JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2009, p. 680–688 Vol. 47, No. 3
0095-1137/09/$08.00+0 doi:10.1128/JCM.01838-08

Changes in Genetic Diversity of the *Bordetella pertussis* Population in the United Kingdom between 1920 and 2006 Reflect Vaccination Coverage and Emergence of a Single Dominant Clonal Type▽†

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Received 23 September 2008/Returned for modification 14 November 2008/Accepted 8 January 2009

Pertussis (whooping cough) is a potentially fatal respiratory disease caused by the bacterium *Bordetella pertussis*. Despite effective vaccination programs, there has been concern in some developed countries that pertussis cases are on the increase. We characterized 703 clinical *B. pertussis* isolates collected in the United Kingdom between 1920 and 2006 using multilocus variable-number tandem repeat analysis (MLVA), pertactin (*prnA*) and pertussis toxin (*ptxA*) genotyping, and serotyping. The results showed that the genetic diversity of the bacterial population decreased during periods of high vaccine coverage. However, it was elevated between 1977 and 1986, when vaccine coverage in the United Kingdom was low and epidemics occurred. A high proportion of MLVA types during this epidemic period were novel, and the *prnA*(2) and *prnA*(3) alleles were seen for the first time in the United Kingdom. MLVA-27 appeared in 1982, was codominant during the 1998-to-2001 period, and comprised ~70% of isolates during both the 2002-to-2004 and the 2005-to-2006 periods. The United Kingdom is dominated currently by an MLVA-27 *prnA*(2) *ptxA*(1) serotype Fim3 clonal type. Even during recent periods dominated by MLVA-27, many novel types were found at low frequencies, suggesting that either there are a large number of uncommon MLVA types circulating at low frequencies or new types are constantly arising. This supports a hypothesis that MLVA-27 is under some form of positive selection conferring increased survival in a highly vaccinated population. There has been no significant change to the bacterial population in the first 2 years since the United Kingdom switched from a whole-cell to an acellular vaccine.

Bordetella pertussis causes potentially fatal pertussis disease (whooping cough) in humans. Despite vaccination programs that are highly effective in reducing serious disease and mortality, there has been some concern recently in developed countries that pertussis cases are on the increase. Various explanations have been proposed, including increased ascertainment due to improved diagnostic methods, poor efficacy of particular batches of vaccine, and antigenic variation occurring in the bacterial population (7, 27, 28, 30, 34). There is evidence of antigenic change in bacterial virulence factors, such as pertactin, pertussis toxin, and fimbriae, and it has been suggested that this might compromise vaccine-mediated immunity against circulating isolates (6, 11, 17, 23, 26, 27, 40).

Mass immunization with a whole-cell vaccine (WCV) was introduced into the United Kingdom in 1957 (14). From 1975 to 1978, there was a dramatic drop in vaccine coverage (to a low of 31%) due to concerns over vaccination side effects. Large epidemics occurred in 1978 and 1982. Coverage gradually recovered as public confidence was restored; it exceeded 80% in 1990 and 90% in 1992 (24; http://www.hpa.org.uk/). In June 1990, the vaccine schedule for primary immunization, including that for pertussis, was altered from 3, 5, and 10 months to 2, 3, and 4 months of age (24). In November 2001, a booster dose of acellular vaccine (ACV; three- or five-component) for children aged 3 to 5 years was added to the schedule (8). In October 2004, the United Kingdom ceased using WCV and switched to using a five-component ACV for primary vaccinations (9). The recent increase of pertussis reported for some other countries has not been observed in the United Kingdom to date.

A number of methods have been used to characterize *B. pertussis* isolates. Most are based on known vaccine antigens or virulence factors. Serotyping with antibodies against three surface antigens divides isolates into four serotypes. Two of these three antigens are fimbrial and are also known as Fim2 and Fim3 (25). DNA sequence-based typing is increasingly being used, and polymorphisms have been found in genes encoding the S1 and S3 subunits of pertussis toxin (*ptxA* and *ptxC*), pertactin (*prnA*), tracheal colonization factor (*tcfA*), fimbrial antigens 2 and 3 (*fim2* and *fim3*), filamentous hemagglutinin (*fhaB*), adenylate cyclase toxin (*cyaA*), outer membrane protein Q (*ompQ*), virulence-activated gene 8 (*vag8*), *Bordetella* autotransporter protein C (*bapC*), and *Bordetella* intermediate-phase protein (*bipA*) (29, 35, 40). However, polymorphisms in most of these genes are rare. In studies, the most commonly analyzed genes are *prnA* and *ptxA*, due to their importance as vaccine antigens (6, 13, 15, 17, 21, 23, 26, 27, 30, 42). Some studies have also included other targets, notably *ptxC*, *tcfA*,

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† Supplemental material for this article may be found at http://jcm.asm.org/.

▽ Published ahead of print on 21 January 2009.

fim2, and *fim3* (1, 4, 22, 29, 36, 37, 40, 41). A multilocus sequence typing (MLST) scheme based on the sequences of *ptxA*, *ptxC*, and *tcfA* has been described (29, 40).

Clinical isolates of *B. pertussis* collected in the United Kingdom between 1920 and 2002 have previously been characterized by our laboratory, using serotyping and DNA sequence-based typing (13, 29). The results revealed that the serotypes of isolates have changed from a mixture of Fim$^-$, Fim2, Fim3, and Fim2,3 before mass vaccination to an almost exclusive phenotype of Fim3 since 2000. Furthermore, isolates carried the same *prnA* allele as the WCV [*prnA*(1)] exclusively until 1982, when *prnA*(2) and *prnA*(3) variants first appeared. The *prnA*(2) allele gradually increased in frequency and comprised 89% (31/35) of isolates collected between 2000 and 2002. The pertussis toxin S1 subunit genotypes have changed from an equal mixture of *ptxA*(1) and *ptxA*(2) alleles in the 1940s (matching the two alleles in the WCV) to the exclusive presence of *ptxA*(1) since the 1990s. The *ptxA*, *ptxC*, and *tcfA* genotypes showed that isolates moved away from MLST types in the WCV (MLST-2 [*ptxA*, *ptxC*, and *tcfA*; allele profile 2,1,2]) and MLST-3 (profile 1,1,2) over time. They were almost all MLST-5 (profile 1,2,2) between 2000 and 2002 (23/24 isolates tested). Overall, the emergences of *prnA*(2) and MLST-5 were consistent with a hypothesis of vaccine-driven evolution, but the changes in *ptxA* and serotyping were not (13, 29).

We sought to characterize our collection of clinical isolates from the United Kingdom further by using a method that offered greater discrimination than MLST. Genetic fingerprinting methods, such as pulsed-field gel electrophoresis and IS*1002*-based fingerprinting, have been used extensively (5, 11, 16, 17, 21–23, 30, 36, 39, 41, 42). However, they are laborious, and comparing results between laboratories can be difficult. We chose to use a multilocus variable-number tandem repeat analysis (MLVA) method recently devised for *B. pertussis* (32). In a previous study, MLVA greatly enhanced the discrimination between isolates in comparison to serotyping and DNA sequence typing (32). We also took this opportunity to extend the serotyping and *prnA* and *ptxA* genotyping of isolates collected up to the end of 2006.

