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Recovery of *Bordetella holmesii* from Patients with Pertussis-Like Symptoms: Use of Pulsed-Field Gel Electrophoresis To Characterize Circulating Strains

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A 4-year retrospective study showing that we isolated *Bordetella holmesii*, but not *Bordetella pertussis*, from patients with pertussis-like symptoms was performed. From 1995 through 1998, we isolated *B. holmesii* from 32 nasopharyngeal specimens that had been submitted from patients suspected of having pertussis. Previously, *B. holmesii* had been associated mainly with septicemia and was not thought to be associated with respiratory illness. A study was undertaken to describe the characteristics of the *B. holmesii* isolates recovered and why we were successful in detecting the organism in nasopharyngeal specimens. *B. holmesii* isolates were characterized for drug sensitivities and for genetic relatedness by pulsed-field gel electrophoresis (PFGE). These isolates, an additional strain of *B. holmesii* isolated from a blood culture and previously confirmed by the Centers for Disease Control and Prevention, Atlanta, Ga., and 14 other clinical isolates of *Bordetella* spp., including 4 of *B. bronchiseptica*, 5 of *B. parapertussis*, and 5 of *B. pertussis*, were studied. They were all separately inoculated on three Bordet Gengou (BG) selective media containing either 0.625 μg of oxacillin per ml, 40 μg of cephalexin per ml, or 2.5 μg of methicillin per ml, on BG agar with no antibiotic (control), and on charcoal agar (CA) with and without 40 μg of cephalexin per ml. We found that cephalexin, the antibiotic commonly incorporated in both CA and BG agar for the recovery of *Bordetella* spp., is inhibitory to the growth of *B. holmesii*. In addition, the genotypic analysis of the 32 *B. holmesii* isolates by PFGE following restriction with *Xba*I and *Spe*I identified the dominant strains circulating during the study period.

Bordetella holmesii is a species representative of a recently described group of bacteria formerly designated as nonoxidizer group 2 by the Centers for Disease Control and Prevention, Atlanta, Ga. (CDC) (9). These organisms are small, oxidase-negative, asaccharolytic, gram-negative coccobacilli that produce a brown soluble pigment (9). Since the organism was first described in 1995, a total of 19 patients infected with *B. holmesii* have been reported in the literature (4, 5, 8, 9). *B. holmesii* has been recovered from patients with several debilitating conditions, including Hodgkin's lymphoma, sickle-cell anemia, pulmonary disease, and asplenia (4, 8, 9). All the reported patients were septic, except one patient with pulmonary failure (8). These observations have led some authors to report that, unlike *Bordetella pertussis*, *B. holmesii* does not cause respiratory disease (6, 9).

However, we isolated *B. holmesii*, but not *B. pertussis*, from 32 nasopharyngeal specimens collected from 1995 to 1998 from patients with pertussis-like symptoms and from a blood specimen. Yih et al. (11) published a report of the epidemiologies of these patients.

Weyant et al. (9) described a biochemical schema to differentiate *B. holmesii* from the other *Bordetella* spp. and from other phenotypically similar organisms. *B. holmesii* can be distinguished from *B. pertussis*, *Bordetella bronchiseptica*, and *Bordetella avium* by its lack of oxidase activity and by its production of a brown soluble pigment on 0.1% tyrosine; a urease test differentiates it from *Bordetella parapertussis*. A DNA transformation assay (3, 10) distinguishes *B. holmesii* from the phenotypically similar nonhemolytic, asaccharolytic species *Acinetobacter calcoaceticus*. This study was designed to answer two questions. First, why were we able to recover *B. holmesii* from nasopharyngeal specimens in our laboratory when others have not? Second, can pulsed-field gel electrophoresis (PFGE) be effectively used as an epidemiological tool for investigating associations among cases of *B. holmesii* infection?

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MATERIALS AND METHODS

Strains. Thirty-two *B. holmesii* isolates from the nasopharynx and one isolate (95R0375) from blood which had been previously confirmed as *B. holmesii* at the CDC were included in the study. In addition, four isolates of *B. bronchiseptica*, five isolates of *B. parapertussis*, and five isolates of *B. pertussis* previously recovered and identified at the Massachusetts State Laboratory Institute (SLI) were used. *Acinetobacter* transformation strain KC1842 was obtained from R. Weyant, Special Bacteriology Reference Laboratory, Division of Bacterial and Mycotic Diseases, CDC, and the positive control strain used for the transformation assay was *Acinetobacter* sp. ATCC 19606.

