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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

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We compared *Bordetella pertussis* isolates collected in France over the last 10 years, the vaccine strains used for more than 30 years, and isolates collected before the introduction of generalized vaccination. The analysis included serotyping, pulsed-field gel electrophoresis of chromosomal DNA after digestion with *Xba*I and *Spe*I, and sequencing of the *pt* S1 gene, encoding the S1 subunit of pertussis toxin, and the *prn* gene, encoding the adhesin pertactin. We found that the incidence of infection increases every 3 years. Ninety-five per cent of the isolates analyzed express type 3 fimbriae. Most of the isolates circulating since 1991, unlike the vaccinal strains, express a type A pertussis toxin and a type 2 pertactin. The isolates could be classified into five major groups by pulsed-field gel electrophoresis. Most of these groups correlated with the pertactin type expressed by the isolates. Pulsed-field gel electrophoresis is more discriminative than sequencing particular genes since it could differentiate isolates expressing type 2 pertactin into two subgroups: those circulating in 1993 to 1997 and those circulating in 1997 to 2001. This observation suggests that there has been continuous evolution of the *B. pertussis* population.

Bordetella pertussis, a gram-negative bacterium, is the agent of whooping cough. Soon after its discovery by Bordet and Gengou in 1906 (9), pertussis whole-cell vaccines were set up, but they were not developed until the 1940s and 1950s. These vaccines, composed of bacterial suspensions inactivated by heat, are effective, although their efficacy is variable and they are generally not well tolerated (17). For these reasons, there has been substantial research worldwide to characterize the bacterial proteins involved in the disease. Over the last 30 years, many *B. pertussis* proteins have been characterized. Most are classified as toxins and adhesins. The toxins include tracheal cytotoxin, a muramyl peptide constitutively secreted by the bacterium, pertussis toxin (PT), an ADP-ribosylating toxin, adenylate cyclase-hemolysin (AC-Hly), a Repeats in Toxins toxin, and dermonecrotic toxin. The adhesins include filamentous haemagglutinin or FHA, pertactin or PRN, and two fimbriae (FIM 2 and FIM 3). After characterization of these bacterial determinants, acellular vaccines, i.e., vaccines composed of purified proteins, were constructed. They all include chemically or genetically detoxified, PT plus one, two, or four adhesins (FHA or FHA plus PRN or FHA plus PRN plus FIM 2 and FIM 3). The efficacy of these acellular vaccines was compared to that of whole-cell vaccines in clinical trials between 1987 and 1997 (26). Two of the major aims were fulfilled: the acellular vaccines were effective in newborns and better tolerated than whole-cell vaccines. The trials confirmed that the efficacy of whole-cell vaccines is variable but also showed that the efficacy of acellular vaccines is similarly variable. The variable efficacy of acellular vaccines could be due to the number or the amount of proteins included in the vaccines, and that of whole-cell vaccines could be due to manufacturing procedures. However, another possibility, suggested a long time ago, is that the strains used to produce the vaccines are antigenically different from the strains circulating in the countries where the vaccines are used (17, 22, 28, 32, 39).

Polymorphism of *B. pertussis* has been described by bacteriologists (18, 27–29, 38–40) but not seriously taken into consideration. Indeed, bacteriologists have argued that it would be better to change vaccine strains regularly to coincide with isolates circulating in the susceptible population. Despite high vaccination coverage in the United States and France, the incidence of pertussis has been increasing since the 1980s (5, 6). Recent reports indicate that this increase is in the 10- to 19-year-old and not the 0- to 4-year-old age group (11). In 1996 to 1997, there was an epidemic in The Netherlands (14), a country where the same locally produced, whole-cell vaccine has been in use for 30 years. It was shown that the isolates currently circulating were different from those circulating before the introduction of the vaccination programme and from the vaccine strains, confirming the previous hypothesis of Kattack and Matthews that showed, using pulsed-field gel electrophoresis (PFGE), differences between circulating isolates and suggested antigenic differences from vaccine strains (27, 28). It was shown that two major virulence factors of *B. pertussis*, namely, PT and PRN, were varying. PT is a secreted ADP-ribosylating toxin composed of five different subunits, and PRN is an outer membrane protein that contains two domains composed of repeated sequences. It was shown that the currently circulating isolates express an S1 subunit of PT and a PRN different from those expressed by the vaccine strains (37). There are various possible causes of epidemics, including a decrease in the vaccine coverage and a decrease in the efficacy of the vaccine. However, the fact that the Dutch clinical iso-

