

Investigations into the emergence of pertactin-deficient *Bordetella pertussis* isolates in six European countries, 1996 to 2012

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Pathogen adaptation has been proposed to contribute to the resurgence of pertussis. A striking recent example is the emergence of isolates deficient in the vaccine component pertactin (Prn). This study explores the emergence of such Prn-deficient isolates in six European countries. During 2007 to 2009, 0/83 isolates from the Netherlands, 0/18 from the United Kingdom, 0/17 Finland, 0/23 Denmark, 4/99 Sweden and 5/20 from Norway of the isolates collected were Prn-deficient. In the Netherlands and Sweden, respectively 4/146 and 1/8 were observed in a later period (2010–12). The Prn-deficient isolates were genetically diverse and different mutations were found to inactivate the *prn* gene. These are indications that Prn-deficiency is subject to positive selective pressure. We hypothesise that the switch from whole cell to acellular pertussis vaccines has affected the balance between ‘costs and benefits’ of Prn production by *Bordetella pertussis* to the extent that isolates that do not produce Prn are able to expand. The absence of Prn-deficient isolates in some countries may point to ways to prevent or delay the spread of Prn-deficient strains. In order to substantiate this hypothesis, trends in the European *B. pertussis* population should be monitored continuously.

Introduction

Introduction of whole cell pertussis vaccines (WCVs) 50 to 60 years ago has greatly reduced the pertussis burden worldwide [1]. However, despite a vaccine coverage between 80% and 96%, pertussis has resurged in many countries [2,3]. In particular, in the last few years, high incidences have been observed in the Netherlands, the United Kingdom (UK) and the United States (US) [4–6]. Several causes have been proposed for the resurgence of pertussis, including waning immunity and pathogen adaptation [7].

In the 1990s, less-reactive acellular pertussis vaccines (ACVs) replaced WCVs. ACVs contain between one and five pertussis proteins: (i) pertussis toxin (Ptx); (ii) pertactin (Prn); (iii) serotype 2 fimbriae (Fim2); (iv) serotype 3 fimbriae (Fim3); and (v) filamentous haemagglutinin (FHA). All ACVs contain Ptx. The effectiveness of the different available WCVs was highly variable [8], which complicates evaluation of the protection that ACVs induce compared with that of WCVs. However, recently, several studies have indicated that the immunity induced by ACVs is less long-lasting compared with effective WCVs, resulting in a higher pertussis burden, particularly in children aged 6 to 10 years [9–12].

The effect of waning immunity may have been aggravated by pathogen adaptation [7], which is reflected in large shifts in *Bordetella pertussis* populations [13–15]. Observed adaptations include antigenic divergence between circulating strains and vaccine strains, and the emergence of strains with a novel promoter for Ptx (*ptxP3*) [16]. In addition, most recently, strains have emerged that do not express Prn, hereafter named Prn-deficient strains [17–22]. Isolates with the *ptxP3* promoter increased in frequency in the 1980s and at present they predominate in many countries, reflecting a global selective sweep [6,13,16,23–26]. The *ptxP3* isolates produce higher amounts of Ptx in vitro (1.6-fold increase), when compared with the *ptxP1* isolates they replaced [16]. It has been suggested that this may result in a more effective suppression of host immunity [16]. In agreement with this, *ptxP3* isolates reach higher bacterial densities than *ptxP1* isolates in mice [27]. Prn-deficient isolates emerged around 2000 and have been found in Finland, France, Japan and the US [18–20,22]. In addition, less frequently, isolates were found that

TABLE 1

Year of introduction, vaccine composition and vaccination coverage of acellular pertussis vaccines in six European countries

Country	Year of ACV introduction ^{a,b}	Primary series Age <13 months		Childhood booster Age 13 months–10 years		Adolescent booster Age 11–20 years		Reference
		ACV ^c	Vaccination coverage	ACV ^c	Vaccination coverage	ACV ^c	Vaccination coverage	
Denmark	1997	1	>90%	–	–	–	–	[39]
	2003	–	–	1	>80%	–	–	
Finland	2003	–	–	3	–	–	–	[20]
	2005	2 or 3	>99%	2 or 3	–	3	–	
Netherlands	2001	–	–	3 or 5	>95%	–	–	[40]
	2005	3 or 5	>95%	–	–	–	–	
Norway	1998	3	>90%	–	–	–	–	[41]
	2006	–	–	2	90%	–	–	
Sweden	1996	1, 2 or 3	>98%	–	–	–	–	[40,42]
	2007	–	–	2 or 3	90%	–	–	
United Kingdom ^{b,d}	2000	3	>90%	–	–	–	–	[43]
	Oct 2001	–	–	3 or 5	–	–	–	
	2004	5	–	3 or 5	–	–	–	

ACV: acellular pertussis vaccine; FHA: filamentous haemagglutinin; *Fim2*: serotype 2 fimbriae; *Fim3*: serotype 3 fimbriae; Prn: pertactin; Ptx: pertussis toxin.

Dashes are used where data are not applicable.

^a The year in which ACVs were introduced as the primary series and as booster.

^b In the United Kingdom, an ACV₃ was used temporarily for the primary series until 2001. Thereafter, the whole cell pertussis vaccines was used again for the primary series, with addition of an ACV₃ or ACV₅ childhood booster in October 2001. Subsequently, in 2004 the ACV₅ was introduced permanently for the primary series.

^c Vaccine compositions are shown. ACV₁: Ptx monocomponent; ACV₂: Ptx, FHA; ACV₃: Ptx, FHA, Prn; ACV₅: Ptx, FHA, Prn, *Fim2*, *Fim3*.

