Evaluation and Validation of a Monoclonal Immunofluorescent Reagent for Direct Detection of Bordetella pertussis

PATRICIA McNICOL,†,* SANDRA M. GIERCKE, MICHAEL GRAY, DENIS MARTIN, BERNARD BRODEUR, MARK S. PEPPLER, TREVOR WILLIAMS, and GREGORY HAMMOND

Cadham Provincial Laboratory, Winnipeg, Manitoba, National Laboratory for Immunology, Laboratory Centre for Disease Control, Ottawa, Ontario, and Department of Medical Microbiology and Infectious Diseases, University of Alberta, Edmonton, Alberta, Canada

Received 31 March 1995/Returned for modification 14 July 1995/Accepted 16 August 1995

An outbreak of pertussis in Manitoba, Canada, provided an opportunity to evaluate the recently developed monoclonal antibody (MAb) BL-5 for the direct detection of Bordetella pertussis. The MAb recognizes a lipoooligosaccharide epitope. A total of 1,507 consecutive nasopharyngeal swabs for culture and companion smears for direct fluorescent-antibody (DFA) detection were evaluated at Cadham Provincial Laboratory between September and November 1994. The cutoff for DFA positivity was four fluorescing organisms with morphology characteristic of B. pertussis. PCR analysis for B. pertussis DNA was performed on a subset of 100 smears by eluting material from the slides after DFA examination. In comparison with culture, the sensitivity, specificity, and positive and negative predictive values of BL-5 were 65.1% (41 of 63 samples), 99.6% (1,438 of 1,444 samples), 87.2% (41 of 47 samples), and 98.5% (1,438 of 1,460 samples), respectively. The sensitivity of culture compared with PCR was 45.5% (10 of 22 samples) for the subset of 100 specimens tested by both procedures. An expanded “gold standard” of positivity by culture or PCR for these 100 specimens resulted in DFA sensitivity, specificity, and positive and negative predictive values of 32.3, 97.1, 83.3, and 76.1%, respectively. The utility of MAb BL-5 for direct detection of B. pertussis in a clinical laboratory setting has been demonstrated by this investigation.

Despite ongoing vaccination programs, pertussis remains an important endemic disease in North America. Epidemic peaks continue to occur at 3- to 4-year intervals (10). The disease burden is greatest among pre-school-age children and infants, with the highest risk for severe disease and death occurring among those less than 6 months of age (25). The annual incidence of reported cases of whooping cough in the United States peaked in 1993 with a rate of 2.6 cases per 100,000, declining to a crude annual incidence of 1.8 cases per 100,000 in 1994 (8), after a long-term trend in increasing overall incidence since 1976 (25). Whether these statistics represent a true increase or are a function of improved reporting is unclear. However, it is estimated that fewer than 12% of pertussis cases are reported (30). Even more disturbing is the recent isolation of an erythromycin-resistant strain of Bordetella pertussis from a 2-month-old infant in Arizona (16). Erythromycin is the drug of choice for treatment of disease related to B. pertussis and for postexposure prophylaxis of household members and other close contacts of patients (3).

B. pertussis infection remains a significant cause of morbidity and mortality in Canada as well. In 1992, over 3,700 cases were reported (7). The incidence increased to 7,049 reported cases of pertussis in 1993 (6). During the late summer and fall of 1994, the Province of Manitoba, Canada, also experienced an outbreak of whooping cough, affording us the opportunity to assess laboratory-based diagnostic methods for detection of B. pertussis.

The criteria on which the clinical diagnosis of pertussis is based include a paroxysmal cough with cough episodes ending in apnea, vomiting, or inspiratory whoop with no other known cause. However, the “gold standard” for diagnosis is the isolation of B. pertussis by culture of nasopharyngeal secretions. Unfortunately, culture suffers from low sensitivity (14, 23, 28) and long incubation times ranging from 3 to 7 days, preclude a rapid diagnosis (11). These shortcomings have prompted the development of several alternative methods for detection of B. pertussis.

