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Surveillance of Circulating *Bordetella pertussis* Strains in Europe during 1998 to 2015

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ABSTRACT One reason for increased pertussis incidence is the adaptation of *Bordetella pertussis* to vaccine-induced immunity by modulating its genomic structure. This study, EUpert IV, includes 265 isolates collected from nine European countries during 2012 to 2015 ($n = 265$) and compares the results to previous EUpert I to III studies (1998 to 2009). The analyses included genotyping, serotyping, pulsed-field gel electrophoresis (PFGE), and multilocus variable-number tandem-repeat analysis (MLVA). Genotyping results showed only small variations among the common virulence genes of *B. pertussis*. The frequencies of serotypes Fim2 and Fim3 varied among the four collections. Genomic analyses showed that MLVA type 27 increased to 80% between the periods of 1998 to 2001 and 2012 to 2015. Two PFGE profiles, BpSR3 (29.4%) and BpSR10 (27.2%), constituted more than 50% of the circulating isolates in the present collection. Our study indicates that the European *B. pertussis* population is changing and became more homogenous after the introduction of acellular pertussis vaccines.

KEYWORDS *Bordetella pertussis*, Europe, genotyping, PFGE, MLVA, serotyping

The introduction of whole-cell pertussis vaccines (WCVs) reduced the numbers of reported pertussis cases significantly during the 1950s. Since the mid-1990s, WCVs have been gradually replaced by acellular pertussis vaccines (ACVs) in many European countries. Although the vaccines and vaccination schedules vary, the vaccination coverage is high (>90%) (1, 2). However, pertussis remains endemic, and many outbreaks have occurred during the past 10 years, including in Australia, the United

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TABLE 1 Pertussis vaccines currently used in European countries

Country	Vaccine[a]
Belgium	ACV3
Denmark	ACV1
Finland	ACV3
France	ACV2, ACV3, or ACV5
Italy	ACV3
Norway	ACV3
Sweden	ACV2 or ACV3
The Netherlands	ACV3
United Kingdom	ACV3 or ACV5

[a]Vaccine compositions: ACV1, PT; ACV2, PT and FHA; ACV3, PT, FHA, and PRN; ACV5, PT, FHA, PRN, Fim2, and Fim3.

Kingdom, and the United States (3–5). One explanation is the adaptation of *Bordetella pertussis* to vaccine-induced immunity. Therefore, monitoring of *B. pertussis* populations is essential for evaluating the impact of bacterial changes on vaccine efficacy.

To investigate the changes in *B. pertussis* populations in Europe with different vaccination histories, vaccines, and schedules and to evaluate the effect of the switch from WCVs to ACVs, the "European Research Programme for Improved Pertussis Strain Characterization and Surveillance" (EUpertstrain) was established (6). So far, three panels of *B. pertussis* isolates (named EUpert I to III) have been collected. EUpert I (1998 to 2001) included 102 strains from five countries, EUpert II (2004 to 2005) included 154 strains from eight countries, and EUpert III (2007 to 2009) included 140 strains from seven countries. The results from these studies were published earlier, including multilocus antigen sequence typing (MAST), fimbrial serotyping, pulsed-field gel electrophoresis (PFGE) profiling, and multilocus variable-number tandem-repeat analysis (MLVA) (7, 8). The results showed that specific allelic types of the genes coding for pertactin (*prn*), *prn2*, pertussis toxin (*ptxA*), *ptxA1*, and the pertussis toxin promoter (*ptxP*), *ptxP3*, and of the fimbrial antigen (Fim), Fim3, were dominant. For *fim3* genotyping, both *fim3-1* and *fim3-2* have been common in all studies. In addition, the dominant PFGE profiles BpSR3, BpSR5, and BpSR10 increased (BpSR3, from 0% to 22%; BpSR5, from 6% to 10%; and BpSR10, from 10% to 20%) in the EUpert III collection, whereas BpSR11, the most prevalent profile in EUpert I and II collections started to decrease (from 26% to 13%). With MLVA types (MT), MT27 has been dominant throughout all studies.

In this study, a fourth panel (EUpert IV) of 265 *B. pertussis* clinical isolates was collected from nine European countries during 2012 to 2015. All study countries are using ACVs (Table 1). Finland, France, the Netherlands, and Sweden also participated in all of the previous three studies. The selection criteria of clinical isolates have remained unchanged for all collections. The typing methods used were as described above. This study provides a unique opportunity to systemically evaluate the changes in the *B. pertussis* bacterial populations over the last 15 years in European countries with different vaccination strategies.

MATERIALS AND METHODS

Isolates. Two hundred sixty-five *B. pertussis* isolates were collected during 2012 to 2015. Most of the isolates were collected during 2013 to 2014 ($n = 236$). The isolates were collected from nine European countries, and the target number of isolates for each country to submit was set at $n = 30$. However, in Denmark, Finland, and Italy, the total numbers of isolates were less than 30 during 2013 to 2014. The following numbers of isolates were received: Belgium, $n = 38$; Denmark, $n = 27$; Finland, $n = 28$; France, $n = 29$; Italy, $n = 20$; the Netherlands, $n = 32$; Norway, $n = 32$; Sweden, $n = 29$; and the United Kingdom, $n = 30$. For Italy, all isolates were collected from the Rome area as no other isolates were available.

