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Surveillance of circulating *Bordetella pertussis* strains in Europe during 1998-2015

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Running title: *Bordetella pertussis* isolates in Europe

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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-III studies (1998-2009). The analyses included genotyping, serotyping and pulsed-field gel electrophoresis (PFGE) and multi-locus variable-number tandem repeat analysis (MLVA). Genotyping results showed only small variation among the common virulence genes of *B. pertussis*. 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 1998-2001 and 2012-2015. 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 more homogenous after the introduction of acellular pertussis vaccines.

Introduction of whole-cell pertussis vaccines (WCVs) dropped the number 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 vaccines and vaccination schedules vary, vaccination coverage is high (>90%) (1, http://vaccine-schedule.ecdc.europa.eu/pages/scheduler.aspx. Accessed 04/04, 2017). However, pertussis remains endemic, and many outbreaks have occurred during the past ten years including Australia, the UK and the USA (2-4). One explanation is 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 changes in *B. pertussis* populations in Europe with different vaccination history, vaccines and schedules and to evaluate the effect of switch from whole cell pertussis vaccine (WCV) to acellular vaccines (ACV), "European Research Programme for Improved Pertussis Strain Characterization and Surveillance" (EUpertstrain) was established (5). So far, three panels of *B. pertussis* isolates (named as EUpert I-III) have been collected. EUpert I (1998-2001) included 102 strains from five countries, EUpert II (2004-2005) included 154 strains from eight countries and EUpert III (2007-2009) included 140 strains from seven countries. Results from these studies have been published earlier, including multi-locus antigen sequence typing (MAST), fimbrial serotyping, pulsed-field gel electrophoresis (PFGE) profiling and multi-locus variable-number tandem repeat analysis (MLVA) (6, 7). 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, 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 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 2012-2015. 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.

MATERIAL AND METHODS

Isolates

265 *B. pertussis* isolates were collected during 2012-2015. Most of the isolates were collected during 2013-2014 (N = 236). 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 number of isolates was less than 30 during 2013-2014. The following number 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.

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123 **Selection criteria and collection of patient data**

124 The selection criteria for EUpert IV study were the same as those used in the previous EUpert
125 I-III studies:

126 1. *B. pertussis* isolates should be selected from different geographical regions and be
127 epidemiologically unrelated
128 2. An equal number of isolates from vaccinated (N=15) and unvaccinated individuals
129 (N=15) should be collected. Selection of isolates should be made from individuals
130 younger than 5 years of age where possible.
131 3. For those countries with large numbers of isolates in their collections, isolates should
132 be randomly selected in addition to the above criteria.

133

134 Data collection included original code of isolate, country, date of collection, city and
135 characteristics of patients from whom *B. pertussis* was isolated. The patient characteristics
136 included gender, age, vaccination status, number of doses received and hospitalization status
137 (7).

138

139 **Culture**

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

143

144 **MAST**

145 Polymorphisms in the genes encoding proteins included in the current ACVs (*ptxA*, *prn* and
146 *fim3*) and the pertussis toxin promoter (*ptxP*) were analyzed as described previously (8-11).
147 Bacterial suspension in deionized H_2O (Ultrapure) was used as a template. In brief, bacterial
148 growth harvested (10 µl loop) from culture plate was suspended in 300µl of deionized H_2O
149 (Ultrapure), vortexed and then heated at + 95°C for 30 minutes. This template was used in the
150 polymerase chain reaction (PCR) assays. Reference strains with known alleles were included
151 as positive controls in each run of each assay. Different alleles of genes mentioned above
152 were determined with size comparison or by sequencing the specific targets in the gene.

153

154 **Serotyping**

155 Fimbrial serotyping (Fim2 or Fim3) was done with specific ELISA as described previously
156 (11). Shortly, specific monoclonal antibodies against Fim2 or Fim3 were used to detect the
157 serotype of each isolate. Reference strains S1 (Fim2) and S3 (Fim3) and monoclonal
158 antibodies [06/124 (Fim2) and 06/128 (Fim3)] (mAbs) were obtained from the National
159 Institute for Biological Standards and Control (NIBSC), Potter´s Bar, England (12).