^d For the purposes of this article, the United Kingdom is considered as one country.

do not produce FHA or Ptx. Hegerle et al. reported one isolate lacking both Prn and FHA, and one isolate lacking Ptx [17,19]. Bouchez et al. have suggested that the emergence of such isolates is related to the introduction of ACVs [17,28]. Prn-deficient isolates have been isolated from pertussis patients and seemed to be as virulent as isolates expressing Prn in infants less than six months of age as well as in a mouse model [17,29]. A better understanding of the epidemiology and virulence of Prn-deficient isolates is crucial for the development of next-generation pertussis vaccines.

In this study, we investigated the emergence and spread of Prn-deficient isolates in Europe. To do so, we combine single nucleotide polymorphism (SNP) typing, antigen expression analysis and DNA sequencing with epidemiological data. The diversity of vaccination programmes in Europe (Table 1) offers a unique opportunity to study the effect, if any, of vaccination policy on the emergence of Prn-deficient strains. Ultimately, we aim to define conditions that could prevent or hamper the emergence of vaccine-adapted strains. This study provides a baseline for future European studies in which the occurrence and spread of Prn-deficient strains will be investigated.

Methods

Analysis of *Bordetella pertussis* isolates

Six countries provided a total of 665 strains isolated from nasopharyngeal swabs from confirmed pertussis patients for this study: Denmark (n=43), Finland (n=56), Netherlands (n=311), Norway (n=20), Sweden (n=197) and the UK (for the purposes of this article, the UK is considered as one country) (n=38) (Table 2). These countries were selected as they are members of European research programme for improved pertussis strain characterisation and surveillance (EUPERTSTRAIN) network and were able to contribute strains. The strains were isolated during 1996 to 2012. In the countries studied, primary immunisation with ACVs was introduced between 1996 and 2005 (Table 1). Furthermore, in these countries, different ACVs and different vaccine strategies were used and the vaccine coverage varied (Table 1). Strains were grouped into three periods, 1996–2006, 2007–09 and 2010–12 (periods I, II and III, respectively). Bacteria were cultured on Bordet Gengou agar plates supplemented with 15% sheep blood and incubated at 35 °C for 3 to 4 days.

PCR and DNA sequencing

DNA isolation was performed using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, Zwijndrecht,

TABLE 2

Bordetella pertussis isolates typed by single nucleotide polymorphisms and analysed for pertactin and pertussis toxin production in six European countries, 1996–2012

Country	Period I 1996–2006			Period II 2007–2009			Period III 2010–2012			Periods I–III 1996–2012			
	SNP typed n	Tested for Prn and Ptx production n	Prn deficient n (%)	SNP typed n	Tested for Prn and Ptx production n	Prn deficient n (%)	SNP typed n	Tested for Prn and Ptx production n	Prn deficient n (%)	SNP typed n	Tested for Prn and Ptx production n	Prn deficient n (%)	Total number of strains provided
Denmark	20	0	–	23	23	0 (0)	0	0	–	43	23	0 (0)	43
Finland	38	0	–	16	17	0 (0)	0	0	–	54	17	0 (0)	56
Netherlands	34	82	0 (0)	16	83	0 (0)	4	146	4 (3)	54	311	4 (1)	311
Norway	0	0	–	20	20	5 (25)	0	0	–	20	20	5 (25)	20
Sweden	27	80	0 (0)	25	99	4 (4)	0	8	1 (13)	52	187	5 (3)	197
United Kingdom ^a	20	0	–	18	18	0 (0)	0	0	–	38	18	0 (0)	38
Total	129	162	0 (0)	118	260	9 (3)	4	154	5 (3)	261	576	14 (2.4)	665

Prn: pertactin; Ptx: pertussis toxin; SNP: single nucleotide polymorphism. Dashes are used where a percentage is not applicable.

^a For the purposes of this article, the United Kingdom is considered as one country.

the Netherlands), following the manufacturer’s instructions for Gram-negative bacteria. For multi-antigen sequence typing (MAST), the pertussis toxin promoter (*ptxP*), regions 1 and 2 of the pertactin gene (*prn*), the genes for serotype 2 fimbriae (*fim2*) and serotype 3 fimbriae (*fim3*) were sequenced in order to determine polymorphisms [16,30].

To determine the location and size of the mutation causing non-expression of Prn, four sets of primers were selected for PCR amplification of the *prn* gene (Table 3). Prn PCR products were visualised and sized by capillary electrophoresis on a QIAxcel instrument (Qiagen Benelux), using the QIAxcel high-resolution kit, QX DNA size marker 250–8,000 base pairs (bp) and QX alignment markers 15/8,000 bp. Each type of mutation was verified in at least one isolate by Sanger sequencing using an ABI 3700 DNA sequencer (Perkin-Elmer Applied Biosystems).

Identification of vaccine antigen-deficient isolates

A microsphere-based Multiplex Immunoassay (Luminex, Austin, TX, US) [31] was developed to measure the expression of Ptx and Prn. Isogenic *ptx* (Boo85) and *prn* (B1686) knockout strains were used as negative controls [32,33]. Analyses of these negative controls resulted in values <5 ng/ml. Therefore, isolates with antigen concentrations <5 ng/ml were considered deficient isolates. All deficient isolates were tested at least twice, starting from independent cultures. In addition, Prn deficiency was verified with an immunoblot using the monoclonal antibody Prn Ab 62, E4D7 [34] (Figure 1).

