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The population structure of *Bordetella pertussis* in The Netherlands in 5 successive periods, encompassing 1949–1996, was analyzed by DNA typing (“fingerprinting”). In 10 years following the introduction of wide-scale vaccination in 1953, a decrease in genotypic diversity (GD) was observed, suggesting clonal expansion of strains that 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. The results herein suggest that vaccination has selected for strains that are adapted to a highly vaccinated population. Similar changes may have occurred in other countries, explaining the reemergence of pertussis in vaccinated populations.

Despite the introduction of large-scale pertussis vaccination in 1953 [1] and high vaccination coverage, pertussis is still an endemic disease in The Netherlands, with epidemic outbreaks occurring every 3–5 years. In 1996, there was an especially severe pertussis epidemic, with an incidence 5-fold higher than in previous epidemic years [2]. Recent pertussis epidemics have also been observed in other countries, such as Australia, Canada, Norway, and the United States, notwithstanding routine vaccination [3–6]. Several explanations have been put forward for the resurgence of pertussis in vaccinated populations [7–9]. 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 that might 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 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 [10]. Pertussis toxin and pertactin variants identical to those found in the Dutch whole cell pertussis vaccine were found in 1996 and 1984, respectively. DNA typing (based on the insertion sequence element IS1002) of Dutch strains revealed little overlap in DNA types when strains from the 1996 epidemic were compared with those from 1978–1994, also providing evidence that shifts had occurred in other countries.

Here we extend our previous studies by IS1002-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 during 1949–1996. Most strains were sent by regional laboratories to the National...
Institute of Public Health and the Environment 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 that, 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 (~30%), either place of isolation or patient name was known, in addition to the date of isolation, allowing us to select strains that 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 periods in which 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 (0048-15-7; Difco, Detroit) supplemented with 1% glycerol and 15% sheep blood, or in Verwey medium [12] supplemented with 200 μg/mL cycloheximide (Sigma, St. Louis) at 35°C for 3 days.

**IS1002-based DNA typing.** Some improvements of the previously described IS1002-based DNA typing method were implemented [11]. 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 using the Puregene DNA isolation kit (D5000A; Gentra Systems, Minneapolis) using the protocol for gram-negative bacteria as provided by the manufacturer. After purification, 1 μg of chromosomal DNA was digested for 2 h, with 2 U of Smal in a final volume of 200 μL, ethanol-precipitated, and dissolved in 10 μL of sample buffer. Samples were applied on a 1% agarose gel (pulse-field certified agarose, Bio-Rad Laboratories, Hercules, CA), and run on the FIGE mapper electrophoresis system (Bio-Rad) for 15 h in 0.5 × 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 s, linear shape, forward voltage 180 V, and reversed voltage 120 V. As a molecular-weight marker, a mixture of the 1 kb-ladder (Life Technologies Gibco BRL, Rockville, MD) and the CHEF marker (Bio-Rad) 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-bp IS1002 probe used for hybridization was generated by polymerase chain reaction (PCR) as follows: 5 μL of a 500-ng/μL DNA stock from strain B24 [13] was amplified with 1 U of SuperTaq (Sphaero-Q, Leiden, The Netherlands) in 1 × SuperTaq buffer, with dimethyl sulfoxide added to a final concentration of 5%, 25 pmol of each primer, and 4 μL of dNTP-mix containing 2.5 mM each dNTP, in a final volume of 50 μL. The PCR program was as follows: 1 cycle of 3 min at 95°C, 35 cycles of 15 s at 95°C, 30 s at 60°C, and 60 s at 72°C, and 1 cycle of 10 min at 72°C. PCR was performed in a Hybaid Touch-Down PCR apparatus (Hybaid Limited, Ashford, UK). The following primers were used: HG1 (5-GCCGATGCGTTCATATA-3) and HG2 (5-AGCCCTTCTTGGTAGAAGGG-3). After PCR, the IS1002 probe was purified with the Qiaquick PCR purification kit (Diagen, Hilden, Germany) and labeled with peroxidase according to the instructions provided with the Enhanced Chemiluminescence Gene Detection System (Amersham-Pharmacia Biotech). Marker DNA was also labeled with peroxidase, and hybridization and detection of IS1002-containing DNA fragments and marker DNA were performed according to the Amersham-Pharmacia protocol. The exposed films were scanned at 190 dpi (HP Scanjet I1cx/T; Hewlett-Packard, Camas, WA). Computer-assisted analysis of the IS1002 patterns was carried out using the GelCompar software (Windows NT version 4.1; Applied Maths, Kortrijk, Belgium). To compare IS1002 patterns on different blots, the 5 bands of the IS1002 type dt-29 (5050 bp, 6580 bp, 8470 bp, 11100 bp, and 15600 bp), which were shared by most DNA types, were used as internal standards to facilitate normalization. The IS1002-hybridizing bands in B. pertussis ranged in size from 2600 to 37,800 bp. Pairwise similarities of IS1002 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 based on DNA typing was calculated by the following equation: genotypic diversity = {n(n−1)(1−Σx²)} / n, where x is the frequency of the i-th DNA type and n is the number of strains [14].

