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Strain Variation among *Bordetella pertussis* Isolates in Finland, Where the Whole-Cell Pertussis Vaccine Has Been Used for 50 Years

Annika Elomaa,[1,3]* Abdolreza Advani,[2] Declan Donnelly,[2] Mia Antila,[1] Jussi Mertsola,[3] Hans Hallander,[2] and Qiushui He[1]

Pertussis Reference Laboratory, National Public Health Institute, Turku, Finland[1]; Department of Immunology and Vaccine Research, Swedish Institute for Infectious Disease Control, Solna, Sweden[2]; and Department of Pediatrics, Turku University Hospital, Turku, Finland[3]

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Pertussis is an infectious disease of the respiratory tract caused by *Bordetella pertussis*. Despite the introduction of mass vaccination against pertussis in Finland in 1952, pertussis has remained an endemic disease with regular epidemics. To monitor changes in the Finnish *B. pertussis* population, 101 isolates selected from 1991 to 2003 and 21 isolates selected from 1953 to 1982 were studied together with two Finnish vaccine strains. The analyses included serotyping of fimbriae (Fim), genotyping of the pertussis toxin S1 subunit (*ptxA*) and pertactin (*prn*), and pulsed-field gel electrophoresis (PFGE) after digestion of *B. pertussis* genomic DNA with XbaI restriction enzyme. Strains isolated before 1977 were found to harbor the same *ptxA* as the strains used in the Finnish whole-cell pertussis vaccine, and strains isolated before 1982 harbored the same *prn* as the strains used in the Finnish whole-cell pertussis vaccine. All recent isolates, however, represented genotypes distinct from those of the two vaccine strains. A marked shift of predominant serotype from Fim serotype 2 (Fim2) to Fim3 has been observed since the late 1990s. Temporal changes were seen in the genome of *B. pertussis* by PFGE analysis. Three PFGE profiles (BpSR1, BpSR11, and BpSR147) were distinguished by their prevalence between 1991 and 2003. The yearly emergence of the three profiles was distributed periodically. Our study stresses the importance of the continuous monitoring of emerging strains of *B. pertussis* and the need to obtain a better understanding of the relationship of the evolution of *B. pertussis* in vaccinated populations.

Bordetella pertussis, a small gram-negative bacterium, is the causative agent of the respiratory infection called pertussis. *B. pertussis* produces many virulence factors that are responsible for the clinical features of the disease (9, 20, 26). The virulence factors of *B. pertussis* are generally divided into two groups, adhesins and toxins. The adhesins, such as filamentous hemagglutinin (FHA) (22, 23), fimbriae (Fim) (6), and pertactin (Prn) (10), facilitate attachment of the bacteria to the host. Toxins, such as pertussis toxin (Ptx) (17) and adenylate cyclase toxin (ACT) (5), enable the bacteria to evade the immune system of the host. Several virulence factors, such as FHA, Fim, Ptx, and Prn, have been used in acellular pertussis vaccines (19). Methods have been developed for the typing of the virulence factors (14); and changes in Fim, Ptx, and Prn have been found in *B. pertussis* isolates around the world (4, 8, 15, 18, 24, 25).

Vaccinations against pertussis have been in use for more than 50 years. Despite the high vaccination coverage rates among children, the incidence of pertussis has increased in countries such as Australia, France, The Netherlands, Poland, and the United States (2, 4, 7, 18, 21, 25). In Finland, vaccination against pertussis has been a part of the National Vaccination Program since 1952. The vaccine is produced at the National Public Health Institute, Helsinki, Finland. Strain 18530 (Prn genotype *prn1*, Ptx subunit S1 genotype *ptxA3*, Fim serotype 3 [Fim3]) has been used since 1962. In 1976, strain 1772 (*prn1*, *ptxA2*, Fim2.3) was added to the vaccine, and the vaccine has not changed since then. The vaccination coverage rate has been high in Finland. The latest survey carried out by the National Public Health Institute showed that the rate of coverage with the four doses of the diphtheria-tetanus-pertussis vaccine was 95.6% among children born in 1999 (16). Still, pertussis has been endemic in Finland, and there has been a notable increase in the numbers of pertussis cases during the last few years: 1,264 (incidence, 24.3/100,000 population) laboratory-confirmed cases were reported in 2003, whereas 315 (6.1/100,000 population) laboratory-confirmed cases were reported in 2001. The laboratory confirmation was done by PCR, culture, or serology.