Selection criteria and collection of patient data. The selection criteria for the EUpert IV study were the same as those used in the previous EUpert I to III studies. (i) *B. pertussis* isolates should be selected from different geographical regions and be epidemiologically unrelated. (ii) Equal numbers of isolates from vaccinated ($n = 15$) and unvaccinated individuals ($n = 15$) should be collected. The selection of isolates should be made from individuals younger than 5 years of age where possible. (iii) For those countries with large numbers of isolates in their collections, the isolates should be randomly selected in addition to the above criteria.

The data that were collected included the original code of the isolate, the country, the date of collection, and the city, and the characteristics of patients from whom *B. pertussis* was isolated. The patient characteristics included sex, age, vaccination status, the number of doses received, and hospitalization status (8).

Culture. Isolates were first cultured in local laboratories and were then shipped in frozen storage tubes to University of Turku, Finland. All isolates were cultured on Regan-Lowe medium (without cephalexin) at 35°C for 48 h.

MAST. Polymorphisms in the genes encoding proteins included in the current ACVs (*ptxA*, *prn*, and *fim3*) and the pertussis toxin promoter (*ptxP*) were analyzed as described previously (7, 9–11). Bacterial suspensions in deionized H_2O (ultrapure) were used as the templates. In brief, the bacterial growth harvested (10-μl loop) from the culture plate was suspended in 300 μl of deionized H_2O (ultrapure), vortexed, and then heated at 95°C for 30 min. This template was used in the PCR assays. Reference strains with known alleles were included as positive controls in each run of each assay. Different alleles of the genes mentioned above were determined with size comparison or by sequencing the specific targets in the gene.

Serotyping. Fimbrial serotyping (Fim2 or Fim3) was performed with specific enzyme-linked immunosorbent assays (ELISAs) as described previously (12). Briefly, specific monoclonal antibodies against Fim2 or Fim3 were used to detect the serotype of each isolate. Reference strains S1 (Fim2) and S3 (Fim3) and monoclonal antibodies ([MAbs] Fim2, 06/124; Fim3, 06/128) were obtained from the National Institute for Biological Standards and Control (NIBSC), Potter's Bar, England (13).

MLVA. For MLVA, the variable numbers of tandem repeats in six loci (VNTR1, VNTR3a, VNTR3b, VNTR4, VNTR5, and VNTR6) were defined as described previously and named according to MLVA profiles described by Schouls et al. (14) and Litt et al. (15). The results were expressed as the MLVA type (MT), e.g., MT18, MT27, etc. Reference strains with known MTs were included as positive controls in each run. New MTs were submitted to the MLVA database (http://www.mlva.net/bpertussis/default.asp) administrator for nomenclature.

PFGE. All isolates were analyzed according to the standardized recommendations for the typing of *B. pertussis* with minor modifications using XbaI (R0145S; New England BioLabs, USA) as a restriction enzyme (8, 16, 17). PFGE profiles were defined as individual profiles with distinct DNA band patterns (at least one band difference) and were designated BpSR1, BpSR2, BpSR3, etc. (18, 19). Isolates with new profiles were designated EU4_1, EU4_2, etc. according to the study name. A cluster analysis was performed with the unweighted pair group method with average linkages (UPGMA), with 1% band tolerance and 1% optimization settings. The same band tolerance and optimization settings were used in the previous EUpert I to III studies (8). For cluster group analysis, UPGMA with 2% band tolerance and 1.5% optimization settings was used as in the previous EUpertstrain studies. Strains 18323 (PFGE cluster I), Tohama I (PFGE cluster II), Bp134 (PFGE cluster III), B902 (PFGE cluster IVα), FIN6 (PFGE cluster IVβ), FIN12 (PFGE cluster IVg), FR287 (PFGE cluster V), and FINR21 (PFGE cluster VII) were included in the dendrogram as reference strains (8, 17).

PRN deficiency. Pertactin (PRN) deficiency was measured by a specific ELISA as described earlier (20). In short, whole bacterial lysates were used as coating antigens. The production of PRN was detected with specific MAbs, kindly provided by the National Institute for Public Health and the Environment (RIVM), the Netherlands. French strain FR3693 (negative for PRN) and purified PRN were used as the controls.

Vaccination status. During the period 2012 to 2015, 130 (49.1%) of the infected individuals were vaccinated and 135 (50.9%) were unvaccinated.

Statistical analysis. BioNumerics software version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) was used to calculate the PFGE cluster analysis. Chi-square tests for *P* values between vaccinated and unvaccinated subjects were calculated using GraphPad Prism version 4.0 (San Diego, CA, USA). Two-tailed *P* values of <0.05 were considered significant. The Simpson diversity index (SDI) was calculated based on the formula $D = 1 - \sum n(n-1)/N(N-1)$, where n indicates the number of individual profiles and N is the number of all profiles.

RESULTS

A summary of EUpert IV study results and isolate characteristics are presented in Table 2. Below, these results are described and compared to previous EUpert I-III studies.

