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Rapid Immunodot Technique for Identifying *Bordetella pertussis*

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We developed and evaluated a rapid test with monoclonal antibodies to identify cultures of *Bordetella pertussis*. Samples of 5 μl of cells suspended in formalin-saline were dried onto a nitrocellulose disk. The disk was placed in a filtration device, and 5-μl volumes of murine monoclonal antibody directed against *B. pertussis* lipooligosaccharide and peroxidase conjugate were added consecutively, with washing after each addition. The disk was removed and immersed in peroxidase substrate solution. All of 66 *B. pertussis* isolates confirmed by direct fluorescent-antibody assay were correctly identified by using four different monoclonal antibodies. One of the monoclonal antibodies did not react with over 20 bacterial species tested, including other *Bordetella*, *Acinetobacter*, *Haemophilus*, *Moraxella*, *Mycobacterium*, *Neisseria*, and *Staphylococcus* spp. This technique detected ≥2 μg of lipooligosaccharide per ml or ≥5 × 10^8 *B. pertussis* cells per ml. This rapid procedure used small amounts of reagents, needed less equipment, and was less subjective and more specific than the direct fluorescent-antibody assay.

Although the reported incidence of pertussis has decreased worldwide since the advent and licensure of whole-cell *Bordetella pertussis* vaccines, demand for the accurate laboratory diagnosis of pertussis persists (3, 15, 16). Laboratory confirmation is indispensible to the clinical and epidemiologic responses to pertussis outbreaks in developed countries and the high morbidity in communities and developing nations with low vaccination compliance (2, 3, 10, 13, 14). The recent evaluations of the immunogenicities and protective efficacies of the latest acellular vaccines exemplify the research applications of diagnostic laboratory methodology.

Primary culture of *B. pertussis* is the most specific laboratory technique commonly used to diagnose pertussis (11). Culture endures as the diagnostic standard, although sensitivity decreases in vaccinated patients with specimens collected later than 21 days after cough onset and in patients treated with erythromycin or sulfamethoxazole (12). Typically, presumptive *Bordetella* colonies are isolated from nasopharyngeal specimens inoculated onto plates of selective agar media. Presumptive isolates are confirmed with biochemical reactions and by a direct fluorescent-antibody assay (6). Because biochemical tests require inoculation of several media and reagents with axenic cultures, results are often deferred for days. The direct fluorescent-antibody assay provides confirmation in about 1 h but requires a microscope with a UV light source. In addition, interpretation of direct fluorescent-antibody assay results is subjective, and false confirmations due to cross-reactive reagents have been described (5).

Gustafsson and Askelof reported the detection of *B. pertussis* on agar plates by a colony blot assay with monoclonal antibodies (MAbs) (7). This technique was sensitive and specific but required lysis of bacterial cells with chloroform, 6 h to complete, and overnight growth of single colonies. In this study, we adapted and evaluated an immunodot procedure previously used to type strains of *Legionella pneumophila* that improves on current methods used to confirm *B. pertussis* cultures (1).

Each of 101 test strains (Table 1) was suspended in 0.6% formalin in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M Na_2HPO_4/NaH_2PO_4 [pH 7.2]) to yield an optical density at 450 nm of approximately 1. The cultures of *B. pertussis* (n = 66) were collected from epidemic and sporadic pertussis cases. The *Bordetella parapertussis* strains were five patient isolates. One *Bordetella bronchiseptica* isolate was the type strain (ATCC 19395), and two were recovered from human clinical specimens. All other cultures were chosen to represent common nasopharyngeal flora, colonizers, or pathogens and were provided by the appropriate reference laboratories at the Centers for Disease Control and Prevention.

We tested four different murine MAb preparations directed against the lipooligosaccharide antigen of *B. pertussis*. Two MAb preparations (9C1G5 and 5H7F2F12) were provided in hybridoma supernatants (4), and two (BPG10 and 6-4H6) were provided in ascites (8, 9). Working concentrations of MAb and conjugate solutions were evaluated by testing appropriate reagent dilutions in two dimensions. In addition, MAb concentrations were normalized by using a kinetic enzyme immunoassay and a pool of human serum samples (U.S. Reference Pertussis Antiserum lot 3; Food and Drug Administration, Center for Biologics Evaluation and Research) from pertussis patients. MAbs 9C1G5, 5H7F2F12, 6-4H6, and BPG10 were used at 2.9 × 10^3, 2.2 × 10^3, 3.2 × 10^3, and 3.6 × 10^3 enzyme immunoassay units per ml, respectively.