The connection between these changes and the reemergence of pertussis has, however, remained unclear. The changes observed in the virulence factors of *B. pertussis* may affect the virulence and immunogenic characteristics of the bacteria. Furthermore, changes in *B. pertussis* might be of importance, especially in the adolescent population with waning immunity, although this might not have an obvious reduction of the efficacy of pertussis vaccines in younger children.

The aim of this study was to study changes in Finnish *B. pertussis* isolates during the 50 years of the whole-cell vaccination, 1953 to 2003. We analyzed 122 Finnish *B. pertussis* isolates and 2 Finnish vaccine strains (18530 and 1772). The analysis included serotyping of Fim and genotyping of *ptxA* and *prn*. Pulsed-field gel electrophoresis (PFGE) analysis of the isolates was performed. We also wanted to compare circulating strains with the vaccine strains.

* Corresponding author. Mailing address: National Public Health Institute, Pertussis Reference Laboratory, Kiinamyllynkatu 13, 20520 Turku, Finland. Phone: 358-2-331 6632. Fax: 358-2-331 6699. E-mail: annika.elomaa@utu.fi.

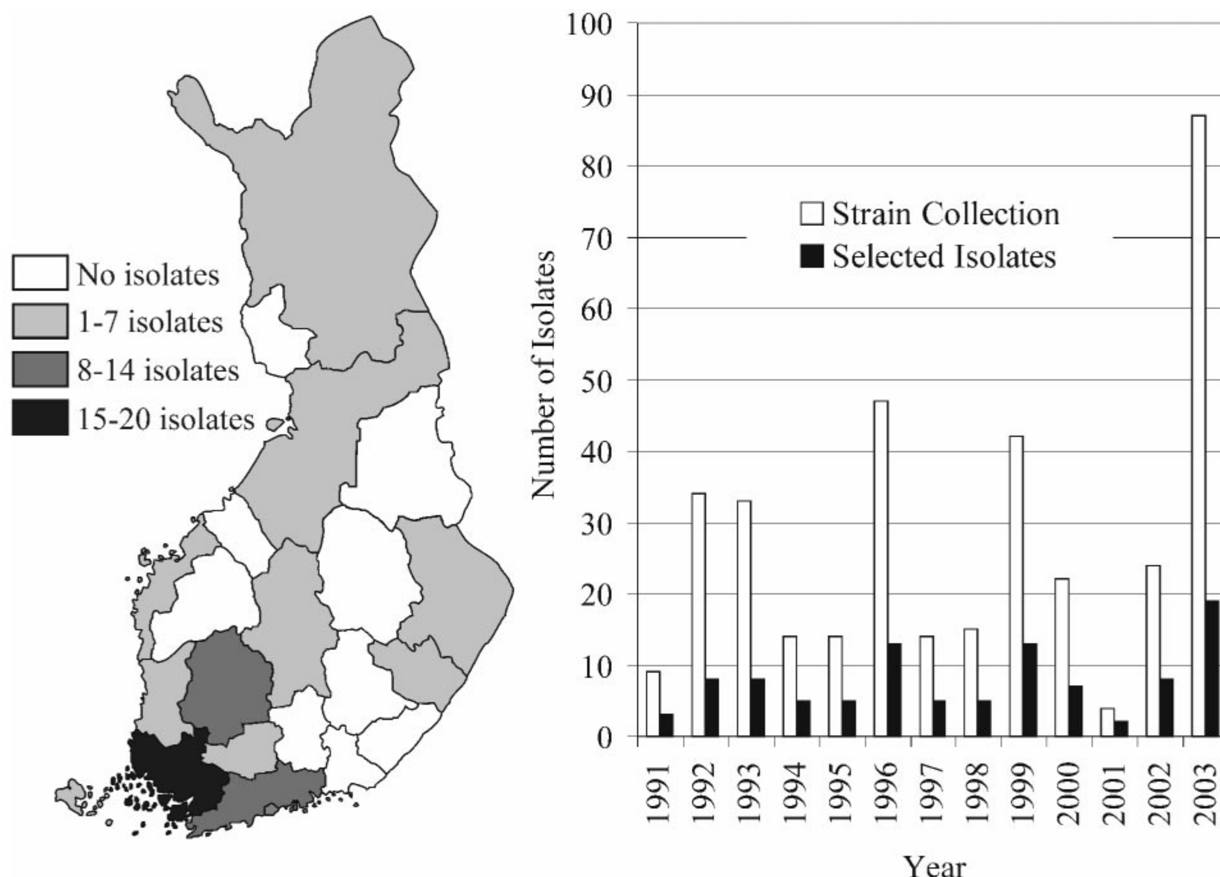

FIG. 1. Distribution of the *B. pertussis* isolates among the 22 hospital districts of Finland. Between 1991 and 2003, the strain collection of the Pertussis Reference Laboratory in the National Public Health Institute of Finland obtained 359 isolates from 13 hospital districts, and 101 isolates were analyzed in this study. The Pertussis Reference Laboratory is located in the region of the hospital district of southwest Finland.

MATERIALS AND METHODS

Bacterial strains. Bacterial isolates were selected from the *B. pertussis* strain collection of the Pertussis Reference Laboratory of the National Public Health Institute, Turku, Finland. The strain collection includes 359 Finnish *B. pertussis* isolates recovered from 1991 to 2003. The isolates had been sent from the local laboratories to the Pertussis Reference Laboratory, which is situated in southwestern