**Statistical analysis.** The statistical significance of the difference in DNA type frequencies between periods was calculated by cross-tabulating frequencies by period (figure 1, tables 1, 2) and applying the χ² test. To comply with the conditions for the χ² test (i.e., >80% of the cells should have an expected cell frequency of ≥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 1949–1954, 1965–1972, 1976–1981, 1982–1990, and 1991–1996, the summed DNA types of the clades A, B, and C were used (figure 1). 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 figure 1 and tables 1 and 2, under the null hypothesis of no differences in frequency of DNA types between the observed number of particular DNA types or the total number of observed DNA types per period or prn group (the so-called “marginal totals”). Under that hypothesis, DNA types essentially occur at random in the different periods or prn groups. This can be computer-simulated 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. 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**

**IS1002-based DNA typing of Dutch strains isolated in 1949–1996.** A collection of Dutch strains, collected in 1949–1996 and comprising 213 isolates, was investigated for IS1002-based restriction fragment length polymorphism (figure 2). The num-
Figure 1. Frequency of DNA types found in Dutch *B. pertussis* population in 5 successive periods; 6 main clades (A–F) are indicated. Bar indicates similarity coefficient. Boxes with DNA types (dt) associated with Dutch vaccine strains (dt-19 and dt-43) are shaded. No. of isolates is in parentheses. GD, genotypic diversity. *P* values for differences in DNA type frequencies: 49–54 vs. 65–72, *P* < .001; 65–72 vs. 76–81, *P* = 0.54; 76–81 vs. 82–90, *P* = 0.89; 82–90 vs. 91–96, *P* = 0.34. *P* values for differences in genotypic diversity: 49–54 vs. 65–72, *P* = .016; 65–72 vs. 76–81, *P* = 0.32; 76–81 vs. 82–90, *P* = .056; 82–90 vs. 91–96, *P* = 0.243.
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 DNA types. To observe temporal trends in DNA types, strains were stratified in 5 periods: 1949–1954, 1965–1972, 1976–1981, 1982–1990, and 1991–1996, respectively (figure 1). Each period comprised 6–9 years. Since large-scale vaccination against pertussis was introduced in The Netherlands in 1953 [1], 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. In contrast, no significant differences were observed between the populations from 1965–1972 and the following periods, 1976–1981, 1982–1990, and 1991–1996. Differences between 1949–1954 and later periods were also evident when the distribution of strains over the different clades was compared. Strains from 1949–1954 were found mainly 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, whereas most strains (56%–82%) were found in clade A, which harbored 4% of the strains from the period 1949–1954.

Statistical analysis based on the distribution of DNA types over the different populations indicated a highly significant difference (\(P < .001\)) between the B. pertussis populations from 1949–1954 and the ensuing period, 1965–1972. In contrast, no significant differences were observed between the populations from 1965–1972 and the following periods, 1976–1981, 1982–1990, and 1991–1996.
Figure 2. Dendrogram of normalized DNA types found in Dutch strains. Branching points of 6 main clades are indicated with letters A–F. Nos. refer to designation of DNA types. Clustering was performed using unweighed pair group method with arithmetic averages. Left and right bars indicate similarity coefficient and size of DNA fragments in kilobases, respectively.
of 2 successive periods after the 1949–1954 period (figure 1). It may be argued that this comparison is incorrect, since the first 2 periods are separated by 10 years, whereas all subsequent periods are separated by 1–3 years. However, the periods 1965–1972 and 1982–1990 are also separated by ~10 years, whereas the B. pertussis populations derived from these periods do not differ significantly with respect to DNA type distributions (P = .17).

