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PFGE and pertactin gene sequencing suggest limited genetic variability within the Finnish *Bordetella parapertussis* population

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The outer-membrane protein pertactin (Prn) of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* is believed to function as an adhesin and is an important immunogen. The emergence of *B. pertussis* and *B. bronchiseptica* Prn variants has been reported. The aim of this study was to determine whether similar variation is found in *B. parapertussis* Prn and to characterize Finnish clinical *B. parapertussis* isolates that were collected in 1982–2000. Of 76 *B. parapertussis* isolates studied, seven (9 %) were found to have silent and non-silent nucleotide changes. In addition, one (1 %) had eight PQP repeats instead of nine. Three closely related *B. parapertussis* *Xba*I PFGE patterns were found. Genetic variation of *B. parapertussis* was found to be very limited, suggesting that *B. parapertussis* is a stable organism that is well-adapted to its own ecological niche.

INTRODUCTION

At the time of writing, the genus *Bordetella* includes eight species. Of these, *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica* are closely related with relatively little genetic variation (Musser *et al.*, 1986). The causative agent of whooping cough (pertussis), *B. pertussis*, is exclusively a human pathogen, whereas *B. parapertussis* and *B. bronchiseptica* may infect both humans and animals. In humans, *B. parapertussis* causes pertussis-like disease that is often milder than pertussis, although severe cases have been reported (Heininger *et al.*, 1994; He *et al.*, 1998). Much less is known about the epidemiology of *B. parapertussis* than about that of *B. pertussis*. When enhanced pertussis surveillance was carried out in Finland in 1994–1997 by using PCR and culture, about one-third of *Bordetella* cases were found to be caused by *B. parapertussis*, suggesting that the incidence of parapertussis is underestimated (He *et al.*, 1998).

B. pertussis, *B. parapertussis* and *B. bronchiseptica* share a number of virulence factors. The most important difference between them is that pertussis toxin is produced only by *B. pertussis*; *B. parapertussis* and *B. bronchiseptica* possess, but do not express, the complete toxin operon. Pertactin (Prn), an outer-membrane protein that is expressed by *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, is an important virulence factor and is known to confer protective immunity to *Bordetella* infection in animals and humans (Charles *et al.*, 1989; Kobisch & Novotny, 1990; Leininger *et al.*, 1992). Thus, it is a component of some acellular pertussis vaccines. Prn is proposed to function as an adhesin by promoting the attachment of bacteria to certain host cells via the RGD motif (Leininger *et al.*, 1991). Prn has two regions that are composed of amino acid repeats (Charles *et al.*, 1991): region 1 includes repeats of GGxxP (Charles *et al.*, 1988), is located near the RGD motif and is polymorphic in *B. pertussis* (Mooi *et al.*, 1998) and *B. bronchiseptica* (Boursaux-Eude & Guiso, 2000; Register, 2001). Region 2 is composed of PQP repeats (Charles *et al.*, 1988) and is polymorphic in *B. bronchiseptica* (Li *et al.*, 1992; Boursaux-Eude & Guiso, 2000; Register, 2001). Prns expressed by *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* are highly similar (>90 %), with the major differences occurring in the number of repeats in regions 1 and 2 (Charles *et al.*, 1988; Li *et al.*, 1991, 1992). Polymorphism of *B. pertussis* and *B. bronchiseptica* Prn has been characterized, but variation of *B. parapertussis* Prn has not

Abbreviation: Prn, pertactin.

The GenBank/EMBL/DDBJ accession numbers for the sequences of polymorphic regions 1 and 2 of *Bordetella parapertussis* pertactin are AF503929 and AF503928, respectively.

been observed previously (Boursaux-Eude & Guiso, 2000). The aim of this study was to characterize the Prn proteins expressed by Finnish *B. parapertussis* isolates by sequencing part of the *prn* gene and to follow the recent evolution of the *B. parapertussis* population in Finland by using PFGE.

METHODS

Clinical isolates. In this study, we sequenced 76 *B. parapertussis* isolates that were collected from patients resident in Finland during the years 1982–2000 (Table 1). Calcium alginate swabs and Regan–Lowe medium that contained charcoal agar and defibrinated sheep blood, supplemented with cephalexin, were used for primary cultures. After collection, swabs were inoculated onto plates at the local health centre or school. Plates were incubated in a humid atmosphere at 35 °C and monitored daily for 7 days. Suspected colonies were Gram-stained and tested by slide agglutination with antisera to *B. pertussis* and *B. parapertussis* (Murex Biotech). The identity of the isolates was further confirmed by GLC and they were stored at −70 °C. Strains were collected from 12 different communities in south-western Finland (Sirkkala school and 11 geographically distinct communities). From two communities, strains were obtained consequentially with 5–6-year intervals. In six communities, there were outbreaks of *B. parapertussis*, which comprised more than three culture-confirmed cases that were considered to be epidemiologically related.