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

Reference strain or isolates	No.	Geographic location	Yr of isolation	Origin or reference[a]
Vaccine 1414 strain	1	United States	Before 1959	A-P
Vaccine 1416 strain	1	United States	Before 1959	A-P
WHO reference strain 18323	1	United States	1947	10
Japanese vaccinal strain Tohama	1	Japan	1954	IP
Reference strain Bp134	1	United States	Before 1960	IP
Reference strain B902	1	The Netherlands	1992	35
Reference strain 287	1	France	1996	10
Isolate CZ	1	France	1992	10
French isolates	9	France	Before 1980	IP
French isolates	878	France	1991–2001	CNR

[a] A-P, Aventis-Pasteur; IP, Institut Pasteur Collection; CNR, Centre National de Référence des Bordetelles-Institut Pasteur-Paris.

lates were antigenically different from the vaccine strains is a possible cause. France, like The Netherlands, has used the same whole-cell vaccine for more than 30 years: vaccination with whole-cell vaccine began in 1959 and was generalized in 1966. The same whole-cell vaccine, produced by Pasteur-Mérieux, now Aventis-Pasteur, has been used for the entire time. In 1991, a study in one pediatric hospital in Paris reported an increase in the numbers of hospitalized infants infected with *B. pertussis* (20). In 1993 to 1994, a study in 22 pediatric hospitals throughout France indicated a resurgence of whooping cough (5) linked to a change in the epidemiology. Parent-to-child transmission was observed rather than the child-to-child transmission observed in countries with no large-scale vaccination programme. This change in the epidemiology was not due to a decrease in the coverage or in the vaccine efficacy (5, 43) but, rather, to waning vaccine-induced immunity (19). As a consequence, the immunization strategy was modified in 1998 with the introduction of a vaccine booster for 11- to 13-year-old children (2). However, although the whole-cell vaccine was shown to be highly effective in France in 1993 to 1994, it is important to analyze the isolates circulating over the last 10 years and to compare them with isolates circulating before the introduction of generalized vaccination and with vaccine strains. Here we report such an analysis involving serotyping with monoclonal antibodies, PFGE analysis, and sequencing of the *ptx* S1 and *prn* structural genes to investigate the influence of temporal and geographic factors on the French population of *B. pertussis* isolates. The *B. pertussis* isolates currently circulating in France are different from the whole-cell vaccine strains used and also from the isolates circulating before vaccination was begun. Analysis of the PFGE profiles using the neighbor-joining method of clustering (42) allowed the classification of the isolates into five major groups. This classification indicates that the population structure of French *B. pertussis* isolates has shifted with time.

MATERIALS AND METHODS

Isolates. A total of 878 isolates of *B. pertussis* (Table 1) were examined. Clinical isolates were collected, either by our laboratory or, since 1994, by hospital laboratories participating in the whooping cough surveillance network, RENACOQ, from nasopharyngeal aspirates mostly from infants hospitalized for whooping cough. They were compared to *B. pertussis* strains 1414 and 1416, components of the whole-cell vaccine used in France for more than 30 years and produced by Aventis-Pasteur, and also to reference strains 18323 (the World Health Organization [WHO] reference strain), the Tohama strain (the Japanese vaccine strain), the U.S. strain Bp134, and isolates collected before or soon after the introduction of vaccination.

Growth of bacteria. Bacteria were grown at 36°C for 72 h on Bordet-Gengou agar supplemented with 15% defibrinated sheep blood (BGA) and subcultured on the same medium for 24 h (35). For Western blot analysis, bacteria grown on BGA were resuspended in saline at 2×10^{10} CFU/ml, diluted in Laemmli buffer, and boiled for 15 min (31).

Adenylate cyclase assay. Adenylate cyclase activity was measured as described previously (30). One unit corresponds to 1 nmol of cyclic AMP formed per min at 30°C and pH 8.

Immune sera. Groups of 10 4-week-old female BALB/c mice received subcutaneous injections of 10 μg of purified *B. pertussis* FHA, or purified detoxified *B. pertussis* PT, or purified *B. pertussis* AC-Hly, adsorbed onto aluminum hydroxide, four times at 4-week intervals. The mice were bled 7 days after the last injection. The specificity of the polyclonal antibodies was checked by Western blotting using purified antigens and whole *B. pertussis* bacterial suspension.