Method for isolating strains used. Our laboratory provides a pertussis culture kit to the healthcare community. Three of the items included in the kit are a sterile nasopharyngeal calgiswab, a tube containing 0.5 ml of 1% casein hydrolysate (CAS) holding medium, and a charcoal agar with cephalexin (CA-LEX) transport agar slant. The clinician is directed to obtain a nasopharyngeal specimen from the patient by using the sterile swab provided and, after it has been taken, to immediately place the swab in the 1% CAS holding medium, in which it may remain for up to 30 min. The clinician is then directed to remove the swab from the 1% CAS, to roll it over the CA-LEX transport agar slant provided, and to return the CA-LEX transport slant to the SLI for testing. It is recommended that the culture kit be transported by same-day courier, although delivery by an overnight transport service on cold packs is acceptable. Cultures that cannot be processed on the day of receipt at the SLI are refrigerated until they are processed. When received at the laboratory, each nasopharyngeal specimen previously streaked onto CA-LEX slants by the health care providers is further subcultured onto a Bordet-Gengou agar plate containing methicillin (BG-MET plate). To do this, a sterile swab is saturated with fresh sterile 1% CAS and swabbed over the slant of the CA-LEX transport agar. It is then streaked onto a BG-MET plate and cross-hatched to obtain isolated colonies. (A wet swab is used to transfer the growth from the slanted culture in order to replenish moisture that may have been lost in transit.) Both the CA-LEX slant and the BG-MET plate are incubated and examined for *Bordetella* spp.

Media and biochemical tests. CA-LEX (charcoal agar with 10% horse blood plus 40 μg of cephalexin per ml) was prepared full strength according to the manufacturer's instructions. Ten milliliters of the sterile molten medium was

TABLE 1. Phenotypic characteristics of *B. holmesii* isolates, other representative *Bordetella* spp., and a nonhemolytic asaccharolytic *Acinetobacter*

Test[a]	% of positive isolates (no. of isolates with delayed reactions)				
	B. holmesii (n = 32 [nasopharyngeal])[b]	*B. pertussis* (n = 5)	*B. parapertussis* (n = 5)	*B. bronchiseptica* (n = 4)	Asaccharolytic nonhemolytic *Acinetobacter*[c]
Fluorescent-antibody test with:					
B. pertussis conjugate	0	100	0	0	NT[d]
B. parapertussis conjugate	0	0	100	0	NT
Beta-like hemolysis	0	100[e]	100	50	22
Acid produced from dextrose	0	0	0	0	0
Growth on MacConkey agar	100[f]	0	100	100	73 (4)
Oxidase	0	100	0	100	0.8[e]
Urea hydrolysis	0	0	100	100	5 (14)
Motility	0	0	0	100	0
Brown soluble pigment	100	0	100	0	10
Acinetobacter transformation assay	0	0	0	0	100

[a] All isolates tested negative by Gram staining. Except for Gram staining, the oxidase test, the *Acinetobacter* transformation assay, and the fluorescent-antibody test, test results were read daily. Final readings were taken after 7 days of incubation.

[b] Isolates include two strains previously confirmed at the CDC as *B. holmesii*. One *B. holmesii* blood isolate (95R0375) was negative by all tests except the oxidase test (it showed a light reaction) and the brown soluble pigment test.

[c] Data are from reference 10.

[d] NT, not tested.

[e] The reaction was weak.

[f] The reaction was light.

