, Sanden G, Wharton M. Evidence for transmission of pertussis in schools, Massachusetts, 1996: epidemiologic data supported by pulsed-field gel electrophoresis studies. *J. Infect. Dis.* 2000;181(1):210-5.
18. **Bronne-Shanbury C**, Miller D, Standfast AF. The serotypes of *Bordetella pertussis* isolated in Great Britain between 1941 and 1968 and a comparison with the serotypes observed in other countries over this period. *J. Hyg. (Lond).* 1976;76(2):265-75.
19. **Cassiday P**, Sanden G, Heuvelman K, Mooi F, Bisgard KM, Popovic T. Polymorphism in *Bordetella pertussis* pertactin and pertussis toxin virulence factors in the United States, 1935-1999. *J. Infect. Dis.* 2000;182(5):1402-8.
20. **Charles IG**, Dougan G, Pickard D, Chatfield S, Smith M, Novotny P, Morrissey P, Fairweather NF. Molecular cloning and characterization of protective outer membrane protein P.69 from *Bordetella pertussis*. *Proc. Natl. Acad. Sci. U.S.A.* 1989;86(10):3554-8.
21. **Charles IG**, Li JL, Roberts M, Beesley K, Romanos M, Pickard DJ, Francis M, Campbell D, Dougan G, Brennan MJ, et al. Identification and characterization of a protective immunodominant B-cell epitope of pertactin (P.69) from *Bordetella pertussis*. *Eur. J. Immunol.* 1991;21(5):1147-53.
22. **Chen I**, Finn TM, Yanqing L, Guoming Q, Rappuoli R, Pizza M. A recombinant live attenuated strain of *Vibrio cholerae* induces immunity against tetanus toxin and *Bordetella pertussis* tracheal colonization factor. *Infect. Immun.* 1998;66(4):1648-53.
23. **Cherry JD**, Gornbein J, Heininger U, Stehr K, Storsaeter J, Hallander HO, Gustafsson L, Olin P, JabbalGill I, Fisher AN, et al. A search for serologic correlates of immunity to *Bordetella pertussis* cough illnesses. *Vaccine* 1998;16(20):2039-46.
24. **Cohen HH**. Establishment of a dried standard pertussis vaccine. *Antonie Van Leeuwenhoek* 1958;24:33-48.
25. **Cohen HH**. Development of pertussis vaccine production and control in the National Institute of Public Health in The Netherlands during the years 1950-1962. *Antonie Van Leeuwenhoek* 1963;29:183-201.
26. **Cohen HH**, Leppink G.J. Selection of *Hemophilus pertussis* strains for vaccine

production in the mouse protection test in a balanced design. *J. Immunol.* 1956;77:299-304.

27. **Cotter PA**, DiRita VJ. Bacterial virulence gene regulation: An evolutionary perspective. *Annu. Rev. Microbiol*. 2000;54:519-65.
28. **Cotter PA**, Miller JF. A mutation in the *Bordetella bronchiseptica bvgS* gene results in reduced virulence and increased resistance to starvation, and identifies a new class of Bvg-regulated antigens. *Mol. Microbiol.* 1997;24(4):671-85.
29. **Cotter PA**, Yuk MH, Mattoo S, Akerley BJ, Boschwitz J, Relman DA, Miller JF. Filamentous hemagglutinin of *Bordetella bronchiseptica* is required for efficient establishment of tracheal colonization. *Infect. Immun.* 1998 Dec;66(12):5921-9.
30. **De Magistris MT**, Romano M, Bartoloni A, Rappuoli R, Tagliabue A. Human T cell clones define S1 subunit as the most immunogenic moiety of pertussis toxin and determine its epitope map. *J. Exp. Med.* 1989;169(5):1519-32.
31. **de Melker HE.** Seroepidemiology of diphtheria, teranus, poliomyelitis and pertussis. *Thesis*, ISBN 90-5808-128-1. 1999.
32. **de Melker HE**, Boshuis HGL, Mommers MPTM, Sundermann LC, Labadie J, Rumke HC, Versteegh FGA, Conyn-van Spaendonck MAE, Schellekens JFP. Serodiagnostics of pertussis: interpretation of high IgG/IgA concentrations in acute phase serum: Re-evaluation of one-point serology. *RIVM Rapport 128507003.* Bilthoven, The Netherlands 1996.
33. **de Melker HE**, Conyn van Spaendonck MA, Rumke HC, van Wijngaarden JK, Mooi FR, Schellekens JF. Pertussis in The Netherlands: an outbreak despite high levels of immunization with whole-cell vaccine. *Emerg. Infect. Dis.* 1997;3(2):175-8.
34. **de Melker HE**, Schellekens JF, Neppelenbroek SE, Mooi FR, Rumke HC, Conyn-van Spaendonck MA. Re-emergence of pertussis in the highly vaccinated population of the Netherlands: observations on surveillance data. *Emerg. Infect. Dis.* 2000;6(4):348-57.
35. **de Moissac YR**, Ronald SL, Peppler MS. Use of pulsed-field gel electrophoresis for epidemiological study of *Bordetella pertussis* in a whooping cough outbreak. *J. Clin. Microbiol.* 1994;32(2):398-402.
36. **De Serres G**, Boulianne N, Douville Fradet M, Duval B. Pertussis in Quebec: ongoing epidemic since the late 1980s. *Can. Commun. Dis. Rep.* 1995;21(5):45-8.
37. **Decker MD**, Edwards KM. The multicenter acellular pertussis trial: an overview. *J. Infect. Dis.* 1996;174(Suppl 3):S270-S275.
38. **Deora R**, Bootsma HJ, Miller JF, Cotter PA. Diversity in the *Bordetella* virulence regulon: transcriptional control of a Bvg-intermediate phase gene. *Mol. Microbiol.* 2001;40(3):669-83.
39. **Dingle KE**, Colles FM, Wareing DR, Ure R, Fox AJ, Bolton FE, Bootsma HJ, Willems

RJ, Urwin R, Maiden MC. Multilocus sequence typing system for *Campylobacter jejuni*. *J. Clin. Microbiol.* 2001;39(1):14-23.

