

Antigenic Divergence between *Bordetella pertussis* Clinical Isolates from Moscow, Russia, and Vaccine Strains[∇]

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We analyzed temporal changes in the frequencies of the *ptxA*, *prn*, *fim2*, and *fim3* alleles in *Bordetella pertussis* strains isolated from pertussis patients in Moscow, Russia, from 1948 to 2004. The three strains used for the whole-cell vaccine harbored the alleles *ptxA2*, *ptxA4*, *prn1*, *fim2-1*, and *fim3A*. Vaccine-type alleles of *ptxA* (*ptxA2* and *ptxA4*) were characteristic for all prevaccination strains and for 96% of the strains isolated in the 1960s and 1970s. At the beginning of the 1970s, *ptxA2* and *ptxA4* were replaced by the *ptxA1* allele. In the 1980s and to the present, strains with *ptxA1* were predominant in the *B. pertussis* population. All prevaccination strains harbored the *prn1* allele, which corresponds to the vaccine-type allele. In subsequent years, the proportion of strains with the *prn1* allele decreased and the proportion of *prn3* and *prn2* strains increased. From 2002 to 2004 strains with *prn2* or *prn3* were predominant in the *B. pertussis* population. The vaccine-type alleles *fim2-1* and *fim3A* were found in all prevaccination strains and in 92% of the strains isolated from 1960 to 1989. The *fim2-2* and *fim3B* alleles were first observed at the beginning of the 1980s. In subsequent years, these strains became predominant. Together with waning immunity, the antigenic divergence between vaccine strains and clinical isolates observed in the Moscow area may explain the persistence of pertussis, despite the high rates of vaccine coverage. The results demonstrate that the selection of *B. pertussis* strains for vaccine manufacturing must be based on a thorough study of the *B. pertussis* population.

In the prevaccination era, pertussis was one of the four leading airborne infections in Russia, along with measles, scarlet fever, and diphtheria. Vaccination for the prevention of pertussis was introduced nationwide in Russia between 1958 and 1960. This subsequently resulted in a significant decrease in the incidence and the severity of the disease. Decreased vaccine uptake, due to concerns about the side effects of pertussis whole-cell vaccines, has resulted in large pertussis outbreaks in the United Kingdom and Japan (1, 11). These experiences clearly indicate that the morbidity and mortality due to pertussis in children far outweigh the risk of postvaccination complications. Infants in Russia are vaccinated with a whole-cell pertussis vaccine (diphtheria, pertussis, and tetanus [DPT] vaccine) at the ages of 3, 4.5, 6, and 18 months. Epidemiological data from Russia have shown that the whole-cell vaccine currently used is not sufficiently effective. We have observed seasonal peaks, periodic increases in incidence, as well as infection clusters in schools and day care facilities. Thus, children who have received a complete vaccination may be susceptible (8). The adverse reactions caused by whole-cell pertussis vaccines have prompted researchers in many countries to develop new, acellular pertussis vaccines. To date, however, an acellular vaccine has not been used in Russia to immunize children.

In the past few years, the pertussis epidemiological situation in Russia has been unfavorable. Regardless of the high rates of vaccination coverage for pertussis, we have registered an increase in the incidence of pertussis among school-aged children, along with persistently high incidences among infants under 12 months of age. In addition, some cases of pertussis have been registered among vaccinated children (8, 23). Similar trends have been observed in many other countries (1, 2, 5, 6, 10). Researchers point to many reasons for the increased incidence of pertussis, including improved surveillance, waning immunity, and pathogen adaptation. The last possibility is supported by the fact that divergence in the protective antigens pertussis toxin (Ptx) and pertactin (Prn) has been observed between vaccine strains and the strains circulating in many countries (2, 7, 9, 12, 13, 17, 18, 20, 24, 31–33). In general, it was found that “non-vaccine-type” alleles of *ptxA* and *prn* (i.e., *ptxA1*, *prn2*, and *prn3*) have gradually replaced the “vaccine-type” alleles (i.e., *ptxA2*, *ptxA4*, and *prn1*) that were in circulation earlier. Analysis of strains isolated in Russia (Moscow and St. Petersburg) from 1998 to 2002 also revealed that strains with the *ptxA1* and the *prn2* or *prn3* alleles prevailed in circulation (12, 14). Possible variations in nucleotide sequences in 15 other genes encoding surface-exposed proteins have also been studied. Polymorphisms were found in the *bipA*, *fhaB*, *fim2*, *fim3*, *ompQ*, *ptxS3*, *tcfA*, and *vag8* genes; but they are especially manifest in *ptxA*, *prn*, and *tcfA* (20, 27, 30, 31).