160

161 **MLVA**

162 For MLVA, the variable number of tandem repeats in six loci (VNTR1, VNTR3a, VNTR3b,
163 VNTR4, VNTR5 and VNTR6) was defined as described previously and named according to
164 MLVA profiles described by Schouls et al (13, 14). Results were expressed as MLVA type
165 (MT) e.g. MT18, MT27 etc. Reference strains with known MT were included as positive

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166 controls in each run. New MTs were submitted for MLVA database
167 (http://www.mlva.net/bpertussis/default.asp) administrator for nomenclature.

168

169 **PFGE**

170 All isolates were analyzed according to the standardized recommendations for typing of *B.*
171 *pertussis* with minor modifications using XbaI (#R0145S, New England Biolabs, the USA) as
172 a restriction enzyme (7, 15, 16). PFGE profiles were defined as individual profiles with
173 distinct DNA band patterns (at least one band difference) and were designated as BpSR1,
174 BpSR2, BpSR3, etc. (17, 18). Isolates with new profiles were designated as EU4_1, EU4_2
175 etc. according to the study name. A cluster analysis was performed with the unweighted-pair
176 group method with arithmetric mean (UPGMA) with 1% band tolerance and 1% optimization
177 settings. The same band tolerance and optimization settings were used in the previous EUpert
178 I-III studies (7). For cluster group analysis, UPGMA with 2% band tolerance and 1.5%
179 optimization settings was used as in the previous EUpertstrain studies. Strains 18323 (PFGE
180 cluster I), Tohama I (PFGE cluster II), Bp134 (PFGE cluster III), B902 (PFGE cluster IVα),
181 FIN6 (PFGE cluster IVβ), FIN12 (PFGE cluster IVg), FR287 (PFGE cluster V) and FINR21
182 (PFGE cluster VII) were included in the dendrogram as reference strains (7, 16).

183

184 **Pertactin (PRN) deficiency**

185 Pertactin deficiency was measured by specific ELISA as described earlier (19). In short,
186 whole bacterial lysate was used as a coating antigen. Production of PRN was detected with
187 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 controls.

Vaccination status

During the period 2012-2015, 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 PFGE cluster analysis. Chi-square tests for *p*-values between vaccinated and unvaccinated subjects were calculated using GraphPad prism 4.0 version (San Diego, CA, USA). Two tailed P values < 0.05 were considered significant. The Simpson diversity index (SDI) was calculated based on the formula $D = 1- \Sigma n(n-1)/N(N-1)$ where n indicates number of individual profiles and N 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

Strains used for production of ACVs contain *ptxA2* and *ptxA4* alleles as described previously (20). In this study, all 265 isolates harbored the *ptxA1* allele (Table 2). All isolates included in EUpert I-III studies also harbored the *ptxA1* allele (6).

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 1998-2001, the frequency of *ptxP1* and *ptxP3* allele were similar (39% vs. 50%). Since then *ptxP3* has become clearly dominant and from the EUpert III study onwards the frequency has been > 95% (6).

prn alleles

Strains used for production of ACVs contain *prn1* or *prn7* (20). 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* with 255 (96.2%) isolates. *Prn1* and *prn9* were both found with three isolates (1.1%) and *prn3* with two isolates (0.8%). The *prn2* allele has been dominant (>75% of tested isolates for *prn*) in the previous EUpert I-III studies (6).

Fim serotype and *fim3* alleles

228 The Fim3 serotype predominated (>67%) in earlier EUpert I-III studies (6). In the current
229 EUpert IV study, 141 (53.2%) isolates were Fim3, 120 (45.3%) Fim2, three (1.1%) were
230 Fim2,3 and one (0.4%) isolate was deficient for Fim2 and Fim3 (Figure 1). In Denmark
231 (22/27, 81.5%) and Finland (22/28, 78.6%) Fim2 was dominant, whereas in France (27/29,
232 93.1%), Sweden (24/29, 82.8%) and the UK (19/30, 63.3%) Fim3 was prevalent. In other
233 study countries distribution between the two serotypes was close to equal. We also compared
234 serotype with vaccination status of the subject. No correlation between serotypes and
235 vaccination status was found (p = 0.709).

236 For *fim3* alleles, 190 (71.7%) isolates carried *fim3-1*, 72 (27.2%) carried *fim3-2*, two
237 (0.8%) carried *fim3-4* and one (0.4%) *fim3-3*. In France, Sweden and the UK, the distribution
238 between *fim3-1* and *fim3-2* were equivalent, whereas in other countries *fim3-1* was dominant.