dispensed into wide-mouthed screw-cap bottles (3.0 by 6.5 cm) and allowed to cool in a slanted position. BG agar plates with 20% sheep blood (Difco Laboratories, Detroit, Mich.) with and without antibiotics were prepared according to the manufacturer's directions and dispensed at 30 ml/plate. One percent CAS (Difco Laboratories) was dispensed at 0.5 ml per tube and sterilized. *B. holmesii* isolates were identified according to the conventional tests and methods of the Special Bacteriology Reference Laboratory, CDC (10). The inoculated selective media were observed after 3 days of incubation and then daily up to 7 days. A final reading was taken after a total incubation of 12 days. Media and biochemical tests were incubated aerobically at 35 to 36°C. The oxidase test was performed on 3-day-old growth obtained from nonselective BG agar plates. Smears were prepared and stained with *B. pertussis* and *B. parapertussis* conjugates (Difco Laboratories). All smears prepared for fluorescent-antibody staining consisted of cells taken from colonies growing on BG selective or nonselective agar plates, which consistently gave satisfactory results at a 1:5 or 1:4 dilution, respectively. It was found that smears prepared from cells growing on CA-LEX gave unsatisfactory results when they were stained with Difco conjugates. The transformation assay procedure as described by Juni (3) was used to determine if the test strains were genetically related to *Acinetobacter*. The transformation assay involves the mixing of crude DNA extracts of the test strain with the viable cellular mass of a specific strain of *A. calcoaceticus* that is an amino acid auxotroph. The mixture is incubated on a heart infusion agar plate for 4 to 6 h and then subcultured to a plate with a minimal medium (lactate mineral agar) upon which only the prototrophic transformants will grow. Conversion of genetically related strains from auxotroph to prototroph is evidenced by the subsequent growth of colonies after a 24-h incubation on the minimal medium (10).

Testing of *Bordetella* spp. on selective media. All isolates were subcultured two consecutive times onto antibiotic-free BG agar plates. After 3 days, a sweep of colonies from the final culture plates was suspended in phosphate-buffered saline (pH 7.2). The cell suspensions were adjusted to a density equivalent to a 0.5 McFarland standard, and subsequent working dilutions were made to yield a final concentration equivalent to 1.5×10^4 CFU/ml. A 10-μl aliquot of the working dilution (1.5×10^2 CFU) of each cell suspension was inoculated onto four culture plates of BG agar under different antibiotic conditions: (i) without antibiotic (BG agar alone), (ii) with 0.625 μg of oxacillin per ml (BG-OXA), (iii) with 40 μg of cephalexin (BG-LEX), and (iv) with 2.5 μg of methicillin per ml (BG-MET). In addition, CA and CA-LEX were inoculated. The plates were incubated aerobically at 35 to 36°C and observed for seven consecutive days. The partial inhibition or absence of bacterial growth was qualitatively observed for each plate. Partial inhibition was defined as a reduction in the colony count on the experimental plate relative to the bacteria's growth on the antibiotic-free BG agar plate.

PFGE of *B. holmesii*. All *B. holmesii* isolates were incubated for 3 days at 37°C on antibiotic-free BG agar. The colonies were harvested with cotton swabs and suspended in 1.5 ml of pH 8.0 cell suspension buffer (CSB) (10 mM Tris, 100 mM EDTA) to a density approximately equal to a 3.5 McFarland standard. The cell suspensions were washed twice with CSB at room temperature, followed by the embedding of the cells in agarose plugs, and processed as previously described by de Moissac et al. (2). Two segments were cut from each plug, and PFGE was performed following restriction with *Xba*I on one segment and *Spe*I on the other. The gels were run using contour-clamped homogeneous electric field MAPPER (Bio-Rad) at 14°C using TBE running buffer (10.9 g of Tris, 6.0 g of boric acid, 14 ml of 0.5 M EDTA, and distilled water to make 1.0 liter [pH 8.0]). PFGE was run for 18 h with initial and final switch times of 2.16 and 35.07 s, respectively.

RESULTS

Phenotypic characteristics. All *B. holmesii* isolates exhibited small gram-negative rod-shaped organisms, predominantly short to medium in length, with some coccobacilli and occasionally longer rods observed. None of the isolates were beta-hemolytic on BG agar, but all subcultures produced a brown discoloration of the medium after 48 h. The colonial morphology of all *B. holmesii* isolates after 3 days of incubation was similar to that of *B. pertussis*. Indeed, each isolate when first detected from the clinical source was being tested as a possible nonhemolytic strain of *B. pertussis*. Colonies were small, convex, entire, and glistening with a pearl to slightly slate-gray coloration. The isolates produced a weak alkaline reaction when they were grown on 10% dextrose agar slants after 7 days of incubation and grew slightly on MacConkey agar after 3 to 5 days. All isolates were oxidase negative using Kovacs' method, did not hydrolyze urea using Christensen's formulation, and were nonmotile. All produced a soluble brown pigment when they were grown on 0.1% tyrosine agar slants, some more strongly than others. In the *Acinetobacter* transformation assay, the DNA extracted from *B. holmesii* did not convert the auxotrophic strain of *Acinetobacter*, KL1842, to prototrophy. The fluorescent-antibody tests using *B. parapertussis* and *B. pertussis* conjugates were negative, although weak cross-reactions with the *B. pertussis* conjugate were observed with some of the strains. The phenotypic characteristics of isolates of *B. holmesii*, *B. pertussis*, *B. parapertussis*, *B. bronchiseptica*, and the asaccharolytic nonhemolytic *Acinetobacter* are shown in Table 1.