Here we present data on the polymorphisms in four genes, *ptxA*, *prn*, *fim2*, and *fim3*, among *Bordetella pertussis* strains isolated before and after the introduction of vaccination in

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TABLE 1. Characteristics of the *B. pertussis* strains used in this study

Period of collection	No. of isolates	Allele(s) (no. of strains) ^a				Serotype(s) (no. of strains)
		<i>ptxA</i>	<i>prn</i>	<i>fim2</i>	<i>fim3</i>	
Before 1958	3 vaccine strains	<i>ptxA2</i> (2), <i>ptxA4</i> (1)	<i>prn1</i> (3)	<i>fim2-1</i> (3)	<i>fim3A</i> (3)	2.3 (1), 3 (1), 2 (1)
1948–1959	13	<i>ptxA2</i> (5), <i>ptxA4</i> (8)	<i>prn1</i> (13)	<i>fim2-1</i> (1)	<i>fim3A</i> (12)	2.3 (13)
1960–1969	20	<i>ptxA2</i> (11), <i>ptxA4</i> (9)	<i>prn1</i> (20)	<i>fim2-1</i> (11), <i>fim2-2</i> (1) ^b	<i>fim3A</i> (11), <i>fim3B</i> (1) ^c	2.3 (12), 3 (7), 2 (1)
1970–1979	28	<i>ptxA2</i> (9), <i>ptxA4</i> (16), <i>ptxA1</i> (3)	<i>prn1</i> (26), <i>prn3</i> (2)			2.3 (4), 3 (23), 2 (1)
1980–1989	27	<i>ptxA1</i> (27)	<i>prn1</i> (19), <i>prn3</i> (5), <i>prn2</i> (3)			2.3 (3), 3 (22), 2 (1), 0 (1)
1990–1999	8	<i>ptxA1</i> (8)	<i>prn1</i> (3), <i>prn3</i> (3), <i>prn2</i> (2)	<i>fim2-1</i> (3), <i>fim2-2</i> (25) ^d	<i>fim3A</i> (10), <i>fim3B</i> (18) ^e	2.3 (2), 3 (4), 2 (1), 0 (1)
2000–2004	62	<i>ptxA1</i> (62)	<i>prn1</i> (1), <i>prn3</i> (19), <i>prn2</i> (42)			2.3 (10), 3 (48), 2 (2), 0 (2)

^a It was not possible to determine the allelic breakdown for all strains according to the time periods defined here. Exceptions are noted.

^b The period of collection for the 11 *fim2-1* strains and the 1 *fim2-2* strain was from 1960 to 1989.

^c The period of collection for the 11 *fim3A* strains and the 1 *fim3B* strain was from 1960 to 1989.

^d The period of collection for the 3 *fim2-1* strains and the 25 *fim2-2* strains was from 1990 to 2004.

^e The period of collection for the 10 *fim3A* strains and the 18 *fim3B* strains was from 1990 to 2004.

Moscow. Clinical isolates were compared with the strains used for vaccine production in Russia.

MATERIALS AND METHODS

Bacterial strains and culture conditions. We studied 62 *B. pertussis* strains isolated in Moscow from 2002 to 2004, as well as 96 *B. pertussis* strains isolated from 1948 to 1999 (from the collection of Gabrichevsky Institute of Epidemiology and Microbiology, Moscow, Russia) and the three vaccine strains currently used to manufacture the DPT vaccine in Russia (Table 1). The strains were grown on casein-charcoal agar with 10% sheep blood for 3 to 5 days at 36°C. Serotyping was performed with polyclonal rabbit antisera to agglutinogens 1, 2, and 3 (Gamelei Institute of Epidemiology and Microbiology, Moscow, Russia) in a slide agglutination assay.