Electrophoresis and immunoblotting methods. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with ready-to-use 8 to 25% polyacrylamide gels and the Pharmacia PhastSystem. After electrophoresis, the proteins were transferred from the gels to Hybond C-Super membranes (Amersham). After blocking, the membranes were incubated at 4°C overnight with polyclonal sera diluted to 10^{-3}. Horseradish peroxidase-labeled sheep anti-mouse immunoglobulins and an enhanced chemiluminescence system were used to reveal binding (Amersham).

Serotyping of the isolates. FIM 2 and FIM 3 production was revealed using monoclonal antibodies as described previously (35).

PFGE. DNA fingerprinting was performed using PFGE because this technique has a great discriminatory power. The conditions used were as described previously (35). PFGE data were analyzed using the neighbour-joining clustering method on representatives of the different profiles generated (42).

Gene typing. Polymorphism was recently described in the genes encoding PRN (*prn*) and the S1 subunit of PT (*ptx* S1) (37). Genotyping was limited to these genes, and the conditions used were as described previously (35).

RESULTS

Description of the isolates. In 1986, obligatory notification of whooping cough stopped in France because of the very substantial decreases in mortality and morbidity that followed the introduction of generalized vaccination in 1966. Laboratory confirmation of the disease was not required, and consequently no isolate was collected thereafter.

In 1990, we set up techniques for biological diagnosis including culture. We first performed cultures in collaboration with Hôpital Trousseau (20, 21, 23); then isolates were collected as part of the National Study coordinated by the Ministry of Health involving 22 pediatric hospitals in 1993 to 1994 (5). Since 1996, isolates have been collected by our surveillance network, RENACOQ, including 43 pediatric hospitals and epidemiologists of the Institut de Veille Sanitaire (4).

Most of the isolates were collected from nonvaccinated infants hospitalized for whooping cough, but some isolates were also collected from members of their families. Figure 1 shows

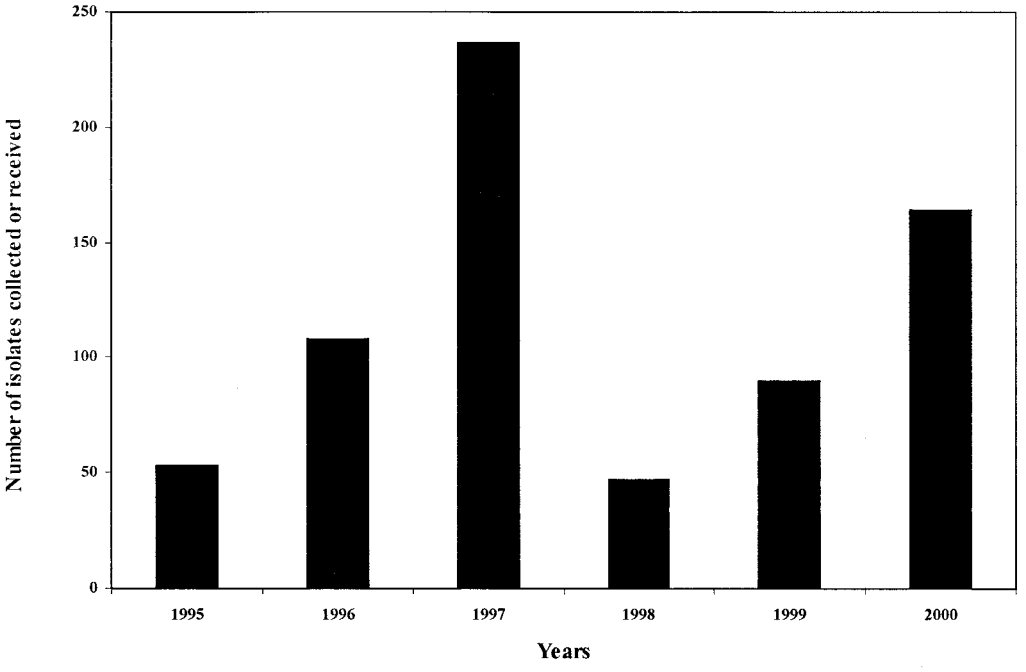

FIG. 1. Graph showing the number of isolates collected and received in our laboratory between 1995 and 2000. Each column represents the number of isolates collected or received in a given year.

the distribution of the number of isolates collected or received each year by our laboratory since 1995. It can be observed that every 3 years there is an increase in the number of isolates collected. The increase observed in 1997 correlated with those observed in other countries including Australia, Canada, The Netherlands, and the United States (1, 6, 14, 16, 24).