Single nucleotide polymorphism typing and clustering analysis

SNP typing was used to establish genetic relationships between *B. pertussis* isolates [35,36]. A total of 113 SNPs that are useful for discriminating between isolates were selected after comparing whole genome data of a selection of 74 worldwide isolates (data not shown). In order to study the origin and spread of European Prn-deficient isolates, SNP typing was performed on 261 isolates from all six countries using the Sequenom technology (Sequenom, San Diego, CA, US) [35]. At least 40 randomly selected strains (using the function RAND in Microsoft Excel) per country were typed, if available. A tree was constructed in Bionumerics 6•6 (Applied Maths, Sint-Martens-Latem, Belgium), treating the SNPs as character data and using the neighbor-joining algorithm. The vaccine strains Boo06 (from the Netherlands, 1950, *ptxP2*), Bo499 (ACV strain 10-536, 1951, *ptxP2*) and Boo05 (from the Netherlands, 1950, *ptxP1*) were included as outgroups.

TABLE 3

Primers used for the characterisation of mutations leading to non-expression of pertactin in *Bordetella pertussis* isolates in six European countries, 1996–2012

PCR	Primer name	Primer sequence (5' to 3')	Position ^a	Reference
1	prn-SPF1	TCC CTG TTC CAT CGC GGT G	1098036–1098054	[44]
	prn-SPR1	CCT GAG CCT GGA GAC TGG	1100878–1100895	
2	prn-BF	AGC TGG GCG GTT CAA GGT	1099489–1099506	[45]
	prn-BR	CGG AT T CAG GCG CAA CTC	1100006–1100024	
3	prn-D1F	CAT GAA ATC CGC CAT CCG CT	1095775–1095794	[this work]
	prn-D1R	ACT GGC CCC CAA CAC AGA CA	1098750–1098769	
4	prn-D2F	TAC TTT TGC TGC GCC CAT T	1098015–1098033	[this work]
	prn-D2R	CTT GAT GGT GGT TCC GCT G	1098283–1098301	

F: forward primer; R: reverse primer.

^a Position of the primer, relative to the origin of replication of reference strain Tohama (National Center for Biotechnology Information (NCBI) reference sequence NC_002929).

Results

Frequency of pertactin-deficient isolates in six European countries

Expression of Prn was assessed by a multiplex Immunoassay assay and immunoblotting (Figure 1) for 576 of the 665 strains from the six countries, of which 14 (2.4%) were shown to be Prn deficient (Tables 2 and 4). All 576 strains tested positive for Ptx production. For period I (1996–2006), only strains from the Netherlands (n=82) and Sweden (n=80) were available for this analysis. One Prn-deficient isolate from Finland was obtained in period I: this isolate was not included in the Prn-deficiency frequency analysis because no other Finnish isolates were analysed for Prn expression in that period. For period II (2007–09), between 17 and 99 strains from each of the six countries were analysed for Prn expression. Prn-deficient strains were observed in Norway (5/20 strains) and Sweden (4/99), but not in Denmark (0/23), Finland (0/17), the Netherlands (0/83) or the UK (0/18). As for period I, for period III (2010–12) only strains from the Netherlands (n=146) and Sweden (n=8) were available for this analysis. In that period, 4/146 Dutch strains and 1/8 Swedish strains were found to be Prn deficient. Thus, Prn-deficient isolates in the frequency analysis were detected in periods II and III. All isolates produced Ptx. The vaccination status of 14 of the 15 patients from whom the Prn-deficient isolates were recovered was unknown (Table 4). Therefore, we cannot relate the infection with a Prn-deficient strain to the degree of immunisation.

Mutations causing non-expression of pertactin

Previous studies observed five types of mutations that inactivate the *prn* gene: inversion, deletion of (part of) the gene, insertion of IS₄₈₁ into *prn*, insertion of a single nucleotide resulting in a frameshift mutation, or a SNP resulting in the introduction of a stop codon [17–22] (Figure 2, Table 5). We used PCR and sequencing of the *prn* gene to determine the cause of non-expression of the Prn-deficient isolates we analysed (Table 4). PCR fragment analysis showed that three Prn-deficient isolates (B3545, B3748, B3865) had an insertion of approximately 1 kilobase pair (kb) proximal to region

2 (Figure 3). We assumed this is the result of an insertion of IS₄₈₁ into this region and no further sequencing analysis was performed on these isolates. Sequence data of the remaining 12 Prn-deficient isolates showed that in two isolates, the promoter region was inverted as described by Pawloski et al. [22]. Another three isolates (B3582, B3640, B3891) had an IS element inserted proximal to one of the two repeat regions of the *prn* gene, designated region 2 (Figure 2). This mutation has also been found previously in French Prn-deficient isolates [17]. Sequence data of a sixth isolate (B3771), which originated from Finland in period I, contained a deletion of 49 bases in the signal sequence. This isolate was not included in the Prn-deficiency frequency analysis because no other Finnish isolates were analysed for Prn expression in period I. The seventh isolate, B3876 from The Netherlands, contained a SNP at position 223 that introduces a premature stop codon in the *prn2* gene. An eighth isolate (B3658), originating from Norway, also contained a SNP that introduces a premature stop codon in *prn2*, but at position 1,273. Sequencing of the *prn* gene around position 1,273 showed that the other four Norwegian Prn-deficient isolates also harboured this SNP.

