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

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Despite the introduction of mass vaccination in 1953 in The Netherlands, pertussis is currently an endemic disease with regular epidemic outbreaks. Changes in the *Bordetella pertussis* population in the first 20 years after the introduction of vaccination were studied by indexing IS1002 fingerprint types, fimbrial serotypes and 15 genes encoding surface proteins. Three periods were compared, the pre-vaccination period (1949–1952) and two subsequent periods, 1953–1958 and 1965–1972. Except for fimbrial serotypes, no changes were observed in the *B. pertussis* population between the first two periods. Mortality decreased fivefold and 543-fold in the periods 1953–1958 and 1965–1972, respectively, compared to the pre-vaccination period. The largest 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 (ft29), 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 may have played a role in driving the observed changes. It is proposed 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.

Keywords: population structure, serotyping, gene polymorphism, fingerprint typing

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 (de Melker *et al.*, 1997, 2000). A remarkable increase in the pertussis incidence has been observed since 1996 (de Melker *et al.*, 1997). In other countries a re-emergence of pertussis has also been observed (Andrews *et al.*, 1997; Bass & Wittler, 1994; De Serres *et al.*, 1995; Guris *et al.*, 1999; Gzyl *et al.*, 2001). Further, although morbidity and mortality is highest in children and newborns, pertussis is now recognized as an important infectious disease of adults (Brennan *et al.*, 2000; Guris *et al.*, 1999; Senzilet *et al.*, 2001; Strebel *et al.*, 2001).

The *Bordetella pertussis* population changed significantly after the introduction of vaccination in a number

of countries (Cassiday *et al.*, 2000; Fry *et al.*, 2001; Gzyl *et al.*, 2001; Mastrantonio *et al.*, 1999; Mooi *et al.*, 1999, 1998; Weber *et al.*, 2001). 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 (van Loo *et al.*, 1999). 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 (Mooi *et al.*, 1998). Thus expansion of escape variants may have contributed to the observed decrease in genotypic diversity. However, only a limited number of isolates and genes encoding immunologically relevant proteins were analysed. 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.

METHODS

Strains. *B. pertussis* isolates were collected in The Netherlands from 1949 to 1972. Most isolates were sent to the RIVM (National Institute of Public Health and the Environment, Bilthoven, The Netherlands) by regional laboratories for serotyping or confirmation of identification. Isolates were stored lyophilized or as a suspension at -70°C . A potential problem was the selection of epidemiologically related isolates. Therefore, whenever possible, we chose those isolates which, based on year or place of isolation, were epidemiologically unrelated. Isolates were revived by growth on Bordet–Gengou agar (Difco, 0048-15-7) supplemented with 1% glycerol and 15% sheep blood at 35°C for 3 days. Additional data concerning the isolates examined are available as supplementary data at <http://mic.sgmjournals.org>.

DNA sequencing. PCR conditions for the amplification of *ptxS1* and *prn* were described previously (Mooi *et al.*, 1998). Amplification of the *tcfA* and *fim2* genes and the 500 bp fragment of *fhaB*, which contained the polymorphic locus (van Loo *et al.*, 2002) was performed in 20 μl , containing 1 μl DNA and 19 μl buffer comprising 50% HotstarTaq Master mix (Qiagen), 1 μM each primer and 5% DMSO. The *tcfA* gene from five isolates was sequenced completely. Two polymorphic regions were identified between bases 1 and 945, which were amplified with the primers *tcfAF* (5'-TTCTTGCGCGTCGTGCTCTTC-3') and *tcfAR3* (5'-GCGGTTGCGGACCTTCAT-3'). A region composed of bases 395–945 was sequenced for all isolates with primers *tcfAR3* and *tcfAF2* (5'-TCGTCTGGCGGACATAACCC-3'). As polymorphism in the second region, composed of bases 1–395, appeared to be linked to polymorphism in region 395–945 (i.e. it was non-informative), it was sequenced for one out of five Dutch isolates with primer *tcfAF*. Primers used for amplification and sequencing of *fim2* were *fim2for* (3'-GCGCCGGCCCTGCGATGCAC-5') and *fim2rev* (3'-GGGGGGTTGGCGATTTC-CAGTTTCTC-5'). For *fhaB* primers *fha9F* (3'-ATCTCCGGCGAGGGG-5') and *fha4450R* (3'-CGGTGTAATCGCCCTGTATC-5') were used for amplification, and primers *fha39R* (3'-GATTGGCGTGCAGCGAGTTC-5') and *fha41R* (3'-GGCTAGCGCTGGCTCTGTCC-5') were used for sequencing. PCR amplification was performed by preheating the samples for 15 min at 95°C . Subsequent amplification of *tcfA* was performed by heating the samples for 15 s at 95°C , initial annealing at 72°C for 15 s and elongation for 1 min at 72°C . The annealing temperature was decreased every two cycles by 2°C until 62°C . Amplification of *fim2* was performed by heating the samples for 15 s at 95°C , initial annealing at 67°C for 15 s and elongation for 1 min at 72°C . The annealing temperature was decreased every cycle by 1°C until 62°C . Amplification of *fhaB* was performed by heating the samples 15 s at 95°C , initial annealing at 65°C for 15 s and elongation