Serological methods for diagnosis are available and include comparison of acute- and convalescent-phase sera to document pertussis-specific seroconversion (15) or detection of an immunoglobulin M or immunoglobulin A response in a single serum specimen (20). A cytotoxicity assay demonstrating good specificity for pertussis toxin has been described; however, the sensitivity remains low (13). The application of the PCR to pertussis diagnosis shows promise (9) but has not been sufficiently validated in a diagnostic setting (19). Direct detection of B. pertussis by fluorescein-conjugated-antibody (direct fluorescent-antibody [DFA]) staining can provide a rapid, presumptive diagnosis; however, several investigations have documented the lack of sensitivity and specificity of the existing commercially available, polyvalent immunological reagent (9, 12, 14, 24). Extensive cross-reaction with normal nasopharyngeal and oral microbial floras makes the test technically difficult to interpret, resulting in false-positive rates as high as 85% (9). Since these limitations might be addressed by using highly specific monoclonal antibodies (MAbs) for DFA analysis, we designed a study to evaluate a newly developed pertussis-specific MAb for direct detection of B. pertussis.

MAb BL-5, developed by the National Laboratory for Immunology, Laboratory Centre for Disease Control, specifically recognizes the lipoooligosaccharide (LOS) of B. pertussis (2, 5). The epitope recognized by this MAb is present on the slowly migrating major LOS A band. The location of this epitope on the surface of intact bacteria suggested that it could be detected directly, without pretreatment of clinical specimens (2,
17). This was proven to be the case by using BL-5 in a dot blot enzyme immunoassay. The MAb recognized all 64 laboratory strains and clinical isolates of *B. pertussis* tested, clearly indicating that this specific epitope is highly conserved. Only very rare LOS-variant laboratory strains do not have this unique epitope and are not recognized by the MAb.

MAb BL-5 is similar to MAb G-10 (21) to the extent that both react with the LOS A band of *B. pertussis* but not with the LOS-variant strains. While both MAb are bacteriolytic, they are idiotypically distinct (5). They also differ in that G-10 affords protection against respiratory colonization in mice (22) and does not cross-react with any *Bordetella* sp., including *Bordetella bronchiseptica* (5).

MAb BL-5 does not cross-react with *Bordetella parapertussis* or other bacterial species normally isolated from the respiratory tract. However, weak reactivity with *Bordetella bronchiseptica*, an animal pathogen rarely causing infections in humans exposed to infected animals, is observed. From 1911 through 1990, only 25 cases of *B. bronchiseptica* infection have been documented; most of these cases were correlated with animal contact (33).

In the present study, we compared direct detection of *B. pertussis* in smears by fluorescein-conjugated MAb BL-5 with culture of nasopharyngeal swabs for 1,507 consecutive specimens sent to Cadham Provincial Laboratory, Winnipeg, Manitoba, Canada, between 1 September and 1 November 1994. Additionally, PCR was used to resolve discordant findings for a subset of these specimens by amplifying *B. pertussis* DNA recovered from the slides after DFA analysis (9). In this report, we detail the validation of BL-5 in a clinical diagnostic setting and demonstrate the utility of this reagent for rapid and direct detection of *B. pertussis*.

### MATERIALS AND METHODS

**Specimen collection.** A single calcium alginate or rayon swab was used to sample the nasopharynx of each child or adult suspected of having whooping cough. In some instances, specimens were collected from contacts of patients. A smear was prepared for DFA analysis by rotating the swab on a glass slide before placing it into Amies charcoal transport medium (1). The swabs were transported in Amies medium to the laboratory at ambient temperature and usually plated within 24 to 48 h of collection onto Regan-Lowe medium (20) containing cephalaxin at a final concentration of 40 μg/ml and 5% lysed sheep blood. The plates were incubated at 35°C in a humidified incubator and examined at 4 and 7 days for colonies typical of *B. pertussis*. The identity of organisms giving rise to colonies was confirmed to be *B. pertussis* by DFA testing with Bacto-FA *B. pertussis* and Bacto-FA *B. bronchiseptica* polyvalent immunoreagents (Difco Laboratories, Detroit, Mich.). In addition, isolates were subcultured on chocolate agar, blood agar, and Regan-Lowe medium to aid in confirmation (data not shown).

