

Development of a Rapid Diagnostic Test for Pertussis: Direct Detection of Pertussis Toxin in Respiratory Secretions

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Monoclonal antibodies (MAb) were produced against the specific *Bordetella pertussis* antigen pertussis toxin (PT). In preliminary studies, one MAb (IB₁₂) was selected and used in an enzyme-linked dot blot immunoassay to evaluate the ability of the method to detect known amounts of PT in control experiments and to test its potential for direct detection of PT in nasopharyngeal secretion (NP) specimens from patients with confirmed cases of whooping cough. The dot blot assay was able to detect PT at levels as low as 10 ng per dot in either buffer or control NP specimens. The assay demonstrated specificity, reacting only with dot blots of whole *B. pertussis* and not *Bordetella bronchiseptica*, *Bordetella parapertussis*, or other bacterial strains. In preliminary studies, NP aspirate, swab, and wash specimens were compared. The specimen of choice was found to be the NP aspirate, for which 100% positive results were found in the assay. These initial studies suggest that the dot blot immunoassay in which a MAb is used for direct detection of PT in NP specimens may be useful as a rapid diagnostic test for pertussis.

Whooping cough, caused by *Bordetella pertussis*, is still a major respiratory disease in children throughout the world today. The disease incidence has increased in the 1980s in the United States, the United Kingdom, and Sweden (5, 7, 34). Recent epidemic outbreaks of pertussis in the United States have occurred in Oklahoma, Maryland, Washington, Idaho, and Arizona (5, 6). Pertussis is difficult to diagnose from only the clinical picture in children and adults because the appearance of the disease is atypical in many cases and because other respiratory infections may present pertussis-like symptoms (36). Definitive diagnosis of pertussis requires culturing of *B. pertussis* from nasopharyngeal (NP) specimens and/or detection of bacteria in the specimen by direct fluorescent-antibody staining (DFA) techniques (11). These current diagnostic methods have been reported to be slow, insensitive, and variable and thus may impede the control of pertussis transmission during outbreaks (5). It is frequently difficult to confirm a clinical diagnosis of pertussis by culture or DFA or both (23). Clearly, there is a need for a more rapid, specific, and sensitive laboratory diagnostic test for the detection of pertussis. The aim of this preliminary study was to produce monoclonal antibodies (MAb) to a specific *B. pertussis* antigen, pertussis toxin (PT). The best MAb of 15 studied, IB₁₂, was selected and used in an enzyme-linked immunosorbent assay (ELISA) to evaluate its ability to detect PT directly in respiratory secretions from children with confirmed cases of pertussis.

MATERIALS AND METHODS

Production and purification of PT. *B. pertussis* BP504 was used to produce PT. Stock cultures were stored as a suspension in a mixture of 50% Stainer-Scholte medium and 50% glycerol at -70°C (19). Cultures were prepared by inoculation of Bordet-Gengou agar (Difco Laboratories, Detroit, Mich.) plates with 2 or 3 drops of thawed stock. The plates were incubated at 37°C in a moist environment for 3 days before use. The purity of cultures was verified by Gram stain morphology and growth characteristics. PT was produced by growth of BP504 in modified Stainer-Scholte medium (19) for

5 days under static culture conditions. Culture supernatant was harvested, and sodium azide was added to 0.04% (wt/vol). PT was purified by the method of Sekura et al. (35). The purity was verified by analytical polyacrylamide gel electrophoresis at pH 4.0 (9) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (37).

Hybridoma production. Six-week-old female BALB/c mice were immunized with PT (detoxified by treatment with 4 M urea) by the method of Cianfriglia et al. (8). The mice were injected intraperitoneally with 50 µg of antigen mixed with an equal volume of Freund complete adjuvant once a week for 2 weeks. At week 3, 5 µg of antigen was injected intraperitoneally, with injections of 2.5 µg intraperitoneally and 2.5 µg intravenously (via the tail vein) given on each of the following 2 days. At the time of the last immunization, a blood sample was obtained by intraorbital puncture, and serum was tested by ELISA for antibody against PT. Animals showing a positive titer were used for hybridoma production.

