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

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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.*, 1998, 1999; 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% Hotstart Taq 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, and gene from five isolates was sequenced completely. Two

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The gene was sequenced for 66 isolates.

The numbers of isolates analysed are shown in parentheses. The distribution of ptxS1 alleles and fimbrial serotypes was analysed by the χ² 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.

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

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<tr>
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<tbody>
<tr>
<td>ptxS1A</td>
<td>30% (20)</td>
<td>0% (0)</td>
<td>0% (0)</td>
<td>71% (20)</td>
</tr>
<tr>
<td>ptxS1B</td>
<td>44% (29)</td>
<td>58% (14)</td>
<td>50% (7)</td>
<td>29% (8)</td>
</tr>
<tr>
<td>ptxS1D</td>
<td>24% (16)</td>
<td>42% (10)</td>
<td>43% (6)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>ptxS1E</td>
<td>2% (1)</td>
<td>0% (0)</td>
<td>7% (1)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Fim2</td>
<td>22% (13)</td>
<td>29% (6)</td>
<td>38% (5)</td>
<td>8% (2)</td>
</tr>
<tr>
<td>Fim2,3</td>
<td>27% (16)</td>
<td>43% (9)</td>
<td>54% (7)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Fim3</td>
<td>52% (31)</td>
<td>29% (6)</td>
<td>8% (1)</td>
<td>92% (24)</td>
</tr>
</tbody>
</table>

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.

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.
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 \( \chi^2 \) test: 1949–1952 vs 1953–1958, \( P = 0.507 \) and 1953–1958 vs 1965–1972, \( P < 0.0001 \).

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<tr>
<td>ft29</td>
<td>A</td>
<td><em>ptxS1A</em></td>
<td>–</td>
<td>–</td>
<td>50% (14)</td>
</tr>
<tr>
<td>ft33</td>
<td></td>
<td><em>ptxS1A</em></td>
<td>–</td>
<td>–</td>
<td>4% (1)</td>
</tr>
<tr>
<td>ft35</td>
<td></td>
<td><em>ptxS1A</em></td>
<td>–</td>
<td>–</td>
<td>4% (1)</td>
</tr>
<tr>
<td>ft38</td>
<td></td>
<td><em>ptxS1A</em></td>
<td>–</td>
<td>–</td>
<td>7% (2)</td>
</tr>
<tr>
<td>ft79</td>
<td></td>
<td><em>ptxS1D</em></td>
<td>8% (2)</td>
<td>–</td>
<td></td>
</tr>
<tr>
<td>ft12</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>4% (1)</td>
<td>–</td>
<td>11% (3)</td>
</tr>
<tr>
<td>ft14</td>
<td>B</td>
<td><em>ptxS1A</em></td>
<td>–</td>
<td>–</td>
<td>7% (2)</td>
</tr>
<tr>
<td>ft15</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>29% (7)</td>
<td>7% (1)</td>
<td>–</td>
</tr>
<tr>
<td>ft78</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>4% (1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ft80</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>–</td>
<td>7% (1)</td>
<td>–</td>
</tr>
<tr>
<td>ft84</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>–</td>
<td>14% (2)</td>
<td>–</td>
</tr>
<tr>
<td>ft85</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>–</td>
<td>7% (1)</td>
<td>–</td>
</tr>
<tr>
<td>ft19</td>
<td>C</td>
<td><em>ptxS1B</em></td>
<td>13% (3)</td>
<td>14% (2)</td>
<td>–</td>
</tr>
<tr>
<td>ft21</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>–</td>
<td>–</td>
<td>4% (1)</td>
</tr>
<tr>
<td>ft22</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>4% (1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ft24</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>4% (1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ft27</td>
<td></td>
<td><em>ptxS1B</em></td>
<td>–</td>
<td>–</td>
<td>14% (4)</td>
</tr>
<tr>
<td>ft43</td>
<td></td>
<td><em>ptxS1D</em></td>
<td>4% (1)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>ft44</td>
<td></td>
<td><em>ptxS1D</em></td>
<td>21% (5)</td>
<td>28% (4)</td>
<td>–</td>
</tr>
<tr>
<td>ft45</td>
<td>E</td>
<td><em>ptxS1D</em></td>
<td>8% (2)</td>
<td>14% (2)</td>
<td>–</td>
</tr>
<tr>
<td>ft48</td>
<td>F</td>
<td><em>ptxS1E</em></td>
<td>–</td>
<td>7% (1)</td>
<td>–</td>
</tr>
</tbody>
</table>

| No. GD*          | 24              | 14       | 28        |
| Mortality†       | 0.87            | 0.90     | 0.73      |
|                  | 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 100000.

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...
Vaccine-driven evolution of *Bordetella pertussis*

Table 3. Linkage between ptxS1 and fimbrial serotypes

<table>
<thead>
<tr>
<th>Serotype</th>
<th>ptxS1D</th>
<th>ptxS1B</th>
<th>ptxS1A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fim 2</td>
<td>14% (2)</td>
<td>30% (8)</td>
<td>11% (2)</td>
</tr>
<tr>
<td>Fim 2,3</td>
<td>71% (10)</td>
<td>22% (6)</td>
<td>0% (0)</td>
</tr>
<tr>
<td>Fim 3</td>
<td>14% (2)</td>
<td>48% (13)</td>
<td>89% (16)</td>
</tr>
</tbody>
</table>

*P* values for the distribution of fimbrial serotypes are (*χ²* test): *P* = 0.121 for ptxS1D, *P* = 0.501 for ptxS1B and *P* = 0.002 for ptxS1A.

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, 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 fabB2 pmn7 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 fabB1 pmn1 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, fabB, pmn and ptxS1). Three allelic combinations were observed in the pre-vaccination period and the period 1953–1958: [fim2-2 fabB2 pmn7 ptxS1D], [fim2-1 fabB1 pmn1 ptxS1B] and [fim2-1 fabB1 pmn10 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 [pmn1 fim2-1 fabB1 ptxS1A], and a novel, fourth, allelic combination was found [pmn1 fim2-1 fabB1 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; Mastrandionio 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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