239

240 **MLVA**

241 MT27 was dominant in the EUpert I and III studies (MLVA not performed in EUpert II). In
242 the EUpert IV, 20 MTs (20/265, 7.5%) were identified. In the EUpert I study 18 MTs
243 (17.6%) out of 102 isolates and in EUpert III 15 MTs (10.7%) among the 140 isolates were
244 identified (Table 3). In the current study, 214 (80.8%) isolates harbored MT27. The second
245 most common type with 18 isolates (6.8%) was MT28 and third most common with seven
246 (2.6%) isolates was MT18. In addition, 15 other MTs (12, 25, 29, 32, 33, 36, 38, 55, 60, 77,
247 95, 114, 158, 312 and 324) were found among 26 (9.8%) isolates, and two new MTs were
248 detected (MT335 and MT336). In all countries, except Denmark, MT27 was dominant. In
249 Denmark, 48.2% carried MT27, whereas other types such as MT28 (29.6%) constituted more
250 than half of the circulating isolates. However, MT27 and MT28 are close to each other as

there is only one difference in number of repeats of variable number of tandem repeat (VNTR) 6. MT18 however, has seven repeats in VNTR3-2, whereas MT27 has none. Otherwise the structures are identical.

PFGE profiles and association to Fim serotype and genotype

42 PFGE profiles were identified among EUpert IV study. The number of different profiles identified in earlier studies is 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, 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 number of isolates with profiles other than the five most common profiles was high (Figure 2). However, in the EUpert III study, the frequencies of BpSR3 and BpSR10 started to increase, whereas frequencies of BpSR11 and other profiles decreased. In the current study, the frequency of BpSR3 (78/265, 29.4%) and BpSR10 (72/265, 27.2%) further increased and number of isolates with BpSR11 (39/265, 14.7%) and other (19.6%) profiles decreased or remained the same as previously reported (6, 7). The most common profile BpSR3 belongs to the cluster IV, BpSR10 to the cluster IVα, BpSR11 to the cluster IVβ, BpSR5 to the cluster IV and BpSR12 to the cluster IVg. From the other PFGE profiles, 21 new profiles were detected (Figure 4). These new profiles belonged mainly to cluster VII, but were also found from clusters IV, IVα, IVβ, IVg and III. When we analyzed 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 distribution of

main PFGE profiles by country and Figure 4 shows all 42 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 Fim2 (66/78, 84.6%) serotype, whereas BpSR10 represented both Fim3 (37/64, 57.8%) and Fim2 (25/64, 39.1%). In previous EUpert I-III studies, however, isolates belonging to profile BpSR3 were associated with Fim3 serotype (Range: 74.2-100% of the isolates), whereas BpSR10 was linked to Fim3 (96.6-100%). With PFGE profiles BpSR5, BpSR11 and BpSR12, 59 out of 60 isolates carried the Fim3 serotype in the current study. With *fim3* genotype, interestingly all BpSR3 and BpSR10 isolates carried *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 association with vaccination status 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 vaccination status of the subject. 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 ($p = 0.1184$).

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298

299 **Pertactin deficiency**

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

303

304 **DISCUSSION**

305

306 In this study we analyzed 265 *B. pertussis* isolates collected from nine European countries
307 during the period 2012-2015, and compared the results to three previous EUpert studies
308 starting in late 1990s. Although the number of participating countries in each study varied,
309 Finland, France, The Netherlands and Sweden have participated in all four studies. Our study
310 showed that the dominant alleles *ptxA1*, *ptxP3* and *prn2* in circulating strains are different
311 from those used for production of ACVs in European countries (for Denmark data not
312 available) (20). However, country based genetic differences of *B. pertussis* isolates were
313 identified especially with PFGE analyses. Serotype has also changed from Fim3 to Fim2 in
314 several countries, although ACVs used in many of these countries do not contain any fimbrial
315 antigens.

316 ACVs contain purified components from strains carrying *ptxA2/4*, *ptxP1* and *prn1/7*
317 alleles (excluding Denmark) (20, 21). In this study we found that almost all circulating
318 isolates harbored different alleles (*ptxA1, ptxP3* and *prn2*) (Table 1). These alleles have been
319 dominant in the previous EUpert I-III studies, suggesting that circulating *B. pertussis* with

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these alleles may have advantages in ACV vaccinated populations. This may have an effect on the vaccine effectiveness (20). Similar findings with the dominant genotypes have been reported in Australia, Japan and the USA (22-24).