for 4 min at 72°C . The annealing temperature was decreased every two cycles by 2°C until 55°C . The cycle with the final annealing temperature was repeated 25 times and a final elongation was performed for 10 min at 72°C in all cases. PCR fragments were purified with Qiaquick (Qiagen). Sequencing reactions were performed with an ABI Prism Big Dye terminator reaction kit and the reactions were analysed 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 autoagglutination the drop of serum was replaced by physiological saline.

IS1002 DNA fingerprinting. IS1002-based DNA fingerprinting was performed as described previously by digesting chromosomal DNA with *SmaI* (van Loo *et al.*, 1999). The samples were analysed on agarose gels (Pulse Field Certified; Bio-Rad), and transferred to Hybond N+ membrane (Amersham Pharmacia Biotech) using standard DNA blotting techniques. A 293 bp IS1002 probe was used for hybridization. Labelling of the probe with peroxidase and detection of hybridizing bands was performed according to the instructions of the Enhanced Chemiluminescence Gene Detection System (Amersham Pharmacia Biotech). The exposed films were scanned at 190 d.p.i. (HP Scanjet IIcx/T; Hewlett Packard). The films were analysed using the Bionumerics software (Applied Maths). Assignment of IS1002 fingerprint patterns to similarity groups was performed by the calculation of pairwise similarities using the Dice coefficient and cluster analysis with the UPGMA algorithm.

Genotypic diversity. This was calculated with the following equation: genotypic diversity = $(n/n-1)(1-\sum x_i^2)$, where n is the number of isolates and x_i is the frequency of i^{th} fingerprint type (Nei & Tajima, 1981).

Statistical analyses. The statistical significance of the *ptxS1* frequencies and differences in fimbrial serotypes and fingerprint types between periods were calculated by applying the χ^2 test. To comply with the conditions for the χ^2 test the analysis was performed on the summed fingerprint types of the similarity groups. The significance of the genotypic diversity was estimated as follows. By computer simulation we randomly generated 1000 cross-tabulations similar to Table 2, 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 encoding surface proteins in the period 1949–1972

Previously we investigated whether polymorphism occurred in 15 genes encoding *B. pertussis* surface proteins (van Loo *et al.*, 2002). 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

	199	583	682	
<i>ptxS1D</i>	CTC	GAA CAT CTG	// TAT TCC AAC GCT	// ATG GCG CCG GTG GTG
	L	E H L	Y S N A	M A P V V
<i>ptxS1E</i>	...	...	// ... C..	// ... .. A..
			P	M
<i>ptxS1B</i>	...C	...	//	// ... .. A..
	D			I
<i>ptxS1A</i>	...C	...	//	// ..A ... A..
	D		I	I

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.

occurred in the period 1949–1972, these genes, or their polymorphic segments, were sequenced for a representative group of isolates. Only one *tcfA* allele was observed in the investigated period ($n=27$). Two *fim2* (*fim2-1* and *fim2-2*), two *fhaB* (*fhaB1* and *fhaB2*), three *prn* (*prn1*, *prn7* and *prn10*) and four *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 two 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* (AJ430832). 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 isolates.