Samples of 5 μl of each test organism suspension were applied to nitrocellulose disks (0.45-μm pore size; 82-mm diameter; Schleicher and Schuell), not exceeding 50 application sites per disk. Filters were dried for 2 h at 25°C and then placed on a colony hybridization filter device (Schleicher and Schuell). Free binding sites were blocked by

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TABLE 1. Reactivity of test microorganisms with MAbs[a] and the immunodot technique

Microorganism (no. of isolates)	Reactivity with MAb:			
	9C1G5	5H7F2F12	BPG10	6-4H6
Acinetobacter calcoaceticus[b] (2)	−	−	−	−
Bordetella bronchiseptica (3)	+	−	+	+[c]
Bordetella parapertussis (5)	w[d]	−	w	w
Bordetella pertussis (66)	+	+	+	+
Candida albicans (2)	−	−	−	−
Haemophilus influenzae[e] (3)	−	−	−	−
Moraxella spp.[f] (4)	−	−	−	−
Mycobacterium spp.[g] (6)	−	−	−	−
Neisseria spp.[h] (7)	−	−	+[i]	−
Oligella urethralis (1)	−	−	−	−
Staphylococcus spp.[j] (2)	−	−	−	w

[a] MAbs were directed against *B. pertussis* lipooligosaccharide.
[b] Includes *A. anitratus* and *A. lwoffii* biovars.
[c] One of the three strains did not react with any MAb tested.
[d] Weakly positive reactions (see the text).
[e] Serotype b.
[f] Includes *M. atlantae*, *M. nonliquifaciens*, *M. osloensis*, and *M. phenylpyruvica*.
[g] Includes *M. avium*, *M. bovis*, *M. chelonei*, *M. fortuitum*, *M. kansasii*, and *M. tuberculosis*.
[h] Includes *N. cinerea*, *N. elongata*, *N. lactamica*, *N. meningitidis*, *N. mucosa*, *N. sicca*, and *N. subflava*.
[i] *N. elongata* only.
[j] Includes *S. aureus* and *S. epidermidis*.

washing with approximately 20 ml of PBS containing 0.3% Tween 20 and 0.25% bovine serum albumin (fraction V). All washes and reagent applications were performed with negative pressure at the filter device from an in-line vacuum or mechanical pump routed through a 2-liter trap.

Samples of 5 μl of the test MAb solutions were applied to appropriate areas of the disk where cell suspensions were spotted, and the disk was washed with PBS–0.3% Tween. Next, 5 μl of goat anti-mouse horseradish peroxidase conjugate (Bio-Rad) diluted 1:10 in PBS was added to each application site. After a final wash, filters were removed from the filter device, placed in petri dishes, and exposed to 10 ml of 4-chloro-1-naphthol solution (Kirkegaard and Perry Laboratories) for 5 min on a Gyrotory shaker (New Brunswick Scientific Co.). Substrate conversion was stopped by rinsing filter disks with deionized water and blotting dry. Development of a dark blue color at the site of application of the bacterial suspension denoted a positive reaction. The appearance of a faint blue color, visually distinguishable from the intense blue of positive reactions, was designated weakly positive. All procedures after antigen binding can be completed within 10 min.

All *B. pertussis* cultures ($n = 66$) reacted with each of the four MAbs evaluated (Table 1). One MAb (5H7F2F12) reacted only with *B. pertussis* suspensions, but three also recognized other bacteria. The reactions of MAb 6-4H6 with *Staphylococcus aureus*, *S. epidermidis*, and *B. parapertussis* and those of 9C1G5 and BPG10 with *B. parapertussis* were weak, and their colors were readily differentiated from the intense blue color of the *B. pertussis* response. In contrast, the reactions of MAb BPG10 with *Neisseria elongata* and *B. bronchiseptica* and those of 9C1G5 and 6-4H6 with *B. bronchiseptica* were indistinguishible from that of the *B. pertussis* cultures. However, these organisms are readily differentiated by Gram stain characteristics and growth on blood agar.

We evaluated the sensitivity of this assay for *B. pertussis* cells. Cells were suspended in PBS as described previously for enumeration by plate count. Appropriate dilutions of test suspensions in PBS were plated in triplicate onto Regan-Lowe agar plates prepared without cephalexin, and the CFU were counted after 5 days of incubation. Each dilution was also tested by the immunodot technique. The minimal detectable level with any of the four test MAbs was 5×10^5 CFU of *B. pertussis*.

Sensitivity was also determined by using purified lipooligosaccharide antigen (no. 300; List Biological Laboratories) diluted in Tris-HCl buffer (0.05 M, pH 8.0) with 1 M NaCl. The minimal concentration of lipooligosaccharide detectable by this technique was 2 μg/ml. This level of sensitivity suggests that direct detection of antigen in respiratory secretions would likely require concentrating the antigen and/or increasing the response of the enzymatic reporter system.

We have described an immunodot technique that uses MAbs directed against lipooligosaccharide to confirm isolates of *B. pertussis*. Although the specificity varied with MAb preparation, all preparations were useful in this technique and one MAb was 100% specific. The sensitivity of this procedure is sufficient for confirming the identity of isolates but is probably insufficient for detecting the antigen directly in respiratory secretions. The immunodot technique improves on current confirmatory tests because it is objective, specific, requires minimal equipment and about 2 h to complete, uses small amounts of reagents, and can be used with primary growth. The technique has clinical, epidemiologic, and research applications.

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