In Europe many countries use ACVs without the Fim2/Fim3 antigens (Table 1). We found that the frequency of Fim2 isolates has markedly increased in several countries compared to previous EUpert II and III studies (Figure 1 and Table 2). Both Denmark and Finland had mostly Fim2 isolates circulating, whereas Fim3 was continuously prevalent in France, Sweden and the UK (Table 1). In Denmark, a mono-component PT vaccine has been used for more than 15 years (25). In Finland, ACV was introduced in 2005 and the vaccine used from 2005 to 2009 contained only PT and FHA. Therefore, the change in frequency from Fim3 to Fim2 is most likely caused by natural infection. It remains to be shown why a high frequency of Fim2 isolates is only observed in certain countries. In Japan, where Fim3 allele has been highly dominating since the 21st century (24), two out of four ACVs in use includes Fim2, which may partly explain why Fim3 is dominant in this country. Similar to Japan, in France and UK where ACVs containing Fim2/3 are in use (Table 1) and prevalent serotype of Fim3 was observed. When we compared these findings to previous EUpert III collection, seven countries were included in both and in Denmark and Finland almost all isolates were Fim3 in EUpert III collection. In addition, similar, but less dramatic increase of Fim2 isolates were noticed in Norway and in the UK (although, Fim3 is still prevalent). This indicates that the numbers are not biased by country changes in different collections, yet they reflect actual change in the circulating strains.

In addition to the serotype of the isolates (Fim3), *fim3-1* allele became prevalent. However, in France, Sweden and the UK where Fim3 isolates were prevalent, both genotypes *fim3-1* and *fim3-2* were common. It is known that the strains used for production of ACVs

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harbor *fim3-1*. As stated above, in France and the UK, 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 USA during the recent outbreaks (26). Since expression of Fim2 or Fim3 of *B. pertussis* might be different between *in vivo* and *in vitro* (13,22), further studies are needed to show whether expression of Fim3 or Fim2 is related to certain alleles of *fim3*, *fim2* or both. However, according to our results, it seems that *fim3-1* allele is frequently found with Fim2 serotype in Denmark and Finland. In addition, in the Netherlands during the period 1995-2008, 99% of the *fim3-2* strains expressed Fim3 (27).

We noticed that MT27 is becoming more dominant in Europe, whereas number of other MTs and SDI, which shows the probability to randomly pick a different isolate from the whole strain population, (Table 3) clearly decreased compared to previous EUpert I and III studies. However, this is not the case in Denmark, the only country in these studies where monocomponent PT vaccine has been used (25). In contrast to other countries, more than 50% of the Danish isolates did not carry MT27. This finding may indicate that effect of population immunity provided by monocomponent and multicomponent vaccines on bacterial populations may differ. In the current study, isolates with MT27 was found equally from vaccinated (N=106) and unvaccinated (N=108) individuals. However, second most common profile MT18 was found in 12 unvaccinated and in six vaccinated individuals, whereas 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 number of other profiles is decreasing (Figure

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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 present findings to earlier results, PFGE profiles BpSR11 and BpSR10 were dominant in Sweden during the EUpert II and III collections (7). Pertussis vaccination was stopped in Sweden in 1979 and was reintroduced in 1996 (28). Therefore, the population immunity may be different compared to other countries in which vaccinations have been continuously used. Similar to 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 similarity within the strains excluding MLVA results (Table 2). In addition, no outbreaks have been reported in these two countries ten years prior to 2015 (a country wide epidemic occurred in Denmark in 2016) (29, 30). In the Netherlands, there has been a shift from BpSR3 to BpSR10, which is currently dominating. This finding is interesting as 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 on the circulating isolates in the EUpert IV collection. Association between PFGE profiles and fimbrial serotype revealed that isolates with BpSR3 and BpSR10 were no longer only associated with Fim3, but were moving towards Fim2 serotype. We also compared vaccination status and 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 number 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 previously.