PCR, sequencing and PFGE. PCR, sequencing and PFGE were performed according to standardized recommendations for typing of *B. pertussis* (Mooi *et al.*, 2000). Bacteria were cultivated on Regan–Lowe medium that contained charcoal agar and defibrinated sheep blood at 35 °C for 2 days. For PFGE, *B. pertussis* strains 134 (USA), 287 (France) and B 902 (Sweden), French *B. parapertussis* strain CIP 64.11^T and *B. parapertussis* ATCC 15311^T were used as reference strains. Briefly, a 1000 bp segment of the *prn* gene (covering regions 1 and 2) was sequenced from the 76 *B. parapertussis* strains. Forty-seven of the strains, including the strains that possessed a variant form of Prn and at least one invariant strain from each community, were genotyped by PFGE with the restriction enzymes *Spe*I and *Xba*I (New England Biolabs), with a CHEF Mapper II apparatus (Bio-Rad). PFGE reference strains and λ ladder PFGE markers (New England Biolabs), used as molecular size standards, were included in each run. Nucleotide sequences were analysed with the Vector NTI Suite 6.0 software (InforMax) and compared by using the Vector NTI AlignX program with the CLUSTAL W algorithm. PFGE patterns were analysed both visually and with the assistance of Bionumerics version 2.5 software (Applied Maths) by using UPGMA with the Dice coefficient and 1 % position tolerance settings for cluster analysis.

Western blotting. Prn expression was demonstrated by Western blotting using an anti-Prn mAb, BPE3 (Brennan *et al.*, 1988) (data not shown). Bacterial cell lysates (20×10^6 bacteria per well) and purified Prn protein (144 ng per well), provided by GlaxoSmithKline, were separated by SDS-PAGE on a 10 % resolving gel. Proteins were transferred to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech), which was blocked with 5 % milk powder/0·1 % Tween 20/PBS at 4 °C overnight. After washing, the membrane was incubated with a 1 : 1000 dilution of BPE3 antibody (obtained from Michael Brennan, FDA, Bethesda, USA) at 25 °C for 1 h. Immunochemical detection was performed with 1 : 1000-diluted horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins (DAKO A/S), using an enhanced chemiluminescence system (Amersham), and analysed visually as positive or negative.

Table 1. *B. parapertussis* clinical isolates used in this study

Community	No. isolates	Year of isolation	No. Prn variants found
Sirkkala school	16	1982–1983	1
Uittamo	1	1990	0
Paimio	1	1993	0
Salo	2	1994	0
Somero	16	1994	3
Littoinen	14	1995	0
Halikko	1	1995	0
Ulvila	1	1996	1
City of Turku	1	1996	0
Masku	5	1996	1
City of Tampere	1	1996	0
Salo	2	1999	0
Raisio	12	2000	1*
Somero	3	2000	0
Total	76	–	7

*This isolate had both the non-silent nucleotide change and eight PQP repeats instead of nine, arbitrarily designated as BPP *prn3*.

RESULTS AND DISCUSSION

Of the 76 *B. parapertussis* isolates studied, seven (9 %) were found to have both silent and non-silent nucleotide changes (arbitrarily designated as BPP Prn2). In addition, one (1 %) had eight PQP repeats instead of nine (arbitrarily designated as BPP Prn3) (Table 1 and Fig. 1). The silent mutation occurred at nucleotide 1078 (C→T). The non-silent mutations change the codon of glutamine (Q311) to serine (S). S and Q are both polar amino acids with uncharged side chains that are usually found at the surface of water-soluble proteins, where they contribute to both the water solubility and formation of binding sites for charged molecules. Therefore, the predicted Q→S change would probably not affect the conformation or function of Prn. However, to confirm this, the functionality of these Prn variants should be studied. In one of the variant isolates, the predicted amino acid sequence has eight PQP repeats instead of nine in region 2. The effect of variation in the number of PQP repeats on protein function is not known. Variation in region 2 has been observed in only two *B. pertussis* isolates, but in several isolates of *B. bronchiseptica* (Boursaux-Eude & Guiso, 2000; Register, 2001). However, variation in region 2 of *B. pertussis* has only been investigated in a limited number of studies.

The presence of Prn in bacterial cell lysates was analysed by Western blotting using the anti-Prn mAb BPE3. It was confirmed that the variant forms of *B. parapertussis* Prn are expressed. However, the method was not quantitative; thus, we do not know whether the level of protein expression was the same in all isolates.

