Polymorphism in *Bordetella pertussis* Pertactin and Pertussis Toxin Virulence Factors in the United States, 1935–1999

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To elucidate the potential role of the etiologic agent in recent increases of pertussis incidence in the United States, we studied the polymorphism in pertactin and pertussis toxin, which are *Bordetella pertussis* proteins important for pathogenesis and immunity. We sequenced regions of their genes (*prn* and *ptx*) in 152 *B. pertussis* strains isolated from 1935 through 1999 and identified 2 *prn* sequences: *prn1* (old), observed continuously since 1935, and *prn2* (new), not recognized until 1981 but seen in 97% of tested isolates in 1999. There were 3 *ptx* S1 subunit sequences: *ptxS1D* (old) was identified in 3 strains (1935 and 1939); *ptxS1B* (old) represented 87% of the strains recovered during 1935–1974; and *ptxS1A* (new) was the most prevalent during 1975–1987 and 1989–1999 (64% and 78%, respectively). Potential association between vaccination and the observed shift from old to new types requires further study. Our results provide the basis for prospectively monitoring for changes among circulating *B. pertussis* that might have epidemiologic relevance.

*Bordetella pertussis*, the etiologic agent of pertussis, remains an important cause of morbidity, not only in developing countries but also in countries with high vaccination coverage [1–4]. Whole-cell pertussis vaccines (WCV) were introduced in the United States in the mid-1940s [1], and, in 1995–1996, the vaccine coverage with ≥3 doses of diphtheria/tetanus toxoids and WCV vaccine (DTP) was 95% among preschool-age children [5]. The WCVs were very effective in preventing serious pertussis but were also frequently associated with pain and swelling at the injection site, fever, and, rarely, serious adverse neurologic events, including seizures [6]. Acellular vaccines (ACV) were licensed in the United States in 1991 for booster doses and were approved for the 3-dose primary series in 1996 to minimize adverse reactions associated with WCV. These ACVs contain inactivated pertussis toxin and may also include ≥1 other bacterial components, such as filamentous hemagglutinin, pertactin, or fimbriae. The inclusion of pertussis toxin in all acellular vaccine formulations reflects its presumed importance in pertussis pathogenesis. This multimeric protein has been suggested to induce lymphocytosis by interfering with G protein signal transduction. Studies have demonstrated that pertactin con-
isolated in the United States over an extended period of time; to
assess potential temporal and geographic trends in these alleles;
and, because pulsed-field gel electrophoresis (PFGE) is currently
the most frequently used method for molecular subtyping of B.
pertussis strains, to evaluate any association between prn or ptxS1
alleles and PFGE profiles.

Material and Methods

Strains. A convenience sample of 152 US B. pertussis strains
isolated from 1935 through 1999 was selected from the Centers for
Disease Control and Prevention (CDC) and US Food and Drug
Administration (FDA) strain collections, to represent the widest
temporal and geographic distribution of available strains and the most
prevalent PFGE profiles. The number of strains from each time
period was as follows: 21 strains (1935–1974), 39 strains (1975–1987),
and 92 strains (1989–1999). A table that lists these strains is available
upon request. In addition, the strain used to prepare the Certiva
ACV (North American Vaccine, Columbia, MD) was tested. B. per-
tussis strains were grown for 4 days at 35°C on Bordet-Gengou agar
(Difco, Detroit) supplemented with 1% glycerol and 15% defibrinated
sheep blood or on charcoal agar (Oxoid, Hampshire, UK) supple-
mented with 10% defibrinated horse blood.

Chromosomal DNA preparation. Chromosomal DNA was iso-
lated with a QIAamp Tissue Kit (QIAGEN, Chatsworth, CA).
DNA sequencing was performed by polymerase chain reaction
(PCR) amplification of DNA, followed by direct sequencing of the
PCR products.

Pertactin. Two previously identified repetitive regions of the
prn gene, designated regions 1 and 2 [12], were chosen for sequenc-
ing. Region 1 was sequenced in all strains; because polymorphism
has been reported infrequently in region 2 [12, 14, 15], only ~20%
of strains were sequenced in this region. Previous studies identified
5 prn alleles, designated prn1–5 [12–15]. The prn primers used were
AF (forward) and BR (reverse), as described by Mooi et al. [12].
The prn gene sequence was amplified in 50 μL containing 1X SuperTaq buffer, 10% dimethyl sulfoxide, 200 μM of each deoxy-
nucleotide, 10 pmol each of primers AF and BR, and 1 U of SuperTaq polymerase (Sphaero-Q, Leiden, The Netherlands). Am-
plification was performed in a Hybaid Touch-Down PCR appa-
ratus (Hybaid Limited, Ashford, UK) or in a Geneamp 9600 PCR
System (Perkin-Elmer Applied Biosystems, Foster City, CA), using
the following program: 95°C for 3 min, then 2 cycles of 95°C for
15 s, 74°C for 30 s, and 72°C for 60 s; 2 cycles of 95°C for 15
s, 72°C for 30 s, and 72°C for 60 s; 2 cycles of 95°C for 15 s, 70°C
for 30 s, and 72°C for 60 s; 2 cycles of 95°C for 15 s, 68°C for 30
s, and 72°C for 60 s; 2 cycles of 95°C for 15 s, 66°C for 30 s, and
72°C for 60 s; and 2 cycles of 95°C for 15 s, 64°C for 30 s, and
72°C for 60 s. The amplification terminated with extension at 72°C
for 10 min.

