Molecular Characterization of Two *Bordetella bronchiseptica* Strains Isolated from Children with Coughs

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During a surveillance program associated with the Italian clinical trial for the evaluation of new acellular pertussis vaccines, two bacterial isolates were obtained in cultures of samples from immunocompetent infants who had episodes of cough. Both clinical isolates were identified as *Bordetella bronchiseptica* by biochemical criteria, although both strains agglutinated with antiserum specific for *Bordetella parapertussis*, suggesting that the strains exhibited some characteristics of both *B. bronchiseptica* and *B. parapertussis*. Both children from whom these strains were isolated exhibited an increase in serum antibody titer to pertussis toxin (PT), a protein that is produced by *Bordetella pertussis* but that is not thought to be produced by *B. bronchiseptica*. We therefore examined whether the clinical isolates were capable of producing PT. Neither strain produced PT under laboratory conditions, although both strains appeared to contain a portion of the *ptx* region that encodes the structural subunits of PT. In order to determine whether the *ptx* genes may encode functional proteins, we inserted an active promoter directly upstream of the *ptx* region of one of these strains. Biologically active PT was produced, suggesting that this strain contains the genetic information necessary to encode an active PT molecule. Sequence analysis of the *ptx* promoter region of both strains indicated that, while they shared homology with the *B. bronchiseptica* ATCC 4617 sequence, they contained certain sequence motifs that are characteristic of *B. parapertussis* and certain motifs that are characteristic of *B. pertussis*. Taken together, these findings suggest that variant strains of *B. bronchiseptica* exist and might be capable of causing significant illness in humans.

*Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* are closely related species that are each capable of producing upper respiratory tract disease in humans. *B. pertussis* is the etiologic agent of whooping cough, which can be especially severe in infants. *B. parapertussis* is usually responsible for milder forms of disease in humans, although parapertussis can also present in a severe form (12). *B. bronchiseptica* primarily produces disease in mammals other than humans. This organism causes disease in both domestic and wild animals including dogs, swine, and rabbits (35). On very rare occasions, however, *B. bronchiseptica* has been known to infect humans. Investigators have reported that *B. bronchiseptica* is associated with upper respiratory tract infections in animal caretakers (9), pertussis-like illnesses in children (24), subacute bacterial endocarditis (17), and infections in immunocompromised patients (5, 9, 29, 30, 35).

These three *Bordetella* spp. share many of the same virulence factors, including toxins such as adenylate cyclase toxin, dermonecrotic toxin, and tracheal cytotoxin (7), as well as adhesins such as filamentous hemagglutinin (FHA) and pertactin (2, 23). One notable exception is the virulence factor pertussis toxin (PT), which is thought to be produced only by *B. pertussis*. While *B. parapertussis* and at least certain strains of *B. bronchiseptica* contain the *ptx* genes that encode the structural subunits for PT (1, 26), the genes are not expressed under conditions that have been examined so far, presumably due to the presence of a cluster of mutations in the promoter region of the *ptx* operons that render those promoters silent (1).

In this report, we describe the isolation and identification of two variant strains of *B. bronchiseptica*. These strains were isolated from two children with episodes of cough who were participating in the clinical trial for the evaluation of acellular pertussis vaccines conducted in Italy. During the course of their illnesses, although culture and PCR of nasopharyngeal aspirates did not reveal the presence of *B. pertussis*, both children showed an increase in serum antibody titers for FHA and, surprisingly, PT. Thus, these strains are interesting in two aspects in that they were isolated from immunocompetent children, instead of one of the more common animal hosts for this pathogen, and they were isolated from children who exhibited increases in antibodies to PT during the course of their illnesses.

Despite the wide host range of *B. bronchiseptica* and despite the fact that many aspects of its biology have been studied, few reports have been written concerning the nature and extent of genetic variation in natural populations. The isolation of two *B. bronchiseptica* strains from children who exhibited increases in antibody titers to PT during the course of their illnesses prompted us to examine the phenotypic and genotypic differences between these two clinical isolates and other *B. bronchiseptica* strains.