(Part of this work was presented as a poster at the Eighth International Symposium, Saga of the genus *Bordetella*, Institut Pasteur, Paris, France, 7 to 10 November 2006.)

MATERIALS AND METHODS

Bacterial strains and culture conditions. A total of 703 clinical *B. pertussis* isolates, collected between 1920 and 2006, were analyzed. This included 325 of 335 previously studied (29) (omitting 1 isolate from 1950, 3 from 1977, 1 from 1998, and 5 from 2000 due to a loss of viability of frozen stocks). It also included the following additional isolates: 1 from 1954, 49 from 1982, 12 from 1983, 1 from 1984, 1 from 1986, 6 from 2001, 25 from 2002, 49 from 2003, 69 from 2004, 109 from 2005, and 57 from 2006. Isolates from before 2001 were obtained from several strain collections from the United Kingdom as previously described (13) or from a recent study of pertussis disease (10). Isolates from 2001 onwards were submitted to the Health Protection Agency's Respiratory and Systemic Infection Laboratory (Health Protection Agency Centre for Infections, London, United Kingdom) by hospital laboratories in the United Kingdom as part of an ongoing enhanced surveillance program. As far as we were able to ascertain, the collection contains only a single isolate from any patient and does not contain more than one isolate from any known outbreaks. Strains CN2992, CN3009, and CN5476 were obtained from the Wellcome Bacterial Collection (held at the National Collection of Type Cultures, London, United Kingdom). The Tohama I strain was obtained from the National Collection of Type Cultures, London, United Kingdom. Strain 10536 was a gift from Aventis Pasteur MSD, Canada. Full typing results for individual isolates are available upon request.

Isolates of *B. pertussis* were cultured on charcoal agar with 10% horse blood (Oxoid, Basingstoke, United Kingdom) at 37°C for up to 5 days in a humid atmosphere. They were archived by preservation in glycerol broth on glass beads at −80°C (18).

Serotyping. Following culture for 48 to 72 h on blood charcoal agar, *B. pertussis* isolates were serotyped in a slide agglutination assay, using rabbit antisera against antigens 1, 2, and 3 (89/596, 89/598, and 89/600, respectively; National Institute for Biological Standards and Controls, Potters Bar, United Kingdom). As antigen 1 is expressed by all isolates of *B. pertussis*, we have chosen to use the alternative notation "Fim$^-$, Fim2, Fim3, and Fim2,3" (17) in place of the historical notation "1, 1,2, 1,3, and 1,2,3."

Preparation of DNA. Following growth on blood charcoal agar, DNA was prepared from *B. pertussis* isolates by using InstaGene Matrix (Bio-Rad Laboratories, Hemel Hempstead, United Kingdom) according to the manufacturer's instructions.

MLVA typing. MLVA typing was performed using the method previously described (32), adapted for analysis using a CEQ8000 genetic analysis system (Beckman Coulter, High Wycombe, United Kingdom). The PCR primers BP-VNTR1-DF, BP-VNTR2-BF, BP-VNTR3-BF, BP-VNTR4-CF, BP-VNTR5-BF, and BP-VNTR6-EF were labeled using WellRED dyes D4, D3, D4, D3, D2, and D2, respectively. Primers were purchased from Sigma-Aldrich. The six PCRs were performed as monoplexes. Each 10-μl reaction mixture contained 5 μl HotStarTaq master mix (Qiagen, Crawley, United Kingdom), 1 μM each PCR primer, 2 μl template DNA, and either 1.0 M betaine (variable-number tandem repeat 1 [VNTR1] to VNTR5 targets) or 1.2 M betaine (VNTR6 target) (stock solution B0300; Sigma-Aldrich, Poole, United Kingdom). The cycling conditions were as previously published (32). Following amplification, PCR products were diluted and mixed into two mixtures in PCR-grade water as follows: mixture 1, 200-μl final volume containing 2 μl VNTR-1 PCR mixture, 2 μl VNTR2 PCR mixture, and 2 μl VNTR5 PCR mixture; mixture 2, 200-μl final volume containing 1 μl VNTR3 PCR mixture, 2 μl VNTR4 PCR mixture, and 3 μl VNTR6 PCR mixture. One microliter of each mixture was then added separately to 30 μl sample loading solution and 0.25 μl CEQ DNA size standard 400 (Beckman Coulter, High Wycombe, United Kingdom) and analyzed using a CEQ8000 genetic analysis system under standard fragment analysis conditions. Analysis of a collection of isolates with known repeats at each VNTR locus allowed for a correction factor to be devised in order to convert the apparent DNA fragment length to the true length for each VNTR target. The number of repeats in each DNA fragment was calculated from the DNA fragment length. The number of repeats at each VNTR locus for each isolate was catalogued and analyzed using the BioNumerics version 4.5 software package (Applied Maths, Sint-Martins-Latem, Belgium).

Each MLVA type was assigned as described previously (32). Novel MLVA profiles were submitted to F. Mooi (Laboratory for Infectious Diseases and Screening, Netherlands Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, The Netherlands) for designation of an MLVA type number.

***prnA* and *ptxA* sequence typing.** Sequencing of the two variable regions of the *prnA* gene was performed using a variation of the method described previously (27). Briefly, a DNA fragment encompassing both variable regions was amplified by PCR on a DNA engine tetrad thermocycler (MJ Research, Inc., MA), using the primers AF and BR in a 20-μl reaction mixture containing 10 μl HotStarTaq master mix (Qiagen, Crawley, United Kingdom), 1 μM each primer, 1 M betaine (stock solution B0300; Sigma-Aldrich, Poole, United Kingdom), and 2 μl target DNA. The thermal cycling conditions were as follows: 15 min at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 58°C, and 1 min 30 s at 72°C. After the last cycle, a final step of 10 min at 72°C was added. The PCR product was purified using a Montage PCR96 filter plate (Millipore, Watford, United Kingdom), and the two variable regions were sequenced in both directions by using primers AF, AR, BF, and BR (27). Further sequencing outside the variable regions was performed where necessary to distinguish between *prnA*(1) and *prnA*(7) genotypes. Sequencing reactions were performed using a CEQ dye terminator cycle sequencing quick start kit and the products analyzed with a CEQ8000 genetic analysis system (Beckman Coulter, High Wycombe, United Kingdom).