DNA sequencing and analysis. PCR products were purified with
a QIAquick PCR purification kit (QIAGEN) and sequenced on both
strands with the primers used for amplification. Sequencing reactions
were carried out with an AB Big Dye Terminator kit (Perkin-Elmer
Applied Biosystems), and the products were analyzed on an AB 377
DNA Sequencer (Perkin-Elmer Applied Biosystems).

PFGE. PFGE was performed essentially as described by Hawes
et al. [16]. PFGE profiles were analyzed using Diversity Database
version 2.0 (BioRad, Hercules, CA).

Results

Pertactin. Analysis of 152 US B. pertussis strains revealed
2 pertactin sequences identical to prn1 and prn2, 2 of the 5
types previously reported in Europe [12–15]. In 1935–1974 and
in 1975–1987, the prn1 allele was identified in all 21 strains and
in 37 (95%) of 39 strains, respectively (table 1). During 1989–
1999, 64 (70%) of 92 strains had the prn2 allele.

S1 subunit of pertussis toxin. Three pertussis toxin alleles,
ptxS1A, ptxS1B, and ptxS1D, were identified in the US B. per-
tussis strains; all were seen previously in Europe and were initially
designated SIA, SIB, and SID. Three (14%) of the 21 strains
during 1935–1974 had the ptxS1D allele, which was seen only in
strains recovered before 1946 (table 1). Type PtxS1A first ap-
ppeared in the study strains in 1970. The other 17 strains from this
time period were type PtxS1B. During 1975–1987, type PtxS1A
comprised 25 (64%) of 39 strains, whereas the frequency of strains
with the ptxS1A allele increased to 72 (78%) of 92 strains during
1989–1999. With a single exception, all strains isolated during
1996–1999 were type PtxS1A (57 [98%] of 58 strains).

Combined pertactin and pertussis toxin types. Because 2 prn
alleles and 3 ptxS1 alleles were identified, a total of 6 theoretical
combinations exist. However, only 5 were observed in the US B.
pertussis strains. The majority of the study strains (n = 118) had either both “old” or both “new” alleles, whereas only 34 were “transitional.” The terms “old,” “new,” and “transi-
tional” refer to the temporal trends observed in The Nether-
lands.

Table 1. Percentage of pertactin and pertussis toxin S1 sequence
types of 152 US Bordetella pertussis isolates, by year of isolation.

<table>
<thead>
<tr>
<th>Years isolated</th>
<th>No. of isolates</th>
<th>Pertactin</th>
<th>S1 subunit of pertussis toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1935–1974</td>
<td>21</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>1975–1987</td>
<td>39</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>1989–1999</td>
<td>92</td>
<td>30</td>
<td>70</td>
</tr>
<tr>
<td>Total</td>
<td>152</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
lands and the United States; they do not refer to time of genesis of these alleles.

As shown in figure 1, most strains isolated before 1993 were either type Prn1/PtxS1A or Prn1/PtxS1B. Strains with type Prn2/PtxS1A became more prevalent in 1993, and, in 1996–1999, comprised almost all (98%) of the strains tested. It is interesting that only 1 US B. pertussis strain was found with type Prn2/PtxS1B (Delaware, 1981). Fifty (33%) strains had the type combination Prn1/PtxS1B (i.e., both markers were defined as old; figure 2). These strains were seen throughout the United States during 1946–1994, but only a single strain of this type was tested over the past 5 years (New Jersey, 1997). Three additional strains had the allele combination in which both markers were also defined as old, but they differed in that the ptxS1 allele was ptxS1D. Two of these strains were isolated in Pennsylvania in 1935, and a single strain was isolated in Michigan in 1939.