***ptxA* alleles.** The strains used for the production of ACVs contain *ptxA2* or *ptxA4* alleles as described previously (21). In this study, all 265 isolates harbored the *ptxA1* allele (Table 2). All isolates included in EUpert I to III studies also harbored the *ptxA1* allele (7).

***ptxP* alleles.** In this study, 253 isolates (95.5%) carried the *ptxP3* allele and 12 (4.5%) carried the *ptxP1* allele. In the EUpert I study during 1998 to 2001, the frequencies of the *ptxP1* and *ptxP3* alleles were similar (39% versus 50%, respectively). Since then, *ptxP3* has become clearly dominant, and from the EUpert III study onwards, the frequency has been >95% (7).

***prn* alleles.** The strains used for the production of ACVs contain *prn1* or *prn7* (21). In this study, four *prn* alleles were detected: *prn1*, *prn2*, *prn3*, and *prn9*. For two isolates, the allele could not be defined because of partial or complete deletion of the *prn* gene. The most common allele was *prn2*, detected in 255 (96.2%) isolates. *prn1* and *prn9* were both

TABLE 2 Overview of the isolate characteristics in EUpert IV study countries

Characteristic	No. of isolates									
	Belgium	Denmark	Finland	France	Italy	Netherlands	Norway	Sweden	United Kingdom	Total
No. of strains	38	27	28	29	20	32	32	29	30	265
Vaccination status										
Vaccinated	14	11	16	15	2	24	24	11	13	130
Unvaccinated/unknown	24	16	12	14	18	8	8	18	17	135
ptxA genotype										
ptxA1	38	27	28	29	20	32	32	29	30	265
ptxP genotype										
ptxP1	1	1	2	3	1	0	1	2	1	12
ptxP3	37	26	26	26	19	32	31	27	29	253
Serotype										
Fim2	17	22	22	2	10	15	16	5	11	120
Fim3	21	5	6	27	9	15	15	24	19	141
Fim2/3	0	0	0	0	1	2	0	0	0	3
Negative	0	0	0	0	0	0	1	0	0	1
fim3 allele										
fim3-1	30	24	22	14	15	24	27	17	17	190
fim3-2	8	3	3	15	5	8	5	12	13	72
fim3-3	0	0	1	0	0	0	0	0	0	1
fim3-4 (*1b*)	0	0	2	0	0	0	0	0	0	2
prn genotype										
prn1	1	0	2	0	0	0	0	0	0	3
prn2	35	26	25	27	20	32	31	29	30	255
prn3	0	0	1	1	0	0	0	0	0	2
prn9	2	1	0	1	0	0	1	0	0	5
PFGE profile										
BpSR3	12	18	16	3	6	2	14	0	7	78
BpSR5	2	0	1	2	0	2	0	3	4	14
BpSR10	16	2	2	7	3	16	8	11	7	72
BpSR11	3	2	2	9	2	7	3	7	4	39
BpSR12	2	1	0	2	1	0	1	1	2	10
Others	3	4	7	6	8	5	6	7	6	52
MLVA type										
MT27	30	13	24	23	18	29	26	24	27	214
Others	8	14	4	6	2	3	6	5	3	51

found in three isolates (1.1%) and *prn3* in two isolates (0.8%). The *prn2* allele has been dominant (>75% of tested isolates for *prn*) in the previous EUpert I to III studies (7).

Fim serotype and *fim3* alleles. The Fim3 serotype predominated (>67%) in earlier EUpert I to III studies (7). In the current EUpert IV study, 141 (53.2%) isolates harbored Fim3, 120 (45.3%) Fim2, three (1.1%) had Fim2/3, and one (0.4%) isolate was deficient for Fim2 and Fim3 (Fig. 1). In Denmark (22/27 [81.5%]) and Finland (22/28 [78.6%]), Fim2 was dominant, whereas in France (27/29 [93.1%]), Sweden (24/29 [82.8%]), and the United Kingdom (19/30 [63.3%]), Fim3 was prevalent. In other study countries, the distributions between the two serotypes were close to equal. We also compared the serotypes with the vaccination statuses of the subjects. No correlation between serotypes and vaccination status was found ($P = 0.709$).

For *fim3* alleles, 190 (71.7%) isolates carried *fim3-1*, 72 (27.2%) carried *fim3-2*, two (0.8%) carried *fim3-4*, and one (0.4%) *fim3-3*. In France, Sweden, and the United Kingdom, the distributions between *fim3-1* and *fim3-2* were equivalent, whereas in other countries, *fim3-1* was dominant.