Finland. The hospital district of southwest Finland has been more active than other areas in sending the isolates, and thus, 75% of the strains in the collection are from that district. Of the 359 isolates recovered from 1991 to 2003, 101 isolates were selected on the basis of the following criteria: (i) that they represented 30% of the isolates collected each year or (ii) that they represented at least two isolates from each year but (iii) not more than two isolates from each town per year. The purposes of the selection criteria were to have an extensive survey of the strains circulating in Finland and exclude the impact of local outbreaks in the final conclusions. The isolates represent 34 communities from 13 hospital districts (Fig. 1). The isolates selected were from patients from 19 days to 69 years of age, with the median age being 6.5 years. The isolates were divided by gender rather evenly, as 55% of the isolates were from female patients and 45% from male patients. The gender of the patient was not reported for three of the isolates.

In addition to the isolates obtained from 1991 to 2003, isolates from earlier decades were also included in the study to obtain more information on the genetic and antigenic changes over a longer time. The numbers of the isolates from 1953 to 1965, 1977, and 1982 were 7, 10, and 4, respectively. The genders and the ages of the patients were not reported for these isolates. The yearly distribution of the isolates is shown in Table 1.

Culture and DNA extraction. The bacteria were cultured on Regan-Lowe medium containing charcoal agar and 10% defibrinated sheep blood at 35°C for 2 or 3 days. The colonies on the plates were harvested in water for DNA isolation. Extraction of DNA from the bacterial suspension was performed with a High Pure PCR template preparation kit (Roche Diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions. DNA concentrations were measured with a SmartSpec 3000 spectrophotometer (Bio-Rad, Richmond, Calif.) and adjusted with water to 3 ng/μl. The DNA solutions were stored at −20°C.