Isolates were stratified in three 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; hence the period 1949–1952

Fig. 2. Fingerprint types found in The Netherlands in the period 1949–1972. Assignment of IS1002 fingerprint patterns to similarity groups was performed by the calculation of pairwise similarities using the Dice coefficient and cluster analysis with the UPGMA algorithm. A–F indicate the similarity groups. Numbers along the bottom indicate size of DNA markers in kb.

represented the pre-vaccination 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

Table 1. Frequencies of *ptxS1* alleles and fimbrial serotypes in The Netherlands in the period 1949–1972

The numbers of isolates analysed are shown in parentheses. The distribution of *ptxS1* alleles and fimbrial serotypes was analysed with the χ^2 test. *P* values for the frequencies of the *PtxS1* variants are: 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, $P<0.0001$.

Allele/serotype	Total	1949–1952	1953–1958	1965–1972
<i>ptxS1A</i>	30% (20)	0% (0)	0% (0)	71% (20)
<i>ptxS1B</i>	44% (29)	58% (14)	50% (7)	29% (8)
<i>ptxS1D</i>	24% (16)	42% (10)	43% (6)	0% (0)
<i>ptxS1E</i>	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)

Table 2. Frequency of fingerprint types found in the Dutch *B. pertussis* population in the period 1949–1972 and their relationship with *ptxS1* alleles

The period 1949–1952 represents the pre-vaccination period. Six similarity groups (A–F) are indicated. The number of isolates is in parentheses. *P* values for differences in fingerprint type frequencies are (χ^2 test): 1949–1952 vs 1953–1958, *P*=0.507 and 1953–1958 vs 1965–1972, *P*<0.0001.

Fingerprint type	Similarity group	<i>ptxS1</i>	1949–1952	1953–1958	1965–1972
ft29	A	<i>ptxS1A</i>	–	–	50 % (14)
ft33		<i>ptxS1A</i>	–	–	4 % (1)
ft35		<i>ptxS1A</i>	–	–	4 % (1)
ft38		<i>ptxS1A</i>	–	–	7 % (2)
ft79		<i>ptxS1D</i>	8 % (2)	–	–
ft12	B	<i>ptxS1B</i>	4 % (1)	–	11 % (3)
ft14		<i>ptxS1A</i>	–	–	7 % (2)
ft15		<i>ptxS1B</i>	29 % (7)	7 % (1)	–
ft78		<i>ptxS1B</i>	4 % (1)	–	–
ft80		<i>ptxS1B</i>	–	7 % (1)	–
ft84		<i>ptxS1B</i>	–	14 % (2)	–
ft85		<i>ptxS1B</i>	–	7 % (1)	–
ft19	C	<i>ptxS1B</i>	13 % (3)	14 % (2)	–
ft21		<i>ptxS1B</i>	–	–	4 % (1)
ft22		<i>ptxS1B</i>	4 % (1)	–	–
ft24		<i>ptxS1B</i>	4 % (1)	–	–
ft27		<i>ptxS1B</i>	–	–	14 % (4)
ft43		<i>ptxS1D</i>	4 % (1)	–	–
ft44	E	<i>ptxS1D</i>	21 % (5)	28 % (4)	–
ft45		<i>ptxS1D</i>	8 % (2)	14 % (2)	–
ft48	F	<i>ptxS1E</i>	–	7 % (1)	–
No. GD*			24 0.87	14 0.90	28 0.73
Mortality†			1.63	0.34	0.003

* 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.

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 (*P*<0.0001; 1953–1958 vs 1965–1972). 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).

Fimbrial serotypes

Fimbrial serotypes (Fim2, Fim2,3 or Fim3) were determined for isolates, 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 isolates 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.