Sequencing of the *ptxA* gene was performed using a modification to the method described previously (27). The entire *ptxA* open reading frame was amplified by PCR using primers S1-F2 and S1-R2 (25) in a 20-μl reaction mixture containing 10 μl HotStarTaq master mix (Qiagen, Crawley, United Kingdom), 1 μM each primer, and 2 μl target DNA. The thermal cycling conditions were as follows: 15 min at 95°C, followed by 40 cycles of 15 s at 95°C, 15 s at 59°C, and 1 min at 72°C. After the last cycle, a final step of 10 min at 72°C

TABLE 1. MLVA typing results for clinical *B. pertussis* isolates in the United Kingdom from different time periods

Period(s)	Description	Yr isolated	No. of isolates	No. of MLVA types	DI[a] (95% CI)	No. of new types (no. of unique types)[b]	%[c]
1	Prevaccination	1920–1956	68	20	0.88 (0.84–0.93)	NA[d] (10)	NA
2	Early postvaccination (WCV)	1963–1967	13	5	0.69 (0.48–0.91)	3 (1)	60
3	Epidemic	1977–1986	161	26	0.84 (0.80–0.89)	19 (13)	73
4	Vaccination recovery	1998–2001	110	16	0.65 (0.60–0.71)	7 (3)	44
5	After ACV booster addition	2002–2004	185	18	0.52 (0.43–0.61)	9 (6)	50
6	After complete switch to ACV	2005–2006	166	24	0.50 (0.40–0.59)	11 (11)	46
1–6	All periods	1920–2006	703	69	0.79 (0.76–0.82)	NA	NA
2–6	All postvaccination periods	1963–2006	635	59	0.75 (0.71–0.78)	NA	NA

[a] Hunter and Gaston's modification of Simpson's DI (19), calculated from MLVA types.
[b] Number of MLVA types first appearing during this period (number unique to this period).
[c] Number of new MLVA types divided by total number of MLVA types seen in the given period, expressed as a percentage.
[d] NA, not applicable.

was added. The PCR product was purified and sequenced as described above, using primers S1-F2 and S1-R2 and (if necessary) S1-MF and S1-MR (25).

DNA sequences were aligned using the BioNumerics version 4.5 software package (Applied Maths, Sint-Martins-Latem, Belgium) and the *prnA* and *ptxA* genotypes assigned as previously described (13, 25).

Division of *B. pertussis* collection into six time periods. The collection of *B. pertussis* isolates was divided into six time periods, around significant events in vaccination history for the United Kingdom, as follows (Table 1): period 1 (prevaccination), before the onset of mass vaccination with a WCV; period 2 (early postvaccination), several years after the onset of mass vaccination, before the fall in vaccine coverage following a health scare over the safety of the WCV; period 3 (epidemic), during the period of low vaccine coverage when epidemics occurred; period 4 (vaccination recovery), several years after vaccine coverage had recovered and the infant vaccination schedule had been changed from 3, 5, and 10 months to 2, 3, and 4 months; period 5 (after ACV booster addition), following the introduction of a three- or five-component ACV booster for 3- to 5-year-olds; and period 6 (after the completion of the switch to ACV), following replacement of the WCV with a five-component ACV for primary vaccinations. The particular years defining each period in Table 1 were determined by the availability of clinical isolates.

Statistical analysis. Diversity was calculated using Hunter and Gaston's modification of Simpson's diversity index (DI) (19). Ninety-five-percent confidence intervals (CIs) were calculated using the method of Simpson (33). Results were generated using an online tool (V-DICE) provided by the Health Protection Agency's Bioinformatics Unit (available via http://www.hpa.org.uk/). DIs in different periods were compared statistically by calculating the differences and associated standard errors. Associated *P* values were calculated for a two-tailed test of significance. The distributions of MLVA types in different periods were compared using Fisher's exact test after aggregating all types occurring five times or fewer (across all time periods) into a single group.

Minimum spanning trees. Minimum spanning trees were generated from the six MLVA loci by using the BioNumerics version 4.5 software package (Applied Maths, Sint-Martins-Latem, Belgium). Links were generated between MLVA types by using a categorical comparison algorithm, with the following priority rules: (i) first, link types that have the maximum number of single-locus variants (SLVs), (ii) then types that have the maximum number of SLVs and double-locus variants (DLVs), (iii) and then types that have the maximum number of entries. No cross-links are shown in the resulting figure (i.e., not all possible SLV and DLV, etc., links between MLVA types are shown).

RESULTS

Seven hundred three clinical *B. pertussis* isolates collected between 1920 and 2006 were typed by MLVA. Those not previously included in earlier studies (13, 29) were also serotyped and *ptxA* and *prnA* genotyped. Genetic diversity was calculated from MLVA types. The collection contained 69 MLVA types (see Table S1 in the supplemental material), some of which were novel. It generated an overall genetic DI of 0.79 (Table 1).

Changes in genetic diversity and major MLVA types over time. The collection was divided into six time periods, around significant events in vaccination history for the United Kingdom (Table 1) (periods are described in Materials and Methods). The group of isolates from the prevaccination period (period 1) possessed the highest genetic diversity of all the periods tested (DI = 0.88). In order to assess whether genetic diversity was changing before the introduction of mass vaccination, the DI was also calculated for isolates from 1941 to 1949 ($n = 59$) and 1950 to 1956 ($n = 17$) separately. The results were 0.87 (95% CI, 0.81 to 0.92) and 0.90 (95% CI, 0.81 to 0.99), respectively. Hence, there was no evidence of a decrease in genetic diversity in the bacterial population before 1957. These results also confirmed that the high DI from this period was not simply an artifact of selecting isolates from a long time period. During the early postvaccination period (period 2), the genetic diversity was noticeably lower (DI = 0.69), but this change was not statistically significant ($P = 0.39$), probably due to the small sample size. However, when the prevaccination period was compared to an amalgamation of all periods following the start of mass vaccination (periods 2 to 6) (Table 1), the fall in DI was found to be highly significant ($P < 0.001$). During the epidemic period (period 3), when vaccine coverage was low, the DI of the bacterial population was as high as that seen before the introduction of vaccination (DI = 0.84; $P = 0.37$ for comparison to period 1). By the late 1990s/early 2000s, when vaccination coverage had recovered (period 4), the DI of the bacterial population had fallen significantly to 0.65 ($P = 0.009$ for comparison to period 3). During the 3 years following the addition of an ACV booster dose to the vaccination schedule (period 5), genetic diversity fell significantly further (DI = 0.52; $P = 0.03$ for comparison to period 4). During the first 2 years after the switch to a five-component ACV for primary immunization (period 6), the genetic diversity fell slightly but insignificantly (DI = 0.50; $P = 0.71$ for comparison to the preceding period).

The relative abundances of the most common types from each period are shown in Table 2. MLVA-10 was the most abundant type in the prevaccination era (27% of isolates). However, it was not seen after the early postvaccination period. (The last isolate was seen in 1966.) MLVA-29 was a minor type in the prevaccination period, became the most

TABLE 2. Frequencies of selected MLVA types over time[a]

MLVA type(s)	Profile[b]	% of total no. of isolates for given period[c]					
		1	2	3	4	5	6
10	7,7,0,7,6,9	27	23	0	0	0	0
16	8,6,0,7,6,7	0	0	0	<1	8	8
23	8,7,0,6,6,10	15	0	0	0	<1	0
25	8,7,0,7,5,7	0	0	<1	2	3	2
27	8,7,0,7,6,7	0	0	9	42	69	71
29	8,7,0,7,6,9	4	54	34	2	0	1
36	8,7,8,7,6,7	0	0	0	2	4	4
44	9,7,0,7,6,9	0	0	11	<1	0	0
64	9,7,0,7,6,6	0	0	12	0	0	0
70	7,6,0,7,6,8	0	0	0	42	2	0
91	7,6,0,7,6,7	1	0	0	2	0	0
92	7,6,0,7,6,9	16	0	0	<1	0	<1
99	7,8,0,8,6,8	0	8	<1	0	0	0
123	8,8,0,8,6,9	0	8	4	0	0	0
125	9,7,0,7,6,10	0	8	0	0	0	0
Other[d]	NA[e]	37	0	28	6	14	14

[a] Amalgamation of the three most common types (or more where the ranking was tied) for each period. Other MLVA types occurring during the study period are given in Table S1 in the supplemental material.
[b] Numbers of repeats at VNTR loci 1, 3a, 3b, 4, 5, and 6, respectively.
[c] The periods are described in detail in Table 1. The numbers of isolates per period are as follows: for period 1, 68; for period 2, 13; for period 3, 161; for period 4, 110; for period 5, 185; and for period 6, 166.
[d] All remaining MLVA types grouped together.
[e] NA, not applicable.

common type in the early postvaccination and epidemic periods (54% and 34%, respectively), but was seen only occasionally thereafter. In contrast, MLVA-27 first appeared at a low frequency (9%) during the epidemic period. (It was first seen in 1983.) It then became codominant with MLVA-70 in the vaccination recovery period (42% each) and then accounted for ~70% of isolates collected during the final two periods. Interestingly, MLVA-70 was seen in high numbers only during the vaccine recovery period.