Serotyping. The serotypes of six control strains (two Fim2 strains, three Fim3 strains, and one Fim2.3 strain) and two Finnish vaccine strains (Fim3 and Fim2.3) obtained by both the microtiter plate and the slide agglutination methods were first compared. The control strains were kindly provided by D. Xing from the National Institute for Biological Standards and Control (NIBSC), United Kingdom. As the two methods gave identical typing results, the *B. pertussis* isolates of this study were serotyped by slide agglutination. Antibody reactions were performed with monoclonal antibodies against the major subunit of Fim. The antibodies against Fim2 and Fim3 were also provided by D. Xing from NIBSC. The antibodies (for Fim2, monoclonal antibody NIBSC 04/154; for Fim3, monoclonal antibody NIBSC 04/156) were produced for research purposes only. They had been produced in mice and were purified partially, and their concentrations were not declared. Thus, appropriate dilutions for Fim2 and Fim3 antibodies were defined experimentally.

Before serotyping, the bacteria were cultured on Regan-Lowe medium containing charcoal agar and defibrinated sheep blood at 35°C for 2 days. The agglutination reaction was done on a glass slide with 40 μl of diluted antibody solution (dilution of 1:100 in phosphate-buffered saline). If the agglutination reaction was obtained with the Fim2 antibody, the Fim3 antibody, or both antibodies, the serotype was defined as Fim2, Fim3, or Fim2.3, respectively. If no reaction was detected, the serotype was defined as untypeable. Autoagglutination was examined with phosphate-buffered saline in parallel with monoclonal antibodies.

Real-time PCR for genotyping. The isolates were analyzed for the pertussis toxin and pertactin genes by LightCycler PCR and gel electrophoresis methods, according to the protocols described by Mäkinen et al. (12, 13).

TABLE 1. Characteristics of the Finnish vaccine strains and *B. pertussis* isolates analyzed in this study

Strain and yr of isolation	No. of isolates	% Isolates with the indicated:											
		ptxA allele			*prn* allele (%)				Serotype[a]			PFGE profile	
		1	2	3	1	2	3	4	Fim2	Fim2.3	Fim3	Total no. identified	Most frequent (%)
Vaccine strains													
18530 (1962)[b]				100	100						100	1	BpFINR13
1772 (1976)[b]			100		100					100		1	BpSR23
1953–1965	7		100		100				43	14	43	2	BpFINR1 (86) BpFINR14 (14)
1977	10	100			90	10			30	20	50	3	BpSR23 (80) BpSR46 (10) BpFINR9 (10)
1982	4	100				100			100			1	BpSR18 (100)
1991–1994[c]	24	100			29	67		4	92		4	9	BpSR1 (38) BpSR127 (17) BpSR175 (13)
1995–1997[c]	23	100			9	83	4	4	91			8	BpSR147 (52) BpSR1 (9) BpSR7 (9) BpFINR16 (9) BpFINR18 (9)
1998–2000[c]	25	100			4	96			60	12	28	11	BpSR147 (24) BpSR11 (16) BpSR1 (12)
2001–2003[c]	29	100				97	3		24	3	72	11	BpSR11 (38) BpSR12 (21) BpSR16 (10)

[a] The serotypes of one isolate recovered in 1994 and two isolates recovered in 1996 were untypeable.
[b] Years when the strains were introduced in the Finnish whole-cell vaccine.
[c] The years 1991 to 2003 are grouped into periods of 3 to 4 years to correlate the incidence peaks in Finland.

Pertussis toxin S1 subunit. Different alleles of the *ptxA* gene were distinguished by two fluorescent resonance energy transfer (FRET) probe assays (12). The assays were performed with a LightCycler DNA Master Hybridization Probes kit (Roche Diagnostics GmbH, Mannheim, Germany). The first assay differentiates the *ptxA1* allele from the *ptxA2*, *ptxA3*, and *ptxA4* alleles. In the second probe assay, the *ptxA4* type of DNA is differentiated from the *ptxA2* and *ptxA3* types. The control strains for *ptxA1*, *ptxA2*, *ptxA3*, and *ptxA4* were PRCB333, 1772, 18530, and 18323, respectively. Negative controls without DNA were included in each run.