40. **Dodhia H**, Miller E. Review of the evidence for the use of erythromycin in the management of persons exposed to pertussis. *Epidemiol. Infect.* 1998;120(2):143-9.
41. **Eldering G**, Holwerda J, Davis A, Baker J. *Bordetella pertussis* serotypes in the United States. *Appl. Microbiol.* 1969;18(4):618-21.
42. **Enright MC**, Spratt BG. Multilocus sequence typing. *Trends Microbiol.* 1999;7(12):482-7.
43. **Everest P**, Li J, Douce G, Charles I, De Azavedo J, Chatfield S, Dougan G, Roberts M. Role of the *Bordetella pertussis* P.69/pertactin protein and the P.69/pertactin RGD motif in the adherence to and invasion of mammalian cells. *Microbiology* 1996;142(Pt 11):3261-8.
44. **Feavers IM**, Gray SJ, Urwin R, Russell JE, Bygraves JA, Kaczmarski EB, Maiden MC. Multilocus sequence typing and antigen gene sequencing in the investigation of a meningococcal disease outbreak. *J. Clin. Microbiol.* 1999;37(12):3883-7.
45. **Fernandez RC**, Weiss AA. Cloning and sequencing of a *Bordetella pertussis* serum resistance locus. *Infect. Immun.* 1994;62(11):4727-38.
46. **Fine PE**, Chen RT. Confounding in studies of adverse reactions to vaccines. *Am. J. Epidemiol.* 1992;136(2):121-35.
47. **Finn TM**, Amsbaugh DF. Vag8, a *Bordetella pertussis* Bvg-regulated protein. *Infect. Immun.* 1998;66(8):3985-9.
48. **Finn TM**, Stevens LA. Tracheal colonization factor: A *Bordetella pertussis* secreted virulence determinant. *Mol. Microbiol.* 1995;16(4):625-34.
49. **Fry NK**, Neal S, Harrison TG, Miller E, Matthews R, George RC. Genotypic variation in the *Bordetella pertussis* virulence factors pertactin and pertussis toxin in historical and recent clinical isolates in the United Kingdom. *Infect. Immun.* 2001;69(9):5520-8.
50. **Gandon S**, Mackinnon MJ, Nee S, Read AF. Imperfect vaccines and the evolution of pathogen virulence. *Nature* 2001;414(6865):751-6.
51. **Geuijen CA**, Willems RJ, Bongaerts M, Top J, Gielen H, Mooi FR. Role of the *Bordetella pertussis* minor fimbrial subunit, FimD, in colonization of the mouse respiratory tract. *Infect. Immun.* 1997;65(10):4222-8.
52. **Geuijen CA**, Willems RJ, Mooi FR. The major fimbrial subunit of *Bordetella pertussis* binds to sulfated sugars. *Infect. Immun.* 1996;64(7):2657-65.
53. **Goldman WE**, Cookson BT. Structure and functions of the *Bordetella* tracheal cytotoxin. *Tokai J. Exp. Clin. Med.* 1988;13 Suppl:187-91.
54. **Greco D**, Salmaso S, Mastrantonio P, Giuliano M, Tozzi AE, Anemona A, Ciofi degli

Atti ML, Giammanco A, Panei P, Blackwelder WC, et al. A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. Progetto Pertosse Working Group. *N. Engl. J. Med.* 1996;334(6):341-8.

55. **Griffith AH**. Permanent brain damage and pertussis vaccination: is the end of the saga in sight? *Vaccine* 1989;7(3):199-210.
56. **Guiso N**, Boursaux-Eude C, Weber C, Hausman SZ, Sato H, Iwaki M, Kamachi K, Konda T, Burns DL. Analysis of *Bordetella pertussis* isolates collected in Japan before and after introduction of acellular pertussis vaccines. *Vaccine* 2001;19(23-24):3248-52.
57. **Gupta S**, Maiden MC. Exploring the evolution of diversity in pathogen populations. *Trends Microbiol.* 2001;9(4):181-5.
58. **Guris D**, Strebel PM, Bardenheier B, Brennan M, Tachdjian R, Finch E, Wharton M, Livengood JR. Changing epidemiology of pertussis in the United States: increasing reported incidence among adolescents and adults, 1990-1996. *Clin. Infect. Dis.* 1999;28(6):1230-7.
59. **Gzyl A**, Augustynowicz E, Loo I, Slusarczyk J. Temporal nucleotide changes in pertactin and pertussis toxin genes in *Bordetella pertussis* strains isolated from clinical cases in Poland. *Vaccine* 2001;20(3-4):299-303.
60. **Hamstra HJ**, Kuipers B, Schijfevers D, Loggen HG, Poolman JT. The purification and protective capacity of *Bordetella pertussis* outer membrane proteins. *Vaccine* 1995;13(8):747-52.
61. **Hausman SZ**, Burns DL. Use of pertussis toxin encoded by *ptx* genes from *Bordetella bronchiseptica* to model the effects of antigenic drift of pertussis toxin on antibody neutralization. *Infect. Immun.* 2000;68(6):3763-7.
62. **Henderson IR**, Navarro-Garcia F, Nataro JP. The great escape: structure and function of the autotransporter proteins. *Trends Microbiol.* 1998;6(9):370-8.
63. **Hethcote HW**. Simulations of pertussis epidemiology in the United States: effects of adult booster vaccinations. *Math. Biosci.* 1999;158(1):47-73.
64. **Hodder SL**, Cherry JD, Mortimer Jr EA, Ford AB, Gornbein J, Papp K. Antibody responses *Bordetella pertussis* antigens and clinical correlations in elderly community residents. *Clin. Infect. Dis.* 2000;31(1):7-14.
65. **Hoe N**, Nakashima K, Grigsby D, Pan X, Dou SJ, Naidich S, Garcia M, Kahn E, Bergmire-Sweat D, Musser JM. Rapid molecular genetic subtyping of serotype M1 group A *Streptococcus* strains. *Emerg. Infect. Dis.* 1999;5(2):254-63.
66. **Hynes RO**. Integrins: a family of cell surface receptors. *Cell* 1987;48(4):549-54.
67. **Kapur V**, Whittam TS, Musser JM. Is *Mycobacterium tuberculosis* 15,000 years old? *J. Infect. Dis.* 1994;170(5):1348-9.
68. **Kimura A**, Mountzouros KT, Relman DA, Falkow S, Cowell JL. *Bordetella pertussis*

filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. *Infect. Immun.* 1990;58(1):7-16.