Identification of the isolates. A total of 878 isolates collected were identified as *B. pertussis* and only 12 (1.4%) were identified as as *B. parapertussis* during the 1993 to 2000 period. All isolates were hemolytic, indicating that they express AC-Hly toxin. They all exhibited the bacteriological characteristics classically described for *B. pertussis* and *B. parapertussis*. FIM 2 and FIM 3 production was tested, as described previously (35). Of the isolates, 95% produced only FIM 3, 1.4% produced only FIM 2, and 3 % produced both FIM 2 and FIM 3.

Analysis of chromosomal DNA by PFGE. Chromosomal DNA from isolates collected each year, from different parts of France, were examined by PFGE. PFGE was performed as described previously (35) using chromosomal DNA from 298 isolates digested with either *Spe*I or *Xba*I. The profiles obtained were compared to those obtained with chromosomal DNA from reference strains (35) and from isolates collected before the introduction of generalized vaccination in France. The neighbor-joining clustering method was used to classify the isolates. The first analysis, performed on a few representatives of each profile, distinguished five major groups (Fig. 2). Group I includes only strain 18323, the WHO reference strain, and one isolate (CZ) collected in 1993 from an unvaccinated infant in Paris; group II is composed of the isolates collected before generalized vaccination and the French vaccine strain 1414; groups III, IV, and V include all the isolates collected between 1993 and 2000 and the Japanese vaccine strain Tohama, the French vaccine strain 1416, the U.S. strain Bp134, and the PFGE reference strains 287 and B902. The isolates collected before and after the introduction of generalized vaccination in France are clearly separated. This observation is similar to those made in other countries. A more precise anal-

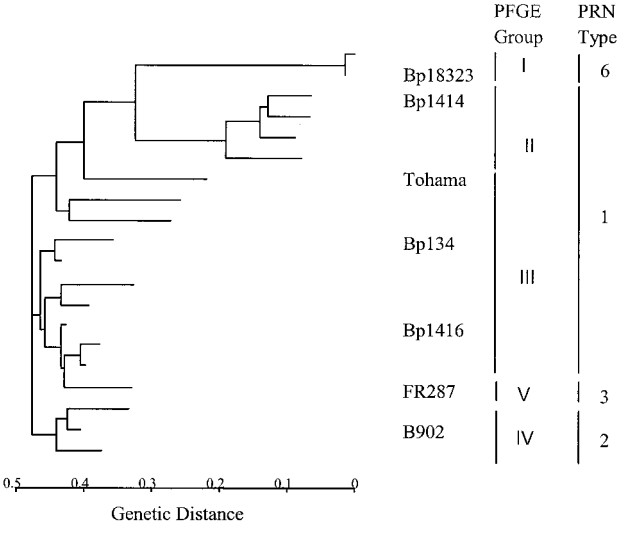

FIG. 2. Classification of isolates by PFGE. DNA was purified from isolates and restricted with *Spe*I and *Xba*I. Fragments were separated by electrophoresis as described previously (35). Classification was performed using the neighbor-joining clustering method with representatives of each PFGE group. Regions encoding the repeats of the *prn* structural gene harbored by selected members of each PFGE group were sequenced and types were assigned as described previously (35).

ysis of each group was performed, taking into account all the isolates. The increased number of isolates analyzed did not change the clear difference between groups I, II, and III (data not shown). For groups IV and V, composed of isolates circulating since 1991, the increase in the number of isolates allowed us to confirm the differences between groups IV and V but also to subdivide group IV in two subgroups : IVα and IVβ (Fig. 3).

Figure 4 shows the distribution of the isolates of groups III, IV, and V according to the year of collection. Group IV is clearly overrepresented among the isolates circulating the last 8 years. Few isolates from groups III and V have been circulating since 1993, although there was an increase in the number of group V isolates in 1996 to 1997, a period when there was an increase in the number of pertussis cases worldwide. Figure 5 shows the distribution of the isolates from groups IVα and IVβ according to the year of collection. Surprisingly, all isolates circulating between 1993 and 1996 were from group IVα, and since then the number of isolates from this group has been decreasing whereas the number of isolates from group IVβ has been increasing.