Pertactin. Eight allelic variants (*prn1* to *prn8*) of the *prn* gene were discriminated in three steps: allele-specific amplification with SYBR Green dye, hybridization assay with FRET probes, and gel electrophoresis (13). In the first step, the *prn6* to *prn8* alleles are distinguished from the *prn1* to *prn5* alleles, as no specific amplification of DNAs with the *prn6* to *prn8* alleles is observed. The allele-specific amplification was done with a LightCycler-FastStart DNA Master SYBR Green I kit (Roche Diagnostics GmbH). The second step, PCR with FRET probes, distinguishes alleles *prn1*, *prn2* to *prn4*, and *prn5* from each other. The hybridization probe assay was performed with the LightCycler DNA Master Hybridization Probes kit (Roche Diagnostics GmbH). The *prn2* to *prn4* types were further distinguished by agarose gel electrophoresis of the PCR products from the hybridization probe assay. The alleles were differentiated by the sizes of the PCR products. The control strains for different *prn* allelic variants were 1772 (*prn1*), PRCB333 (*prn2*), RUS4 (*prn3*), PRCB9 (*prn4*), B935 (*prn5*), and 18323 (*prn6*).

PFGE. Isolates were analyzed by the PFGE protocol described by Advani et al. (1). The PFGE analyses were performed in The Swedish Institute for Infectious Disease Control, Solna, Sweden. Genomic DNA was digested with the restriction enzyme XbaI (Amersham Biosciences, Little Chalfont, United Kingdom). The electrophoresis analyses were performed on a DRIII contour-clamped homogeneous electric field apparatus (Bio-Rad). The band patterns obtained were analyzed with BioNumerics, version 3, software (Applied Maths, Sint-Martens-Latem, Belgium). Different PFGE profiles were defined by one or more band differences in the DNA band patterns. The clustering method used was the unweighted pair group with arithmetic clustering (UPGMA) dendrogram type with the Dice similarity coefficient, 1% optimization, and 1% tolerance. The nomenclature was based on the defined profiles already observed in Sweden (BpSR). Profiles assigned BpFINR have been found only among the Finnish isolates analyzed.

RESULTS

Serotyping. All three serotypes, Fim2, Fim2.3, and Fim3, were observed among the *B. pertussis* isolates (Table 1). However, the proportion of each type has changed over time. In the 1980s and 1990s, the predominant serotype of the isolates was Fim2. Since 1999, the Fim3 type started to replace Fim2 in the Finnish isolates. All the isolates from the year 1998 produced Fim2, but in 2003 the proportion of Fim2 serotype isolates decreased to 16% ($n = 3$). The Fim2.3 type was also seen more often than it had been in the earlier years.

Genotyping. The *ptxA* genotype of Finnish pertussis vaccine strain 18530 was *ptxA3*. The isolates from 1953 to 1965 and strain 1772, added to the vaccine in 1976, represented the *ptxA2* genotype. All other isolates represented the *ptxA1* ge-

notype (Table 1). Thus, the change in the prevalent *ptxA* type of the circulating *B. pertussis* strains had already occurred in 1970s.

In the 1950s, the prevalent *prn* allele was *prn1*, which is also represented in the Finnish vaccine strains (Table 1). The change from *prn1* to *prn2* was already seen in the isolates from 1982, as all of them represented *prn2*. In 1991 and 1992, one-third of the isolates still represented *prn1* allele (N = 4), but *prn1* was last seen in Finnish isolates in 1999 (n = 1). All the recent isolates demonstrated the *prn2* allele. The *prn3* and *prn4* alleles occurred only twice among the isolates tested.

PFGE. A total of 34 different profiles were found among the strains studied (Table 1 and Fig. 2). The profile of vaccine strain 18530 (BpFINR13) was not found among the isolates. Two profiles, BpFINR1 and BpFINR14, were found among the isolates recovered from 1953 to 1965. Those profiles have not appeared since then. Vaccine strain 1772, added to the Finnish pertussis vaccine in 1976, and 8 of the 10 isolates from 1977 represented the profile BpSR23, which was also found in 1 isolate recovered in 1999. The profiles of other isolates from 1977 (BpFINR9 and BpSR46) have not occurred since then. The isolates from 1982 represented profile BpSR18, which still appeared occasionally, in five isolates, between 1992 and 2003.