69. **King AJ**, Berbers B, Van Oirschot HFLM, Hoogerhout P, Knipping K, Mooi FR. Role of the polymorphic region 1 of the *Bordetella pertussis* protein pertactin in immunity. Microbiology 2001;147:2885-95.
70. **Kinnear SM**, Boucher PE, Stibitz S, Carbonetti NH. Analysis of BvgA activation of the pertactin gene promoter in *Bordetella pertussis. J. Bacteriol.* 1999;181(17):5234-41.
71. **Kinnear SM**, Marques RR, Carbonetti NH. Differential regulation of Bvg-activated virulence factors plays a role in *Bordetella pertussis* pathogenicity. *Infect. Immun.* 2001;69(4):1983-93.
72. **Ladant D**, Ullmann A. *Bordatella pertussis* adenylate cyclase: a toxin with multiple talents. *Trends Microbiol.* 1999;7(4):172-6.
73. **Lapin**. Whooping cough. Springfield Illinois, Charles C Thomas 1943.
74. **Leininger E**, Ewanowich CA, Bhargava A, Peppler MS, Kenimer JG, Brennan MJ. Comparative roles of the Arg-Gly-Asp sequence present in the *Bordetella pertussis* adhesins pertactin and filamentous hemagglutinin. *Infect. Immun.* 1992;60(6):2380-5.
75. **Leininger E**, Roberts M, Kenimer JG, Charles IG, Fairweather N, Novotny P, Brennan MJ. Pertactin, an Arg-Gly-Asp-containing *Bordetella pertussis* surface protein that promotes adherence of mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* 1991;88(2):345-9.
76. **Li ZM**, Hannah JH, Stibitz S, Nguyen NY, Manclark CR, Brennan MJ. Cloning and sequencing of the structural gene for the porin protein of *Bordetella pertussis*. *Mol. Microbiol.* 1991;5(7):1649-56.
77. **Locht C**, Keith JM. Pertussis toxin gene: nucleotide sequence and genetic organization. *Science* 1986;232(4755):1258-64.
78. **Long SS**, Welkon CJ, Clark JL. Widespread silent transmission of pertussis in families: antibody correlates of infection and symptomatology. *J. Infect. Dis.* 1990;161(3):480-6.
79. **Major**. A history of medicine. V. I. Springfeld, IL: Charles C. Thomas 1954;423.
80. **Marques RR**, Carbonetti NH. Genetic analysis of pertussis toxin promoter activation in *Bordetella pertussis. Mol. Microbiol.* 1997;24(6):1215-24.
81. **Martinez de Tejada G**, Cotter PA, Heininger U, Camilli A, Akerley BJ, Mekalanos JJ, Miller JF. Neither the Bvg$^-$ phase nor the Vrg6 locus of *Bordetella pertussis* is required for respiratory infection in mice. *Infect. Immun.* 1998;66(6):2762-8.
82. **Mastrantonio P**, Spigaglia P, van Oirschot H, van der Heide HG, Heuvelman K, Stefanelli P, Mooi FR. Antigenic variants in *Bordetella pertussis* strains isolated from

vaccinated and unvaccinated children. *Microbiology* 1999;145(Pt 8):2069-75.

83. **Mattoo S**, Foreman-Wykert AK, Cotter PA, Miller JF. Mechanisms of *Bordetella* pathogenesis. *Front. Biosci.* 2001;6:E168-E186.
84. **Merkel TJ**, Barros C, Stibitz S. Characterization of the BvgR locus of *Bordetella pertussis*. *J. Bacteriol.* 1998;180(7):1682-90.
85. **Miller D**, Madge N, Diamond J, Wadsworth J, Ross E. Pertussis immunisation and serious acute neurological illnesses in children. *B.M.J.* 1993;307(6913):1171-6.
86. **Miller E**, Vurdien JE, White JM. The epidemiology of pertussis in England and Wales. *Commun. Dis. Rep. CDR. Rev.* 1992;2(13):R152-R154.
87. **Moissenet D**, Valcin M, Marchand V, Grimprel E, Begue P, Garbarg-Chenon A, Vu-Thien H. Comparative DNA analysis of *Bordetella pertussis* clinical isolates by pulsed-field gel electrophoresis, randomly amplified polymorphism DNA, and ERIC polymerase chain reaction. *FEMS Microbiol. Lett.* 1996;143(2-3):127-32.
88. **Mooi FR**, He Q, van Oirschot H, Mertsola J. Variation in the *Bordetella pertussis* virulence factors pertussis toxin and pertactin in vaccine strains and clinical isolates in Finland. *Infect. Immun.* 1999;67(6):3133-4.
89. **Mooi FR**, Jansen WH, Brunings H, Gielen H, van der Heide HG, Walvoort HC, Guinee PA. Construction and analysis of *Bordetella pertussis* mutants defective in the production of fimbriae. *Microb. Pathog.* 1992;12(2):127-35.
90. **Mooi FR**, van der Heide HG, ter Avest AR, Welinder KG, Livey I, van der Zeijst BA, Gaastra W. Characterization of fimbrial subunits from *Bordetella* species. *Microb. Pathog.* 1987;2(6):473-84.
91. **Mooi FR**, van Loo IH, King AJ. Adaptation of *Bordetella pertussis* to vaccination: A cause for its re-emergence? *Emerg. Infect. Dis.* 2001;7(3 Suppl):526-S28.
92. **Mooi FR**, van Oirschot H, Heuvelman K, van der Heide HGJ, Gaastra W, Willems RJL. Polymorphism in the *Bordetella pertussis* virulence factors p.69/Pertactin and pertussis toxin in the Netherlands: temporal trends and evidence for vaccine-driven evolution. *Infect. Immun.* 1998;66(2):670-5.
93. **Mu HH**, Cooley MA, Sewell WA. Studies on the lymphocytosis induced by pertussis toxin. *Immunol. Cell Biol.* 1994;72(3):267-70.
94. **Munoz JJ**, Arai H, Bergman RK, Sadowski PL. Biological activities of crystalline pertussigen from *Bordetella pertussis*. *Infect. Immun.* 1981;33(3):820-6.
95. **Murray PR**, Drew WL, Kobayashi GS, Thompson JH. in *Medical Microbiology* 1990.
96. **Musser JM**, Hewlett EL, Peppler MS, Selander RK. Genetic diversity and relationships in populations of *Bordetella* spp. *J. Bacteriol.* 1986;166(1):230-7.
97. **Nagel J**, de Graaf S, Schijf-Evers D. Improved serodiagnosis of whooping cough caused by *Bordetella pertussis* by determination of IgG anti-LPF antibody levels. *Dev. Biol. Stand.* 1985;61:325-30.