Hybridomas were produced by the procedure of Oi and Herzenberg (28). Spleen cells were isolated and fused with the P₃ × 63 Ag 8.653 nonsecreting myeloma cell line for production of hybridomas. Fused cells were initially cultured in thymocyte cell-enriched hypoxanthine-aminopterin-thymidine (HAT)-RPMI 1640 selection medium with 15% fetal calf serum and plated into six 24-well tissue culture plates (28). Cells were maintained in this medium for 2 weeks, and then the medium was changed to HAT-RPMI-15% fetal calf serum for an additional 2 weeks to complete the hybridoma selection. Cells from wells producing MAb against PT were cloned as single cells in individual wells by limiting-dilution methods in RPMI-10% fetal calf serum (28). Hybridomas were allowed to grow, and culture supernatants were retested for the presence of anti-PT MAb. Positive clones were isotyped by using a MonoAb-ID EIA Kit (Zymed Laboratories, South San Francisco, Calif.).

Ascites production and purification of MAb. For ascites production of anti-PT MAb, mice were primed with 0.5 ml of pristane and 9 days later given a radiation dose of 500 R. They were injected with 6 × 10⁶ to 8 × 10⁶ hybridoma cells the following day. Ascites fluid was collected by passive

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drainage (using an 18-gauge needle), cleared by centrifugation, and delipidified with silicone dioxide (Cab-O-Sil; Eastman Kodak Co., Rochester, N.Y.) in Vernal buffer (27). MAb were purified by DEAE-Sephacel anion-exchange column chromatography (17).

Clinical specimens. Twenty-five NP specimens were obtained from children with whooping cough confirmed by either positive culture or DFA or both. Five NP aspirates were received from Kenneth Ryan, University Medical Center, University of Arizona, Tucson. Also, 25 NP aspirate specimens from adult inpatients were obtained to be used as control samples in the study. Four samples collected by NP swabs diluted in transport medium (veal infusion broth) were received from David Bemis, Department of Microbiology, University of Tennessee, Knoxville. Sixteen specimens (one NP swab and fifteen NP washes [diluted in transport medium]) were received from John Ogle, Department of Pediatrics, University of Colorado Medical Center, Denver. All NP specimens were stored frozen at -70°C until used in experiments. NP specimens showing great viscosity were treated by the addition of 15 to 20 mg of crystalline *N*-acetyl-L-cysteine (NALC) to liquefy the specimen for use in the dot blot immunoassay (25).

Dot blot immunoassay. For the assays, various preparations were applied (dot blotted) onto strips of nitrocellulose paper (BA85; Schleicher & Schuell, Inc., Keene, N.H.) by using either a Pipetman or a Drummond microdispenser. Samples (from 2 to 20 μl) were dotted 2 μl at a time and allowed to dry between applications. After dotting, the strips were dried at 37°C for 30 min and then blocked for an additional 30 min at 37°C with a solution containing 50 mM Tris hydrochloride–50 mM NaCl (pH 7.4) buffer (TBS) and 5% skim milk. The strips were washed in TBS, anti-PT MAb was added at various dilutions in a 5-ml final volume, and the strips were incubated for 3 h at 37°C in corked test tubes with mixing. The strips were washed with TBS, and rabbit anti-mouse antibody–horseradish peroxidase conjugate (RAMA-PO) (Bio-Rad Laboratories, Richmond, Calif.) was added at a 1:3,000 dilution in 5 ml of TBS and incubated for 1 h at 37°C . The strips were washed with TBS, and the enzyme reaction was started by the addition of 5 ml of TBS containing 4-chloro-1-naphthol and H_2O_2 (18). Positive reactions appeared as blue dots on the strips. When the reaction was complete (30 min), the strips were washed with water and stored in the dark.