Phylogenetic relationships of European pertactin-deficient *Bordetella pertussis* isolates

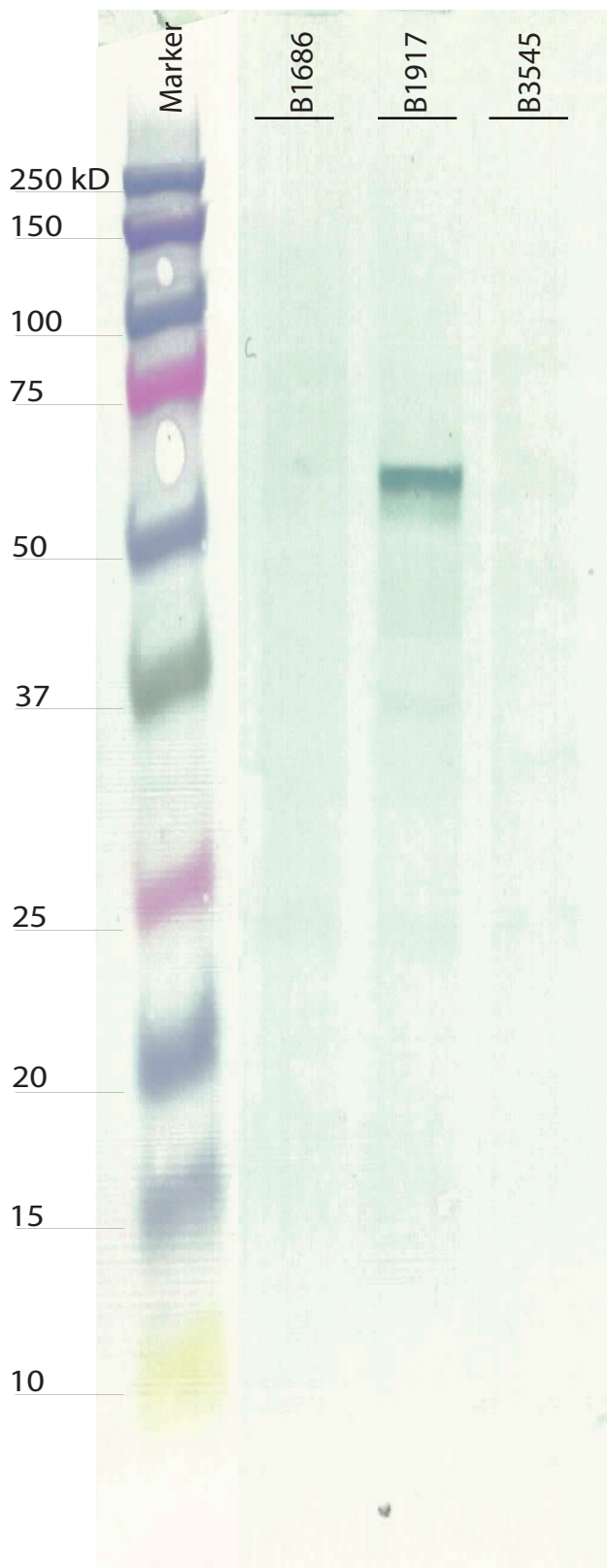
Typing of 261 isolates from the six different European countries using 113 SNPs allowed us to distinguish 20 STs.

Phylogenetic analysis revealed a closely related European *B. pertussis* population (Figure 4). The tree topology showed two distinct clades containing *ptxP1* and *ptxP3* isolates. In addition, a *fim3.1* and a *fim3.2* cluster could be observed within the *ptxP3* clade (Figure 4).

The 12 European Prn-deficient isolates included in the phylogenetic analyses were found in four different STs: ST13, ST14, ST17 and ST19 (Figure 4). These STs were among the predominant types and contained 70% (184/264) of the isolates analysed.

FIGURE 1

Immunoblot to confirm absence of pertactin (69 kD) expression in *Bordetella pertussis* isolates in six European countries, 1996–2012



The result of isolate B3545 (fourth lane) is shown here as an example. A Kaleidoscope marker (161-0375, Bio-Rad, Hercules, US) was loaded in the first lane. B1686 is an isogenic pertactin knock-out strain, which was used as a negative control. A commonly used reference strain, B1917, was used as a positive control.

All Prn-deficient isolates analysed in this study were found in the *ptxP3* clade but were associated with both *fim3.1* and *fim3.2* alleles. ST14 contains two types of *prn* mutations (Table 4). In contrast, the five Prn-deficient isolates that belong to ST13 all harboured the same mutation in *prn*: a SNP resulting in premature translational termination of *prn*. All five isolates were from Norway. The *prn* mutation, in which IS481 was inserted proximal to region 2, was found in three STs that were dispersed across the *ptxP3* clade. This mutation was associated with both the *fim3.1* and *fim3.2* alleles. Thus, identical mutations were observed in different STs, while within a single ST, different mutations were found.

Discussion

The diversity in immunisation programmes in Europe offers an opportunity to explore the effect of vaccination strategies on adaptation of *B. pertussis* in populations with a vaccine coverage between 80% and 96%. In this study, the overall mean of Prn-deficient strains from six European countries isolated between 2007 and 2012 was 3.4% (14/414 isolates). Bouchez et al. suggested that the emergence of French Prn-deficient isolates was driven by the switch from WCVs to ACVs [17]. Indeed, Prn-deficient isolates were rarely found before the introduction of ACVs, when WCVs were used [37]. Compared with the mean of Prn-deficient strains found in the six countries studied here (3.4%), higher percentages of Prn-deficient isolates were observed in France (13% in 2011), Japan (32% in 2005–09) and the US (53% in 2012) [18,19,22]. In Japan, ACVs were introduced in 1981 [18], and in the US, the first ACV was marketed in 1991. Thus, these countries have a longer history of ACV immunisation compared with the six countries included in this study, where ACVs vaccines for the primary series were introduced between 1996 and 2005 (Table 1). Interestingly, the early Prn-deficient strains found in this study were mainly from Norway and Sweden, where ACVs for the primary series were introduced in 1996 and 1998, respectively, which is earlier than in the UK (in 2000), Finland and the Netherlands (both in 2005) (Table 1). Notably, in France an ACV booster was introduced in 1998, while the WCV for the primary series was replaced with an ACV in 2004 [29]. The timing of these changes is similar to that in Norway which, as in France, has a relatively high percentage of Prn-deficient strains. In Denmark, an ACV was introduced relatively early in 1997, while Prn-deficient strains were not detected. Unlike the vaccine in the other five countries in this study, which use ACVs containing Prn (Table 1), the Danish ACV contains Ptx only. As the Danish vaccine does not induce Prn antibodies, it is conceivable that Prn-deficient strains will not emerge in Denmark, if Prn-deficiency does not contribute to strain fitness, although overflow of Prn-deficient strains from neighbouring countries is possible.