New MLVA types were seen during every period following the onset of vaccination, even those possessing low genetic diversity (Table 1). The highest proportion of new types was seen during the epidemic period (73% types). Every period contained unique MLVA types (Table 1).

Combination of MLVA, *prnA*, and *ptxA* types. The combinations of MLVA, *prnA*, and *ptxA* types from each period are shown in Fig. 1. There was no obvious correlation between MLVA and *prnA* or *ptxA* genotypes during either the prevaccination period (Fig. 1a) or the early postvaccination period (Fig. 1b). All isolates from these two time periods possessed *prnA*(1) and either *ptxA*(1) or *ptxA*(2) genotypes. Although these two periods contained similar MLVA types, comparison of the distribution of types between the two periods revealed a significant difference ($P < 0.001$).

Two of the three strains making up the WCV for the United Kingdom (Table 3) were of MLVA types (MLVA-6 and -105) seen during the prevaccination period, although both were minor types in this sample. Neither MLVA-6 nor MLVA-105 was seen outside this time period. The third vaccine type, MLVA-122, was not seen during any time period.

The isolates collected during the epidemic period (Fig. 1c) possessed distributions of MLVA types significantly different from those for both the preceding period ($P = 0.005$) and the prevaccination period ($P < 0.001$). The *ptxA*(2) genotype was very rare during this period (1% [2/161] of isolates) (Fig. 1c). From 1986 onwards, all isolates carried *ptxA*(1). In contrast, the *prnA*(2) and *prnA*(3) alleles first appeared in 1982. During the epidemic period as a whole, they were seen in 20% (32/161) and 7% (12/161) of isolates, respectively. MLVA-27 was first seen in 1983 in isolates carrying both *prnA*(1) and *prnA*(2). MLVA-27 is an SLV of six other MLVA types present during the epidemic period (MLVA-25, -28, -29, -30, -32, and -60; not all SLV connections are shown in the figure), suggesting that it could have evolved from closely related strains via a number of routes.

During the vaccine recovery period, there were strong correlations between MLVA-27 and *prnA*(2) and between MLVA-70 and *prnA*(1) (100% [46/46] and 93% [43/46] of isolates, respectively) (Fig. 1d). The distribution of MLVA types during this period was significantly different from those during the previous period ($P < 0.001$). As mentioned earlier, MLVA-70 was not seen before this period and subsequently was seen only at low frequencies between 2002 and 2004. It is not closely related to MLVA-27 or MLVA-29, the two most common types from the same and the preceding period (Table 2).

After the addition of the ACV preschool booster, 93% (172/185) of isolates possessed the *prnA*(2) genotype (Fig. 1e). Ninety-six percent (122/127) of the dominant MLVA-27-type isolates were *prnA*(2). Overall, the percentages of *prnA*(1), -(2), and -(3) alleles in the population during this time were 3%, 93%, and 4%, respectively. Comparison of the distribution of MLVA types with that for the previous period showed a significant difference ($P < 0.001$).

There was no striking change in distribution of major MLVA, *ptxA*, and *prnA* types following the complete switch to the ACV (Fig. 1f). In fact, there was a borderline statistical difference between the distribution of MLVA types from this period and the previous one ($P = 0.05$). Overall, the percentages of *prnA*(1), -(2), and -(3) alleles in the population during this period were 5%, 91%, and 4%, respectively. Ninety-seven percent (114/117) of MLVA-27 isolates were *prnA*(2).

Serotypes. There was no obvious correlation between serotype and MLVA, *prnA*, or *ptxA* type during the prevaccination, early postvaccination, or epidemic period (not shown). There was a predominance of Fim2 in the prevaccination period but an increasing proportion of Fim3 in the next two periods (Fig. 1a to c).

There was a clear correlation between serotype and either pertactin type or MLVA type during the vaccine recovery period (Fig. 1d). The concordance between *prnA* type and serotype was found to be 99% when the newer *prnA*(2) and *prnA*(3) alleles were grouped together [46% of isolates were *prnA*(1) and Fim2, 50% were *prnA*(2) and Fim3, 3% were *prnA*(3) and Fim3, and 1% were *prnA*(2) and Fim2]. The overall concordance between serotype and the two major MLVA types was 82% (41% of isolates were MLVA-27 and Fim3, and 41% were MLVA-70 and Fim2). All but one of the MLVA-27 isolates possessed both *prnA*(2) and serotype Fim3. All but three of the MLVA-70 isolates possessed both *prnA*(1) and serotype Fim2 (Fig. 1d).

Ninety-seven percent of isolates (including all of the MLVA-27 isolates) were serotype Fim3 in the period following the introduction of the ACV booster (Fig. 1e). Four MLVA-70