Sequencing the structural genes encoding the S1 subunit of the pertussis toxin and repeated regions of pertactin. *ptx* S1 and *prn* genes in selected isolates from the five PFGE groups were sequenced as described previously (35). There was no correlation between PFGE group and S1 PT type, but there was a correlation with the year of collection: the isolates collected before generalized vaccination was started express type B or D PT whereas the isolates collected after 1991 express type A, as already observed in other countries (12, 36, 37). As previously published, the reference strain 18323 and isolate CZ are very different and express type E (10).

However, the PFGE groups correlated surprisingly well with the PRN type. Isolates from group I express type 6 PRN, those from groups II and III express type 1, those from group IV express type 2, and those from group V express type 3 (Fig. 2). Type 4 PRN (accession number, AJ309315) was expressed by only three isolates, one collected before 1960 and two collected in 1993 and 1994. These three isolates are displayed in PFGE groups II, III, and V.

Detection of toxins and adhesins. Specific polyclonal sera were used to test some isolates from each of the five PFGE groups for AC-Hly, PT, FHA, and PRN. All isolates but one expressed the two toxins and the two adhesins examined. The one exception was an isolate collected before the introduction of vaccination, which does not express FHA. Concerning AC-Hly, some isolates exhibited a higher hemolytic activity in the samples collected from the infant nasopharyngeal aspirate than after subculture. This higher hemolytic activity was linked to a higher adenylate cyclase activity in the corresponding bacterial suspension (data not shown).

Isolates type according to vaccine status of the patient and city in which they were collected. Most of the isolates were collected from unvaccinated infants. However, isolates were also collected from members of their families or contact individuals in nine cases. Among the isolates analyzed by PFGE, two were from vaccinated children. These isolates were not different from those collected from unvaccinated patients and were not part of a special group. However, their number is too small to allow any significant conclusion.

There was no correlation between PFGE group and geographical region. However, as expected, in several cases isolates from an infected infant and its mother had identical profiles. There was also a cluster of six isolates, collected over a 4-month period from a city in the southwest of France: three were from an infant and his parents and three were from other infants. All six isolates had an identical PFGE profile that was not found elsewhere. This confirmed transmission between household members, as previously observed (8, 15), and spread to susceptible individuals in the surrounding community.

We did not find any association between PFGE profiles and age or season.

DISCUSSION

Despite the introduction of large-scale vaccination with pertussis whole-cell vaccine in many countries, whooping cough is still an endemic disease with outbreaks every 3 to 5 years. There was a major epidemic in 1996 to 1997 in The Netherlands, and there have been epidemics in other countries with large-scale vaccination programs (1, 6, 14, 16). There are several possible causes: improved surveillance, changes in case definitions, new diagnosis techniques, decrease in vaccination coverage (which could be due to a decrease in the tolerability of the whole-cell vaccine), changes in whole-cell vaccine manufacturing procedures affecting its efficacy, and waning vaccine-induced immunity. Epidemics may also be caused by an antigenic change in the circulating isolates in such a way that vaccine strains will not induce an immunity that is able to protect against these new isolates. Isolates circulating in Canada, Italy, The Netherlands, and Finland have been characterized, but few data were available from France (7, 10, 22, 33, 34, 36, 41, 44), a country that has used a single whole-cell vaccine for more than 30 years in its high-coverage vaccination programme.

Obligatory notification of whooping cough was stopped in 1986, and consequently few isolates (stored in the Institut Pasteur collection) are available from 1985 until 1991, when epidemiologic studies were initiated. Cultures were performed in very few centers in 1991. We reintroduced culture as a diagnostic method slowly thereafter, in particular during the 1993 to 1994 study (5). It is generally accepted that peaks of whooping cough incidence occur every 3 to 5 years (13). In France, as shown in Fig. 1, since 1995 an increase in the number of isolates has been observed every 3 years.

Isolates collected or received by our laboratory since 1991 or stored in our Institute collection were all hemolytic and displayed similar biochemical characters. However, phenotypically, some freshly collected isolates expressed a higher hemolytic activity, linked to a higher adenylate cyclase activity, than did others. The significance of this observation is unknown and is under investigation.