**Direct MAb BL-5 detection.** The smears were fixed with methanol and stained with fluorescein-conjugated MAb BL-5 after the reagent, containing approximately 0.3 mg of protein per ml, was diluted 40-fold in phosphate-buffered saline (pH 7.2) with 0.004% Evans blue and 0.5% bovine serum albumin. This working stock of MAb BL-5 provided a 4+ fluorescence. Each group of 40 clinical slides was accompanied by a positive and a negative control. A minimum of four fluorescing organisms per slide, with morphology characteristic of *B. pertussis*, was required for the specimen to be called positive. These stringent criteria are the same as those routinely used for interpretation of results of DFA tests using the commercial, polyvalent immunoreagent. Each of the slides was read for 3 min by the same experienced, registered technologist.

**PCR amplification of *B. pertussis* DNA.** Following DFA analysis, a subset of 100 slides, including those with discordant findings relative to culture, were analyzed for the presence of *B. pertussis* DNA by PCR. The DNA was recovered from the slides essentially as described previously (9), by resuspending the material in 50 μl of 12 mM Tris-HCl (pH 7.6) and then transferring the suspension to a microcentrifuge tube. Proteinase K (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 0.2 mg/ml. The samples were incubated at 55°C for 90 min, and then the enzyme was inactivated by being boiled for 20 min. A 20-μl aliquot of this material was used directly in the PCR. In addition to the digested material eluted from the slide, each reaction mixture contained, as final concentrations, 200 nM (each) sense and antisense *B. pertussis* primers (9), 50 μM deoxyribonucleotide triphosphates (Pharmacia Biotechnology, Baie-D’Urfé, Quebec, Canada), 2 U of Taq poly-

### RESULTS

The performance of the fluorescein-conjugated MAb BL-5 for direct detection of *B. pertussis* was evaluated relative to the gold standard of culture for 1,507 consecutive nasopharyngeal swab specimens sent to Cadham Provincial Laboratory between September and November 1994. Of the 1,507 specimens, 22 (1.5%) were positive by confirmed culture only, 6 (0.4%) were positive by DFA staining only, and 41 (2.7%) were positive by both procedures, for a total positivity rate for either technique of 4.6% (Table 1). On the basis of these results, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the DFA procedure relative to culture were 65.1% (41 of 63 samples), 99.6% (1,438 of 1,444 samples), 87.2% (41 of 47 samples), and 98.5% (1,438 of 1,460 samples), respectively.

A subset of 100 smears was analyzed by PCR for *B. pertussis* DNA. Representative results are displayed in Fig. 1. All of these specimens were positive for amplification of the 268-bp human β-globin gene sequence. The coamplification of human DNA controlled for the adequacy of the specimen and the presence of PCR inhibitors in the suspended material. The *B. pertussis*-positive specimens were distinguished by the presence of a 424-bp amplicon in conjunction with the 268-bp human β-globin gene sequence. The coamplification of human DNA controlled for the adequacy of the specimen and the presence of PCR inhibitors in the suspended material.

### Table 1. Culture and DFA results for 1,507 specimens received for examination for *B. pertussis*

<table>
<thead>
<tr>
<th>DFA result</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>41</td>
<td>6</td>
<td>47</td>
</tr>
<tr>
<td>Negative</td>
<td>22</td>
<td>1,438</td>
<td>1,460</td>
</tr>
<tr>
<td>Total</td>
<td>63</td>
<td>1,444</td>
<td>1,507</td>
</tr>
</tbody>
</table>

The performance of MAb BL-5 was evaluated against an expanded gold standard of positivity by culture or PCR, since relative to PCR, the sensitivity, specificity, PPV, and NPV of culture were only 45.5% (10 of 22 samples), 88.5% (69 of 78 samples), 52.6% (10 of 19 samples), and 85.2% (69 of 81 samples), respectively (Table 2). By this criterion, the corrected sensitivity, specificity, PPV, and NPV of MAb BL-5 for the 100 specimens were 32.3% (10 of 31 samples), 97.1% (67 of 69 samples), 83.3% (10 of 12 samples), and 76.1% (67 of 88 samples), respectively.
DISCUSSION

Laboratory diagnosis of pertussis is a challenge that can be addressed by utilizing improved reagents. Clearly, the advantage of DFA staining relative to other diagnostic methods, including PCR, is the provision of a rapid result. When BL-5 is used for direct detection of B. pertussis, the diagnosis is not only rapid, but also accurate. In comparison with culture, the sensitivity, specificity, PPV, and NPV of the DFA test utilizing BL-5 are 65.1, 99.6, 87.2, and 98.5%, respectively. These results are in contrast to those reported recently for the commercially available polyclonal conjugate. Relative to culture of nasopharyngeal secretions, the sensitivity, specificity, PPV, and NPV for the commercial, polyclonal immunoreagent were reported as 61, 95, 53, and 96%, respectively. These findings call into question the utility of results obtained with the commercial reagent (29).