In 2007–09, five of the 20 Norwegian strains were Prn-deficient. Although the number of isolates tested was

TABLE 4

Pertactin-deficient *Bordetella pertussis* isolates analysed from four European countries, 2004–12 (n=15)

Isolate name	Patient's age	Patient's country of residence	Patient's vaccination status	Year isolate recovered	Sequence type ^a	Mutation ^b
B3771 ^c	2 years	Finland	Vaccinated	2004	ST19	¶6: <i>prn2</i> (32_80Δ[49])
B3645	7 months	Norway	Unknown	2007	ST13	¶12: <i>prn2</i> (1273STOP:C>T)
B3652	14 years	Norway	Unknown	2008	ST13	¶12: <i>prn2</i> (1273STOP:C>T)
B3654	14 years	Norway	Unknown	2008	ST13	¶12: <i>prn2</i> (1273STOP:C>T)
B3545	Unknown	Sweden	Unknown	2008	ST17	<i>prn2</i> (PCRregion2::1kb)
B3657	2 months	Norway	Unknown	2009	ST13	¶12: <i>prn2</i> (1273STOP:C>T)
B3658	3 months	Norway	Unknown	2009	ST13	¶12: <i>prn2</i> (1273STOP:C>T)
B3594	1 month	Sweden	Unknown	2009	ST14	¶1: <i>prn2</i> (-20892_-75<)[22kb])
B3582	0 months	Sweden	Unknown	2009	ST17	¶4: <i>prn2</i> (1613^1614::IS481[1049])
B3748	11 months	Sweden	Unknown	2009	ST17	<i>prn2</i> (PCRregion2::1kb)
B3640	13 years	Netherlands	Unknown	2010	ST14	¶4: <i>prn2</i> (1613^1614::IS481[1049])
B3865	Unknown	Netherlands	Unknown	2011	ST14	<i>prn2</i> (PCRregion2::1kb)
B3876	2 years	Netherlands	Unknown	2011	NI	¶10: <i>prn2</i> (223STOP:C>T)
B3891	Unknown	Sweden	Unknown	2011	NI	¶4: <i>prn2</i> (1613^1614::IS481[1049])
B3977	69 years	Netherlands	Unknown	2012	NI	¶1: <i>prn2</i> (-20892_-75<)[22kb])

NI: not included in the SNP analysis; SNP: single nucleotide polymorphism; ¶: mutation number.

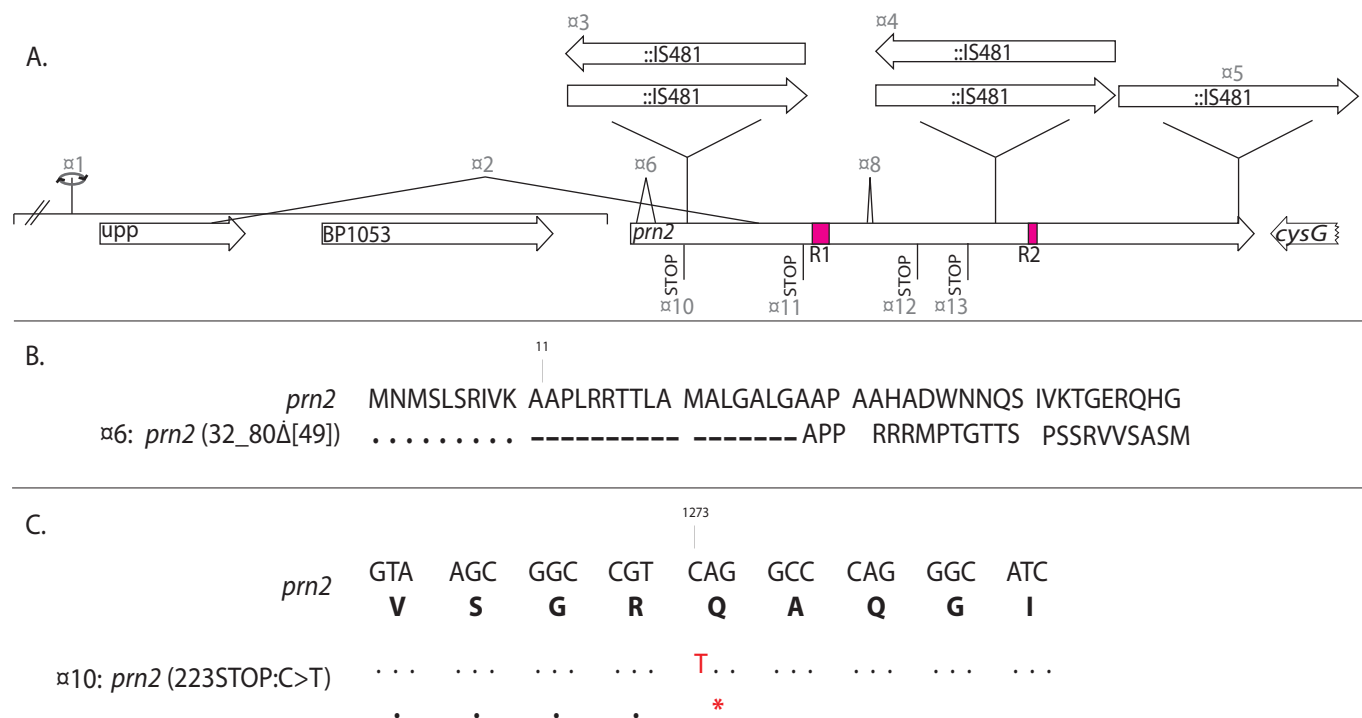
^a Sequence type as presented in Figure 2A.