Specificity of dot blot immunoassay. To evaluate the specificity of PT MAb used in the dot blot assay, a panel of microorganisms that might be present in NP specimens were screened for potential cross-reactivity. Organisms tested were *B. pertussis* BP504, BP165, and BP338 (from C. Manclark, U.S. Food and Drug Administration, Bethesda, Md.); *Bordetella parapertussis* 230, 231, and 176 (from Per Askelöf, National Bacteriology Laboratory, Sweden); and *Bordetella bronchiseptica* SW 878, SW 1262, and SW 1330 (from A. Bertschinger, Veterinary Institute, University of Zurich, Zurich, Switzerland). Other microorganisms listed in Table 1 were received from Kenneth Ryan. Bacteria were grown on appropriate plate media, harvested, washed, and heat killed. The bacteria were suspended in phosphate-buffered saline (PBS) to $10^{11}/\text{ml}$ via direct counting by using a Petroff-Hausser counting chamber. Dilutions were made to yield organisms at 10^7 , 10^6 , and $10^5/\mu\text{l}$, with 1- μl volumes dotted onto nitrocellulose strips, dried, and prepared for screening with PT MAb as described above.

TABLE 1. Reactivity of IB₁₂ MAb by dot blot assay against various organisms

Microorganism or control	No. of strains ^a tested	Reaction
Pertussis toxin		+
PBS-Tween		—
<i>B. pertussis</i>	4	+
<i>B. bronchiseptica</i>	4	—
<i>B. parapertussis</i>	2	—
Group A streptococcus	4	—
Group B streptococcus	2	—
<i>Streptococcus pneumoniae</i>	2	—
<i>Neisseria meningitidis</i>	6	—
<i>Staphylococcus aureus</i>	6	—
<i>Haemophilus influenzae</i> (type b)	5	—
<i>Haemophilus influenzae</i> (not type b)	3	—
<i>Pseudomonas aeruginosa</i>	2	—
<i>Candida albicans</i>	3	—
Diphtheroids	2	—

^a The strains were prepared via direct counts by using a Petroff-Hausser counting chamber and tested at 10^7 , 10^6 , and $10^5/\mu\text{l}$, volumes which were blotted on nitrocellulose paper strips, dried, blocked, and screened with IB₁₂ MAb.

RESULTS

Production of MAb to PT. Spleen cells from two mice immunized with PT were fused with the myeloma cell line. Forty-eight wells from the initial fusion were found by ELISA to be positive for the production of MAb against PT. Cells from anti-PT MAb-positive wells were cloned, and the resulting MAb were isotyped. Fifteen clones were found to be of the immunoglobulin G1 subclass. These 15 clones were expanded in tissue culture flasks, and the culture supernatants were reanalyzed by ELISA. Supernatants from hybridomas IB₁₂ and IIA₁₂ gave the strongest reactions in the ELISA (optical density at 405 nm, >1.0) and when reacted with native PT blotted directly onto nitrocellulose paper strips. Western immunoblot analysis of PT separated into its five subunits (37) showed that MAb IB₁₂ and IIA₁₂ reacted only with the S-1 subunit of PT (data not shown).

Both hybridomas were injected into mice for ascites production. IIA₁₂ was found to be a poor secretor in vivo, while IB₁₂ produced a high level of MAb, which was recovered in the harvested ascites fluid. IB₁₂ MAb was purified as described in Materials and Methods and used for the development of the dot blot immunoassay.

Sensitivity of the dot blot assay. The sensitivity of the dot blot assay was evaluated by blotting serial dilutions of purified PT from 10,000 to 0.1 ng per dot onto nitrocellulose paper strips. The blots were dried, blocked with skim milk, and incubated with a 1:10, 1:20, 1:30, or 1:40 dilution of a 16-mg/ml stock of the purified IB₁₂ MAb at 37°C for 3 h. The dot blots were washed, and RAMA-PO was added for the detection of bound MAb. All dilutions of IB₁₂ MAb reacted strongly with PT down to 10 ng per dot (Fig. 1). The potential inhibitory effect of NP specimens and NALC treatment on the sensitivity of detection of PT in the dot blot assay was assessed by adding known amounts of purified PT to duplicate normal NP specimens (a total of 10 were tested). One duplicate specimen was assayed directly, and the other was treated first with NALC. Detection of PT was as sensitive in the normal NP specimens as in PBS (10 ng per dot). NALC treatment of NP specimens with added PT had no effect on the sensitivity of the assay.