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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 UK 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 (2). Another recent WGS study from Australia have identified five single nucleotide polymorphisms which were common in the epidemic isolates and differentiated them from pre-epidemic isolates, stressing the role of WGS in studying of B. pertussis (23). However, little is still known about the impact 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 to vaccine efficacy and to opinions towards 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, 1) We have a serial collection of isolates during the last 15 years, 2) Selection criteria has been the same for all collections, 3) isolates have been isolated from infants, young children and adults (range: 0·01 – 62·30 years), 4) the place of origin is known for all isolates and shows that they were not collected from local outbreaks and 5) all analyses for the EUpert IV panel strains were done by one laboratory. The limitations included 1) the number of isolates from each country was relatively low (range: 20-38). However, they do comprise almost all available isolates in many countries such as

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Denmark, Finland and Italy, where the use of culture is diminishing, 2) The epidemiological pressure of pertussis varies in European countries, which could have an effect on the spread of new emerging strains, and 3) Even though the total number of vaccinated and unvaccinated individuals included in this study was comparable (Table 2), the difference in numbers between vaccinated and unvaccinated subjects in individual countries existed. To avoid such effect, a study with a large number of isolates and equal number 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 (78.9-90.6%) 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 selection pressure 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 towards homogeneity in European countries. To get more deep insight of the *B. pertussis* strain diversity in Europe, whole genome sequencing could be applied for surveillance of *B. pertussis*.

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Alex-Mikael Barkoff is a PhD student at the University of Turku, Finland. His main research interests include diagnostics, molecular typing and surveillance of *B. pertussis* and other related respiratory bacteria.

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The funders of the study (GlaxoSmithKline Biologicals and Sanofi Pasteur MSDP) had no role in study design, data collection, data analysis, data interpretation, or in report writing.

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457 The corresponding (QH) and first (AMB) author had full access to all data. In addition, the
458 corresponding author had the final decision where to submit the data for publication.

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460 **Declaration of interests**

461 We declare no competing interests

462 **References**

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TABLES AND FIGURES

Table 1. Pertussis vaccines currently used in European countries*

Country	Vaccine
Belgium	ACV3
Denmark	ACV1
Finland	ACV3
France	ACV2, ACV3 or ACV5
Italy	ACV3
Norway	ACV3
Sweden	ACV2 or ACV3
The Netherlands	ACV3
UK	ACV3 or ACV5

584 *Vaccine compositions: ACV1: PT; ACV2: PT and FHA; ACV3: PT, FHA and PRN; ACV5:

585 PT, FHA, PRN, Fim2 and Fim3

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597 **Table 2**. Overview of the isolate characteristics in EUpert IV study countries

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	COUNTRY									
	Belgium	**Denmark**	**Finland**	**France**	**Italy**	**Netherlands**	**Norway**	**Sweden**	**UK**	**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

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Table 3. Number of PFGE profiles and MLVA types dentified in the four study periods

	Percentage (%) of PFGE or MLVA types (identified/total no of isolates)			
Method	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)	N/A†	10.7 (15/140)	7.5 (20/265)

*The Simpson diversity index calculated for each study period was 0.91, 0.88, 0.88 and 0.83 for PFGE and 0.70, 0.47 and 0.34 for MLVA.

†*N/A, not available.

Figure legends

Figure 1. Frequency of fimbrial serotypes among the EUpert I-IV studies

Figure 2. Distribution of PFGE profiles among the EUpert I-IV studies (1998-2015)

Figure 3. Distribution of PFGE profiles among the EUpert IV study countries (2012-2015)