^b Mutation causing the non-expression of pertactin. Numbers correspond to those in Table 5 and Figure 2A.

^c This isolate was not included in the frequency analysis, because it was a single analysed Finnish strain that was isolated before 2007, but was included in SNP analysis and *prn* mutation analysis.

FIGURE 2

Mutations in *prn2* causing non-expression of pertactin in *Bordetella pertussis* isolates in six European countries, 1996–2012



A. *prn2* is shown with the surrounding genes. The arrowheads indicate the transcription direction. The variable regions 1 (R1) and 2 (R2) of *prn* are indicated in red. Numbers 1 to 13 refer to mutations (¶) in *prn2* and correspond to the numbers in Table 5.

B. Detailed representation of mutation ¶6, a deletion of 49 bases in the signal sequence: *prn2* (32_80-149): • identical bases/amino acids, - deleted bases/amino acids.

C. Detailed representation of mutation ¶10: a SNP leading to a stop codon: *prn2* (223STOP:C>T). Base and amino acid numbering is relative to the *prn2* initiation codon.

TABLE 5

Mutations in the *pertactin* gene causing loss of pertactin expression

Number of the mutation ^a	Name of the mutation ^b	Note	Reference
#1	<i>prn2</i> (-20892_-75<>[22Kb])	Inversion of about 22 kb in the promoter	[21, this work]
#2	<i>prn2</i> (-1846_-553Δ[2399])	Deletion of the first part of <i>prn2</i> and upstream <i>BP1053</i>	[16]
#3	<i>prn2</i> (245^246::IS481[1049])	Insertion of IS481 in <i>prn2</i> , between position 245 and 246	[20]
#4	<i>prn2</i> (1613^1614::IS481[1049])	Insertion of IS481 in <i>prn2</i> , between position 1,613 and 1,614	[16, this work]
#5	<i>prn2</i> (2735^2736::IS481[1049])	Insertion of IS481 in <i>prn2</i> , between position 2,735 and 2,736	[21]
#6	<i>prn2</i> (32_80Δ[49])	Deletion in <i>prn2</i> signal sequence	[this work]
#7	<i>prn2</i> (Δprn)	Deletion of the entire prn gene	[18]
#8	<i>prn2</i> (1043_1067Δ[25])	25 bp deletion in first repeat region	[18]
#9	<i>prn2</i> (Δ[89])	89 bp deletion at 5' of prn	[18]
#10	<i>prn2</i> (223STOP:C>T)	SNP C>T, leading to a stop codon	[this work]
#11	<i>prn2</i> (760STOP:C>T)	SNP C>T, leading to a stop codon	[21]
#12	<i>prn2</i> (1273STOP:C>T)	SNP C>T, leading to a stop codon	[20, this work]
#13	<i>prn2</i> (1479STOP)	SNP leading to a stop codon	[18]
#14	<i>prn1</i> (1598^1599::IS481[1049])	Insertion of IS481 in <i>prn1</i> , also found in reverse direction	[17]
#15	<i>prn1</i> ΔSS	Deletion in <i>prn1</i> signal sequence	[17,19,21]

bp: base pairs; IS: insertion element; kb: kilobase pairs; SNP: single nucleotide polymorphism; #: mutation number.

^a As presented in Figure 2, except for #14 and #15.

^b The name is composed as follows: pertactin gene (prn) allele (position from open reading frame of the prn gene/type of mutation/[size of the mutation]).

x_y	from position x to y
x^y	between position x and y
<>	inversion
::IS481	insertion of IS481
Δ	deletion
STOP:C>T	SNP (C to T) leading to a premature stop codon

low, this proportion is relatively high compared with the overall mean of the six countries in this period (3.4%, 14/414). This implies that in Europe, besides France, Norway may also have elevated levels of Prn-deficient isolates. Comparisons of frequencies of Prn-deficient isolates should, however, be made with caution, as differences in the period investigated, vaccination policies and isolate collection are among the factors that can influence the outcome. It seems valid, though, to conclude that the fraction of Prn-deficient strains in several countries of Europe is relatively low, with the exception of France [19] and Norway, as stated above. In order to confirm these findings, further studies are required that include more isolates for each country. The analyses of larger amounts of Prn-deficient strains may also reveal if these strains are found in higher frequencies in particular age groups. Future studies should include more southern European countries.