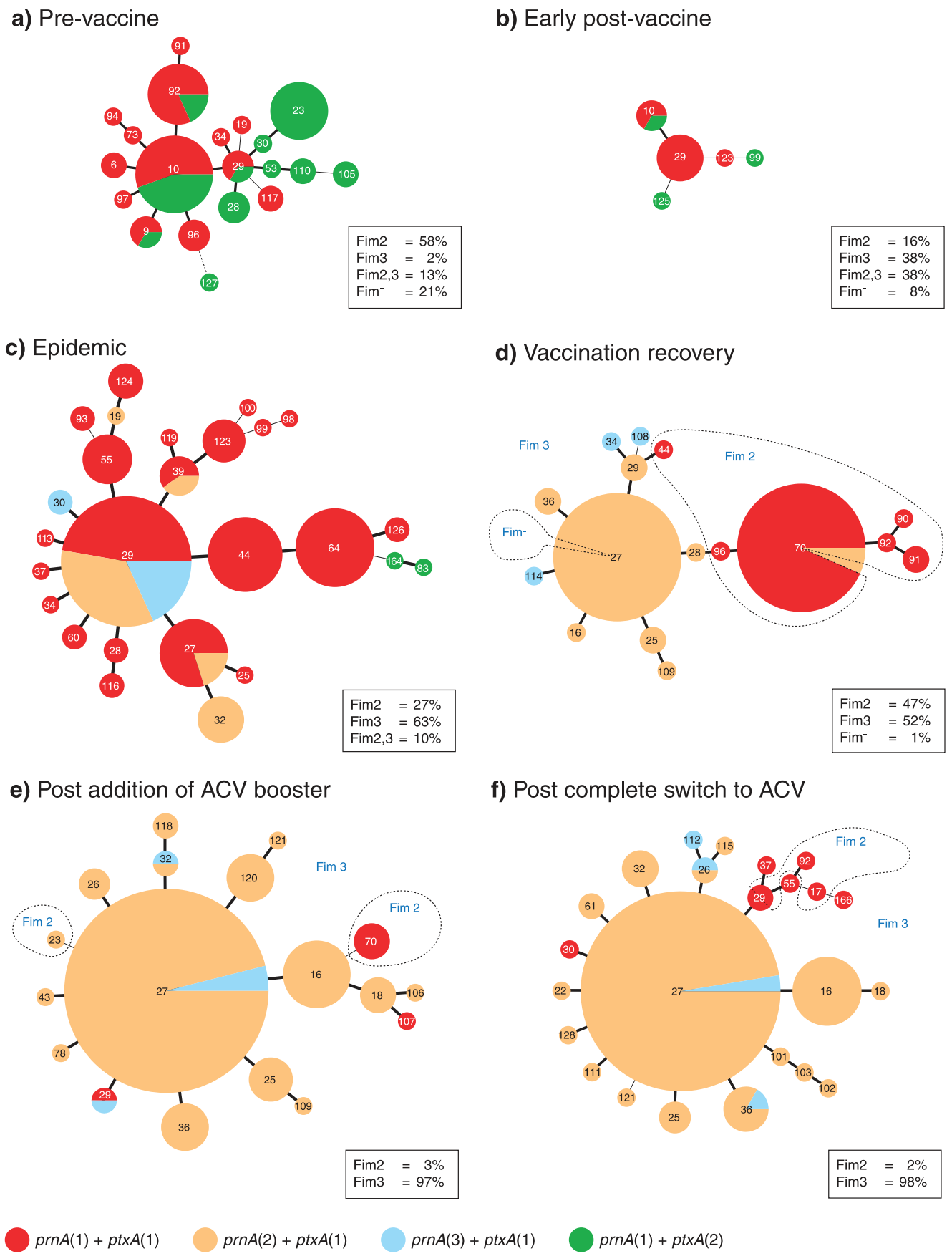

TABLE 3. MLVA types, *prnA* and *ptxA* genotypes, and serotypes of *B. pertussis* strains used to derive vaccines deployed in the United Kingdom

Strain	Vaccine	MLVA type (profile)	*prnA* genotype	*ptxA* genotype	Fim type[a]
CN2992	WCV[b]	105 (8,6,7,6,6,11)	*prnA*(1)	*ptxA*(2)	Fim2,3
CN3099	WCV[b]	6 (6,7,0,7,6,9)	*prnA*(1)	*ptxA*(1)	Fim2
CN5476	WCV[b]	122 (8,8,0,7,7,9)	*prnA*(1)	*ptxA*(1)	Fim3
10536	ACV(5)[c]	167 (8,5,0,7,12,7)	*prnA*(7)	*ptxA*(3)	Fim2,3
Tohama-I	ACV(3)[d]	83 (9,7,0,9,7,11)	*prnA*(1)	*ptxA*(2)	Fim2[e]

[a] Expressed fimbrial type (data derived from serotype).
[b] WCV comprising three strains.
[c] Five-component acellular vaccine (Sanofi Pasteur MSD).
[d] Three-component acellular vaccine (GlaxoSmithKline).
[e] Fimbriae are not included in the three-component ACV.

strains and one MLVA-23 strain were the only isolates with serotype Fim2 (Fig. 1e).

The serotyping results following the complete switch to the ACV (Fig. 1f) were similar to those for the previous period. All isolates from this time period possessed the Fim3 serotype, apart from three (Fig. 1f).

Correlation between MLVA and MLST. MLST data (*ptxA*, *ptxC*, and *tcfA* alleles) from an earlier study (29) were available for a subset of 132 randomly selected *B. pertussis* isolates collected in the United Kingdom between 1920 and 2002. Comparison of the results of MLVA and MLST for these and 4 additional isolates (i.e., 136 isolates in total) revealed seven MLST types, subdivided by a total of 34 MLVA types (Table 4).

Interestingly, the MLVA-27 genotype was first seen in this subset of samples in isolates with the rare MLST-10 type (profile 1,1,6). From 1998 to 2002, it was seen predominantly in isolates of MLST-5 (profile 1,2,2), although it also occurred in MLST-3 (profile 1,1,2) and MLST-4 (profile 1,1,3) strains.

DISCUSSION

Our present study extends earlier studies of *prnA* and *ptxA* genotypes and serotypes of *B. pertussis* clinical isolates collected in the United Kingdom (13, 29) from 2002 to the end of 2006. During these 5 years, all clinical isolates have continued to possess solely the *ptxA*(1) allele and have been dominated by the *prnA*(2) allele. The *prnA*(1) and *prnA*(3) types have persisted at low frequencies. During this time period, isolates with serotype Fim3 have also consistently dominated. The remaining isolates have been solely serotype Fim2. Hence, the population of strains from the United Kingdom, as defined by serotype and *prnA* and *ptxA* typing, has not changed significantly during a period immediately after the introduction of an ACV booster dose (November 2001) and one during which the WCV was replaced with a five-component ACV (October 2004). Our results continue the trend seen during the early 2000s, not only in the United Kingdom but also in Sweden, France, Germany, The Netherlands, and Finland (although the trend toward serotype Fim3 was delayed in Finland) (1, 4, 11, 12, 17, 37). Similar trends have also been seen outside Europe, in Russia, Taiwan, Australia, and the United States (3, 6, 22, 30). Fim3 and *prnA*(2) have both been proposed as providing a survival advantage to *B. pertussis* isolates in a WCV-vaccinated human population (23, 27, 31).

The main purpose of the present study was to analyze changes in the genetic composition and diversity of the *B. pertussis* population in the United Kingdom by using a six-locus MLVA typing scheme (32). This MLVA scheme greatly increased discrimination between strains in comparison to our earlier analysis using a combination of serotyping and genotyping of virulence-related genes (13, 29).

We divided our collection of *B. pertussis* isolates into six time periods, around significant events in the epidemiology of pertussis in the United Kingdom (Table 1). The group of isolates collected before the onset of mass vaccination (period 1) possessed the highest genetic diversity of all the time periods (Table 1). Genetic diversity fell in the decade following the introduction of the WCV (period 2), rose again during the epidemic period, when vaccine coverage was low (period 3), and fell repeatedly during successive periods of consistently high coverage (periods 4 to 6). A drop in genetic diversity following the introduction of mass vaccination was also seen in studies of strains from The Netherlands using MLVA or IS*1002* fingerprinting (32, 41) and is consistent with the hypothesis that vaccine-mediated immunity causes a drop in the genetic diversity of the bacterial population by selecting for a subset of isolates that are less affected by it.