**MATERIALS AND METHODS**

Patients and sampling. Nasopharyngeal aspirates (NPAs) and capillary blood samples (obtained 6 to 8 weeks apart) were collected by established procedures (10) from two infants participating in the Italian clinical trial for the efficacy of acellular pertussis vaccines.

Clinical and epidemiological data for each child were recorded. Both infants lived in Piemonte, one of the four regions involved in the clinical trial, but in two
different locations. The first infant, code 03145, coughed for 15 days without paroxysms at the age of 18 months, 10 months after having completed the vaccination cycle with three doses of diphtheria and tetanus toxoids and acellular pertussis vaccine manufactured by Chiron-Biocine (Siena, Italy). At the onset of the cough he was treated with azithromycin for 2 days and amoxicillin for 7 days. The second infant, code 15374, had 19 days of paroxysmal cough at the age of 14 months, 9 months after having completed the vaccination cycle with three doses of diphtheria and tetanus toxoids and acellular pertussis vaccine manufactured by SmithKline Beecham Biologicals (Rixensart, Belgium). Starting at the onset of the cough, the child was treated for 10 days with rokitamycin. This infant was admitted to the hospital because of a high temperature 1 day after the onset of cough and remained in the hospital for 8 days; the diagnosis was pertussis.

No immunocompromised status and no household contacts suffering from pertussis was recorded for either of the infants. The NPAs were collected 13 days after the onset of cough for study infant 03145 and 12 days after the onset of cough for the second infant, infant 15374. No animal contacts were reported for either infant.

Primary isolation and identification of Bordetella spp. For primary isolation from NPAs, bacteria were grown on charcoal agar plates supplemented with cephalin (20 µg/ml; Unipath, Milan, Italy), incubated at 37°C in a moist atmosphere, and observed for up to 7 days. All suspected colonies were identified by biochemical tests including tests for oxidase and urease production and mobility, and they were evaluated by the API 20 NE system (bioMérieux, Marcy l’Etoile, France) testing for agglutination with antisera for B. pertussis and B. parapertussis (Murex Diagnostics, Dartford, England). For confirmation by PCR, Bordetella strains which had grown for 48 h on charcoal agar plates were suspended in 1 ml of distilled water, heated for 10 min at 100°C, and diluted to a concentration of 10^2 colony-forming units/mL in PBS.

Description of bacterial strains. The following strains were used as reference strains: the American Type Culture Collection (ATCC) strains B. pertussis ATCC 9797 (18-32D), B. parapertussis ATCC 9305, and B. bronchiseptica ATCC 4617. B. pertussis RB50 was obtained from Jeff Miller (University of California, Los Angeles) and has been described previously (4). B. parapertussis 10978 was obtained from James Cherry (University of California, Los Angeles) (11).

PCR for strain confirmation. Two oligonucleotide primer pairs from the sequence of the 465-bp region were selected for amplification of the ptx and ptp region numbering as described in Nicosia et al. (31), and primers PtxM and PtxN flank positions 745 and 1035. The two sets of primers define two amplification products of 406 and 346 bp, respectively (32). Both PCR products are detected when 10^5 and 10^5 CFU/mL of bacteria are used as the template.

PCR with GAPDH. As with other NPAs collected during the trial, PCR was performed to detect B. pertussis DNA by using the procedure previously described by Manstrantonio et al. (27). Two primers derived from the PT promoter region were used to amplify a 191-bp PCR product specific for B. pertussis. To investigate the presence of B. parapertussis DNA, primers BPP1 and BPP2 were used to amplify a 196-bp fragment. Both PCR products are detected when either B. parapertussis or B. bronchiseptica DNA is used as the template. PCR analysis and detection of PCR products were carried out as described previously (32).