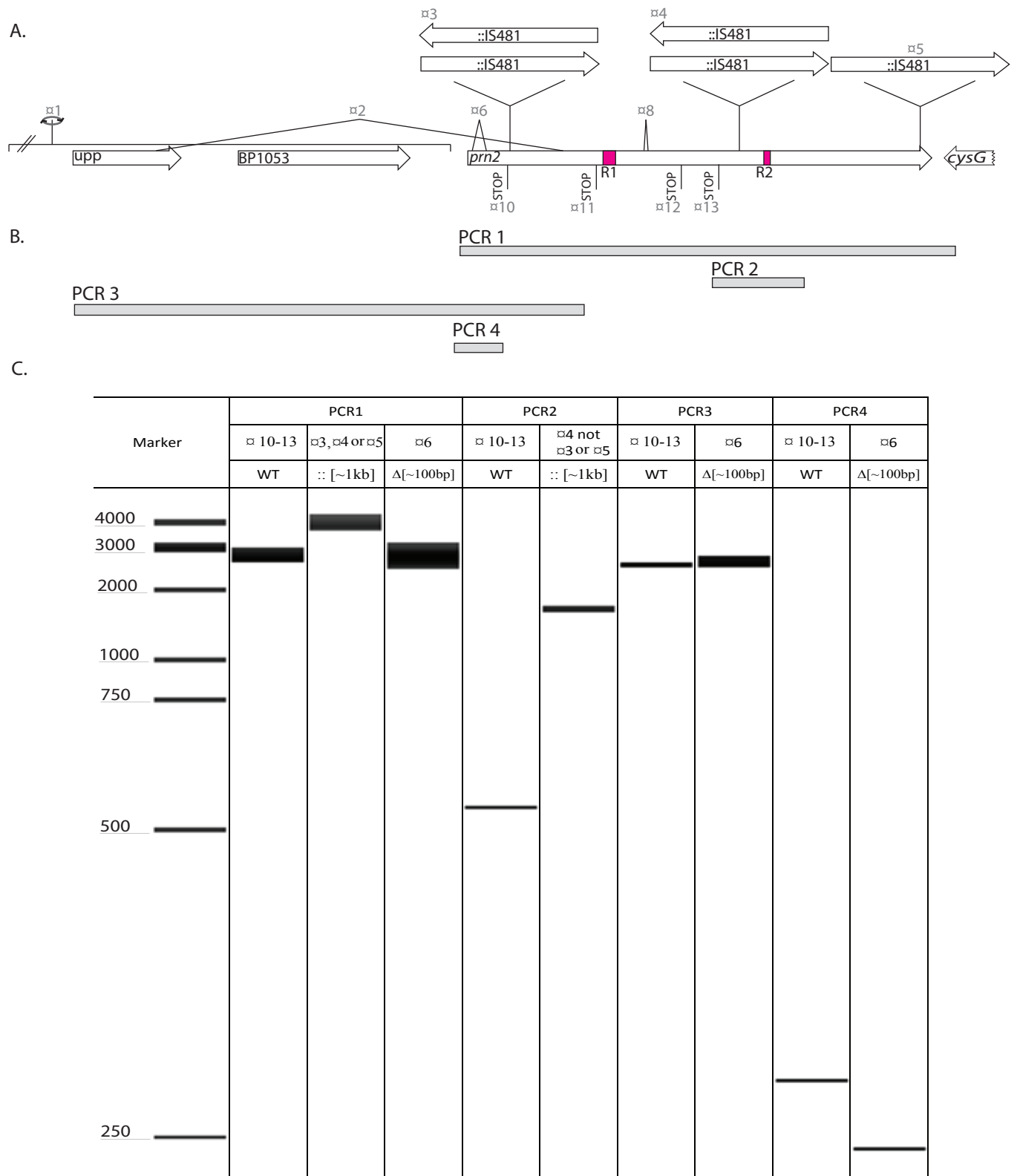
Mutations that were previously found to inactivate the *prn* gene [17,19,21] were also observed in the Prn-deficient isolates analysed in this study. We found that the mutations in *prn* occurred several times independently in different lineages, which is an indication that it has been fixed in the population by positive

selection. The European Prn-deficient isolates seem to have originated from distinct lineages, but within the diverse nature of the mutations causing non-expression, we also observed possible signs of clonal expansion of a Prn-deficient strain in Norway. Notably, the specific point mutation that occurred in five Norwegian isolates was also found in seven of 12 Prn-deficient isolates collected in Philadelphia in the US [21]. It was previously suggested that the spread of Prn-deficient isolates in Japan was the result of clonal expansion [18].

The production of bacterial virulence factors, such as pertactin, involves 'costs and benefits' for the bacteria. Costs include the energy used to express the virulence factors and activation of the host immune response against these antigens. Benefits comprise attachment to host tissues and manipulation of host defences. We hypothesise that the switch from WCV to ACV has affected the balance between these costs and benefits of Prn production to the extent that strains that do not produce Prn are able to expand. In support of this, our phylogenetic analysis indicates Prn-deficiency is subject to positive selection. We postulate that, because non-expression of one of the

FIGURE 3

Characterisation of the *prn* gene from pertactin-deficient *Bordetella pertussis* isolates by polymerase chain reaction, from six European countries, 1996–2012



bp: base pairs; kb: kilobase pair; PCR: polymerase chain reaction; WT: wild type.