Genotyping. DNA isolation, *ptxA* gene amplification, and sequencing of the *B. pertussis* strains were done as described previously (16, 18). Polymorphism in the *prn* gene was studied by PCR-based methods (19), and *prn* gene sequencing was done as described previously (16). The coordinates of the primers are based on the published genome sequence of the Tohama I strain (GenBank accession number NC_002929). The sequencing of the *fim2* and *fim3* genes of the *B. pertussis* strains was performed as follows. PCR amplification and sequencing of chromosomal DNA or bacterial lysate were performed by adding 1 µl DNA

or lysate to 19 µl of a PCR mixture. For *fim2*, the PCR mixture consisted of 50% HotstarTaq Mastermix (QIAGEN), 4 µl 5 M betain (Sigma-Aldrich), 10 pmol PCR primer F (GCGCCGGCCCTGCATGCAC; coordinates 1176626 to 1176607), 10 pmol of primer R (GGGGGGTTGGCGATTTCCAGTTTCTC; coordinates 1170431 to 1170406), and 3 µl H₂O. Thermal cycling for *fim2* was as follows: 15 min at 95°C and 30 cycles of 30 s at 95°C, 30 s at 65°C, and 1 min at 72°C. A final extension was performed for 10 min at 72°C. For *fim3*, the PCR mixture consisted of 50% HotstarTaq Mastermix (QIAGEN), 5% dimethyl sulfoxide (ICN), 10 pmol PCR primer F (GACCTGATATTCTGATGCCG; coordinates 1647541 to 1647560), 10 pmol of primer R (CGCAAGGCTTGCCGGT TTTTTTTGG; coordinates 1648255 to 1648231), and 6 µl H₂O. Thermal cycling of *fim3* was as follows: 15 min at 95°C and 30 cycles of 30 s at 95°C, 30 s at 56°C, and 1 min at 72°C. A final extension was performed for 10 min at 72°C. The *fim2* and *fim3* PCR products were purified with ExoSap-IT (U.S. Biochemicals, Cleveland, OH) and sequenced with the same forward and reverse primers used for amplification. The sequencing reactions were performed with an ABI Prism BigDye Terminator reaction kit, and the reaction products were analyzed with a model 3700 ABI DNA sequencer (Perkin-Elmer Applied Biosystems). Sequence trace data were analyzed by using the Kodon program (Applied Maths, Sint-Martens-Latem, Belgium). The nucleotide sequences of the alleles in the *ptxA*, *prn*, *fim2*, and *fim3* genes of the *B. pertussis* strains were compared with sequences in the EMBL/GenBank database.