Three closely related *B. parapertussis Xba*I PFGE patterns were found among the 47 isolates studied. The isolates were considered to be closely related, as the PFGE patterns differed

							1075								1939			1947			
BPP *prn1*	TCG	ATC	GTC	GAG	GCG	CCG	CAG	CTG	GGC	GCC	-//-	CCG	CAG	CCG	CCG	CAG	CCG	CCA	CAG	AGG	CAG
BPP Prn1	S	I	V	E	A	P	Q	L	G	A	-//-	P	Q	P	P	Q	P	P	Q	R	Q
BPP *prn2*	...	...	...	...	...	...	AGC	T..	...	...	-//-	...	...	...	...	...	...	...	...	...	...
BPP *prn3*	...	...	...	...	...	...	AGC	T..	...	...	-//-	...	...	...	..-	---	---	--.	...	...	...
BPP Prn3	S	I	V	E	A	P	S	L	G	A	-//-	P	Q	P	-	-	-	P	Q	R	Q

Fig. 1. Finnish *B. parapertussis* pertactin variants. DNA and corresponding amino acid sequences of polymorphic regions are depicted. Dots indicate identical bases; lines indicate deletion of bases. Numbers refer to position of bases in the pertactin gene. Complete sequences can be obtained from GenBank, accession numbers AF503929 (polymorphic region 1) and AF503928 (polymorphic region 2).

by only one or two bands (there was 96·21 % similarity between the profiles). These changes are consistent with a single genetic event, i.e. a point mutation or an insertion or deletion of DNA (Tenover *et al.*, 1995). The isolates collected in 1982–1995 ($n = 30$) exhibit an A1 pattern (Fig. 2a). The majority of the isolates collected in 1996 ($n = 6$) exhibit an A2 pattern, like reference strains ATCC 15311^T and CIP 64.11^T, and only one isolate exhibited the A1 pattern. The A2 pattern is slightly different from the A1 pattern and is characterized by a fragment of 262 kb instead of the 250 kb fragment (Fig. 2a). In 1999–2000, all isolates ($n = 15$) exhibited an A2 pattern. The isolate that harbours eight