Figure 4. Dendogram of PFGE profiles identified in the EUpert IV study

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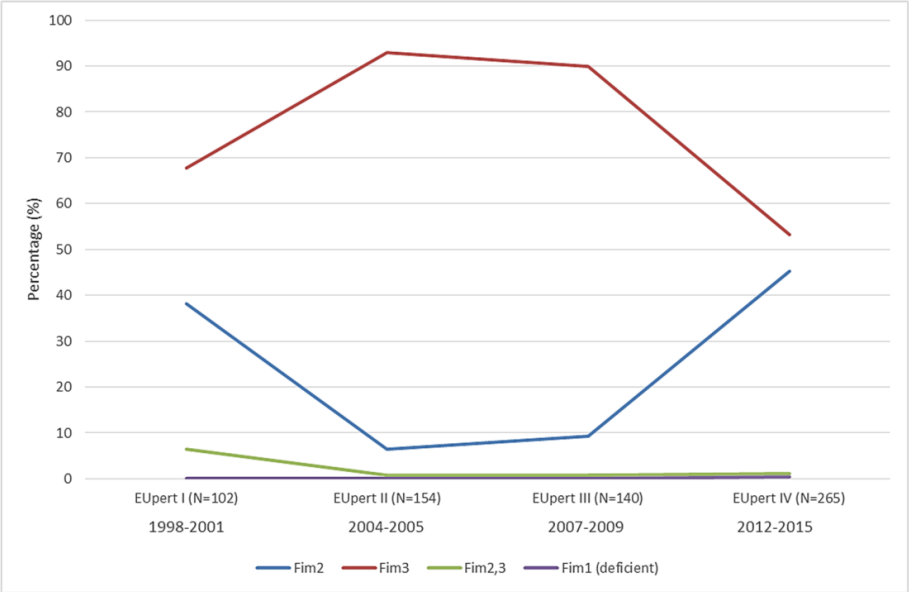
663 Figure 1.



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682 Figure 2.