MLVA. MT27 was dominant in the EUpert I and III studies (MLVA not performed in EUpert II). In the EUpert IV, 20 MTs (20/265 [7.5%]) were identified. In the EUpert I study,

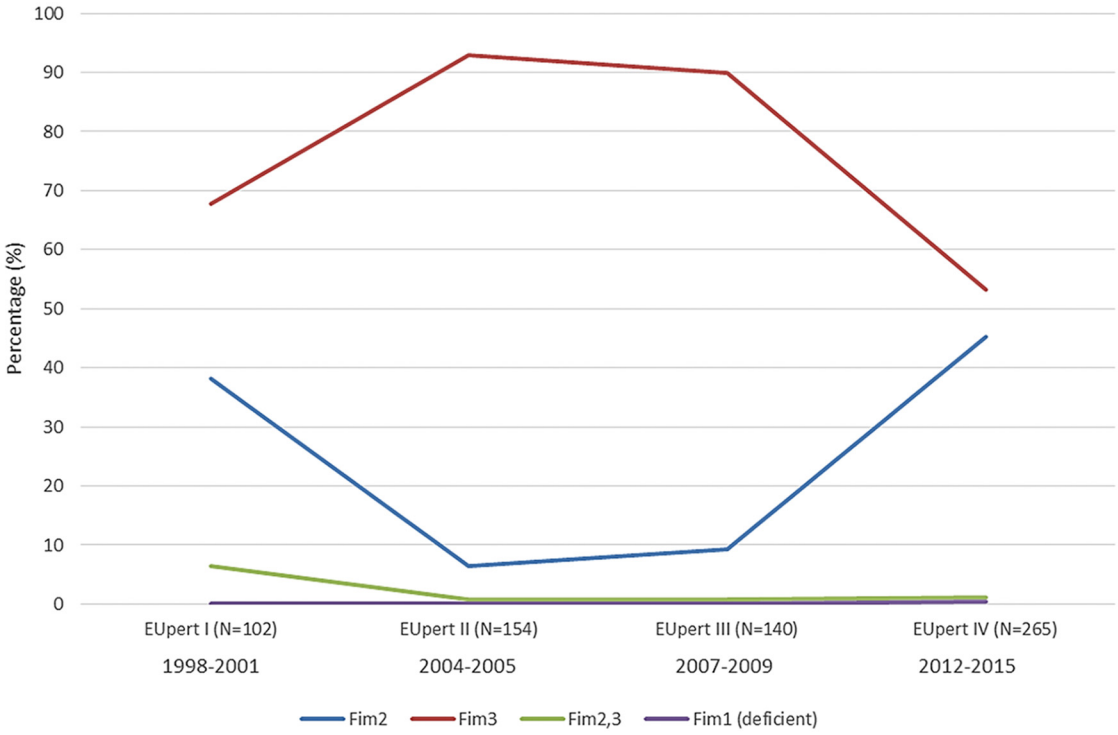

FIG 1 Frequencies of fimbrial serotypes among the EUpert I to IV studies.

18 MTs (17.6%) among 102 isolates were identified, and in EUpert III, 15 MTs (10.7%) among the 140 isolates were identified (Table 3). In the current study, 214 (80.8%) isolates harbored MT27. The second most common type with 18 isolates (6.8%) was MT28 and third most common with seven (2.6%) isolates was MT18. In addition, 15 other MTs (12, 25, 29, 32, 33, 36, 38, 55, 60, 77, 95, 114, 158, 312, and 324) were found among 26 (9.8%) isolates, and two new MTs were detected (MT335 and MT336). In all countries except Denmark, MT27 was dominant. In Denmark, 48.2% carried MT27, whereas other types, such as MT28 (29.6%), constituted more than half of the circulating isolates. However, MT27 and MT28 are close to each other, as there is only one difference in the numbers of repeats of the variable number of tandem repeat (VNTR) 6. However, MT18 has seven repeats in VNTR3-2, whereas MT27 has none. Otherwise, the structures are identical.

PFGE profiles and association to Fim serotype and genotype. Forty-two PFGE profiles were identified in the EUpert IV study. The numbers of different profiles identified in earlier studies are as follows: EUpert I, 33 of 102 (32.4%), in EUpert II, 36 of 154 (23.4%), and in EUpert III, 29 of 140 (20.7%) isolates (Table 3). Throughout the studies, the five most common PFGE profiles have been BpSR3, BpSR5, BpSR10, BpSR11, and BpSR12. In the previous EUpert I and II studies, BpSR11 was the dominant profile, and the numbers of isolates with profiles other than the five most common profiles

TABLE 3 Percentages of PFGE profiles and MLVA types identified in the four study periods[a]

Method	% (no. identified/total no. of isolates) of isolates with types identified			
	1998–2001	2004–2005	2007–2009	2012–2015
PFGE	32.4 (33/102)	23.4 (36/154)	18.8 (29/140)	15.8 (42/265)
MLVA	17.6 (18/102)	NA[b]	10.7 (15/140)	7.5 (20/265)

[a]The Simpson diversity indices calculated for each study period were 0.91, 0.88, 0.88, and 0.83 for PFGE and 0.70, 0.47, and 0.34 for MLVA.
[b]NA, not available.