The number of profiles found from 1991 to 2003 was 29. Most of the profiles appeared in one to seven isolates, but three profiles were clearly distinguished by their higher prevalence. Profiles BpSR1, BpSR11, and BpSR147 were represented in 14, 15, and 18 isolates from 1991 to 2003 (Table 2). The yearly emergence of those profiles was not, however, evenly distributed but periodic. All the BpSR1 profiles appeared from 1991 to 1998, and all BpSR11 profiles appeared from 1999 to 2003. The appearance of BpSR147 overlapped with those of BpSR1 and BpSR11, as the isolates with the BpSR147 profile were found from 1996 to 2000. There were also 13 BpFINR profiles among the total of 16 isolates recovered from 1991 to 2003. These profiles have not been isolated in any of the countries within the European Union collaboration (Eupertstrain; France, Germany, The Netherlands, and Sweden).

The most common PFGE profiles, BpSR1, BpSR11, and BpSR147, were clearly correlated with serotypes Fim2, Fim3, and Fim2, respectively (Table 2). Genotype *ptxA2* was found in old isolates, which harbored PFGE profiles BpFINR1 and BpFINR14. Neither those profiles nor *ptxA2* has appeared since then. Isolates with the *prn1* genotype (n = 26) were shown to harbor nine PFGE profiles. Seven of the nine profiles are correlated with *prn1*. *Prn2* was observed in 92 isolates harboring 24 PFGE profiles, including the most common profiles, BpSR1, BpSR11, and BpSR147. *Prn3* appeared in two isolates harboring unique PFGE profiles, BpFINR8 and BpFINR23, respectively, which were seen only in these isolates. *Prn4* was also detected in two isolates, but the PFGE profiles of those isolates were also found in isolates with the *prn2* genotype.

DISCUSSION

During the last decade, the number of pertussis cases has increased in countries with high vaccination coverage rates (4, 7, 18, 21, 25), including Finland. The reason for this reemergence has been of concern. One proposed cause is the antigenic variation of *B. pertussis*, especially in regard to the virulence factors such as Fim, Prn, and Ptx. Furthermore, these antigens have been included in many acellular vaccines. We have carried out research among 122 Finnish *B. pertussis* isolates collected since 1953. The virulence factors analyzed here were Fim, *prn*, and *ptxA*. The isolates were analyzed by PFGE analysis.

The results of this study concur with previously published suggestions on the continuous evolution of *B. pertussis* and its virulence factors (4, 24, 25). In Finland, the first known change in virulence factors had already occurred in the S1 subunit of pertussis toxin in the 1970s, leading to *ptxA1* being the predominant allele. This transition has been seen in many countries (4, 7, 11, 18, 24, 25) and is suggested to be vaccine driven (15), as the strains used in the whole-cell pertussis vaccines do not contain the *ptxA1* variant. The second noticeable change was seen from 1980 to the 1990s, as the prevalent pertactin allele shifted from *prn1* to *prn2*. A similar shift has been reported in the United States (4). An increase in the frequency of the *prn3* genotype was not seen in Finland in those days, which is in contrast to the situation in France and The Netherlands (15, 25), as only two Finnish isolates represented *prn3*. In Australia, *prn1* changed to *prn3*, and *prn2* did not appear until the mid-1990s (18). Our results correspond to the shift observed in the United States (4), indicating that the changes in *ptxA* and *prn* happened through a transition period. The prevalent genotype, *ptxA2* or *ptxA3* and *prn1*, first changed to *ptxA1* and *prn1*, followed by a second modification to *ptxA1* and *prn2*. This was the genotype of all Finnish isolates included in our study from 2003. Ten isolates recovered from 1991 to 1999 were found to harbor *prn1*. In Italy, the frequency of *prn1* was shown to be higher in an unvaccinated population than in the vaccinated population (11), which may partially explain the low number of *prn1* isolates in Finland and other countries with high vaccination coverage rates. We also confirm the findings of van Loo et al. (24), which indicate that serotypes Fim2, Fim2.3, and Fim3 have been evident in the *B. pertussis* population throughout the decades and which showed differences only in the frequencies of the different serotypes. Recently, the predominant serotype has changed from Fim2 to Fim3, which has also been the case in Australia (18) and The Netherlands (24).