98. **Nagel J**, Poot-Scholtens EJ. Serum IgA antibody to *Bordetella pertussis* as an indicator of infection. *J. Med. Microbiol.* 1983;16(4):417-26.
99. **Natl. Adv. Comm. Imm**., Adv. Com. Epid., Can. Ped. Soc. Statement on management of persons exposed to pertussis and pertussis outbreak control. *Can. Med. Ass. J.* 1995;152:712-6.
100. **Nei M**, Tajima F. DNA polymorphism detectable by restriction endonucleases. *Genetics* 1981;97(1):145-63.
101. **Nennig ME**, Shinefield HR, Edwards KM, Black SB, Fireman BH. Prevalence and incidence of adult pertussis in an urban population. *JAMA* 1996;275(21):1672-4.
102. **Nicosia A**, Rappuoli R. Promoter of the pertussis toxin operon and production of pertussis toxin. *J. Bacteriol.* 1987;169(6):2843-6.
103. **Nygren M**, Reizenstein E, Ronaghi M, Lundeberg J. Polymorphism in the pertussis toxin promoter region affecting the DNA-based diagnosis of *Bordetella* infection. *J. Clin. Microbiol.* 2000;38(1):55-60.
104. **Olin P**, Rasmussen F, Gustafsson L, Hallander HO, Heijbel H. Randomised controlled trial of two-component, three-component, and five-component acellular pertussis vaccines compared with whole-cell pertussis vaccine. Ad Hoc Group for the Study of Pertussis Vaccines. *Lancet* 1997;350(9091):1569-77.
105. **Pierce C**, Klein N, Peters M. Is leukocytosis a predictor of mortality in severe pertussis infection? *Intensive Care Med.* 2000;26(10):1512-4.
106. **Pittman M**. The concept of pertussis as a toxin-mediated disease. *Pediatr. Infect. Dis.* 1984;3(5):467-86.
107. **Poolman J**, Hamstra H-J, Barlow A, Kuipers B, Loggen H, Nagel J. Outer membrane vesicles of *Bordetella pertussis* are protective antigens in the mouse intracerebral challenge model. In: *Proceedings of the Sixth International Symposium on Pertussis* 1990;202-6.
108. **Preston NW**. Prevalent serotypes of *Bordetella pertussis* in non-vaccinated communities. *J. Hyg. (Lond).* 1976;77(1):85-91.
109. **Preston NW**, Carter EJ. Serotype specificity of vaccine-induced immunity to pertussis. *Commun. Dis. Rep. CDR. Rev.* 1992;2(13):R155-R156.
110. **Rambow AA**, Fernandez RC, Weiss AA. Characterization of BrkA expression in *Bordetella bronchiseptica*. *Infect. Immun.* 1998;66(8):3978-80.
111. **Rappuoli R**, Podda A, Pizza M, Covacci A, Bartoloni A, de Magistris MT, Nencioni L. Progress towards the development of new vaccines against whooping cough. *Vaccine* 1992;10(14):1027-32.
112. **Relman D**, Tuomanen E, Falkow S, Golenbock DT, Saukkonen K, Wright SD. Recognition of a bacterial adhesion by an integrin: macrophage CR3 (alpha M beta 2, CD11b/CD18) binds filamentous hemagglutinin of *Bordetella pertussis*. *Cell*

1990;61(7):1375-82.

113. **Roberts M**, Fairweather NF, Leininger E, Pickard D, Hewlett EL, Robinson A, Hayward C, Dougan G, Charles IG. Construction and characterization of *Bordetella pertussis* mutants lacking the vir-regulated P.69 outer membrane protein. *Mol. Microbiol.* 1991;5(6):1393-404.

114. **Roberts M**, Tite JP, Fairweather NF, Dougan G, Charles IG. Recombinant P.69/pertactin: immunogenicity and protection of mice against *Bordetella pertussis* infection. *Vaccine* 1992;10(1):43-8.

115. **Robinson A**, Gorringe AR, Funnell SG, Fernandez M. Serospecific protection of mice against intranasal infection with *Bordetella pertussis*. *Vaccine* 1989;7(4):321-4.

116. **Romanus V**, Jonsell R, Bergquist SO. Pertussis in Sweden after the cessation of general immunization in 1979. *Pediatr. Infect. Dis. J.* 1987;6(4):364-71.

117. **Rota PA**, Bloom AE, Vanchiere JA, Bellini WJ. Evolution of the nucleoprotein and matrix genes of wild-type strains of measles virus isolated from recent epidemics. *Virology* 1994;198(2):724-30.

118. **Roy CR**, Falkow S. Identification of *Bordetella pertussis* regulatory sequences required for transcriptional activation of the *fhaB* gene and autoregulation of the *bvgAS* operon. *J. Bacteriol.* 1991;173(7):2385-92.

119. **Scarlato V**, Prugnola A, Arico B, Rappuoli R. The Bvg-dependent promoters show similar behaviour in different *Bordetella* species and share sequence homologies. *Mol. Microbiol.* 1991;5(10):2493-8.

120. **Scarselli M**, Esposito G, De Magistris MT, Domenighini M, Rappuoli R, Burroni G, Bernini A, Niccolai N. NMR studies on the structure/function correlations of T-cell-epitope analogs from pertussis toxin. *Eur. J. Biochem.* 1998;254(2):313-7.

121. **Schmitt HJ**. Acellular pertussis vaccines: the final countdown. *Eur. J. Pediatr.* 1996;155(2):74-6.

122. **Senzilet LD**, Halperin SA, Spika JS, Alagaratnam M, Morris A, Smith *Bordetella pertussis* is a frequent cause of prolonged cough illness in adults and adolescents. *Clin. Infect. Dis.* 2001;32(12):1691-7.

123. **Shahin RD**, Amsbaugh DF, Leef MF. Mucosal immunization with filamentous hemagglutinin protects against *Bordetella pertussis* respiratory infection. *Infect. Immun.* 1992;60(4):1482-8.

124. **Simon R**, Priefer U, Puhler AA. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technol.* 1983;1:784-91.

125. **Skorupski K**, Taylor RK. Positive selection vectors for allelic exchange. *Gene* 1996;169(1):47-52.

126. **Smith LW**. The pathologic anatomy of pertussis. *Archives of Pathology and*

*Laboratory Medicine* 1927;732-42.