Various techniques of DNA fingerprinting have been used to study the polymorphism of *B. pertussis* populations. We found that the most appropriate method for *B. pertussis* is currently PFGE, since it has a good discriminatory power and reproducibility for isolates not repeatedly subcultured before storage (35).

PFGE revealed numerous different DNA profiles. The differences between profiles were mostly small, confirming that *B.*

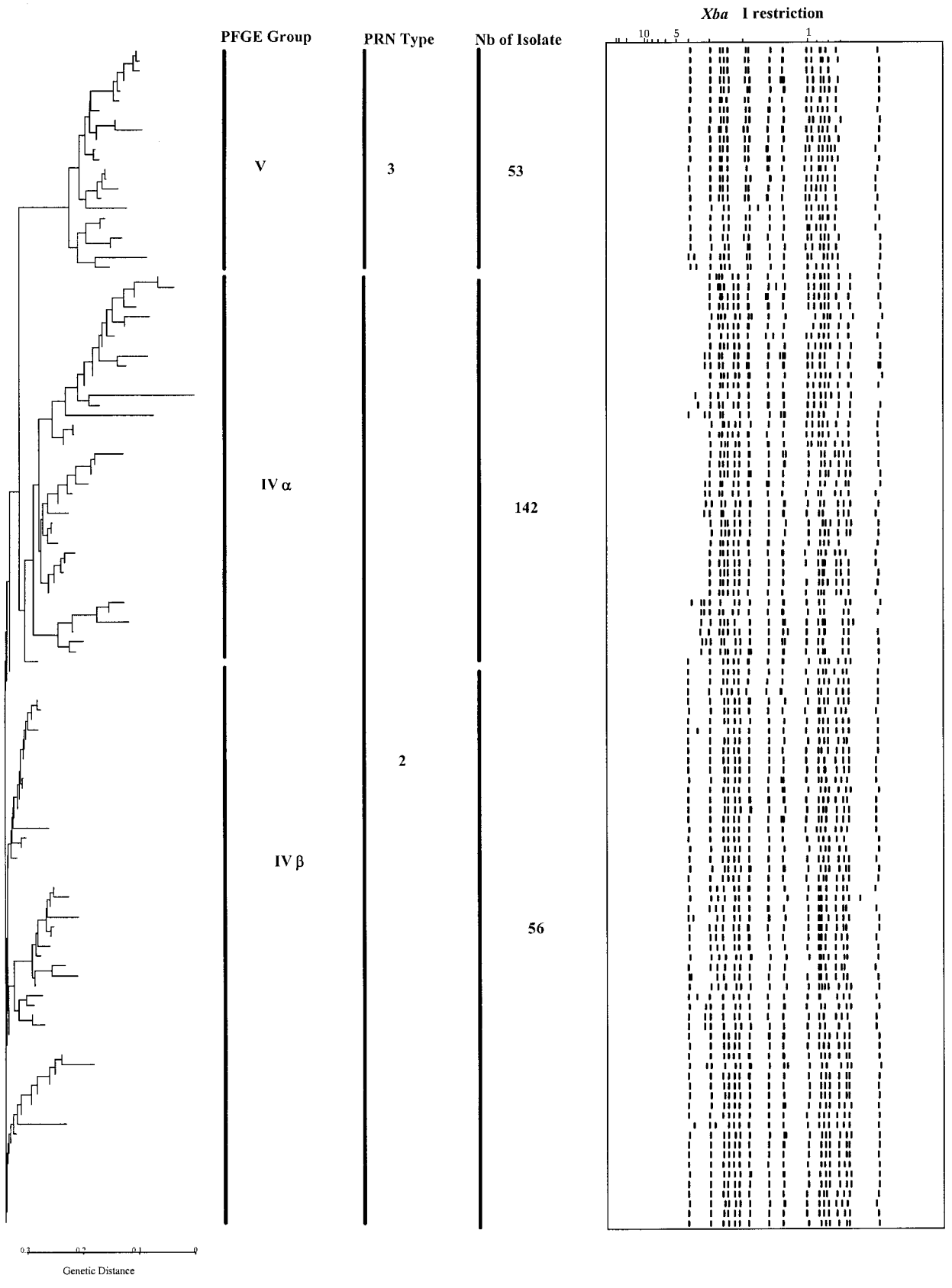

FIG. 3. Classification of recently collected isolates by PFGE. DNA was purified from isolates and restricted with *Spe*I and *Xba*I. Fragments were separated by electrophoresis as described previously (35). Classification was performed using the neighbor-joining clustering method with representatives of each PFGE group. Regions encoding the repeats of the *prn* structural gene harbored by selected members of each PFGE group and subgroups were sequenced and types assigned as described previously (35). In this figure, only DNA profiles obtained after *Xba*I restriction are shown.