Between 1975 and 1989, vaccine coverage fell below 80% (to a low of 31% in 1978) due to a health scare in the United Kingdom, and large epidemics occurred in 1978 and 1982 (24). Strains collected during this period (period 3) showed an in-

FIG. 1. Minimum spanning trees showing the genetic diversity of the *B. pertussis* population from 1920 to 1956 (a), 1963 to 1967 (b), 1977 to 1986 (c), 1998 to 2001 (d), 2002 to 2004 (e), and 2005 to 2006 (f). Trees were derived from the six MLVA alleles. Each circle represents a unique MLVA type (shown by the number in the circle). The size of each circle illustrates the proportion of strains with that MLVA type (the smallest circle in each tree represents one isolate). Thick lines separate SLVs, thin lines separate DLVs, and dotted lines signify a more distant relationship. Colors illustrate the combination of *prnA* and *ptxA* (see key). When more than one combination is present for a given MLVA type, the circle is divided proportionally in the form of a pie chart. The distribution of serotypes is given in an inset panel for each time period. It is also shown graphically in panels d to f (serotypes are separated by dotted lines).

TABLE 4. Comparison of MLST and MLVA types for 136 randomly selected *B. pertussis* isolates collected between 1920 and 2002

MLST type (profile)[a]	MLVA type	Frequency for indicated period[b]				
		1	2	3	4	5
2 (2,1,2)	10	5				
	23	2				
	28	1				
	29			2		
	39			1		
	83			1		
	98			1		
	100			1		
	105	1				
	125		1			
	127	1				
	164			1		
3 (1,1,2)	10	4	1			
	19	1				
	27				2	
	29		2	5	1	
	32			1		
	55			1		
	70				19	1
	73	1				
	91				1	
	92	3			1	
	96	1			1	
	97	1				
	108				1	
	117	1				
	123		1	1		
4 (1,1,3)	27				1	
	34				1	
5 (1,2,2)	16				1	6
	25				1	1
	26					1
	27				19	16
	28				1	
	32					1
	36					2
	118					1
	120					4
6 (1,1,4)	44			1	1	
	64			6		
10 (1,1,6)	27			2		
11 (2,1,5)	23	1				

[a] MLST type derived from allelic profile of *ptxA*, *ptxC*, and *tcfA* genotypes (29, 40).

[b] The periods are described in detail in Table 1. None of the isolates in this analysis were from period 6. The numbers of isolates analyzed per period were as follows: for period 1, 23; for period 2, 5; for period 3, 24; for period 4, 51; and for period 5, 33.

crease in genetic diversity compared to those from the previous period, to a level comparable to that seen before vaccination (Table 1). This is in contrast to the Dutch study (32), in which genetic diversity in the bacterial population fell around the times of two epidemics. In the Dutch case, however, epidemics did not occur as the result of a large drop in vaccination and were attributed to the clonal expansion of a limited number of strains. It appears that in the United Kingdom, the fall in vaccine coverage either allowed new bacterial genotypes to emerge or coincided with the import of novel genotypes from outside the United Kingdom. Our analysis confirmed that, although the bacterial populations present before the onset of mass vaccination (period 1) and during this later epidemic period possessed similar genetic diversities, their distributions of MLVA types were significantly different.

New MLVA types and the *prnA*(2) and *prnA*(3) genotypes appeared during the epidemic period (period 3). In favor of the hypothesis that these new types arose out of the existing bacterial population is the observation that many of the new MLVA types are SLVs of a common type from the preceding periods (MLVA-29). In support of the theory that they may have been imported instead is the fact that several of the new MLVA types have also been seen in other European countries. MLVA-27 and -44 were seen among a collection of 13 isolates from before 1953 in The Netherlands (32), and MLVA-25, -27, -32, -37, -39, and -44 were subsequently seen in The Netherlands, Sweden, Finland, Germany, and France during the 1990s (20, 32, 37). Similarly, the *prnA*(2) and/or *prnA*(3) variant also coincidentally arose during the 1970s or 1980s in The Netherlands, Russia, Australia, the United States, and Finland (3, 6, 11, 27, 30). While Russia experienced a period of low vaccine coverage similar to that in the United Kingdom, the other countries did not.

In the United Kingdom, MLVA-27 was first seen in small numbers during the epidemic period in 1983, in strains possessing both *prnA*(1) and *prnA*(2) genotypes and a range of serotypes. MLST results for selected MLVA-27 isolates revealed that they included the rare MLST-10 lineage in 1983 and a mixture of MLST-3, -4, and -5 lineages between 1988 and 1999, with exclusivity of MLST-5 by 2002. As mentioned earlier, MLVA-27 isolates could have arisen from closely related existing strains or been imported to the United Kingdom during the 1980s. These data suggest that the MLVA-27 *prnA*(2) *ptxA*(1) Fim3 clone that came to dominate later did not arise in a single genetic event but was probably selected from a pool of MLVA-27 variants during the 1990s.

MLVA-27 was a major type by the end of the 1990s (period 4) and accounted for ~70% isolates in the 2002-to-2004 and 2005-to-2006 periods (periods 5 and 6). MLVA-27 was also the most common MLVA type by the end of the 1990s/beginning of the 2000s in several European countries [often in conjunction with *prnA*(2) and Fim3] (32, 37). Hence, MLVA-27 has become the dominant type in countries with various vaccination policies and epidemiological histories.

Following the epidemic period (period 3), vaccine coverage recovered during the 1980s and has remained above 90% since 1992 (http://www.hpa.org.uk/). The genetic diversity of the bacterial population in the United Kingdom dropped significantly between the epidemic period and the vaccination recovery period (period 4) and decreased further during subsequent periods. The MLVA-27 type has become gradually more dominant during this time of high vaccine coverage, leading us to speculate that it is a marker for some unknown genetic trait conferring increased survival in a highly vaccinated population. In general, a drop in genetic diversity could be caused by more than one pattern of change in the distribution of types in the population. In this instance, the drop is caused by the domi-

nance of a single type, even though a relatively large number of types still occur at low frequencies. Furthermore, even during each of the later periods of low genetic diversity, approximately half of all MLVA types found were novel (Table 1). This suggests that there is either a large number of slowly evolving MLVA types circulating in the *B. pertussis* population in the United Kingdom at low frequencies (and we have taken a different random selection in each period) or a smaller but more rapidly evolving pool of types. Thus, MLVA-27 has increased its dominance against a rich background of potential competitors.

Following the introduction of an ACV booster dose to the vaccination schedule in November 2001, genetic diversity fell in comparison to that in the previous period (period 5 versus period 4) (Table 1). The number of notifications (in England and Wales) also reached an all-time low (totaling 409) in 2003, with notifications in the 5- to 9-year-old age group falling to less than a third of that for the preceding year (from 230 to 68) (http://www.hpa.org.uk/). During the first 2 years following the replacement of WCV by a five-component ACV (period 6), genetic diversity in the bacterial population fell slightly (although not statistically significantly) and still comprised ~70% isolates with the clonal type of MLVA-27 *prnA*(2) *ptxA*(1) Fim3. Although notifications rose slightly (504 in 2004, 594 in 2005, and 550 in 2006), they were lower in 2006 in the <1-year-old age group (140 in 2004, 185 in 2005, and 135 in 2006) (http://www.hpa.org.uk/). Hence, our data in conjunction with notifications suggest that the recent switch to ACV has not had any effects on disease incidence or the makeup of the bacterial population in its first 2 years.