IS1002-based DNA fingerprinting

Isolates for which the *ptxS1* allele was identified were also analysed by IS1002-based DNA fingerprinting. Twenty-one fingerprint types were observed, which were grouped into six (A–F) similarity groups (Fig. 2). To observe temporal trends, isolates were stratified into three periods: 1949–1952, 1953–1958, 1965–1972 (Table 2). The first two periods did not significantly differ in fingerprint frequencies (*P*=0.507). In these two periods most isolates were found in similarity group B (37 % and

Table 3. Linkage between *ptxS1* and fimbrial serotypes

P values for the distribution of fimbrial serotypes are (χ^2 test): *P*=0.121 for *ptxS1D*, *P*=0.501 for *ptxS1B* and *P*=0.002 for *ptxS1A*.

Serotype	<i>ptxS1D</i>	<i>ptxS1B</i>	<i>ptxS1A</i>
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)

35 %, respectively) and similarity group 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 two periods were very similar, there was a significant difference in fingerprint types and frequencies between the periods 1953–1958 and 1965–1972 (*P*<0.0001). In the period 1965–1972, most (65 %) fingerprint types were found in similarity group A, while in the two previous periods 9 % and 0 % of the fingerprint types were found in this similarity group. Further, in this period 50 % of the isolates were associated with one fingerprint type, ft29, a type that was not detected previously. The expansion of ft29 strains was reflected in the genotypic diversity, which decreased to 0.73 in the period 1965–1972 (*P*=0.004, 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 3). Most notable were the high frequencies of linkage between *ptxS1D* with Fim2,3 (71 %) and *ptxS1A* with Fim3 (89 %).

Linkage between *ptxS1* alleles and fingerprint types

There was congruence between clustering based on fingerprint types and *ptxS1* alleles (Table 2). In similarity group A, four out of five fingerprint types were associated with *ptxS1A*. The major fingerprint type in this similarity group, ft29, was uniquely associated with *ptxS1A*. In similarity group B, six out of seven fingerprint types were associated with *ptxS1B*. Similarity groups C, D, E and F harboured only a single *ptxS1* allele, *ptxS1B*, *ptxS1D* and *ptxS1E*, respectively.

DISCUSSION

In 1953 large-scale pertussis vaccination was introduced in The Netherlands. Since then the pertussis whole-cell vaccine has been changed several times (Cohen, 1958; 1963; Cohen & Leppink, 1956). Initially, vaccine strains were grown on solid media. However, in 1958 production was switched to liquid media and a new strain was chosen (strain 509, *fim2-2 fhaB2 prn7 ptxS1D* Fim2,3), which showed a high potency when grown in

liquid media (Cohen, 1958, 1963; Cohen & Leppink, 1956). It is not clear which strain(s) were used before 1958. In the early 1960s a second strain was added to the vaccine (strain 134, *fim2-1 fhaB1 prn1 ptxS1B* Fim3). Strains 509 and 134 are still used nowadays.

Of the 15 investigated genes, four were found to be polymorphic in the investigated period (*fim2*, *fhaB*, *prn* and *ptxS1*). Three allelic combinations were observed in the pre-vaccination period and the period 1953–1958: [*fim2-2 fhaB2 prn7 ptxS1D*], [*fim2-1 fhaB1 prn1 ptxS1B*] and [*fim2-1 fhaB1 prn10 ptxS1E*]. The first two combinations were present in the two vaccine strains, used since the early 1960s, while the third combination of alleles was observed in one isolate only. In the 1960s, only one allelic combination from the pre-vaccination period was observed [*prn1 fim2-1 fhaB1 ptxS1B*], and a novel, fourth, 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 (Tables 1 and 2). However, changes in frequencies of fimbrial serotypes occurred. The pre-vaccination *B. pertussis* population was characterized by equal frequencies of Fim2 and Fim3 isolates (29 %), while Fim2,3 isolates were found at a higher frequency (43 %) (Table 1). Similar frequencies were found in other countries in the pre-vaccination period (Bronne-Shanbury *et al.*, 1976; Eldering *et al.*, 1969). In the period 1953–1958 a decrease in Fim3 isolates 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 (Tables 1 and 2). The *ptxS1A* allele, not previously detected, was found in 89 % of the isolates, while *ptxS1B* was observed in 29 % of the isolates. 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 ft29, associated with 50 % of the isolates. 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 fivefold lower in the periods 1965–1972 and 1953–1958, respectively, compared to the pre-vaccination period (Cohen, 1963). 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.