Transitional allele combinations were identified, in which 1 of the markers was defined as old and the other as new. Thirty-three (22%) strains had a combination of an old prn (prn1) and a new ptxS1 gene (ptxS1A). They originated in 9 US states and, with a single exception (Arizona, 1998), were collected in 1978–1993. However, only a single strain (Delaware, 1981) had the reverse combination (i.e., a new prn [prn2] and an old ptxS1 gene [ptxS1B]). The timeline presented in figure 1 shows that the old and transitional allele combinations existed concurrently until 1993. Since then, strains in which both markers are defined as new (prn2/ptxS1A) were seen almost exclusively, and 65 (43%) of our strains were characterized by this combination: a single isolate from Washington, DC, dated back to 1983, whereas the remaining 64 were all isolated in 1993–1999 in 15 US states. This type comprised almost all (98%) of the tested strains isolated in the last 4 years.

PFGE. Forty PFGE profiles (figure 2) were identified in the US B. pertussis strains. Forty (26%) of these strains had profile CYXXI-010.

Alleles versus PFGE types. We compared prn and ptxS1 alleles with 5 PFGE profiles seen frequently in the United States (figure 3). Most strains of a particular PFGE profile also shared the same Prn type. This was also true for PtxS1 types, except for PFGE CYXXI-003 and PFGE CYXXI-006, which were comprised almost evenly of types PtxS1A and PtxS1B. All 10 strains with PFGE profile CYXXI-004 were type Prn1/PtxS1B, whereas 39 (98%) of 40 strains with PFGE profile CYXXI-010 were type Prn2/PtxS1A.

In a phylogenetic tree (figure 2) created using the unweighted,
pair-group method using arithmetic averages (UPGAMA) cluster method, all strains of type Prn2/PtxS1A clustered in a relatedness group, whereas strains of types Prn1/PtxS1A and Prn1/PtxS1B were distributed throughout the tree. Two of the most prevalent PFGE profiles (CYXXI-002 and CYXXI-010) were almost exclusively identified in strains with the prn2/p1txS1A combination. Profiles CYXXI-002 and CYXXI-010 were first identified in 1989 and 1983, respectively, and, from the very beginning, were associated only with this new allele combination. Two other prevalent PFGE profiles (CYXXI-004 and CYXXI-006) were identified in strains with the old allele combination (prn1/p1txS1B). It is of interest that all but 1 of the strains with these 2 PFGE profiles were only identified in 1989–1993 in Ohio. The PFGE profile CYXXI-003, identified in the US B. pertussis strains in 1978–1994, was not associated with a single allele, as seen in some other PFGE profiles. Three different allele combinations were seen among the 8 strains with this profile; half of the strains had the prn1/p1txS1B allele (old) combination, and the other half had transitional allele combinations (prn1/p1txS1A and prn2/p1txS1B).

Discussion

Countries with long-standing pertussis vaccination, such as the United States and The Netherlands, have recently been experiencing an increase in pertussis incidence. The highest number of cases in the United States since 1967 occurred in 1996, although vaccination coverage with ≥3 doses among US preschool-age children was 95% in 1995–1996 [1]. This increase may be the result of improved reporting or the expanded use of diagnostic serology (as in Massachusetts). However, it probably reflects the general trend during the 1990s: an increasing incidence in adolescents and adults, among whom immunity induced by vaccination has waned, coincident with consistent incidence among
Figure 3. Distribution of combined pertactin and pertussis toxin types by 5 pulsed-field gel electrophoresis (PFGE) profiles in US Bordetella pertussis strains, 1978–1999. Prn, pertactin; PtxS1, pertussis toxin S1 subunit.

children 1–4 years old [1]. In contrast, in the Netherlands, where immunization coverage is >90%, pertussis incidence increased primarily among children 1–4 years old in 1996. An investigation of the population structure of Dutch B. pertussis isolates demonstrated a divergence in the Ptx and Prn proteins between vaccine strains and clinical isolates. Reports of similar trends from Finland, Germany, and Italy [12–15] prompted us to address the dynamics of the US B. pertussis population.

We identified an apparent shift in the prevalence of Prn types over time comparable with that in The Netherlands [12], but, unlike in The Netherlands, where types Prn2 and Prn3 now comprise 90% of the strains, no US B. pertussis strains, to date, have been reported to contain the prn3 allele. Also, allele prn2 is seen much more frequently in recent US B. pertussis strains (98%) than in recent Dutch, Finnish, or Italian B. pertussis strains (36%, 72%, and 41%, respectively) [12, 14, 15]. Three ptxS1 subunit sequences identical to ptxS1A, ptxS1B, and ptxS1D, alleles previously seen in Europe [12–15], were identified: the new PtxS1A type that emerged during 1975–1987 was seen almost exclusively (98%) between 1996 and 1999, nearly achieving the prevalence (100%) reported in Finland and Italy [14, 15].

PFGE analysis of >1000 B. pertussis isolates from the CDC strain collection showed predominance of PFGE profiles CYXXI-010 and CYXXI-002 [16], which were identified in 40 and 12 strains, respectively, in this study, but no specific association of Prn or PtxS1 type with a particular PFGE profile was observed. However, 2 PFGE relatedness groups contained 42 of 53 strains that had the old allele combinations, as well as 26 of 34 strains with the transitional allele combinations. In contrast, all strains with the new allele combination clustered in a third PFGE relatedness group (figure 2).