127. **Spratt BG**, Maiden MC. Bacterial population genetics, evolution and epidemiology. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 1999;354(1384):701-10.
128. **Sreevatsan S**, Pan X, Stockbauer KE, Connell ND, Kreiswirth BN, Whittam TS, Musser JM. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proc. Natl. Acad. Sci. U.S.A.* 1997;94(18):9869-74.
129. **Stibitz S**, Black W, Falkow S. The construction of a cloning vector designed for gene replacement in *Bordetella pertussis*. *Gene* 1986;50(1-3):133-40.
130. **Stockbauer KE**, Fuchslocher B, Miller JF, Cotter PA. Identification and characterization of BipA, a *Bordetella* Bvg intermediate phase protein. *Mol. Microbiol.* 2001;39(1):65-78.
131. **Storsaeter J**, Hallander HO, Gustafsson L, Olin P, JabbalGill I, Fisher AN, Rappuoli R, Davis SS, Illum L. Levels of anti-pertussis antibodies related to protection after household exposure to *Bordetella pertussis.* *Vaccine* 1998;16(20):1907-16.
132. **Strebel P**, Nordin J, Edwards K, Hunt J, Besser J, Burns S, Amundson G, Baughman A, Wattigney W. Population-based incidence of pertussis among adolescents and adults, Minnesota, 1995-1996. *J. Infect. Dis.* 2001;183(9):1353-9.
133. **Syedabubakar SN**, Matthews RC, Preston NW, Owen D, Hillier V. Application of pulsed field gel electrophoresis to the 1993 epidemic of whooping cough in the UK. *Epidemiol. Infect.* 1995;115(1):101-13.
134. **Tamin A**, Rota PA, Wang ZD, Heath JL, Anderson LJ, Bellini WJ. Antigenic analysis of current wild type and vaccine strains of measles virus. *J. Infect. Dis.* 1994;170(4):795-801.
135. **Taranger J**, Trollfors B, Bergfors E, Knutsson N, Sundh V, Lagergard T, Lind-Brandberg L, Zackrisson G, White J, Cicirello H, et al. Mass vaccination of children with pertussis toxoid-decreased incidence in both vaccinated and nonvaccinated persons. *Clin. Infect. Dis.* 2001;33(7):1004-10.
136. **Taranger J**, Trollfors B, Lagergard T, Lind L, Sundh V, Zackrisson G, Bryla DA, Robbins JB. Unchanged efficacy of a pertussis toxoid vaccine throughout the two years after the third vaccination of infants. *Pediatr. Infect. Dis. J.* 1997;16(2):180-4.
137. **Taranger J**, Trollfors B, Lagergard T, Sundh V, Bryla DA, Schneerson R, Robbins JB. Correlation between pertussis toxin IgG antibodies in postvaccination sera and subsequent protection against pertussis. *J. Infect. Dis.* 2000;181(3):1010-3.
138. **Tasman A**. Het verband tussen de aard van de gebruikte entstof, de wijze van inspuiten en de ent-reacties na immunisatie tgen kinkhoest, difterie en tatanus. *Ned. T. Geneesk.* 1959;103(20):1049-57.

139. **Thalen M**, van den IJ, Jiskoot W, Zomer B, Roholl P, de Gooijer C, Beuvery C, Tramper J. Rational medium design for *Bordetella pertussis*: basic metabolism. *J. Biotechnol.* 1999;75(2-3):147-59.

140. **Toyota T**, Kai Y, Kakizaki M, Sakai A, Goto Y, Yajima M, Ui M. Effects of islet-activating protein (IAP) on blood glucose and plasma insulin in healthy volunteers (phase 1 studies). *Tohoku. J. Exp. Med.* 1980;130(2):105-16.

141. **Tuomanen E**, Towbin H, Rosenfelder G, Braun D, Larson G, Hansson GC, Hill R. Receptor analogs and monoclonal antibodies that inhibit adherence of *Bordetella pertussis* to human ciliated respiratory epithelial cells. *J. Exp. Med.* 1988;168(1):267-77.

142. **Tuomanen E**, Weiss A. Characterization of two adhesins of *Bordetella pertussis* for human ciliated respiratory-epithelial cells. *J. Infect. Dis.* 1985;152(1):118-25.

143. **Uhl MA**, Miller JF. Central role of the BvgS receiver as a phosphorylated intermediate in a complex two-component phosphorelay. *J. Biol. Chem.* 1996;271(52):33176-80.

144. **Urwin G**, Krohn JA, Deaver-Robinson K, Wenger JD, Farley MM. Invasive disease due to *Haemophilus influenzae* serotype f: clinical and epidemiologic characteristics in the *H. influenzae* serotype b vaccine era. The *Haemophilus influenzae* Study Group. *Clin. Infect. Dis.* 1996;22(6):1069-76.

145. **van Boven M**, de Melker HE, Schellekens JF, Kretzschmar M. A model based evaluation of the 1996-7 pertussis epidemic in The Netherlands. *Epidemiol. Infect.* 2001;127(1):73-85.

146. **van Buynder PG**, Owen D, Vurdien JE, Andrews NJ, Matthews RC, Miller E. *Bordetella pertussis* surveillance in England and Wales: 1995-7. *Epidemiol. Infect.* 1999;123(3):403-11.

147. **van den Berg BM**, Beekhuizen H, Willems RJ, Mooi FR, van Furth R. Role of *Bordetella pertussis* virulence factors in adherence to epithelial cell lines derived from the human respiratory tract. *Infect. Immun.* 1999;67(3):1056-62.

148. **van den Hof S**, Conyn-van Spaendonck MAE, de Melker HE, Geubbels ELPE, Suijkerbuijk AWM, Talsma E, Plantinga AD, Rumke HC. The effects of vaccination, the incidence of the target diseases. *RIVM Rapport* 213676008 1998.

149. **van der Zee A**, Groenendijk H, Peeters M, Mooi FR. The differentiation of *Bordetella parapertussis* and *Bordetella bronchiseptica* from humans and animals as determined by DNA polymorphism mediated by two different insertion sequence elements suggests their phylogenetic relationship. *Int. J. Syst. Bacteriol.* 1996;46(3):640-7.

150. **van der Zee A**, Mooi F, van Embden J, Musser J. Molecular evolution and host adaptation of *Bordetella* spp.: phylogenetic analysis using multilocus enzyme

electrophoresis and typing with three insertion sequences. *J. Bacteriol.* 1997;179(21):6609-17.

