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 antisera 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 20 μg/ml of pertussis vaccine manufactured by Chiron-Biocine (Siena, Italy). At the onset of the cough, the child was treated for 10 days with rokitamycin. The second infant, code 15374, had 19 days of paroxysmal cough at the age of 18 months, 10 months after having completed the vaccination cycle with 3 μg of PT. A standardized enzyme-linked immunoabsorbent assay (ELISA) was used to detect antibodies to PT. The interval between the onset of the cough and the child was treated for 10 days with rokitamycin.

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 35°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 motility, and they were evaluated by the API 20 NE system (bioMerieux, Marcy l’Etoile, France) testing for agglutination in antiserum 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 10^3 colony-forming units per ml.

PCR for strain confirmation. Two oligonucleotide primer pairs from the sequence of the ptx promoter region were selected for amplification of the ptx promoter and a S4 subunit of PT. The precipitates were suspended in sodium dodecyl sulfate (SDS) sample buffer containing the primer-S1 region of B. pertussis (ps15184) into the chromosome of B. bronchiseptica 03145 essentially as described previously (11).

Electrophoresis and immunoblotting analysis of B. bronchiseptica cell extracts and cellular fractions. Cell extracts were prepared by suspending bacteria, which had grown for 5 days on Bordet-Gengou agar (in the presence or the absence of 20 mM MgSO₄ and nicotinic acid), in phosphate-buffered saline to an A₅₀₀ of 2. MgSO₄ and nicotinic acid modulate the cells from the Bvg phase to the Bvg phase such that they do not express genes regulated by the Bvg (Bordetella virulence genes) locus. The bacterial growth from the NPAs on Bordet-Gengou agar showed the presence of small hemolytic colonies at the age of 120 h. The gel was stained with ethidium bromide (0.5 μg/ml) and was photographed under UV light.

Antimicrobial susceptibility assay. Antimicrobial susceptibility testing was performed by the disk agar diffusion method with Mueller-Hinton agar (Unipath) and eight different antimicrobial agents: ampicillin, amikacin, cephaloridine, imipenem, piperacillin, and sulfamethoxazole-trimethoprim. Staphylococcus aureus ATCC 29233 was used as a quality control strain; B. bronchiseptica ATCC 4617 was used to compare the performance of B. bronchiseptica isolates. The interpretative criteria were those recommended by the National Committee for Clinical Laboratory Standards (NCCLS) for rapidly growing aerobic organisms.

Construction of B. bronchiseptica 03145:psSZH8. The ptx promoter and a portion of the gene for the S1 subunit of the PT of B. bronchiseptica 03145 were replaced by the corresponding region of B. pertussis by introducing a plasmid containing the promoter-S1 region of B. pertussis-ps15184 into the chromosome of B. bronchiseptica 03145 essentially as described previously (11).

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 grew with the Bordet-Gengou agar 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

Boehringer). Lysis buffer was substituted with 10 ml of ESP buffer (0.5 M EDTA [pH 8.0], 1% N-lauroylsarcosine, protease K [2 mg/ml; Sigma]), and the plugs were incubated for 48 h at 50°C. The blocks were then washed three times for at least 15 min each time with 20 ml of TE buffer. Restriction digestion was performed (England Biolabs) in a final volume of 200 μl per plug, and the plug mold was incubated overnight at 37°C. Pulsed-field gel electrophoresis (PFGE) was performed with a CHEF Mapper II (Bio-Rad). A 1% agarose gel (15 cm) was prepared in 0.5 X Tris-borate-EDTA buffer (TBE). The agarose blocks were loaded with wells along with lambda ladder PFGE Markers (New England Biolabs). Electrophoresis was performed with 5- to 45-s ramping times for 27 h at a field strength of 6.0 V/cm, and an included angle of 120°. The gel was stained with ethidium bromide (0.5 μg/ml) and was photographed under UV light.

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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* antiserum but showed a strong reactivity with *B. parapertussis* antiserum. In contrast, *B. bronchiseptica* ATCC 4617 did not agglutinate with either antiserum.

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 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 between the acute- and convalescent-phase serum samples, 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*
straining 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 bordetelae 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 bordetelae 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-

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**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>PT IgG</td>
<td>PT 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.

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**FIG. 3.** Immunoblot analysis of PT subunits. Cell extracts of B. bronchiseptica 03145::pSZH8 (lane 1) and B. pertussis 536 (lane 2) were examined for the presence of the S2 subunit of PT (A) and the S4 subunit of PT (B) by using subunit-specific monoclonal antibodies and immunoblot analysis as described in Materials and Methods.
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 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. Recent studies are needed to determine whether the pertussis toxin genes of Bordetella parapertussis might be expressed under certain, as yet unknown, conditions.

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