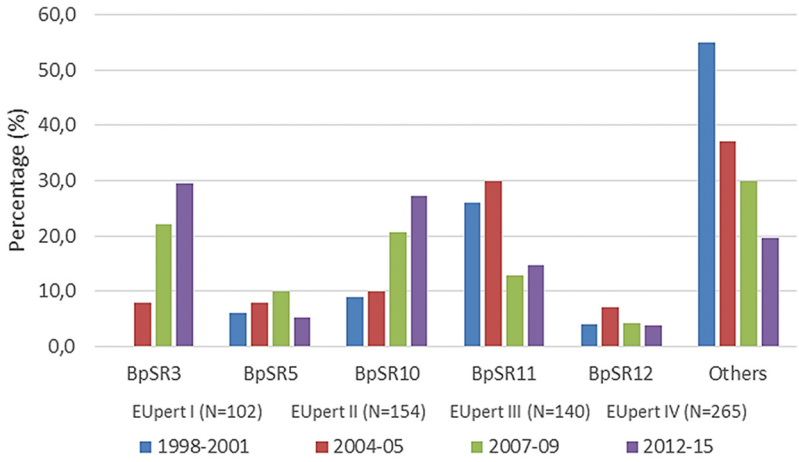

FIG 2 Distributions of PFGE profiles among the EUpert I to IV studies (1998 to 2015).

were high (Fig. 2). However, in the EUpert III study, the frequencies of BpSR3 and BpSR10 started to increase, whereas the frequencies of BpSR11 and other profiles decreased. In the current study, the frequencies of BpSR3 (78/265 [29.4%]) and BpSR10 (72/265 [27.2%]) further increased, and the numbers of isolates with BpSR11 (39/265 [14.7%]) and other (19.6%) profiles decreased or remained the same as previously reported (7, 8). The most common profile, BpSR3, belongs to cluster IV, BpSR10 to cluster IVα, BpSR11 to cluster IVβ, BpSR5 to cluster IV, and BpSR12 to cluster IVg. From the other PFGE profiles, 21 new profiles were detected (see Fig. 4). These new profiles belonged mainly to cluster VII, but were also found from clusters IV, IVα, IVβ, IVg, and III. When we analyzed the country-based data, BpSR3 was dominant in Denmark (18/27 [66.7%]), Finland (16/28 [57.1%]), and Norway (14/32 [43.8%]). However, no BpSR3 profile was found from Sweden. BpSR10 was dominant in the Netherlands (14/32 [43.8%]) and Sweden (11/29 [37.9%]). In Belgium, the frequencies of both BpSR3 (12/38 [31.6%]) and BpSR10 (14/38 [36.8%]) were high. Figure 3 shows the distributions of the main PFGE profiles by country, and Fig. 4 shows all 42 PFGE profiles identified in the EUpert IV study.

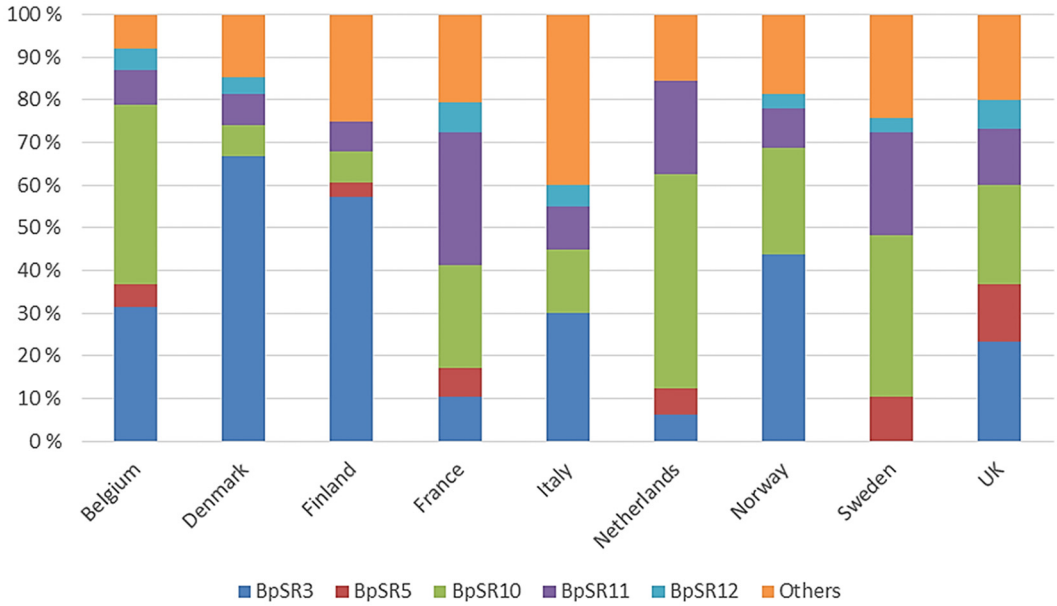

FIG 3 Distributions of PFGE profiles among the EUpert IV study countries (2012 to 2015).