151. **van der Zee A**, Vernooij S, Peeters M, van Embden J, Mooi FR. Dynamics of the population structure of *Bordetella pertussis* as measured by IS*1002*-associated RFLP: comparison of pre- and post-vaccination strains and global distribution. *Microbiology* 1996;142(Pt 12):3479-85.
152. **van Embden JD**, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, Hermans P, Martin C, McAdam R, Shinnick TM, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J. Clin. Microbiol.* 1993;31(2):406-9.
153. **van Loo IH**, van der Heide HG, Nagelkerke NJ, Verhoef J, Mooi FR. Temporal trends in the population structure of *Bordetella pertussis* during 1949-1996 in a highly vaccinated population. *J. Infect. Dis.* 1999;179(4):915-23.
154. **van Loo IHM**, Heuvelman CJ, King AJ, Mooi FR. Multilocus sequence typing of *Bordetella pertussis* based on surface proteins: Identification of epidemic types? *J. Clin. Miocrobiol.* Accepted.
155. **van Loo IHM**, Heuvelman CJ, Mooi FR. Effect of variation in pertactin and pertussis toxin on fitness of *Bordetella pertussis* in mice. This thesis Chapter 5.
156. **Verwey WF**, Thiele EH, Sage DN, Suchardt LT. A simplified liquid culture medium for the growth of *Haemophilus pertussis*. *J. Bacteriol.* 1949;58:127-34.
157. **Weber C**, Boursaux-Eude C, Coralie G, Caro V, Guiso N. Polymorphism of *Bordetella pertussis* isolates circulating for the last 10 years in France, where a single effective whole-cell caccine has been used for more than 30 years. *J. Clin. Microbiol.* 2001;39:4396-403.
158. **Willems R**, Paul A, van der Heide HG, ter Avest AR, Mooi FR. Fimbrial phase variation in *Bordetella pertussis*: a novel mechanism for transcriptional regulation. *EMBO J.* 1990;9(9):2803-9.
159. **Willems RJL**, Mooi FR. From whole cell to acellular pertussis vaccines. *Rev. Med. Microbiol.* 1996;7(1):13-21.
160. **Wirsing von Konig CH**, Postels-Multani S, Bock HL, Schmitt HJ. Pertussis in adults: frequency of transmission after household exposure. *Lancet* 1995;346(8986):1326-9.
161. **Witvliet MH**, Vogel ML, Wiertz EJ, Poolman JT. Interaction of pertussis toxin with human T lymphocytes. *Infect. Immun.* 1992;60(12):5085-90.

# Nederlandse samenvatting

Kinkhoest is een ernstige infectie aan de bovenste luchtwegen, veroorzaakt door de bacterie *Bordetella pertussis*. Kinkhoest was in het begin van de 20e eeuw een van de meest voorkomende kinderziekten, totdat er in de 50er jaren grootschalige vaccinatie werd ingevoerd. Kinkhoest verloopt het meest ernstig in pasgeborenen en kinderen jonger dan 1 jaar. In de kinderleeftijd heeft de ziekte een karakteristiek beloop. In het begin kenmerkt de ziekte zich door algemene symptomen van verkoudheid en koorts, gevolgd door een fase, waarin de kenmerkende "kinkende" hoest zich presenteert, een periode die enkele weken tot maanden kan duren. Pasgeborenen en kleine kinderen kunnen in deze fase ernstig ziek worden a.g.v. ademhalingsmoeilijkheden, die ontstaan omdat zij het taaie slijm niet kunnen ophoesten, soms met de dood tot gevolg. Recentelijk is echter gevonden dat kinkhoest ook veel voorkomt bij volwassen, die de ziekte kunnen overdragen op (niet-immune) kinderen.

De vaccins, die in de 50er jaren geïntroduceerd zijn, bestonden uit hele gedode bacteriën (in Nederland onderdeel van het DKTP vaccin) en waren zeer effectief in het bestrijden van de ziekte. In de 70er jaren zorgde negatieve publiciteit over de bijwerkingen van het kinkhoestvaccin voor de ontwikkeling van nieuwe vaccins. Deze acellulaire vaccins bestaan uit een aantal immunogene bestanddelen van de bacterie en geven minder bijwerkingen. In veel landen zijn de hele cel vaccins door deze nieuwe acellulaire vaccins vervangen. In Nederland wordt nog steeds het hele cel vaccin uit de jaren 50 gebruikt.

Ondanks de hoge vaccinatiegraad in Nederland is het aantal kinkhoestgevallen enorm toegenomen sinds 1996 met epidemieën in 1996, 1999 en 2001 (Fig. 1. p.13). In Nederland nam vooral het aantal kinkhoestpatiënten toe in de leeftijdsgroepen 1-4 jaar en 5-9 jaar. Om deze kinderen beter te beschermen zijn de DKTP vaccinaties met 1 maand vervroegd (van 3, 4, 5 en 12 naar 2, 3, 4 en 11 maanden) en worden kinderen van 4 jaar sinds 2001 gevaccineerd met een acellulair vaccin. Het is echter niet duidelijk waarom het aantal kinkhoest gevallen zo is toegenomen. Het doel van dit proefschrift was om een verklaring te vinden voor de toegenomen kinkhoest incidentie in landen met een hoge vaccinatiegraad, Nederland in het bijzonder.

Er zijn een aantal oorzaken waarom de incidentie van kinkhoest zou kunnen toenemen, zoals veranderingen in de surveillance en diagnostiek, veranderingen in de vaccinproductie en een verlaging van de vaccinatiegraad. Deze verklaringen kunnen echter de toename in incidentie in Nederland niet verklaren. Een van de factoren die verder van belang is, is het feit dat de bacterie zich aangepast kan hebben aan het vaccin.
Om dit te kunnen onderzoeken hebben wij bacteriestammen bestudeerd, die bij patiënten zijn geïsoleerd in de periode 1949-2000, en deze vergeleken met de stammen, die voor het vaccin gebruikt worden. In dit proefschrift wordt aangetoond dat er grote veranderingen in de *B. pertussis* populatie zijn ontstaan na de introductie van vaccinatie. Opmerkelijke verschillen werden gevonden in 2 perioden: de jaren 60, 10 jaar na de introductie van vaccinatie, en de jaren 90, de periode waarin de kinkhoestincidentie in Nederland weer is toegenomen (hoofdstukken 2 en 4).