The genotypic diversity of the strains isolated in the 5 periods was calculated (figures 1, 3), and the highest (0.89) and lowest (0.60) genotypic diversities were observed, respectively, during 1949–1954 and the succeeding period, 1965–1972. The difference in genotypic diversity between these 2 periods was significant (P = .016; figure 1). After the period 1965–1972, the genotypic diversity increased to 0.69, 0.88, and 0.79, respectively, in the periods 1976–1981, 1982–1990, and 1991–1996. The difference in genotypic diversity between 1976–1981 and 1982–1990 was marginally significant (P = .056). P values of other, successive, periods were not significant.

Interesting differences between the 5 periods were also observed when the DNA types were grouped on the basis of the number of IS1002 copies (figure 3). In the prevaccination period, strains with 4 and 6 copies of IS1002 predominated (frequencies, respectively, =33% and 46%). In the subsequent period (1965–1972), all strains harbored 5 IS1002 copies, whereas 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.

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 3 types coexisted (previously, the products of the prn1, prn2, and prn3 alleles were referred to as P.69A, P.69B, and P.69C, respectively) [10]. The frequency distributions of the DNA types within the three 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 (25% and 32%, respectively). The frequencies of dt-12 in the three 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 2 closely related DNA types dt-9 and dt-10 (figure 2), 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 the prn2 and prn3 populations were significant (P = .044 and <.001, respectively). The difference in DNA type frequencies between the prn1 and prn2 populations was not significant (P = .83; table 1).

The genotypic diversities, 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).

Discussion

As in 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 [15]. A second study, which focused on the measles virus, provided evidence that vaccination has affected the population structure of the virus and selected for strains that 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 [16, 17]. Here we extend our previous studies [10, 11] on the effect of vaccination on the population structure of B. pertussis.

We analyzed temporal trends in the population structure of B. pertussis during 1949–1996 in The Netherlands. Strains were stratified in 5 periods encompassing 1949–1954, 1965–1972, 1976–1981, 1982–1990, and 1991–1996 (figure 1). Large-scale vaccination against pertussis was introduced in The Netherlands in 1953 [1], and isolates from the earliest period analyzed, 1949–1954, probably represent a B. pertussis population that has not been significantly affected by vaccination. Differences were found between the populations from the 5 periods, reflected in the frequency of DNA types (figure 1), the number of IS1002 copies (figure 3), and the genotypic diversity (figures 1, 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 = .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.

During 1965–1996, 3 DNA types (dt-12, dt-29, and dt-35) were predominant, comprising 60%–73% of the isolates. Strain typing with pulsed-field gel electrophoresis (PFGE) also revealed a predominance of 2 or 3 PFGE types in the B. pertussis population in Canada and England [18, 19]. A single PFGE type was observed during an epidemic in the United States that occurred in a small rural community [20]. B. pertussis is thought to be a very homogeneous species. For example, multilocus enzyme electrophoresis (MLE) has revealed only 4 electrophoretic types [11, 21]. In contrast, IS1002-based DNA typing revealed 45 DNA types for 213 isolates. Thus, IS1002-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 [22, 23].

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 [10]. 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, identical to that 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; 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 allele 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 humans and animals [24–26]. 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, 1965–1972, and 1949–1954, respectively (table 3). Thus, the largest difference in prn1 frequency (i.e., the largest increase in the frequencies of prn2 and prn3) is found between the last 2 periods. 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 that was not statistically significant (P = .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 with the prn1 group (P = .106 and <.001, respectively), is consistent with the observation that prn1 strains represent the oldest lineage [10]. The prn3 group shows the lowest genotypic diversity, suggesting it represents a younger lineage than the prn2 group (P = .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 [2]. 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 that 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 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 reemergence of pertussis in vaccinated populations [3–6]. Our results predict that whole cell vaccines will protect less well against recent isolates compared with strains from the 1950s. Preliminary studies in a mouse model have confirmed this prediction (unpublished data). Most pertussis vaccines are derived from strains isolated >50 years ago [11]. It seems plausible that such vaccines may be made more effective by using contemporary strains.

References