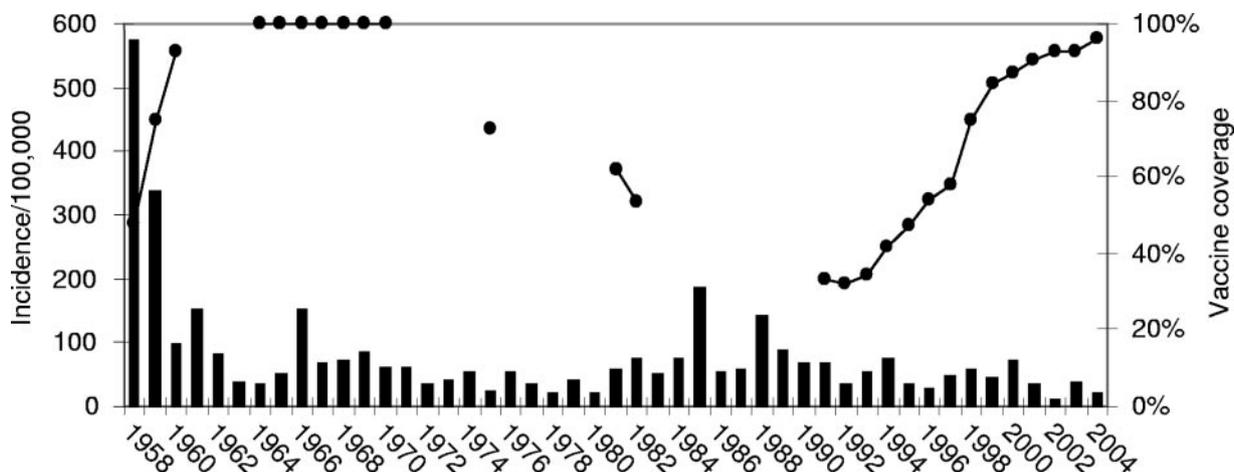


FIG. 1. Vaccination coverage (lines) and pertussis incidence rates (columns) in Moscow from 1958 to 2004. Vaccination was introduced in 1958.