Testing of *Bordetella* spp. on selective media. Cephalexin, as demonstrated by incorporation in both the CA-LEX slant and the BG-LEX plate, was found to inhibit all isolates of *B. holmesii* tested and to partially restrict the growth of the other *Bordetella* spp. tested. Substitution of methicillin or oxacillin

TABLE 2. Recovery of *Bordetella* species on selective and nonselective media

Species (no. tested)	% of isolates recovered on:					
	CA	CA-LEX	BG agar	BG-LEX	BG-MET	BG-OXA
B. holmesii (33)	100	0	100	0	100	100
B. pertussis (5)	100	100	100	NT[a]	100	100
B. parapertussis (5)	100	100	100	NT	100	100
B. bronchiseptica (4)	100	100	100	NT	100	100

[a] NT, not tested (the effect of cephalexin in BG agar on *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica* was not evaluated since all of them were originally recovered from a medium containing cephalexin).

TABLE 3. *Bordetella* spp. isolated at the SLI from nasopharyngeal cultures

Organism	No. of specimens isolated in:				Total
	1995	1996	1997	1998	
B. holmesii	3	6	8	15	32
B. pertussis	140	325	129	146	740
B. parapertussis	20	32	11	33	96
Total	2,462	3,677	2,343	2,484	10,966

for cephalexin in the BG agar selective medium allowed the growth of all strains of *Bordetella* spp. in the study (Table 2).

PFGE patterns. Five distinct patterns were observed from PFGE of the 32 nasopharyngeal *B. holmesii* isolates when they were restricted with *Xba*I, and three patterns were observed when they were restricted with *Spe*I. The blood isolate of *B. holmesii* demonstrated banding patterns, with each of the enzymes, that were different from the patterns observed among nasopharyngeal isolates. The images of these banding patterns and their frequencies are presented in Fig. 1. Restriction with *Xba*I resulted in 22 distinct genomic bands ranging in size from 50 to 600 kb; *Spe*I restriction produced 13 distinct genomic bands in the 50- to 600-kb range. Of the 32 nasopharyngeal isolates of *B. holmesii* restricted with *Xba*I, the most common pattern, pattern A, occurred in 17 (53%) isolates. The remaining patterns comprised seven isolates (22%), five isolates (16%), 2 isolates (6%), and 1 isolate (3%), designated patterns B to E, respectively. The distribution of patterns for restriction with *Spe*I was 24 isolates (75%), 7 isolates (22%), and 1 isolate (3%). The most common *Spe*I pattern corresponded to *Xba*I strains A and D.

DISCUSSION

From 1 January 1995 to 31 December 1998 a total of 10,996 nasopharyngeal cultures were examined for the presence of *Bordetella* spp. Of these, 32 were positive for *B. holmesii*, 740 were positive for *B. pertussis*, and 96 were positive for *B. parapertussis*. No *B. bronchiseptica* strains were isolated. A year-by-year breakdown is given in Table 3. Because this was a retrospective study, we were not able to look for *B. holmesii* in any controls.

Cephalexin, the antibiotic currently recommended for the isolation of *Bordetella* spp., is widely used in various transport media. We found the antibiotic to have an inhibitory effect on *B. holmesii*, which may explain why many laboratories do not isolate this organism from respiratory specimens. The original nasopharyngeal *B. holmesii* isolates included in this study were recovered predominantly from the BG-MET plate. Indeed, after the first few initial isolations of *B. holmesii*, a presence of pertussis-like colonies on BG-MET plates with a conspicuous