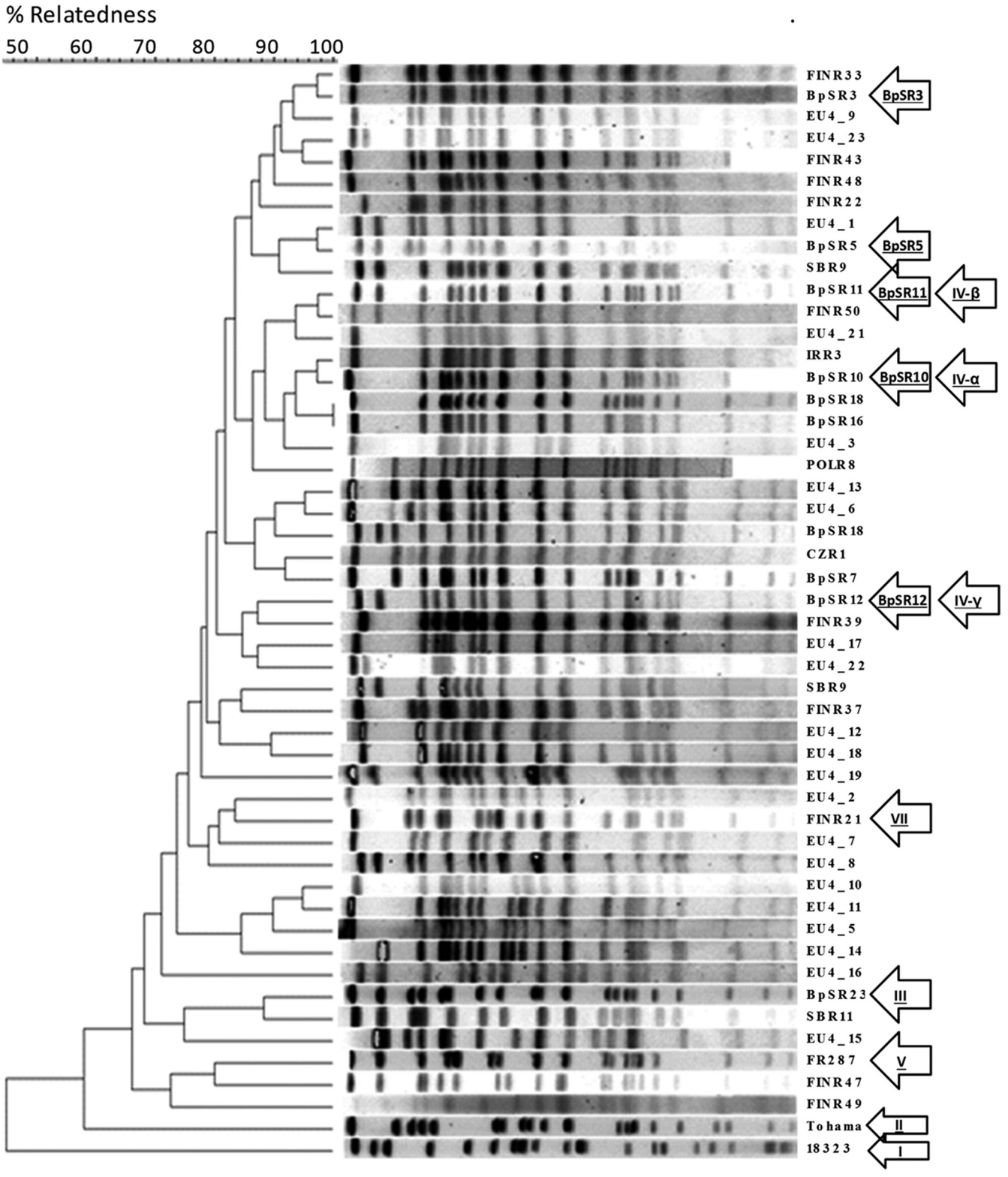

FIG 4 Dendrogram of PFGE profiles identified in the EUpert IV study.

When we compared the two most common PFGE profiles, BpSR3 and BpSR10, found in the EUpert IV study with Fim2 and Fim3 serotypes, we observed that most of the isolates with BpSR3 profiles were the Fim2 (66/78 [84.6%]) serotype, whereas BpSR10 represented both Fim3 (37/64 [57.8%]) and Fim2 (25/64 [39.1%]). However, in previous EUpert I to III studies, the isolates belonging to profile BpSR3 were associated with the Fim3 serotype (range, 74.2 to 100% of the isolates), whereas BpSR10 was linked to Fim3 (96.6 to 100%). With PFGE profiles BpSR5, BpSR11, and BpSR12, 59 of 60 isolates carried the Fim3 serotype in the current study. Interestingly, with the *fim3* genotype, all BpSR3 and BpSR10 isolates carried the *fim3-1* genotype and almost all BpSR5, BpSR11, and BpSR12 isolates carried *fim3-2* (60/63 [95.2%]).

Combined analyses of MAST, MLVA, and PFGE profiles and their associations with vaccination statuses of study subjects. The most common profile among the study isolates was *ptxP3-prn2*-Fim2-MT27-BpSR3, with 50 (18.9%) of 265 isolates. Second was *ptxP3-prn2*-Fim3-MT27-BpSR10, with 37 (13.9%) isolates. Third was *ptxP3-prn2*-Fim3-MT27-BpSR11, with 34 (12.8%) isolates and fourth, *ptxP3-prn2*-Fim2-MT27-BpSR10, with 20 (7.5%) isolates.

We compared these main profiles with the vaccination statuses of the subjects. We did not find any significant difference (all $P > 0.1$) between vaccinated and unvaccinated individuals, e.g., the most common profile, *ptxP3-prn2*-Fim2-MT27-BpSR3, was found in isolates from 30 vaccinated and 20 unvaccinated subjects ($P = 0.1184$).