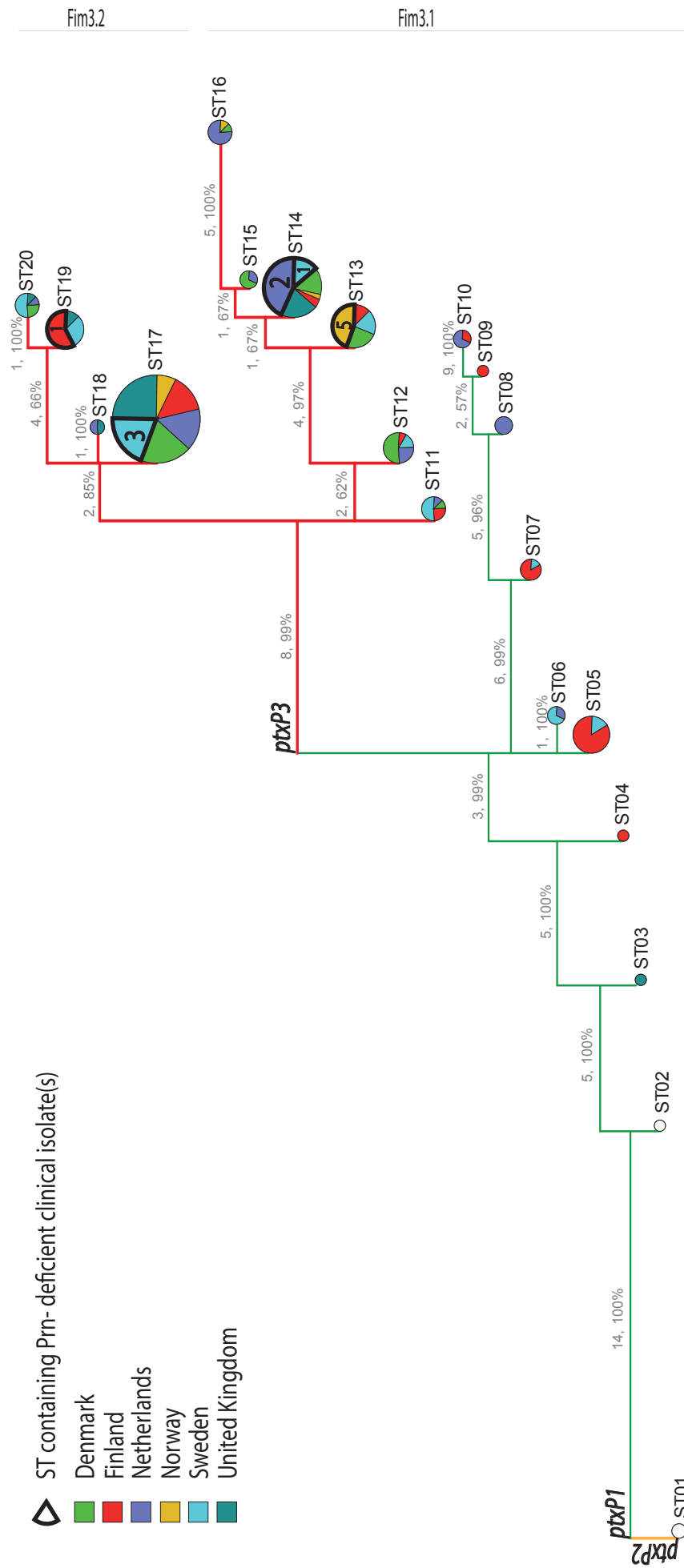
A. The *prn* gene and surrounding genes.

B. The grey bars represent the regions targeted in the PCR that were used to identify insertion and deletion mutations in *prn*. For the primers, see Table 3.

C. The PCR products of the WT *prn* allele were compared with PCR products derived from *Prn*-deficient strains. PCR fragments were sized by capillary electrophoresis. On the basis of the primers used and the size of the PCR fragments, the type of mutation was derived, as shown above the lanes. Designation of PCR fragments as in (B) and mutations as in Table 5. The first lane contains the size marker DNA (in bp). PCR2 distinguishes $\alpha 4$ from $\alpha 3$ or $\alpha 5$, which does not result from PCR1. Mutations 10–13 yielded WT products for the PCRs.

FIGURE 4

Genetic relationship between European *pertactin*-deficient *Bordetella pertussis* isolates



SNP: single nucleotide polymorphism; ST: sequence type.

A total of 113 predefined SNPs were used to construct a neighbor-joining tree of 261 European strains collected between 1998 and 2011. The STs are listed next to the branch tips and circle sizes are scaled to the number of isolates in each ST. Colours indicate the country from which the isolates originate. The bold sectors are STs containing Prn-deficient strains and the number in the centre of the segment represents the number of Prn-deficient isolates. The *ptxP* alleles are indicated next to the tree arms (*ptxP1*: green line; *ptxP2*: orange line; *ptxP3*: red line) and the *fim3* types are presented at the tips of the branches. Branch labels give the distance between the STs followed by the bootstrap values as percentages (200 replicates). ST01 and ST02 respectively consist of two and one vaccine strain(s), which are used as an outgroup.

vaccine antigens contributes relatively little to immune evasion of a broad immune response, vaccination with WCVs limits the advantage of Prn-deficient isolates. Conversely, if the immune response is directed against a limited number of antigens (one to five in the case of ACVs), non-expression of one of the vaccine antigens may confer a considerable selective advantage. This is especially the case when high antibody titres are induced, as is observed for ACVs [38]. However, further studies are required to substantiate this hypothesis.

The emergence of Prn-deficient strains raises questions about its role in pertussis. An analysis of clinical symptoms caused by Prn-positive and Prn-negative isolates in infants did not reveal major differences between the two groups, which suggests that lack of Prn in *B. pertussis* does not affect virulence in infants substantially [29]. Other important questions that should be addressed in the future are the effect, if any, of Prn-deficiency on the efficacy of pertussis vaccines and whether Prn-deficient strains prevail more in groups with a particular level of immunity. This study establishes a baseline for investigations into the emergence and spread of Prn-deficient strains in Europe.

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Conflict of interest

None declared.

Authors' contributions

FRM obtained funding and supervised the study. QH and JM organised the EUPERTSTRAIN 2 and 3 collections from the network members. Isolates, epidemiological data and isolate characteristics were provided by all authors. AZ, MvG, KH, HvdH, MB and FRM were responsible for the study concept and practical work. AZ and FRM analysed and interpreted data and wrote the manuscript. All authors were responsible for the critical revision of the manuscript.

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