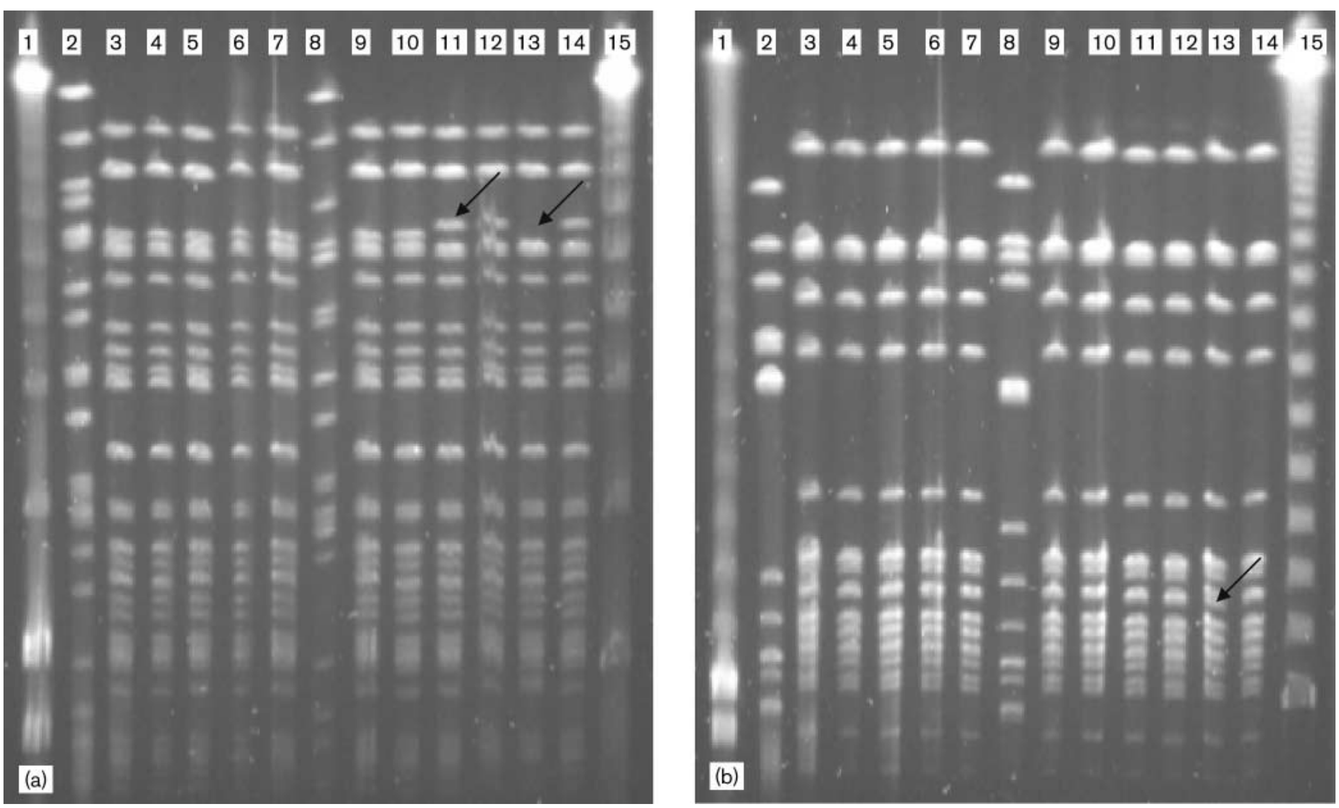

Fig. 2. PFGE profiles of (a) *Xba*I- and (b) *Spe*I-digested chromosomal DNA of *B. parapertussis* isolates. Lanes 1 and 15, molecular size markers; 2 and 8, *B. pertussis* PFGE reference strains 134 and 287; 14, *B. parapertussis* PFGE reference strain CIP 64.11^T; 3–7, 9, 10, DNA from isolates harbouring PFGE pattern A1 (isolated in 1982–1995); 11 and 12, DNA from isolates harbouring pattern A2 (isolated in 1999 and 2000); 13, DNA from the isolate harbouring pattern B (isolated in 2000).

PQP repeats exhibits a unique PFGE type, arbitrarily designated as pattern B (Fig. 2a). Other isolates from the same outbreak that were submitted to PFGE ($n = 6$) exhibit the predominant pattern, A2. *Xba*I was found to be more discriminatory than *Spe*I, as patterns A1 and A2 could not be distinguished when examined with the restriction enzyme *Spe*I (Fig. 2b). The isolate that harbours the *Xba*I B pattern also produced a unique *Spe*I restriction pattern. As we only observed one B-type isolate, which contained eight PQP repeats, it remains to be seen whether the B-type strains will emerge in Finland and whether this PFGE pattern correlates with Prn type. However, there is no correlation between PFGE types A1 and A2 and *prn* sequences.

We do not know the importance of differences between groups A1 and A2 on the pathogenicity of *B. parapertussis* isolates. However, the same *Xba*I PFGE patterns were observed during a vaccine trial in 1992 and 1993 in Italy (Mastrantonio *et al.*, 1998); the PFGE patterns of the isolates did not correlate with duration of coughing or severity of illness. In Italy, both strain types were co-circulating at the time, but the A1 isolates were found to be limited to the northern regions of the country (Mastrantonio *et al.*, 1998). In Finland, it seems that type A2 has recently replaced type A1. However, to confirm this, historical Finnish *B. parapertussis* isolates would be needed. Unfortunately, Finnish *B. parapertussis* isolates collected before 1982 are not available.

In conclusion, analysis of the *B. parapertussis* isolates obtained from various communities in Finland during the past 20 years shows that the *B. parapertussis* population is very homogeneous, confirming the results of previous studies (Yuk *et al.*, 1998; Boursaux-Eude & Guiso, 2000). In this study, we showed that *B. parapertussis* Prn of seven of 76 isolates (9 %) had both non-silent and silent mutations and that one of the seven isolates had eight PQP amino acid repeats instead of nine in the second domain that contains repeated sequences (a domain that is very polymorphic in *B. bronchiseptica*, but not in *B. pertussis*). Several studies indicate that natural selection favours residue charge changes in the surface proteins of pathogens (Hughes, 1999), possibly allowing the pathogen to escape host defences. This might not be the case with *B. parapertussis* Prn, where Q311 can be changed to a similar amino acid, S. Our data confirm that repeated regions of *B. parapertussis* are very important for protein function, as they are conserved. It has been suggested that *B. pertussis* Prn variants have emerged as a result of vaccine-driven evolution, as strains used for whole-cell vaccines harbour a different Prn allele from that of most of the currently circulating *B. pertussis* strains (Mooi *et al.*, 1998). The low level of variation in *B. parapertussis* Prn could indicate that *B. parapertussis* is not under similar selection pressure and has therefore remained stable. However, PFGE analysis clearly shows that the *B. parapertussis* population that infects humans is highly clonal and, thus, differences seen in the level of antigenic variation probably reflect differences in the overall stability of these organisms, rather than different selection pressures. Currently, acellular vaccines that contain only a few *B. pertussis* antigens are replacing whole-cell vaccines in the industrialized world. Surveillance should be continued to address whether the vaccine change affects the incidence of infection by *B. parapertussis*.

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