Pertactin deficiency. Of the 265 isolates included in the EUpert IV collection, 66 (24.9%) were found to be PRN negative, whereas the corresponding frequency was only 6.4% in the EUpert III collection (data not shown).

DISCUSSION

In this study, we analyzed 265 *B. pertussis* isolates collected from nine European countries during the period 2012 to 2015 and compared the results to those from three previous EUpert studies starting in late 1990s. Although the numbers of participating countries in each study varied, Finland, France, the Netherlands, and Sweden have participated in all four studies. Our study showed that the dominant alleles *ptxA1*, *ptxP3*, and *prn2* in circulating strains are different from those used for the production of ACVs in European countries (for Denmark, data not available) (21). However, country-based genetic differences of *B. pertussis* isolates were identified especially with PFGE analyses. The serotype has also changed from Fim3 to Fim2 in several countries, although ACVs used in many of these countries do not contain any fimbrial antigens.

ACVs contain purified components from strains carrying *ptxA2* or *ptxA4*, *ptxP1*, and *prn1* or *prn7* alleles (excluding Denmark) (21, 22). In this study, we found that almost all circulating isolates harbored different alleles (*ptxA1*, *ptxP3*, and *prn2*) (Table 1). These alleles were dominant in the previous EUpert I to III studies, suggesting that circulating *B. pertussis* organisms with these alleles may have advantages in ACV-vaccinated populations. This may have an effect on the vaccine effectiveness (21). Similar findings with the dominant genotypes have been reported in Australia, Japan, and the United States (23–25).

In Europe, many countries use ACVs without the Fim2/Fim3 antigens (Table 1). We found that the frequencies of Fim2 isolates have markedly increased in several countries compared to those in previous EUpert II and III studies (Fig. 1 and Table 2). Both Denmark and Finland had mostly Fim2 isolates circulating, whereas Fim3 was continuously prevalent in France, Sweden, and the United Kingdom (Table 2). In Denmark, a monocomponent pertussis toxin protein (PT) vaccine has been used for more than 15 years (26). In Finland, ACV was introduced in 2005, and the vaccines used from 2005 to 2009 contained only PT and filamentous hemagglutinin (FHA). Therefore, the change in frequency from Fim3 to Fim2 is most likely caused by natural infection. It remains to be shown why high frequencies of Fim2 isolates are only observed in certain countries. In Japan, where the Fim3 allele has been highly dominating since the beginning of the 21st century (25), two of the four ACVs in use include Fim2, which may partly explain why Fim3 is dominant in this country. Similar to that in Japan, in France and the United Kingdom where ACVs containing Fim2/3 are in use (Table 1), a prevalent serotype of

Fim3 was observed. When we compared these findings to the previous EUpert III collection, seven countries were included in both, and in Denmark and Finland, almost all isolates were Fim3 in the EUpert III collection. In addition, similar but less dramatic increases of Fim2 isolates were noticed in Norway and in the United Kingdom (although Fim3 is still prevalent). This indicates that the numbers are not biased by country changes in different collections, yet they reflect actual changes in the circulating strains.

In addition to the serotype of the isolates (Fim3), the *fim3-1* allele became prevalent. However, in France, Sweden, and the United Kingdom where Fim3 isolates were prevalent, both genotypes *fim3-1* and *fim3-2* were common. It is known that the strains used for the production of ACVs harbor *fim3-1*. As stated above, in France and the United Kingdom, ACVs containing Fim2/3 are in use, which could partly explain why strains with *fim3-2* were circulating. In addition, natural infections caused by *B. pertussis* with different genotypes of *fim3-1* and *fim3-2* can also have a selective pressure on circulating isolates. *fim3-1* has also been dominant in the United States during recent outbreaks (27). Since the expression of Fim2 or Fim3 of *B. pertussis* might be different *in vivo* and *in vitro* (14, 23), further studies are needed to show whether the expression of Fim3 or Fim2 is related to certain alleles of *fim3*, *fim2*, or both. However, according to our results, it seems that the *fim3-1* allele is frequently found with the Fim2 serotype in Denmark and Finland. In addition, in the Netherlands during the period 1995 to 2008, 99% of the *fim3-2* strains expressed Fim3 (28).

We noticed that MT27 is becoming more dominant in Europe, whereas the numbers of other MTs and SDIs, which show the probability to randomly pick a different isolate from the whole strain population, (Table 3) clearly decreased compared to those from previous EUpert I and III studies. However, this is not the case in Denmark, the only country in these studies where a monocomponent PT vaccine has been used (26). In contrast to other countries, more than 50% of the Danish isolates did not carry MT27. This finding may indicate that the effects of population immunity provided by monocomponent and multicomponent vaccines on bacterial populations may differ. In the current study, isolates with MT27 were found equally from vaccinated ($n = 106$) and unvaccinated ($n = 108$) individuals. However, the second most common profile, MT18, was found in 12 unvaccinated and in 6 vaccinated individuals, whereas the third most common profile, MT28, was equal between vaccinated ($n = 3$) and unvaccinated ($n = 4$) individuals.