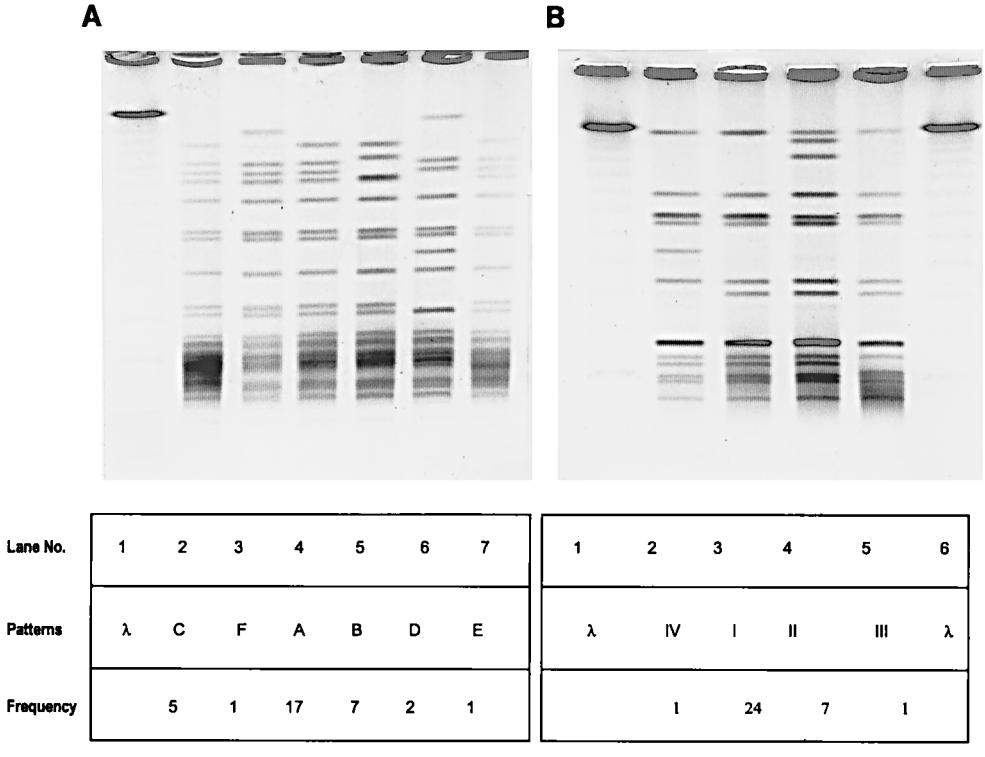

FIG. 1. *Xba*I (A) and *Spe*I (B) *B. holmesii* restriction patterns.

absence of similar colonies on CA-LEX led us to a consideration of *B. holmesii*.

A second possibility for the limited number of isolations from the respiratory tract may pertain to a lack of awareness. Since this organism is not generally regarded as an inhabitant of the respiratory tract, this identification may not be considered when it is encountered.

We demonstrated that *B. holmesii*, like other *Bordetella* spp., can be isolated from the respiratory tracts of patients with clinical illness. Although *B. holmesii* had previously been isolated from a sputum sample (8), this study is the first to analyze an extensive series of nasopharyngeal *B. holmesii* isolates. The recovery of *B. holmesii* can be achieved by the inclusion of a selective medium containing methicillin or oxacillin and not cephalexin in primary or secondary culture. Studies under way in our laboratory indicate that increasing the concentration of oxacillin in BG agar from 0.625 to 1.0 μg/ml reduces the level of normal floral breakthrough to that observed when BG agar is used with methicillin at a concentration of 2.5 μg/ml. This level appears to be noninhibitory to *Bordetella* spp.

The numbers of distinct nasopharyngeal DNA profiles generated after restriction with *Xba*I and *Spe*I endonucleases were 5 and 3, respectively. The small sample size in the study does not enable us to draw conclusions about *B. holmesii* genetic characteristics. Pattern A (*Xba*I) may represent the dominant strain circulating in Massachusetts. The limited number of banding patterns observed suggests that the *B. holmesii* genome may be highly conserved or that the endonucleases used are not sufficiently discriminating for this organism. The PFGE profile of the *B. holmesii* recovered from blood was *Xba*I pattern F and *Spe*I pattern IV.

PFGE using *Xba*I and *Spe*I endonucleases identified predominant strains circulating in Massachusetts during this study period. The occurrence of indistinguishable PFGE profile patterns did not correlate temporally or geographically.

It was not determined whether cephalexin was bacteriostatic or bactericidal to *B. holmesii*. Additional studies to resolve this issue would be of value so that a more accurate account on the incidence of *B. holmesii* in the nasopharynx could be determined. Future studies should address the presence of virulence factors responsible for the pathogenicity of *B. holmesii* in nasopharyngeal and/or blood specimens and the prevalence of asymptomatic carriage in the nasopharynxes of patients.

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