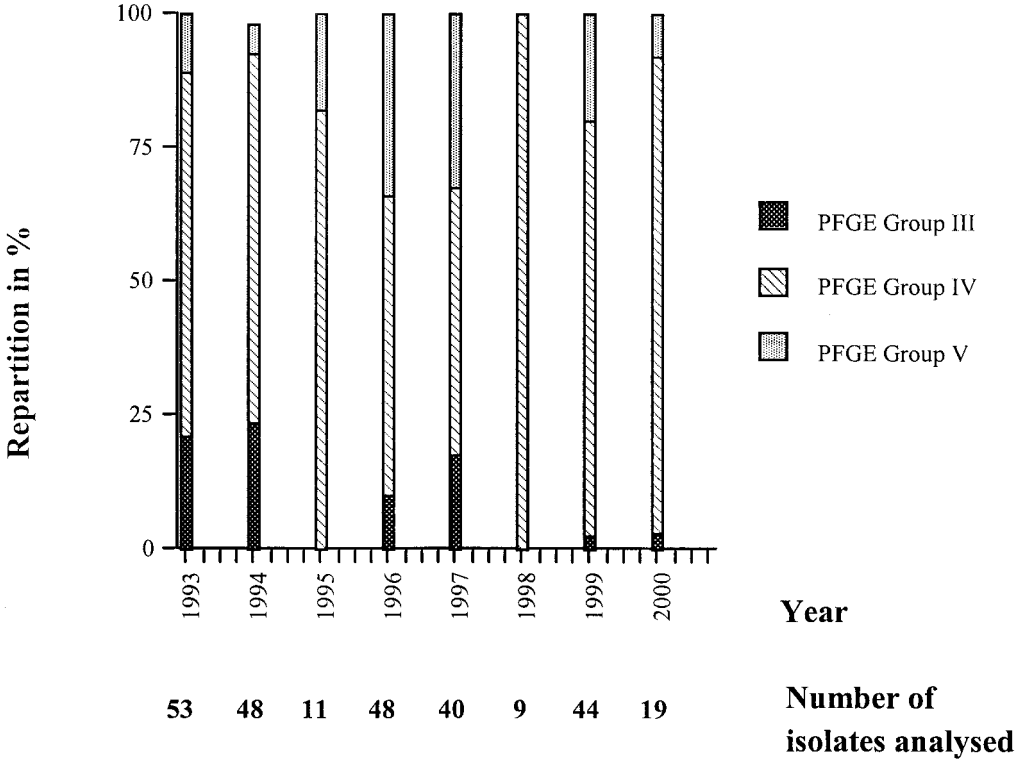

FIG. 4. Distribution of isolates of PFGE groups III, IV, and V according to the year of collection.

pertussis variability as assessed by this technique is limited. The neighbor-joining method of clustering classified the different profiles into five major groups (I to V).

PFGE group I only contains two isolates (18323 and CZ), one collected before generalized vaccination in United States and one collected after generalized vaccination in France. The 18323 strain (the WHO reference strain) was previously clearly shown to be different from all other isolates whether assessed by phenotypic characteristics, genetic characteristics, PT and PRN sequences, or virulence in animal models (3, 27). Since this type of isolate has not spread, whole-cell vaccination presumably induced a protective immunity against it. It could be of interest to determine whether this type of isolate is circulating in countries where vaccination is not used.

Group II contains isolates collected before the introduction of generalized vaccination and one vaccine strain. The isolates in this group, expressing type B or D S1 PT and type 1 PRN, are clearly different from the isolates currently circulating in France. This observation is consistent with those made in The Netherlands, Finland, and the United-States (12, 37, 44).