Isolates with *ptxS1D* disappeared before *ptxS1B* isolates (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 (Cohen, 1963). 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 isolates from the pre-vaccination era in the United Kingdom and detected in low frequencies (14%) in the United States (Fry *et al.*, 2001; Cassiday *et al.*, 2000). In the same period, *ptxS1B* was found at frequencies of 50% and 81%, respectively, in these countries. Although *ptxS1A* was not detected in the Dutch pre-vaccination population, we presume that it was present at low frequencies, as it was found in the pre-vaccination period in both the United Kingdom and the United States (frequencies 50% and 5%, respectively) (Fry *et al.*, 2001; Cassiday *et al.*, 2000). Strains with *ptxS1A* predominate in many countries with a high vaccination coverage (Cassiday *et al.*, 2000; Fry *et al.*, 2001; Gzyl *et al.*, 2001; Mastrantonio *et al.*, 1999; Mooi *et al.*, 1999, 1998; Weber *et al.*, 2001). Most likely, *ptxS1A* strains are less affected by immunity induced by the vaccine strains, which harbour *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 (in the 1960s) was observed not only in The Netherlands, but also in other countries (Blaskett *et al.*, 1971; Bronne-Shanbury *et al.*, 1976; Eldering *et al.*, 1969; Preston, 1976). It has been suggested that this phenomenon is due to the fact that Fim3 is less immunogenic than Fim2 (Preston, 1976; Preston & Carter, 1992). 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 homopolymeric C-tract (Willems *et al.*, 1990). Thus strains can switch between fimbrial serotypes with relatively high frequency (Robinson *et al.*, 1989).

In general, there was congruence between clustering based on fingerprint type and *ptxS1* type (Table 2). Two exceptions were observed, isolates with *ptxS1D* and *ptxS1A* were found in similarity groups A and B, in which *ptxS1A* and *ptxS1B* predominated, respectively. Especially the presence of *ptxS1D* in similarity group A was striking, as this allele was normally found in similarity group E, which showed a deep branching point with similarity group A. This observation could be explained by horizontal transfer of *ptxS1D* to a similarity group A strain, or chromosomal rearrangements resulting in a change in fingerprint type. 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* isolate found in similarity group A harboured *fim2-2*, *fhaB2* and *prn7*, it seems likely that its close relationship with *ptxS1A* isolates is due to chromosomal rearrangements. Weber *et al.* (2001) and Cassiday *et al.* (2000) studied the relationship between *B. pertussis* isolates with PFGE. 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* (Mooi *et al.*, 1998), this may suggest that fingerprinting based on IS1002-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 an effect resulting from the introduction of a vaccine. In the period 1965–1972, 50% of the isolates belonged to one fingerprint type (ft29), and all of these isolates contained the *ptxS1A* allele, indicating that clonal expansion had occurred. The 114-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 harbouring PtxS1A. Strains with PtxS1A may have been introduced by import or mutation, or may have existed at a low frequency before the introduction of vaccination. Although we favour 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 of the polymorphisms observed in *ptxS1* were non-synonymous, which was consistent with the emergence of these alleles as a consequence of positive immune 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 (De Magistris *et al.*, 1989; Scarselli *et al.*, 1998). Hausman & Burns (2000) did not find a difference in the ability of antibodies raised with an acellular vaccine to neutralize pertussis toxin variants derived from *B. pertussis* and *Bordetella bronchiseptica*. 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 three *B. pertussis* genes since the introduction of vaccination: *ptxS1*, *tcfA* and *prn* (van Loo *et al.*, 2002). Mutations in *prn* have been shown to affect efficacy of a whole-cell vaccine in the mouse model (King *et al.*, 2001). Together, these changes may act synergistically and reduce efficacy of pertussis whole-cell vaccines.

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