M.b.v. IS*1002* fingerprint typering werden grote verschillen gevonden in fingerprint types in de jaren 60. Tien jaar na de start van vaccinatie verdwenen de fingerprint types uit de prevaccinatieperiode en werden nieuwe fingerprint types gevonden. Met name de frequentie van 1 fingerprint type (ft 29) nam toe, tot 50% van de isolaten uit de 60er jaren. A.g.v. de opkomst van nieuwe fingerprint types nam de genetische diversiteit af in de jaren 60 (Fig. 1. p. 122).
Omdat variatie in buitenmembraaneiwitten (antigene variatie) de onderliggende oorzaak kan zijn van de opkomst van bepaalde fingerprint typen, onderzochten wij variatie in genen, die coderen voor deze buitenmembraaneiwitten. Het enige eiwit dat variatie vertoonde tussen vaccinstammen en klinisch isolaten in de jaren 60 was subeenheid 1 van pertussis toxine (PtxS1). Het nieuwe type, *ptxS1A* werd in 50% van de stammen gevonden, terwijl de frequentie van de vaccin types *ptxS1B* en *ptxS1D* afnam. Ondanks dat er antigene variatie werd gevonden in vaccinstammen en klinisch isolaten, hetgeen suggereert dat vaccinatie heeft geselecteerd voor stammen waarop vaccin geïnduceerde immuniteit minder effect heeft, was vaccinatie in de jaren 60 erg effectief, aangezien het sterftecijfer een 543-voudige daling vertoonde t.o.v. de prevaccinatieperiode.

De 2e periode, waarin grote verschillen in de *B. pertussis* populatie werd gevonden is de jaren 90. In deze periode werd er variatie gevonden in 2 buitenmembraan geassocieerde eiwitten, pertactine (Prn) en tracheaal kolonisatie factor (TcfA). Voor Prn zijn de vaccintypes Prn1 en Prn7, vervangen door Prn2 en Prn3. Voor TcfA is het vaccintype TcfA2 vervangen door TcfA3 (hoofdstuk 3). Verder is er variatie gevonden in genen, die betrokken zijn bij regulatie van expressie van eiwitten, o.a. de promoter regio van pertussis toxine (*ptxP*). De vaccintypes *ptxP1* en *ptxP2* zijn vervangen door *ptxP3* (hoofdstuk 6).
In Nederland is er een toename van kinkhoestpatiënten geregistreerd sinds 1996. Vergelijking van de perioden voor en na 1996 leverde echter geen verschillen op in fingerprint types en frequenties van allelen.
Een meer gedetailleerde analyse van de periode 1990-2000 leverde interessante trends op in frequenties van allelen van de polymorfe eiwitten (Fig. 2. p.123). De 2-voudige toename in kinkhoestaangiften in 1994 t.o.v. 1993 en de 10-voudige toename in 1996 t.o.v. 1995 was geassocieerd met een toename in frequentie van stammen met het niet-vaccintype TcfA3. Dus, de toename in incidentie kan verklaard worden door verandering in de *B. pertussis* populatie. Deze aanname komt overeen met mathematische modellering van Boven et al., die suggereert dat de toename in incidentie verklaard kan worden door een veranderde *B. pertussis* populatie. Na 1996 nam de frequentie van stammen met TcfA3 af, hetgeen verklaard kan worden door de toename in immuniteit tegen deze stammen.
In 1998 en 1999 was er een toename in aangiften t.o.v. 1997 (1,9 en 3,2 x, respectievelijk)(Fig. 2. p.123). Deze toename is geassocieerd met de toename in frequentie van *ptxP3* stammen, opnieuw suggererend dat epidemische jaren zijn geassocieerd met clonale expansie van bepaalde stammen (hoofdstuk 6).
Het aantal doden t.g.v. kinkhoest is sinds 1996 toegenomen in Nederland. In de periode 1996-1999 werden 8 sterfgevallen geregistreerd t.o.v. van 0, 1, 0, 0 and 2 sterfgevallen, respectievelijk, in de perioden 1976-1979, 1980-1983, 1984-1987, 1988-1991 and 1992-1995 (Fig. 3. p.125). In 88% van de sterfgevallen betrof het kinderen jonger dan 3 maanden, hetgeen suggereert dat het aantal doden t.g.v. kinkhoest toeneemt in niet-geïmmuniseerde personen. Gandon et al. toonde aan in een mathematisch model dat sommige vaccins

selecteren voor meer virulente stammen. Een toename in frequentie van zulke stammen veroorzaakt vervolgens een toename in mortaliteit in naïeve personen. Ook de letaliteit (het aantal sterfgevallen / aantal ziekenhuisopnames) toonde een toename van 0.0000, 0.0022, 0,0000, 0.0000 and 0,0024 in de perioden 1976-1979, 1980-1983, 1984-1987, 1988-1991 and 1992-1995, respectievelijk, naar 0.0046 in de periode 1996-1999 (Fig. 3. p.125). De toename in letaliteit was geassocieerd met een toename in frequentie van *ptxP3* stammen, hetgeen suggereert dat *ptxP3* stammen een ernstiger ziektebeeld veroorzaken door verhoogde pertussis toxine productie. Muisexperimenten tonen aan dat het aantal witte bloed cellen (leukocyten) in muizen, die geïnfecteerd zijn met een *ptxP3* stam significant hoger waren t.o.v. muizen, die geïnfecteerd waren met een *ptxP1* stam (hoofdstuk 6). In kinderen is de hoogte van leukocytose een maat voor de ernst van infectie en letaliteit. Een ziekte, die verloopt met ernstigere klinische symptomen, met name hoesten, heeft een hogere overdraagbaarheid van de bacterie tot gevolg. Verder wordt transmissie verhoogd door het betere koloniserende vermogen van *ptxP3* stammen (hoofdstuk 6). Onze hypothese is dat een verhoogde pertussis toxine productie, gevonden in *ptxP3* stammen (hoofdstuk 6), gunstig is voor transmissie door het veroorzaken van een ernstiger ziektebeeld en betere kolonisatie. Beide factoren kunnen mortaliteit beïnvloeden.