All isolates collected since 1991 express a type A S1 PT, but they fell into three of the major PFGE groups. Surprisingly, the differentiation of the isolates by PFGE correlates with the PRN type expressed by the isolates. However, the PFGE groups have very different numbers of isolates. Group IV, composed of isolates expressing PRN type 2, is the largest and contains more than half of the isolates collected since 1993 and more than 85% of those collected since 1998. Group III, including the isolates expressing PRN type 1, and group V, including isolates expressing PRN type 3, are much smaller. About 20% of the isolates collected in 1993 to 1994 and only 2% of those collected in 1999 to 2000 are in group III; and 10% of the isolates collected in 1993 to 1994, 35% of those collected in 1996 to 1997, and less than 10% of those collected in 2000 are in group V. The types of isolates circulating in France are similar to those circulating in The Netherlands (44; C. Weber, unpublished data). An increase in the proportion of isolates

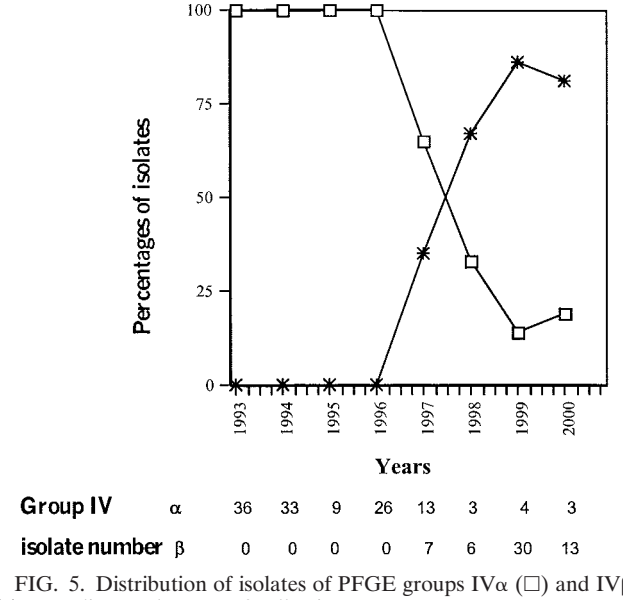

FIG. 5. Distribution of isolates of PFGE groups IVα (□) and IVβ (*) according to the year of collection.

expressing type 3 PRN was observed in both countries in 1996 to 1997, although there were more such isolates in The Netherlands (60%) than in France (35%).

PFGE group IV can be subdivided into two groups, with one group being composed of isolates circulating between 1993 and 1996 and one group being composed of isolates circulating since 1997. This change was not associated with the expression of new PRN or S1 PT types. This confirms that PFGE is more discrimative than typing based on these characteristics for *B. pertussis* isolates. This observation is also consistent with a shift in the circulating *B. pertussis* population every 3 years. The significance of this change is not known but might correspond to the periodicity of pertussis incidence observed in all countries. The 1996 to 1997 period saw epidemics in various countries, and there was at that time in France a change between PFGE type IVα and PFGE type IVβ (Fig. 5). However, this evolution in the *B. pertussis* population does not seem to correspond to major antigenic changes. The French whole-cell vaccine was shown to be very effective (vaccine efficacy, 94%) during the national study in 1993 to 1994 (5). Indeed, patients were mostly nonvaccinated infants contaminated by siblings or parents, and very few vaccinated young children were infected. The surveillance network (RENACOQ) set up by our Ministry of Health following the national study has not observed changes in pertussis epidemiology between 1993 and 1999, although there have been changes in the population of isolates as assessed by PFGE (4). Furthermore, our data correlate with those on the effects of antigenic drift of PT or PRN obtained using either neutralizing antibodies induced by vaccination or animal models (10, 25). There is therefore no evidence that genetic variability necessarily interferes with the ability of the antigen to induce protective immunity; in contrast, the results indicate that a certain variability can be tolerated.

However, drift is observed. Is this drift due to the use of a whole-cell vaccine for more than 30 years, or does it also occur in countries where vaccination is not used? Drift is probably ubiquitous, but this has yet to be demonstrated. Analysis of the *B. pertussis* population in a single geographical area for at least 10 years in regions with and without an extensive vaccination program would help to answer this question. Such an analysis would be valuable, since new acellular vaccines are replacing the traditional whole-cell vaccines in most industrialized countries. These new vaccines are composed of bacterial antigens expressed by strains collected before generalized vaccination and which are therefore different from the proteins expressed by circulating isolates. For these reasons, surveillance of currently circulating isolates using standardized techniques (35) and the development of new typing techniques, more sensitive and faster to perform than PFGE, must continue.

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