DNA analysis of clinical isolates. Identification of clinical isolates. Between April and May 1994, two bacterial strains were isolated from two infants with suspected pertussis participating in the clinical trial for the evaluation of new acellular pertussis vaccines conducted in Italy (10). The bacteria were grown on buffered charcoal-yeast extract-Genger agar, which showed the presence of small hemolytic colonies composed of gram-negative rods. Both clinical isolates were identified as variants of B. bronchiseptica on the basis of biochemical tests as well as use of the PCR, as outlined below. Upon further passage, there appeared a second, distinctly

RESULTS

Identification of clinical isolates. Between April and May 1994, two bacterial strains were isolated from two infants with suspected pertussis participating in the clinical trial for the evaluation of new acellular pertussis vaccines conducted in Italy (10). The bacteria were grown on buffered charcoal-yeast extract-Genger agar, which showed the presence of small hemolytic colonies composed of gram-negative rods. Both clinical isolates were identified as variants of B. bronchiseptica on the basis of biochemical tests as well as use of the PCR, as outlined below. Upon further passage, there appeared a second, distinctly...
larger colony phenotype which was nonhemolytic on Bordet-Gengou agar. These larger colonies were found to be the modulated form of the organism in which virulence genes controlled by the bvg locus (15, 20, 34) were no longer expressed. Therefore, care was taken to isolate the small colonies and to use only those colonies for further analyses.

The two clinical isolates were urease positive, oxidase positive, and motile. B. bronchiseptica strains are motile, whereas B. pertussis and B. parapertussis are nonmotile (35). Analysis of the two clinical isolates with the commercial API 20 NE system yielded, for both strains, the numerical code 1200067, which was compatible with the identification of B. bronchiseptica (percent identification, 95.6).

The two strains did not agglutinate with B. pertussis antisera but showed a strong reactivity with B. parapertussis antisera. In contrast, B. bronchiseptica ATCC 4617 did not agglutinate with either antisera.

Identification of strains was confirmed by PCR, revealing on the gel only the presence of the 346-bp amplified product, indicative of B. parapertussis or B. bronchiseptica DNA. Attempts to identify B. pertussis or B. parapertussis directly from NPAs by PCR did not yield positive results.

Characterization of the B. bronchiseptica isolates. The macrorestriction fingerprinting by PFGE performed with the two B. bronchiseptica isolates showed different patterns. DNAs of the B. bronchiseptica isolates from infants 15374 and 03145 (the DNAs had been digested with XbaI) exhibited 11 to 13 bands in the 90- to 400-kb range, along with multiple small bands not sufficiently resolved around 97 kb. Figure 1 shows the restriction profiles of the two isolates compared with that of B. bronchiseptica ATCC 4617. The DNA patterns differed among all three strains. Differentiation between B. bronchiseptica ATCC 4617 and clinical B. bronchiseptica isolates was based on the absence of a band corresponding to a molecular size of about 350 kb and on the presence of only one band at 161 kb instead of the double band visible for the reference strain. Moreover, the B. bronchiseptica strain from infant 15374 showed a new band corresponding to 240 kb. The DNAs were then digested with SpeI, using the same conditions, and each of the isolates exhibited different patterns (data not shown).

No differences in behavior were detected in the antibiotic susceptibility test between the two B. bronchiseptica isolates and B. bronchiseptica ATCC 4617. As determined by the disk agar diffusion method, all the strains were sensitive to amikacin, imipenem, gentamicin, piperacillin, and sulfamethoxazole-trimethoprim and were resistant to ampicillin, cefazidime, and cephaloridine.

The clinical isolates were examined for the ability to produce virulence factors that are found in the virulent phase of the organism (Bvg+ phase) or to produce flagellin, which is produced in the Bvg− phase by motile strains of B. bronchiseptica. As in the case of the control strain B. bronchiseptica RB50, both clinical isolates produced adenylate cyclase toxin as well as FHA in the Bvg− phase and flagellin in the Bvg+ phase (Fig. 2). In Fig. 2, multiple bands are observed for both adenylate cyclase toxin and FHA. The presence of multiple bands is likely due to the fact that both of these proteins are easily proteolyzed, generating lower-molecular-weight forms.

Analysis of the ptx locus of the clinical isolates. Table 1 shows the serology results obtained by ELISA and the CHO cell assay for infants 15374 and 03145. For both infants, a 100% or more increase in IgG titer to PT and FHA was evident while a positive result for IgA was obtained only against FHA. Positive results were observed also for PT neutralizing antibody on CHO cells, with at least a fourfold increase in the neutralizing antibody titers between the acute- and convalescent-phase sera.