Wij vonden een associatie tussen bepaalde *prn* allelen en allelen van andere polymorfe genen, b.v. *tcfA3* was geassocieerd met *prn3* en *ptxP3* met *prn2* (hoofdstuk 3). Dit was onverwacht, omdat variatie in Prn is gebaseerd op recombinatie in repeterende eenheden DNA, hetgeen met een hoge frequentie gebeurt en reversibel is. Gupta et al. heeft aangetoond dat bacterie populaties zich opsplitsen in stammen met niet-overlappende antigene structuren, wanneer deze antigenen immunodominant zijn. De associatie tussen *prn* allelen en allelen van andere polymorfe genen geeft een sterke immuunselectie aan gericht tegen Prn, TcfA en andere nog niet bekende *B. pertussis* antigenen.
Verschillende studies tonen aan dat Prn, TcfA en Ptx belangrijk zijn voor immuniteit. Sero-epidemiologische studies tonen een correlatie aan tussen de titer van anti-Prn antistoffen en bescherming tegen klinische symptomen.

Vaccinatie met een monocomponent pertussis toxoid vaccin induceert significante bescherming voor tenminste 2 jaar en verminderde de transmissie van *B. pertussis* in een populatie. Vaccinatie met TcfA verminderde significant de kolonisatie van trachea in muizen. Het feit dat polymorfe gebieden in Ptx en Prn B- en T-cel epitopen bevat, geeft aan dat deze gebieden belangrijk zijn voor de immuunrespons. Verder was variatie in Prn van invloed of de efficacy van een hele cel vaccin in muizen. Bescherming was het best tegen het vaccintype, Prn1 en minder tegen de niet-vaccintypen Prn2 en Prn3. Wij vonden dat stammen met de niet-vaccintypen Prn en PtxS1 minder goed in staat waren om naïeve muizen te infecteren vergeleken met de vaccintype stam (hoofdstuk 5). Deze data geven aan dat vaccinatie de competitieve balans tussen stammen heeft verschoven, hetgeen heeft geleid tot een toename in frequentie van stammen, die minder goed kunnen overleven in niet immune personen.
De volgende vraag is hoe pathogenen met polymorfe antigenen, zoals *B. pertussis*, beter bestreden kunnen worden. Mathematische modellering benadrukt dat het van belang is om geconserveerde antigenen te gebruiken, zelfs als deze minder immunogeen zijn. In dit proefschrift hebben wij een aantal geconserveerde antigenen beschreven, zoals BrkA, FHA, Fimbriae, OmpP, OmpQ en Vag8, die geschikt kunnen zijn voor toekomstige kinkhoestvaccins (hoofdstuk 3). Vanwege hun rol in immuniteit tegen *B. pertussis* zijn de polymorfe antigenen TcfA en Prn potentiële vaccinkandidaten, vooral wanneer de immuunrespons wordt gericht naar geconserveerde epitopen.

De data gepresenteerd in dit proefschrift geven een verklaring voor de wederopkomst van kinkhoest in Nederland en een beter begrip van de evolutie van pathogenen na de introductie van vaccinatie. Wij hebben aangetoond dat vaccinatie mogelijk heeft geresulteerd in de selectie van meer virulente stammen. Wij hopen dat deze kennis bijdraagt tot de ontwikkeling van betere kinkhoestvaccins.

# Dankwoord

# Curriculum vitae

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Inge van Loo werd geboren op 21 januari 1972 in Maastricht, waar zij opgroeide en in 1990 het Gymnasium B diploma behaalde aan De Stedelijke Scholengemeenschap te Maastricht. Vervolgens ging zij geneeskunde studeren in Utrecht en behaalde haar propedeuse in 1991. Tijdens de daaropvolgende doctoraalstudie heeft zij o.a. stage gelopen bij de vakgroep Fysiologie en Sportgeneeskunde te Utrecht (Dr. Inge Huisveld) en bij het Laboratorium voor Medische Microbiologie te Lund (Dr. Åsa Ljungh) in Zweden. In 1995 behaalde zij het doctoraaldiploma en doorliep vervolgens de coschappen, waarbij het keuze-coschap werd gevolgd bij de afdelingen Medische Microbiologie en Infectieziekten van het Academisch Ziekenhuis Utrecht en het Wilhelmina Kinderziekenhuis. Zij behaalde het artsendiploma in 1997 en kon in ditzelfde jaar beginnen als assistent in opleiding bij het Eijkman-Winkler Instituut voor Medische Microbiologie van het Academisch Ziekenhuis Utrecht (Prof. Dr. Jan Verhoef). Dit promotieonderzoek is uitgevoerd o.l.v. Prof. Dr. Frits Mooi bij het Laboratorium voor Infectieziekten Onderzoek bij het Rijksinstituut voor Volksgezondheid en Milieu te Bilthoven en heeft geleid tot het schrijven van dit proefschrift. Zij zal haar loopbaan vervolgen op de afdeling Medische Microbiologie van het Elizabeth ziekenhuis te Tilburg, waar zij bij Dr. Marcel Peeters als arts-assistent de opleiding tot arts-microbioloog gaat volgen.

# Abbreviations

| | |
|---|---|
| Ac-Hly: | Adenylate cyclase hemolysin |
| ACV: | Acellular vaccine |
| BG: | Borduet Gengou |
| BipA: | *Bordetella* intermediate phase protein |
| bp: | basepair |
| BrkA: | *Bordetella* resistance to killing |
| Bvg: | *Bordetella* virulence genes |
| CFU: | Colony forming units |
| DTPP: | Diphtheria, tetanus, polio and pertussis |
| dt (=ft): | DNA-type |
| Fim: | Fimbriae |
| FHA: | Filamentous hemagglutinin |
| ft (=dt): | fingerprint type |
| GD: | genotypic diversity |
| Gen: | Gentamycin |
| Kan: | Kanamycin |
| ko: | knock-out |
| MLEE: | Multi-locus enzyme electrophoresis |
| MLST: | Multi-locus sequence typing |
| OmpP/Q: | Outermembrane protein P/Q |
| PFGE: | Pulse field gel electrophoresis |
| Prn: | Pertactin |
| Ptx(S1-S5)/(P): | Pertussis toxin (subunits S1-S5)/(promoter) |
| RF: | Relative Fitness |
| RFLP: | Restriction fragment length polymorphism |
| st: | serotype |
| TcfA: | Tracheal colinization factor |
| TCT: | Tracheal cytotoxin |
| *vag/vrg*: | Virulence activated genes/virulence repressed genes |
| WCV: | Whole cell vaccine |
| wt: | wild-type |