Specificity of the dot blot assay. To test the specificity of IB₁₂ MAb in the dot blot assay, the strains shown in Table 1

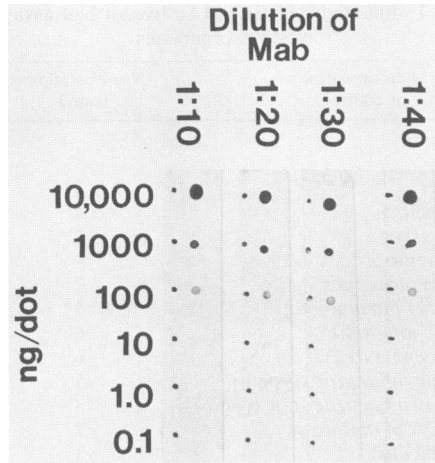


FIG. 1. Sensitivity of the dot blot assay for detection of PT in PBS. Purified PT was diluted in PBS, and various amounts were dot blotted onto nitrocellulose strips. The strips were dried, blocked, and screened with dilutions of IB₁₂ MAb to detect PT. Bound MAb was detected by the addition of RAMA-PO and by the enzyme color reaction (see Materials and Methods).

were screened in the assay. Dilutions of microorganisms at 10^7 , 10^6 , and $10^5/\mu\text{l}$ were made, and 1- μl samples were blotted onto nitrocellulose strips. The strips were processed and screened with IB₁₂ MAb. The IB₁₂ MAb gave strong positive reactions with the *B. pertussis* strains at all dilutions tested. All of the *B. bronchiseptica* and *B. parapertussis* strains tested were negative in the dot blot assay (Table 1). All other microorganisms screened in the assay for cross-reactivity with IB₁₂ MAb were negative. These results demonstrate that the IB₁₂ MAb is highly specific for *B. pertussis* and does not react with other *Bordetella* spp. or other microorganisms which may be found in NP specimens.

Screening of clinical specimens. A total of 25 NP specimens collected from children with whooping cough, confirmed by either culture or DFA, were screened in the dot blot assay to evaluate the ability of the method to detect cases of pertussis. Of the NP specimens, 6 were aspirates, 4 were swabs, and 15 were washes. Twenty-five NP aspirate specimens from adult inpatients were used as controls (Table 2). A 2- to 4- μl sample of each specimen was blotted onto the nitrocellulose paper strips and screened in the assay. Typical results observed in the assays are shown in Fig. 2. All six NP

TABLE 2. Detection of PT in various NP specimens from confirmed cases of pertussis by using IB₁₂ MAb in the dot blot assay

Type of specimen (no. tested)	No. (%) of specimens giving a positive result in:	
	Dot blot assay	Culture
NP aspirate ^a (6)	6 (100)	6 (100)
NP swab ^b (4)	3 (75)	4 (100)
NP wash ^c (15)	7 (47)	8 (53)
NP control ^d (25)	3 (12)	

^a NP aspirates from positive cases of pertussis.

^b NP swabs from positive cases of pertussis.

^c NP washes from positive cases of pertussis.

^d NP specimens obtained from adult inpatients at University Medical Center, Tucson, Ariz.; clinical history not known.