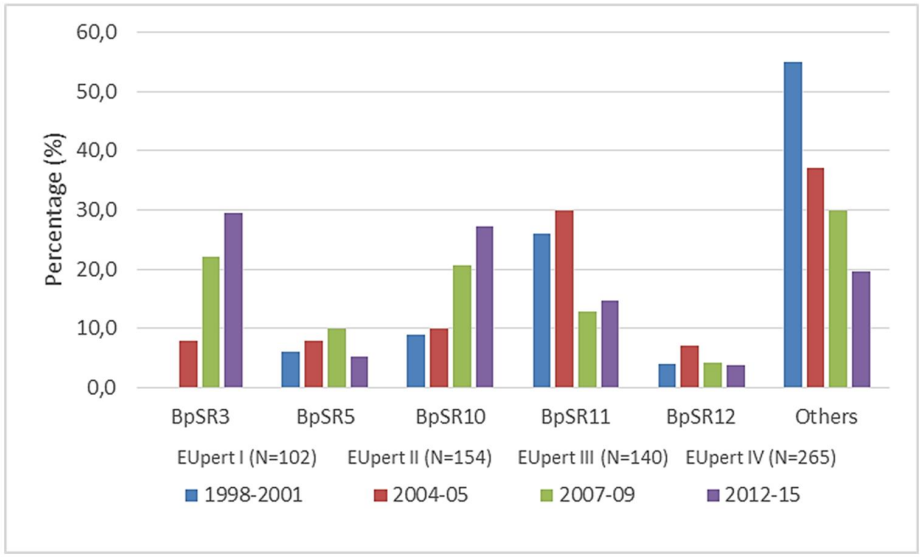

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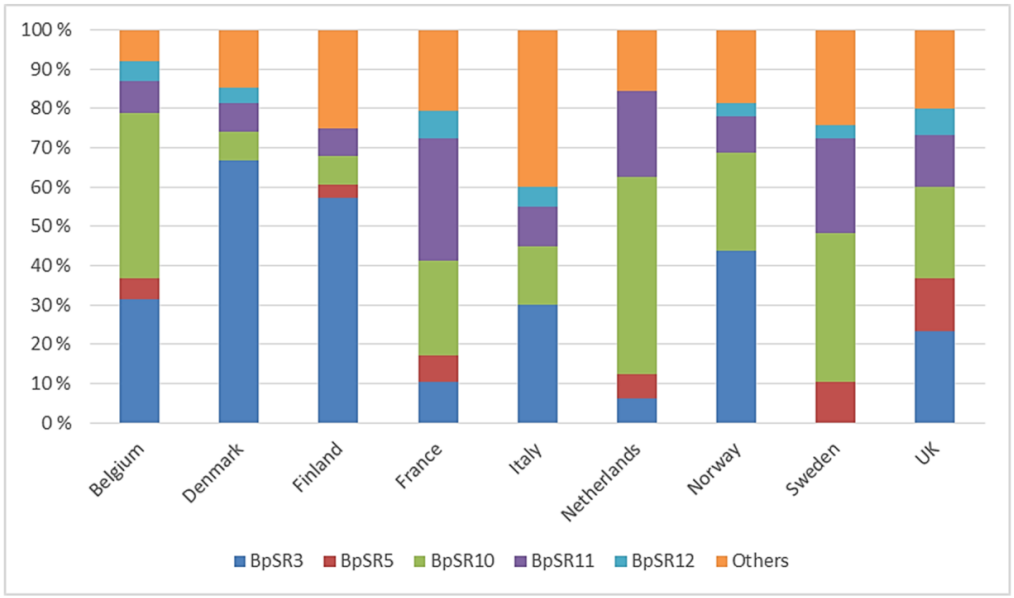
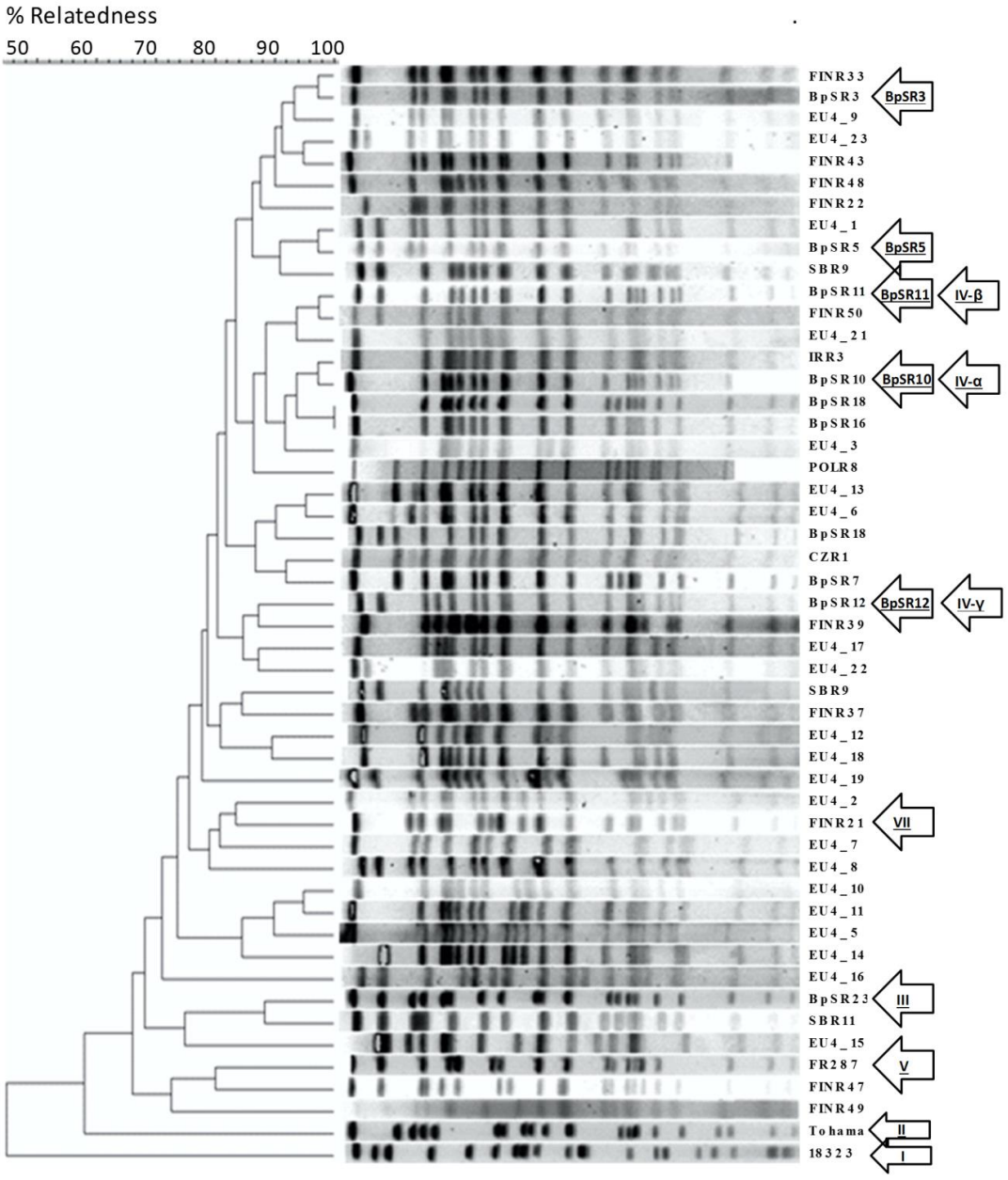
Figure 3.



721 Figure 4.



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