An earlier study of pertussis disease cases in England and Wales between 1995 and 1997 found a significant association between serotype Fim2 and increased disease severity (as judged by likelihood of hospital admission and incidence of complications), and it was proposed that serotype Fim2 *B. pertussis* strains caused more-severe disease than Fim3 or Fim2,3 serotypes (38). There have been no other reports in support of this hypothesis, and a recent Swedish study of cases from 1997 to 2004 showed no association between serotype and clinical outcome (2). In our study, we observed a strong correlation between serotype and pertactin type among isolates from 1998 to 2001 (period 4). Furthermore, we saw a notable correlation between serotype and MLVA type in the same period. We have no bacterial isolates from 1995 to 1997 in our collection, but if our data for 1998 to 2001 are comparable to those for 1995 to 1997, the possibility that serotype may have simply been acting as a surrogate marker for pertactin type or MLVA type arises in the study by Van Buynder et al. (38). In support of this hypothesis, strains carrying the *ptxP3* pertussis toxin promoter allele have recently been reported to be associated with increased virulence during the resurgence of pertussis in The Netherlands during the 1990s, and many of these possessed the MLVA-27 *prnA*(2) genotype (20). Similarly, a significant correlation was found between the most common pulsed-field gel electrophoresis profile (BpSR11) and an increased duration of hospital stay in 2,646 cases in the recent Swedish study (2). Whether serotype, pertactin type, some gene associated with MLVA type, or another unknown linked virulence factor affects clinical outcome requires further investigation. Statistical associations may be difficult to test using more-recent isolates from the United Kingdom, however, due to the strong skewing of serotype, pertactin type, and MLVA in the bacterial population. For example, a strong association between *prnA*(2) and serotype Fim3 continued between 2002 and 2006 [323/324 *prnA*(2) isolates]. However, only 14 *prnA*(1) isolates were seen during this time and were evenly split between serotypes Fim2 and Fim3. Whatever the true cause, if the association of serotype Fim2 with increased disease severity seen by van Buynder et al. (38) holds true, then the recent increase in cases caused by MLVA-27 *prnA*(2) Fim3 isolates would be predicted to be associated with a general decrease in disease severity in the United Kingdom. Interestingly, this is counter to the results of the recent Swedish and Dutch studies described above (2, 20).

ACKNOWLEDGMENTS

We are very grateful to Frits Mooi, Leo Schouls, and Han van der Heide for assistance and advice in relation to MLVA; to Steve Platt for expert assistance with BioNumerics; to Tim Harrison, Robert George, and Elizabeth Miller for helpful discussions and critical review of the manuscript; to John Duncan and Sally Flynn for assistance in the laboratory; to Ayoub Saei and Nick Andrews for statistical expertise; to Jon White for generating Fig. 1; to Aventis Pasteur MSD for the gift of strain 10536; and to the clinicians and laboratory staff from the hospitals in the United Kingdom for submitting *B. pertussis* isolates to our laboratory.

REFERENCES

1. **Advani, A., D. Donnelly, L. Gustafsson, and H. O. Hallander.** 2007. Changes of the Swedish *Bordetella pertussis* population in incidence peaks during an acellular pertussis vaccine period between 1997 and 2004. APMIS **115:**299–310.
2. **Advani, A., L. Gustafsson, R.-M. Carlsson, D. Donnelly, and H. O. Hallander.** 2007. Clinical outcome of pertussis in Sweden: association with pulsed-field gel electrophoresis profiles and serotype. APMIS **115:**736–742.
3. **Borisova, O., S. Yu Kombarova, N. S. Zakharova, M. van Gent, V. A. Aleshkin, I. Mazurova, and F. R. Mooi.** 2007. Antigenic divergence between *Bordetella pertussis* clinical isolates from Moscow, Russia, and vaccine strains. Clin. Vaccine Immunol. **14:**234–238.
4. **Caro, V., A. Elomaa, D. Brun, J. Mertsola, Q. He, and N. Guiso.** 2006. *Bordetella pertussis*, Finland and France. Emerg. Infect. Dis. **12:**987–989.
5. **Caro, V., E. Njamkepo, S. C. M. Van Amersfoorth, F. R. Mooi, A. Advani, H. O. Hallander, Q. He, J. Mertsola, M. Riffelmann, C. Vahrenholz, C. H. W. von König, and N. Guiso.** 2005. Pulsed-field gel electrophoresis analysis of *Bordetella pertussis* populations in various European countries with different vaccine policies. Microbes Infect. **7:**976–982.
6. **Cassiday, P., G. Sanden, K. Heuvelman, F. Mooi, K. M. Bisgard, and T. Popovic.** 2000. Polymorphism in *Bordetella pertussis* pertactin and pertussis toxin virulence factors in the United States, 1935–1999. J. Infect. Dis. **182:**1402–1408.
7. **Celentano, L. P., M. Massari, D. Paramatti, S. Salmaso, and A. E. Tozzi.** 2005. Resurgence of pertussis in Europe. Pediatr. Infect. Dis. J. **24:**761–765.
8. **Chief Medical Officer.** 2001. Current vaccine and immunisation issues. Letter PL CMO (2001)5. United Kingdom Department of Health, London, United Kingdom.
9. **Chief Medical Officer.** 2004. New vaccinations for the childhood immunisation programme. Letter PL CMO (2004)3. United Kingdom Department of Health, London, United Kingdom.
10. **Crowcroft, N. S., R. Booy, T. Harrison, L. Spicer, J. Britto, Q. Mok, P. Heath, I. Murdoch, M. Zambon, R. George, and E. Miller.** 2003. Severe and unrecognised: pertussis in UK infants. Arch. Dis. Child. **88:**802–806.
11. **Elomaa, A., A. Advani, D. Donnelly, M. Antila, J. Mertsola, H. Hallander, and Q. He.** 2005. Strain variation among *Bordetella pertussis* isolates in Finland, where the whole-cell pertussis vaccine has been used for 50 years. J. Clin. Microbiol. **43:**3681–3687.
12. **Elomaa, A., A. Advani, D. Donnelly, M. Antila, J. Mertsola, Q. He, and H. Hallander.** 2007. Population dynamics of *Bordetella pertussis* in Finland and Sweden, neighbouring countries with different vaccination histories. Vaccine **25:**918–926.
13. **Fry, N. K., S. Neal, T. G. Harrison, E. Miller, R. Matthews, and R. C. George.** 2001. Genotypic variation in the *Bordetella pertussis* virulence factors pertactin and pertussis toxin in historical and recent clinical isolates in the United Kingdom. Infect. Immun. **69:**5520–5528.
14. **Grant, C. C., and J. D. Cherry.** 2002. Keeping pace with the elusive *Bordetella pertussis*. J. Infect. **44:**7–12.