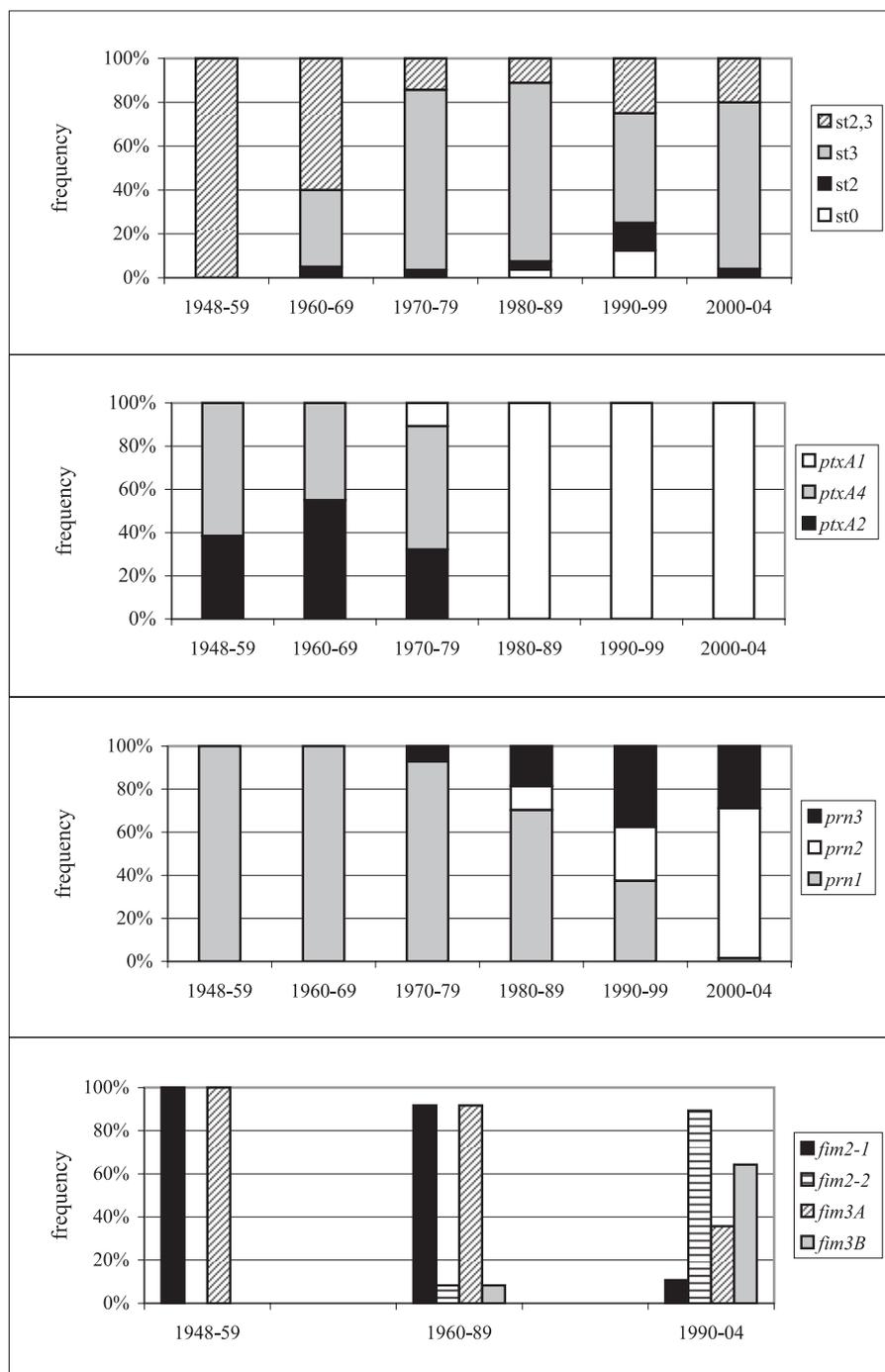


FIG. 2. Temporal trends in serotypes and alleles for pertussis toxin, pertactin, and fimbriae in Moscow.

RESULTS AND DISCUSSION

Epidemiology of pertussis in Moscow. The area of Moscow studied encompasses 1,081 square kilometers and harbors a population of approximately 11 million people. The incidence rate (IR) of pertussis per 100,000 persons was 574 in the year that vaccination was introduced (Fig. 1). After the introduction of pertussis vaccination, IRs decreased, although in the first 9 years of mass immunization, some sharp increases in IRs were observed (IRs, 152 and 153 in 1961 and 1966, respectively), as

were sharp decreases (IRs, 36 and 35 in 1963 and 1964, respectively). Between 1970 and 1979, IRs of pertussis infection stabilized (IRs, 53 and 19 in 1976 and 1978, respectively). This period was characterized by a slow decrease in IRs, with periodic peaks every 2 years. It is important to mention that the decreased IRs in the 1970s in Moscow were occurring against a backdrop of reduced vaccine coverage. An increase in IRs was observed in the 1980s, presumably an effect of the decreased vaccine coverage mentioned earlier (8, 23, 25) (Fig. 1).

In the 1980s, high IRs were registered in 1985 and 1988 (IRs, 186 and 140, respectively). Epidemics in the middle and late 1990s were less intense than those in the early and mid-1980s (4). Periodic peaks and troughs in the incidence of pertussis were then occurring against the backdrop of a sufficiently high rate of vaccination (DPT vaccine) coverage among 6- to 12-month-old children (Fig. 1). In the 1990s and 2000s, the rate of vaccine coverage increased from 33% in 1991 to 96% in 2004. In 2004, the percentage of children who had received a booster for pertussis by the age of 4 years was 96%. The booster at 18 months of age was given on time for 95% of the cases. Despite the high vaccine coverage rate, the average IR for the years from 2000 to 2004 was 35 (Fig. 1). We studied 158 *B. pertussis* strains isolated from pertussis patients. Of these, 13 strains were isolated from patients in prevaccination periods (1948 to 1959), 20 strains were isolated from 1960 to 1969, 28 strains were isolated from 1970 to 1979, 27 strains were isolated from 1980 to 1989, 8 strains were isolated from 1990 to 1999, and 62 strains were isolated from 2000 to 2004 (Table 1).

Temporal trends in serotype frequencies. The three *B. pertussis* vaccine strains were of serotypes 2.3, 3, and 2; i.e., they expressed Fim2 and Fim3, Fim3 only, or Fim2 only, respectively. Serotyping with polyclonal sera showed that all *B. pertussis* strains isolated during the prevaccination period belonged to serotype 2.3 (Fig. 2). After the introduction of vaccination, serotype 2.3 strains were gradually replaced by serotype 3 strains. The frequencies of the serotype 3 strains were 35%, 82%, 81%, 50%, and 76% in the periods 1960 to 1969, 1970 to 1979, 1980 to 1989, 1990 to 1999, and 2000 to 2004, respectively. The increase in the frequency of serotype 3 after the introduction of vaccination has been described before and has been attributed to the lower immunogenicity of Fim3 and to the higher level of cross-reacting antibodies induced by Fim3 than by Fim2 (21, 22). Serotypes 0 and 2 were found at low frequencies (0% to 13%) throughout the whole period.