Evaluating the dynamics of B. pertussis in the United States is critically dependent on evaluating isolates representing the natural geographic and temporal distribution of this population. We recognized 2 sources of strain sampling bias in our study. Because the test strains were limited to B. pertussis isolates maintained at clinical and public health laboratories, fewer strains collected before 1975 were available, and strains from 1993 were primarily associated with an outbreak in the Cincinnati metropolitan area [17]. Also, isolates cultured after 1989 were chosen for their PFGE pattern prevalence, as well as for their temporal and geographic diversity. The changes observed in the Prn and PtxS1 types in our study, however, appear to have been fundamental and consistent with trends in several European countries, which suggest that they were sufficiently pronounced to overcome any sampling bias.

Although both the Dutch and Finnish vaccines are composed of B. pertussis strains containing the same (old) alleles (ptxs1B, ptxs1D, and prn1), evaluation of the strains used to manufacture US vaccines was confounded, because the vaccines distributed currently and in the past were very diverse. Our analysis of the strain used to prepare the Certiva ACV (North American Vaccines) and reports on 2 other currently available ACVs (Infanrix; SmithKline Beecham Biologicals, Philadelphia; Tripedia; Aventis Pasteur, Swiftwater, PA) and previously used whole-cell US vaccines derived from the B. pertussis To-
hama strain show that they are all of the old combination of prn1/ptxsI1B alleles [18]. We have been unable to obtain a culture or relevant sequence information for the strain used to prepare the Acel-Imune vaccine (Wyeth-Lederle, Philadelphia). In contrast to the vaccine strains, the great majority of B. pertussis strains currently circulating in the United States have the new allele combination, and similar divergence was previously recognized in Finland, Italy, and The Netherlands [12, 14]. Together with the failure to show variation in gene sequence types among isolates from the years before the introduction of wholecell vaccines in several countries, these observations imply that this divergence may be driven by selective pressure from vaccination. In contrast, the population structure of B. pertussis in Italy was similar to that in The Netherlands, although vaccination coverage was comparatively low, which suggests that the recent Prn and PtxS1 types diverged from the vaccine type in the absence of strong vaccine selection [15].

In the United States, however, the epidemiology of pertussis may be confounded by additional factors. Changes in vaccines instituted by manufacturers to decrease reactogenicity could potentially impact the performance of the vaccines [9]. Furthermore, insufficient data exist to characterize the number of vaccine doses administered by manufacturer (prospective enhanced surveillance programs that address this issue are ongoing). Consequently, changes in vaccines, such as agglutinin composition, cannot be associated with changes in incidence. Finally, ≥4 WCVs have been available in the United States, and, since 1997, 4 ACVs have been approved that differ significantly in their compositions, concentrations of individual components, and efficacies [19].

As pertussis incidence among children has stabilized in recent years, it appears that the effects of the antigenic shifts in Prn and PtxS1 on vaccine efficacy probably have been minimal. Conversely, finding old vaccine-type Prn variants in lower frequencies among vaccinated people compared to unvaccinated people would be expected if the vaccine protects differentially against strains with distinct Prn types [12]. In this regard, it is notable that the Prn3 type has dramatically increased in frequency, from 0% to 20% in the few years since the introduction of ACVs in Sweden (H. Hallander, personal communication) with concomitant decreases in Prn1 (old). In addition, a dominant role recently has been suggested for pertactin and pertussis toxin in protection [18], and we have shown in an animal model that the variable region of Prn harbors an immunodominant epitope that provides protection (authors’ unpublished data) and that variation in Prn affects vaccine efficacy (authors’ unpublished data). Conversely, Hausman and Burns [20] suggested that significant amino acid changes could occur in the pertussis toxin sequence without affecting functional antibody responses. Further studies to evaluate the distribution of Prn and PtxS1 types in unvaccinated and vaccinated populations by age and to compare the epitope specificity of pertussis- and vaccine-induced antibodies should be considered, to elucidate the effects of polymorphisms in Prn and PtxS1 in the pertussis epidemiology in the United States.

Our results are serving as the comparative basis for prospectively monitoring the variations of the Prn and PtxS1 among US isolates, through the Enhanced Pertussis Surveillance Program recently initiated by the CDC, with investigators in the state health departments in Arizona, Georgia, Illinois, Massachusetts, Minnesota, and New York. This program serves as a significant source of prospective isolates and related clinical and epidemiologic information for monitoring the B. pertussis population structure for the impact of epidemiologically relevant changes in vaccination coverage, type of vaccine used, severity of the disease, and trends in the disease incidence.

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