15. **Gzyl, A., E. Augustynowicz, I. van Loo, and J. Slusarczyk.** 2001. Temporal nucleotide changes in pertactin and pertussis toxin genes in *Bordetella pertussis* strains isolated from clinical cases in Poland. Vaccine **20:**299–303.
16. **Hallander, H., A. Advani, M. Riffelmann, C. H. W. von König, V. Caro, N. Guiso, F. R. Mooi, A. Gzyl, M. S. Kaltoft, N. K. Fry, J. Mertsola, and Q. He.** 2007. *Bordetella pertussis* strains circulating in Europe in 1999 to 2004 as determined by pulsed-field gel electrophoresis. J. Clin. Microbiol. **45:**3257–3262.
17. **Hallander, H. O., A. Advani, D. Donnelly, L. Gustafsson, and R.-M. Carlsson.** 2005. Shifts of *Bordetella pertussis* variants in Sweden from 1970 to 2003, during three periods marked by different vaccination programs. J. Clin. Microbiol. **43:**2856–2865.
18. **Harrison, T. G., and A. G. Taylor.** 1988. A laboratory manual for *Legionella*, p. 157–158. John Wiley and Sons, Ltd., Chichester, United Kingdom.
19. **Hunter, P. R., and M. A. Gaston.** 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. **26:**2465–2466.
20. **King, A. J., T. van Gorkom, J. L. A. Pennings, H. G. J. van der Heide, Q. He, D. Diavatopoulos, K. Heuvelman, M. van Gent, K. van Leeuwen, and F. R. Mooi.** 2008. Comparative genomic profiling of Dutch clinical *Bordetella pertussis* isolates using DNA microarrays: identification of genes absent from epidemic strains. BMC Genomics **9:**311.
21. **Kourova, N., V. Caro, C. Weber, S. Thiberge, R. Chuprinina, G. Tseneva, and N. Guiso.** 2003. Comparison of the *Bordetella pertussis* and *Bordetella parapertussis* isolates circulating in Saint Petersburg between 1998 and 2000 with Russian vaccine strains. J. Clin. Microbiol. **41:**3706–3711.
22. **Lin, Y.-C., S.-M. Yao, J.-J. Yan, Y.-Y. Chen, M.-J. Hsiao, C.-Y. Chou, H.-P. Su, H.-S. Wu, and S.-Y. Li.** 2006. Molecular epidemiology of *Bordetella pertussis* in Taiwan, 1993–2004: suggests one possible explanation for the outbreak of pertussis in 1997. Microbes Infect. **8:**2082–2087.
23. **Mastrantonio, P., P. Spigaglia, H. van Oirschot, H. G. J. van der Heide, K. Heuvelman, P. Stefanelli, and F. R. Mooi.** 1999. Antigenic variants in *Bordetella pertussis* strains isolated from vaccinated and unvaccinated children. Microbiology **145:**2069–2075.
24. **Miller, E., J. E. Vurdien, and J. M. White.** 1992. The epidemiology of pertussis in England and Wales. Commun. Dis. Rep. CDR Rev. **2:**R152–R154.
25. **Mooi, F. R., H. Hallander, C. H. Wirsing von König, B. Hoet, and N. Guiso.** 2000. Epidemiological typing of *Bordetella pertussis* isolates: recommendations for a standard methodology. Eur. J. Clin. Microbiol. Infect. Dis. **19:**174–181.
26. **Mooi, F. R., Q. He, H. van Oirschot, and J. Mertsola.** 1999. Variation in the *Bordetella pertussis* virulence factors pertussis toxin and pertactin in vaccine strains and clinical isolates in Finland. Infect. Immun. **67:**3133–3134.
27. **Mooi, F. R., H. van Oirschot, K. Heuvelman, H. G. J. van der Heide, W. Gaastra, and R. J. L. Willems.** 1998. 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. **66:**670–675.
28. **Ntezayabo, B., G. De Serres, and B. Duval.** 2003. Pertussis resurgence in Canada largely caused by a cohort effect. Pediatr. Infect. Dis. J. **22:**22–27.
29. **Packard, E. R., R. Parton, J. G. Coote, and N. K. Fry.** 2004. Sequence variation and conservation in virulence-related genes of *Bordetella pertussis* isolates from the UK. J. Med. Microbiol. **53:**355–365.
30. **Poynten, M., P. B. McIntyre, F. R. Mooi, K. J. Heuvelman, and G. L. Gilbert.** 2004. Temporal trends in circulating *Bordetella pertussis* strains in Australia. Epidemiol. Infect. **132:**185–193.
31. **Preston, N. W., and E. J. Carter.** 1992. Serotype specificity of vaccine-induced immunity to pertussis. Commun. Dis. Rep. CDR Rev. **2:**R155–R156.
32. **Schouls, L. M., H. G. J. van der Heide, L. Vauterin, P. Vauterin, and F. R. Mooi.** 2004. Multiple-locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. J. Bacteriol. **186:**5496–5505.
33. **Simpson, E. H.** 1949. Measurement of diversity. Nature **163:**688.
34. **Tanaka, M., C. R. Vitek, F. B. Pascual, K. M. Bisgard, J. E. Tate, and T. V. Murphy.** 2003. Trends in pertussis among infants in the United States, 1980–1999. JAMA **290:**2968–2975.
35. **Tsang, R. S., A. K. H. Lau, M. L. Sill, S. A. Halperin, P. Van Caeseele, F. Jamieson, and I. E. Martin.** 2004. Polymorphisms of the fimbria *fim3* gene of *Bordetella pertussis* strains isolated in Canada. J. Clin. Microbiol. **42:**5364–5367.
36. **Tsang, R. S., M. L. Sill, I. E. Martin, and F. Jamieson.** 2005. Genetic and antigenic analysis of *Bordetella pertussis* isolates recovered from clinical cases in Ontario, Canada, before and after the introduction of the acellular pertussis vaccine. Can. J. Microbiol. **51:**887–892.
37. **van Amersfoorth, S. C. M., L. M. Schouls, H. G. J. van der Heide, A. Advani, H. O. Hallander, K. Bondeson, C. H. W. von König, M. Riffelmann, C. Vahrenholz, N. Guiso, V. Caro, E. Njamkepo, Q. He, J. Mertsola, and F. R. Mooi.** 2005. Analysis of *Bordetella pertussis* populations in European countries with different vaccination policies. J. Clin. Microbiol. **43:**2837–2843.
38. **van Buynder, P. G., D. Owen, J. E. Vurdien, N. J. Andrews, R. C. Matthews, and E. Miller.** 1999. *Bordetella pertussis* surveillance in England and Wales: 1995–7. Epidemiol. Infect. **123:**403–411.
39. **van Loo, I. H., H. G. van der Heide, N. J. Nagelkerke, J. Verhoef, and F. R. Mooi.** 1999. Temporal trends in the population structure of *Bordetella pertussis* during 1949–1996 in a highly vaccinated population. J. Infect. Dis. **179:**915–923.
40. **van Loo, I. H. M., K. J. Heuvelman, A. J. King, and F. R. Mooi.** 2002. Multilocus sequence typing of *Bordetella pertussis* based on surface protein genes. J. Clin. Microbiol. **40:**1994–2001.
41. **Van Loo, I. H. M., and F. R. Mooi.** 2002. Changes in the Dutch *Bordetella pertussis* population in the first 20 years after the introduction of whole-cell vaccines. Microbiology **148:**2011–2018.
42. **Weber, C., C. Boursaux-Eude, G. Coralie, V. Caro, and N. Guiso.** 2001. Polymorphism of *Bordetella pertussis* isolates circulating for the last 10 years in France, where a single effective whole-cell vaccine has been used for more than 30 years. J. Clin. Microbiol. **39:**4396–4403.