The PFGE profiles of the Finnish isolates show that the prevalent PFGE profiles change temporally, as suggested by Weber et al. (25). The profiles represented in the Finnish isolates from past decades only occasionally appear in the recent isolates. All of the BpFINR profiles that were not seen in the recent Swedish study, based on a large number of isolates (1), and most of the BpSR profiles appear in only a few isolates within 1 to 3 years. The isolates with the BpFINR profiles represent unique subtypes that for some reason do not seem to spread as well as some other strains. Three of the PFGE profiles among the Finnish isolates were distinguished by their frequencies. The BpSR1, BpSR11, and BpSR147 profiles were found in 14 to 18 isolates within a few years. The BpSR147 profile was represented in 42% of the isolates during the years of its occurrence. All other profiles appeared in no more than four isolates during the period. Thus, few PFGE profiles are represented in a considerable proportion of circu-

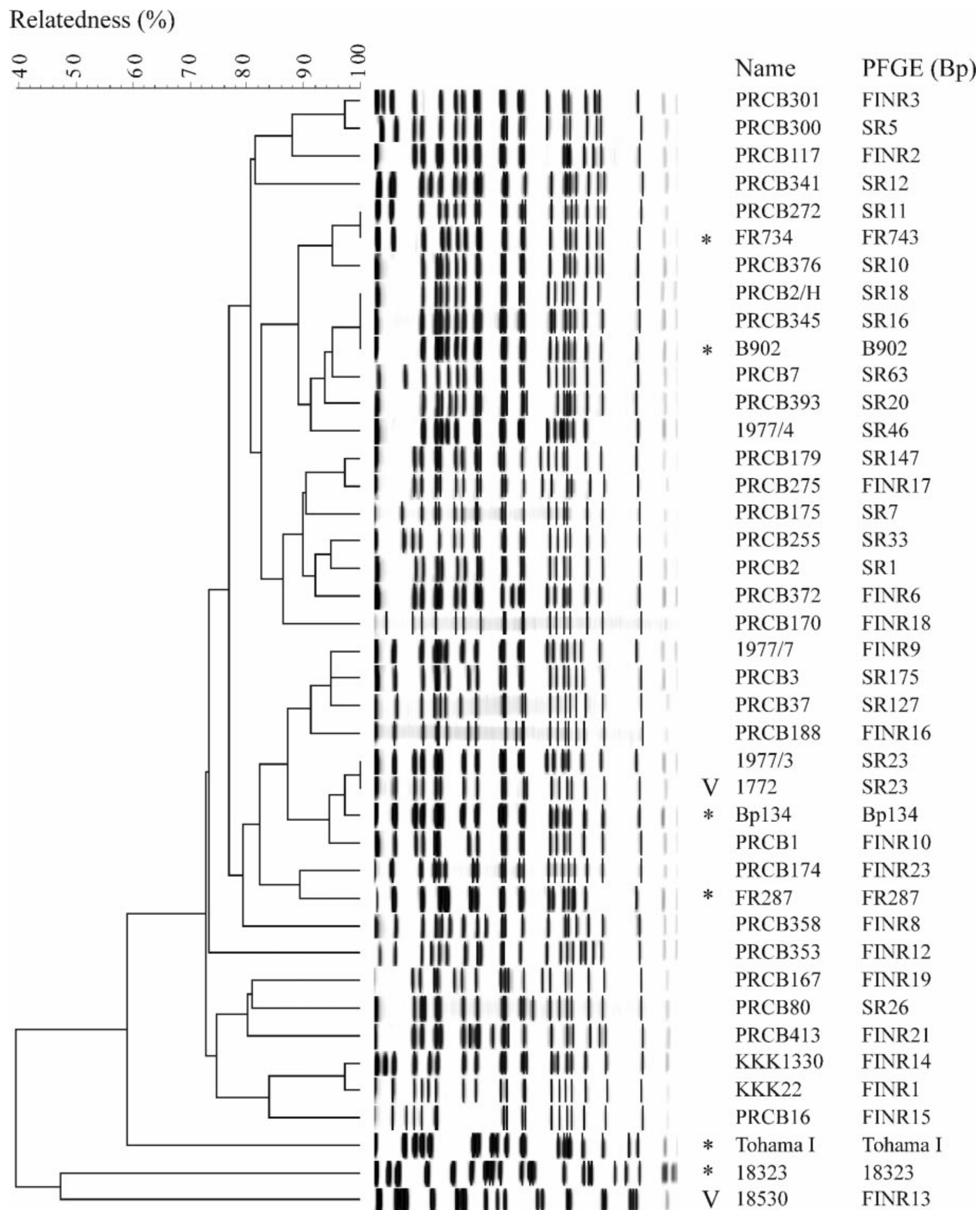

FIG. 2. Classification of the 34 PFGE profiles represented in the Finnish *B. pertussis* isolates and vaccine strains (indicated by "V") and the reference strains of PFGE (indicated by asterisks). Classification was performed by using the UPGMA dendrogram type with the Dice similarity coefficient, 1% optimization, and 1% tolerance.

lating isolates, as also described earlier (3). Even though the research material used in our study was limited, the isolates representing the BpSR1, BpSR11, and BpSR147 profiles were from three to four hospital districts in Finland and, thus, do not represent local outbreaks. As the changes in the PFGE profiles and virulence factors are merged, it is seen that the recent increase in serotype Fim3 observed since 1999 interfaces with the appearance of new PFGE profiles. Eight PFGE profiles were represented among 30 isolates with the Fim3 serotype, which were detected in 1999 or later, including the BpSR11

TABLE 2. Characteristics of the three most frequent PFGE profiles among the isolates analyzed in this study

PFGE profile	No. of isolates	Yr of isolation	% Isolates with the indicated:									
			ptxA allele			*prn* allele				Serotype[a]		