Temporal trends in frequencies of *ptxA*, *prn*, *fim2*, and *fim3* alleles. Among the *B. pertussis* strains that we studied, *ptxA* gene sequencing yielded three allelic variants identified previously, *ptxA1*, *ptxA2*, and *ptxA4* (16). The vaccine strains harbored the alleles *ptxA2* and *ptxA4*. The presence of the *ptxA2* or the *ptxA4* allele was characteristic of *B. pertussis* strains isolated in the prevaccination period (1948 to 1959), when they were found at frequencies of 38% and 62%, respectively (Fig. 2). The *ptxA1* allele was first detected from 1970 to 1979 (frequency, 11%), and the frequency of detection increased to 100% from 1980 to 1989 and subsequent periods.

Three *prn* alleles were identified in this study: *prn1*, *prn2*, and *prn3* (16). The vaccine strains harbored the *prn1* allele. The *prn1* allele was found at a frequency of 100% in 1948 and from 1960 to 1969 (Fig. 2). In subsequent periods it gradually decreased in frequency to the low level of 2% from 2000 to 2004. The *prn3* allele was first observed from 1970 to 1979 (frequency, 7%). In the subsequent periods, 1980 to 1989, 1990 to 1999, and 2000 to 2004, it was found at frequencies of 19%, 38%, and 29%, respectively. The *prn2* allele was first detected from 1980 to 1989 (frequency, 11%) and increased in frequency in the periods from 1990 to 1999 (25%) and 2000 to 2004 (70%).

The characteristics of *B. pertussis* strains isolated in Moscow with respect to serotype and the *ptxA* and *prn* alleles agree with

the data obtained from the analysis of *B. pertussis* strains isolated in St. Petersburg, Russia (12, 29).

There is a paucity of data on polymorphisms in the serotype 2 and 3 fimbrial genes, although the levels of antibodies against fimbriae have been implicated in protective immunity (3, 15, 26). Two *fim2* alleles (*fim2-1* and *fim2-2*) and two *fim3* alleles (*fim3A* and *fim3B*) have been identified in *B. pertussis* populations (27, 31). The Russian vaccine strains were found to harbor *fim2-1* and *fim3A*. These alleles were found at frequencies of 100% from 1948 to 1959. In the subsequent period, 1960 to 1989, *fim2-2* and *fim3B* were found at low frequencies (8% for both alleles). In the next period analyzed, 1990 to 1994, *fim2-2* and *fim3B* rose to predominance (frequencies, 89% and 64%, respectively). Shifts in *fim2* alleles have been observed in the United Kingdom (20). When 80 strains isolated in the United Kingdom between 1920 and 2002 were studied, 75% were found to harbor *fim2-1* and 25% were found to harbor *fim2-2*. The United Kingdom vaccine strains harbored *fim2-1*, and the *fim2-2* allele was not found in isolates prior to 1998. Temporal trends in the frequencies of the *fim3* alleles have been studied in Canada, where *fim3B* was detected in 1994, although isolates from earlier periods were not analyzed (27, 28).

Despite a high vaccination coverage rate in Moscow of 87% to 96% from 2000 to 2004, the average IRs were 35 and 224 for all age categories and those 0 to 14 years old, respectively. The antigenic divergence between vaccine strains and circulating strains may have contributed to the persistence of pertussis in Moscow, despite high rates of vaccine coverage. The mismatch between vaccine strains and clinical isolates raises the possibility that the current vaccine can be improved by the use of modern strains. However, it should be noted that waning immunity may also be an important cause for the reemergence of pertussis in Moscow, and pertussis vaccines which induce long-term immunity could reduce the pertussis burden significantly. The results obtained by us clearly demonstrate that the process of selection of *B. pertussis* strains for vaccine manufacture must be based on a thorough study of the *B. pertussis* population. Analyses of polymorphisms in *B. pertussis* genes other than those investigated here remain an important area of research. Such analyses will allow the identification of the evolutionary changes in *B. pertussis* which allow it to persist in immunized populations.

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