We evaluated BL-5 by using the criterion of four fluorescing organisms with typical morphology for DFA positivity. This is the standard when a polyclonal reagent is used; a less stringent criterion may be more appropriately applied to a monoclonal immunoreagent. By lowering this limit, the value obtained for the sensitivity of MAb BL-5 would have improved. Indeed, the application of this criterion may be responsible for some of the discordant findings observed for the subset of 100 specimens analyzed by culture, PCR, and DFA test.

Identical results were obtained by the three procedures for 71 of the 100 specimens. An additional 14 samples were positive by PCR but negative by the DFA procedure. Six of the 14 were also positive by culture. In order for the PCR analysis to be positive, B. pertussis DNA must have been eluted from the smears following the DFA procedure. However, for PCR positivity, the bacteria need not be intact, while at least four whole organisms with characteristic morphology were required for the DFA procedure to be called positive.

Other discordant findings for this subset of 100 specimens can be reconciled on the basis of anecdotal clinical information or laboratory-based observations. For example, both of the specimens positive by DFA only were collected from the sibling of a culture-positive patient. Therefore, these specimens are likely to have been falsely negative by culture and PCR. Most of the seven specimens that were positive for B. pertussis by culture only demonstrated scant growth on the culture plates, suggesting that the companion smears may have been inadequate for DFA and PCR analyses. It should be noted that DFA staining was the only procedure evaluated in our investigation that did not employ amplification of either the viable organism in transit and/or after plating) or target genomic DNA sequences.

The four specimens found to be B. pertussis negative by culture only may have been collected late in the course of treatment or after the initiation of antibiotic therapy. Both of these factors are known to compromise culture (28).

The remaining two specimens providing negative results by PCR only doubt reflect the limitations of the PCR procedure under the conditions of our study. Several investigators have shown PCR to be more sensitive than culture for the detection of B. pertussis (27, 31). While in general this is true of our study as well, in our investigation the PCR assay was compromised by amplifying fixed material eluted from slides rather than sampling the clinical specimen directly. Indeed, a previous study demonstrated that the sensitivity of PCR drops 10-fold when it is performed with material eluted from slides rather than directly on suspensions of B. pertussis bacteria (9). In our study, there may have been an additional negative bias since the slides used for the PCR procedure were examined initially by the DFA test. Also, some specimens were collected with calcium alginate swabs; both the fibers and the aluminum shaft have been shown to have inhibitory effects on PCR-based assays for B. pertussis (32). However, this did not appear to be a factor in our investigation, since coamplification of human DNA sequences was consistently observed in the specimens undergoing PCR analysis.

The most important outcome of our investigation is the validation of a new immunological reagent for rapid, direct detection of B. pertussis. We observed the specificity and PPV and NPV of MAb BL-5 to be superior to those of the existing polyclonal reagent. While definitive diagnosis of B. pertussis will remain dependent on a combination of clinical findings and laboratory techniques, the utility of the DFA procedure will be greatly improved by incorporating this new monoclonal reagent.

TABLE 2. DFA, culture, and PCR results for 100 specimens received for examination for B. pertussis

<table>
<thead>
<tr>
<th>DFA result</th>
<th>Culture result</th>
<th>PCR result</th>
<th>No. of specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>−</td>
<td>2</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>−</td>
<td>2</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>−</td>
<td>7</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>+</td>
<td>8</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>−</td>
<td>67</td>
</tr>
</tbody>
</table>

ACKNOWLEDGMENT

We gratefully acknowledge the expert technical assistance provided by the Clinical Microbiology Staff, Cadham Provincial Laboratory.

REFERENCES

6. Bureau of Communicable Disease Epidemiology, Laboratory Centre for Disease Control, Health Canada. Personal communication.