The most common PFGE profiles observed were BpSR3 and BpSR10, showing an increase in Europe. In contrast, the numbers of other profiles are decreasing (Fig. 2). Interestingly, the most common profile, BpSR3, was not found in Sweden, whereas other profiles were commonly found among the Swedish isolates, suggesting a shift in the *B. pertussis* population in this country. When we compared the present findings to earlier results, PFGE profiles BpSR11 and BpSR10 were dominant in Sweden during the EUpert II and III collections (8). Pertussis vaccination was stopped in Sweden in 1979 and was reintroduced in 1996 (29). Therefore, the population immunity may be different compared to that in other countries in which vaccinations have been continuously used. Similar to that in Sweden, PFGE profiles BpSR11 and BpSR10 were common in France and the Netherlands. However, in other Nordic countries, Denmark, Finland, and Norway, BpSR3 was the most prevalent PFGE profile. Denmark and Finland have high similarities within the strains, excluding MLVA results (Table 2). In addition, no outbreaks have been reported in these two countries 10 years prior to 2015 (a country-wide epidemic occurred in Denmark in 2016) (30, 31). In the Netherlands, there has been a shift from BpSR3 to BpSR10, which is currently dominating. This finding is interesting, as a similar change was not detected, e.g., in France or Sweden. However, in Belgium, both BpSR3 and BpSR10 were prevalent. This may indicate transmission of the isolates from neighboring countries or country-specific differences in ACVs or in vaccination policies. In addition, outbreaks prior to 2015 most likely affected the circulating isolates in the EUpert IV collection. An association between PFGE profiles and fimbrial serotype revealed that isolates with BpSR3 and BpSR10 were no longer only associated with Fim3 but were moving toward the Fim2 serotype. We also

compared the vaccination status and the three most common PFGE profiles, BpSR10, BpSR3, and BpSR11. These profiles were found almost equally among vaccinated and unvaccinated individuals. Still, we noticed that the numbers of profiles were decreasing and BpSR3 and BpSR10 were clearly dominant. In addition, the SDI was decreasing for PFGE (Table 3), which also indicates that the strains are more similar than they were previously.

Although we did not find any significant differences in the *B. pertussis* strains isolated from vaccinated and unvaccinated individuals, it does not signify that vaccination has not guided the strains to evolve more homogenously. However, it seems that PFGE and serotyping have the most discriminating power in this study, whereas MLVA is losing its power as shown by the SDI. Therefore, the use of whole-genome sequencing (WGS) should be considered to have more insight on the strains. Indeed, one WGS study from the United Kingdom showed that mutations in the ACV antigen genes have significantly increased after the introduction of ACVs, but variations in other surface antigen genes are minor (3). Another recent WGS study from Australia identified five single nucleotide polymorphisms which were common in the epidemic isolates and differentiated them from preepidemic isolates, stressing the role of WGS in studying *B. pertussis* (24). However, little is known about the impacts of all mutations in the *B. pertussis* genome.

The number of PRN-deficient isolates is alarming. In this study, we found that approximately 25% of the study strains did not produce this antigen. How this will affect vaccine efficacy and the opinions toward pertussis vaccination remains to be seen. A detailed description of PRN-deficient isolates and the mechanisms behind the deficiency observed in this study is currently under consideration for publication elsewhere.

The strengths of this study are that (i) we have a serial collection of isolates during the last 15 years, (ii) the selection criteria have been the same for all collections, (iii) the isolates have been isolated from infants, young children, and adults (range, 0.01 to 62.30 years), (iv) the place of origin is known for all isolates and shows that they were not collected from local outbreaks, and (v) all analyses for the EUpert IV panel strains were done by one laboratory. The limitations include that (i) the numbers of isolates from each country were relatively low (range, 20 to 38). However, they do comprise almost all available isolates in many countries such as Denmark, Finland, and Italy, where the use of culture is diminishing. (ii) The epidemiological pressure of pertussis varies in European countries, which could have an effect on the spread of new emerging strains. (iii) Even though the total numbers of vaccinated and unvaccinated individuals included in this study were comparable (Table 2), the difference in numbers between vaccinated and unvaccinated subjects in individual countries existed. To avoid such an effect, a study with a large number of isolates and equal numbers of those from vaccinated and unvaccinated individuals in participating countries is needed.

In conclusion, common MLVA types and PFGE profiles were identified in *B. pertussis* populations circulating in European countries with different vaccination programs. The prevalent MT types and PFGE profiles contain the *ptxA1-prn2-ptxP3* alleles. However, in contrast to the high prevalence (78.9 to 90.6%) of MT27 in most European countries using two- and three-components ACVs, the prevalence in Denmark (PT monocomponent ACV) represented only 48.1% of the circulating strains, suggesting a difference in the selection pressures induced between these ACVs. In addition, the shift in serotype from Fim3 and Fim2 is ongoing in several countries. This study suggests that the *B. pertussis* population is moving toward homogeneity in European countries. To obtain a deeper insight of the *B. pertussis* strain diversity in Europe, whole-genome sequencing could be applied for the surveillance of *B. pertussis*.

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We declare no competing interests.

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