The serology results were surprising since B. bronchiseptica

![FIG. 1. PFGE patterns of Bordetella chromosomal DNA digested with the restriction enzyme XbaI. The results of macrorestriction analysis are shown for DNAs of B. bronchiseptica ATCC 4617 (lane 1), the B. bronchiseptica strain from infant 03145 (lane 2), and the B. bronchiseptica strain from infant 15374 (lane 3). Lane M, Lambda Ladder molecular size marker for PFGE (New England Biolabs). Fragment sizes are indicated on the left (in kilobases).](http://jcm.asm.org/)

![FIG. 2. Immunoblot analysis for the production of adenylate cyclase (A), FHA (B), and flagellin (C) by clinical B. bronchiseptica isolates. Cell extracts were prepared from the B. bronchiseptica strain from infant 03145 (lanes 1 and 2), the B. bronchiseptica strain from infant 15374 (lanes 3 and 4), B. bronchiseptica RB50 (lanes 5 and 6), and B. parapertussis 10978 (lanes 7 and 8) which had been grown in either the presence (lanes 1, 3, 5, and 7) or the absence (lanes 2, 4, 6, and 8) of MgSO4 and nicotinic acid. (A) Lanes 1 and 3 contain immunoblot artifacts that do not represent protein bands.](http://jcm.asm.org/)
strains are not thought to produce PT. Therefore, we investigated whether these strains were capable of producing PT. We were unable to detect the production of PT by these strains either using immunoblot analysis or analyzing the ability of culture supernatants to cluster CHO cells, a sensitive assay for PT.

Since we were unable to detect the production of PT, we next examined whether these strains contain the genes encoding this toxin and, if so, whether the genes would encode an active toxin. While many *B. bronchiseptica* strains contain the genes for PT, some strains do not (26). PCR analysis had suggested that both strains contained at least a portion of the *ptx* region since we were able to amplify a region corresponding to nucleotides 749 to 1053 of the *ptx* locus in the strains. We examined the *B. bronchiseptica* strain from infant 03145 in some detail to determine whether this strain contained the entire *ptx* region and, if so, whether the genes would encode a functional toxin. In order to do this, we replaced the *ptx* promoter region and a portion of the S1 subunit of this strain with the corresponding region from *B. pertussis* using homologous recombination, as described previously (11). Since the *ptx* promoter from *B. pertussis* is active under normal laboratory conditions, replacement of the promoter of the *B. bronchiseptica* strain from infant 03145 with the *B. pertussis* promoter should result in active transcription of the *ptx* locus of this strain. As shown in Fig. 3, the engineered strain which contained the active promoter did produce an S2 subunit and an S4 subunit which resembled the corresponding subunits from *B. pertussis*. Moreover, analysis of the culture supernatant from this engineered strain showed that it contained active PT, as determined by the CHO cell assay. Dilutions of the supernatant as high as 1:128 were capable of clustering CHO cells, demonstrating the presence of active PT. Thus, this strain contains all *ptx* genes needed for the production of an active toxin, but apparently lacks a promoter that is active under normal laboratory conditions.

We examined the sequences of the *ptx* promoter regions of the strains from infants 03145 and 15374 in order to determine whether they differed from the published sequence of the *B. bronchiseptica* *ptx* promoter region. Both clinical isolates were found to have identical sequences (Fig. 4). The sequences, however, differed in certain respects from the published sequence of this region for *B. bronchiseptica* ATCC 4617 (1). At four positions, nucleotides 339, 434, 438, and 479, the sequence resembled that of *B. pertussis* rather than that of *B. bronchiseptica*. Interestingly, at four positions (nucleotides 349, 393, 427, and 506), the sequence resembled that of *B. parapertussis* rather than that of either *B. bronchiseptica* or *B. pertussis*. Finally, three new changes not seen in the other *Bordetella* spp. occurred at positions 340, 471, and 484.