			1	2	3	1	2	3	4	Fim2	Fim2.3	Fim3
BpSR1	14	1991–1994, 1997–1998	100				93		7	93		
BpSR147	18	1996–2000	100				100			89		
BpSR11	15	1999–2000, 2002–2003	100				100				7	93

[a] The serotypes of one isolate with the BpSR1 profile and two isolates with the BpSR147 profile were untypeable.

profile, which was the second most common PFGE profile of all time in Finland. Among the 30 isolates, 28 were Fim3 and 2 were Fim2.3. The other common PFGE profiles, BpSR1 and BpSR147, detected during earlier epidemics correlate with serotype Fim2. Weber et al. (25) questioned the connection between PFGE profiles and antigenic changes. Our results, however, suggest that the serotype change may be correlated with the appearance of new PFGE profiles.

Typing of the virulence factors of *B. pertussis* isolates helps to validate the idea of the continuous evolution of the bacteria. However, when additional information is craved, the PFGE reference system published by Advani et al. (1) is very profitable and provides more precise evidence of the changes at the molecular level. Our results show that *ptxA* and *prn* have changed before the recent reemergence of pertussis. However, the role of the Fim3 type of strains with new PFGE profiles in the increased incidence of pertussis should be studied further.

In this study, PFGE analysis of *B. pertussis* isolates representing the six decades of experience with whole-cell vaccination showed the emergence of new PFGE profiles and the disappearance of the former ones, in parallel with changes in the virulence factors. In Finland, the whole-cell pertussis vaccine has been replaced with an acellular vaccine in 2005. The effects of acellular vaccines on the circulating *B. pertussis* strains should be closely monitored. This study lays a good background for further monitoring of the circulating *B. pertussis* isolates in Finland. The exceptionally stable vaccination history with a high vaccination coverage rate makes Finland a good location for monitoring of the changes in the *B. pertussis* population after the introduction of a new vaccination program with acellular pertussis vaccines in Finland in 2005.

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