**DISCUSSION**

In the present report, we provide information about two variant strains of *B. bronchiseptica* isolated from the NPAs of two infants during the follow-up of suspected cases of pertussis in the Italian clinical trial for the evaluation of new acellular pertussis vaccines (10). The isolation of *B. bronchiseptica* from the two infants was interesting since *B. bronchiseptica* is rarely isolated from immunocompetent humans. Both isolates showed many morphological and biochemical properties characteristic of *B. bronchiseptica* strains; however, they were not typical *B. bronchiseptica* strains since they both cross-agglutinated with *B. parapertussis* antiserum. Moreover, sequence analysis of the *ptx* promoter region of these strains indicated that, while their sequences shared homology with the *B. bronchiseptica* ATCC 4617 sequence, their sequences were not identical to that sequence and contained certain sequence motifs that are characteristic of *B. parapertussis* and certain motifs characteristic of *B. pertussis* rather than *B. bronchiseptica* ATCC 4617. These results would suggest that these strains are variants of *B. bronchiseptica* that share certain characteristics with other *Bordetella* spp. These findings are of interest since they suggest that a broader spectrum of bordetellae exists than was previously thought and that these “intermediate strains” may be able to cause significant illness in humans. Future studies of these strains will be needed to determine whether the host specificity of the two clinical isolates is typical of *B. bronchiseptica* or whether these strains may represent *B. bronchiseptica* strains that have adapted to a human host. Moreover, careful surveillance of strains isolated from patients affected by whooping cough-like disease should be continued to monitor the existence and emergence of variants of bordetellae that might be capable of causing disease in humans.

Although neither culture nor PCR with aspirates revealed the presence of *B. pertussis*, both infants exhibited a significant increase in PT neutralizing antibodies and IgG to PT between the acute- and convalescent-phase sera. While neither *B. bronchiseptica* variant was capable of producing PT under the normal laboratory conditions examined, both contained at least a portion of the *ptx* locus. Detailed analysis of the *ptx* region of the *B. bronchiseptica* strain from infant 03145 revealed that this strain contained all *ptx* genes essential for the production of an active toxin, although under normal laboratory conditions the promoter does not seem to be active.

Interestingly, both children from whom these strains were isolated had been vaccinated with acellular pertussis vaccines that each contained inactivated PT as one of their major com-

**TABLE 1. Serology results between acute- and convalescent-phase serum samples by ELISA and CHO cell assay**

<table>
<thead>
<tr>
<th>Patient code</th>
<th>Sample</th>
<th>Antibody titers (EUs)</th>
<th>PT neutralizing antibody titer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IgG</td>
<td>IgA</td>
</tr>
<tr>
<td>03145</td>
<td>Acute phase</td>
<td>41</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Convalescent phase</td>
<td>240</td>
<td>2</td>
</tr>
<tr>
<td>15374</td>
<td>Acute phase</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Convalescent phase</td>
<td>30</td>
<td>4</td>
</tr>
</tbody>
</table>

* Values are reciprocals of end dilutions.
ponents. Thus, both children likely were immunologically primed with this antigen. Infection of the children with bacteria that transiently produce even small quantities of PT might not be sufficient to boost their antibody responses during the course of their illness, although such quantities of PT might not be able to elicit a robust primary antibody response.

Recently, several strains of \textit{B. parapertussis} were isolated from children who had a small but significant antibody rise to PT during the course of their illness (11). Like \textit{B. bronchiseptica}, \textit{B. parapertussis} is not thought to produce PT. These clinical isolates of \textit{B. parapertussis} were shown to contain \textit{ptx} genes that encode a functional toxin, although production of PT was not detected when these strains were examined in the laboratory. The present study reports very similar findings for two variant strains of \textit{B. bronchiseptica}. Taken as a whole, these findings bring up the intriguing question of whether the \textit{ptx} genes of \textit{B. bronchiseptica} and \textit{B. parapertussis} might be expressed under certain, as yet unknown, conditions.

Recently, others have reported the finding that certain bacterial genes are expressed only during infection of the host (3). Further studies are needed to determine whether the \textit{ptx} genes of \textit{B. bronchiseptica} and \textit{B. parapertussis} are expressed transiently during the infection process.

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