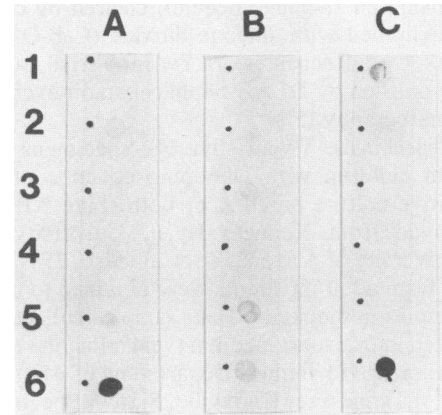


FIG. 2. Screening of NP specimens from pertussis patients by the dot blot assay with IB₁₂ MAb. Four NP swab and six aspirate specimens were prepared, and 2 or 4 μl was blotted onto nitrocellulose strips. The strips were screened for the presence of PT by using IB₁₂ MAb. Lanes: A, 2 μl of NP swab specimens no. 1 to 4 (A1 to A4) and 2 μl of NP aspirate specimens no. 1 and 2 (A5 and A6); B, 4 μl (B1, B3, and B5) or 2 μl (B2, B4, and B6) of NP aspirate specimens no. 3, 4, and 5, respectively; C, 4 μl (C1) or 2 μl (C2) of NP aspirate no. 6, 4 μl of PBS (C3 and C4), control NP specimen (C5), 2 μl of purified PT at 1 mg/ml (C6). All samples tested positive except one swab specimen (A3), PBS (C3 and C4), and the control NP specimen (C5).

aspirates gave strong positive color reactions, whereas three of four NP swab specimens gave weak but positive color reactions above background control NP specimens (Fig. 2). Of the 15 NP wash specimens screened, 7 were positive by the dot blot assay. Twenty-two of the control NP specimens were negative in the assay, with two showing weak reactions and one showing a strong reaction. Five NP aspirates from normal individuals were also tested and gave negative results (data not shown).

DISCUSSION

A major problem in the identification of pertussis cases is the insensitivity of the presently available diagnostic methods. Culture methods require a special medium and are slow, needing 4 to 7 days for initial growth of *B. pertussis*. Reported rates of recovery of *B. pertussis* by culture from clinically diagnosed cases vary greatly, with reported isolation rates of 20, 42, 50, and 83% (3, 10, 22). Introduction of the Regan-Lowe transport and isolation media has improved isolation rates (32). Still, culture remains difficult and subject to numerous factors affecting the viability of *B. pertussis* (1, 2).

DFA, which uses polyclonal rabbit sera, provides more rapid results but has limited sensitivity owing to the number of organisms needed for microscopic visualization (30). DFA staining also suffers from variability and difficulty in interpretation, which accounts for a high percentage of false-positives (ranging from 6.7 to 40%) (3, 5, 20).

Various ELISAs have been developed to detect antibodies to *B. pertussis* in acute- or convalescent-phase serum from patients with pertussis (4, 11, 16, 24, 26, 29, 33, 38). A twofold or greater rise in *B. pertussis* specific immunoglobulin A antibody titer suggests a recent infection, whereas immunization induces mainly immunoglobulin M and immunoglobulin G antibody responses (4, 26). Goodman et al. reported the development of an ELISA for detection of *B.*

pertussis-specific immunoglobulin A antibody in NP secretions as an indicator of recent infection (12). ELISAs which detect the humoral response of a patient to pertussis infection are most useful in epidemiological studies and can be used only retrospectively for clinical diagnosis of whooping cough (11, 12, 26, 29).

In this preliminary study, we have investigated the hypothesis that during a *B. pertussis* infection, bacterial antigens will be produced, secreted, and deposited in the NP secretions of the patient. If this is true, secretions could be collected and screened by immunoassay, using MAb to specifically detect the presence of these antigens.

MAb against PT were used in these initial studies to investigate the potential of the method in rapid diagnosis of pertussis, since the toxin is produced only by *B. pertussis* and not by other *Bordetella* spp. (13, 31, 40). Studies have also reported that PT produced by various strains of *B. pertussis* are antigenically similar (21, 23, 39).

A dot blot immunoassay was developed for the detection of PT by using a MAb against PT, IB₁₂ MAb. In control experiments in which known amounts of purified PT were suspended in either PBS or NP specimens, IB₁₂ MAb was able to detect PT at a level as low as 10 ng per dot (Fig. 1). Pretreatment of NP specimens with the mucus-liquefying agent NALC had no effect on the sensitivity of the assay.

The dot blot assay was specific only for *B. pertussis* when various bacterial strains were screened (Table 1). IB₁₂ MAb demonstrated no cross-reactivity with other microorganisms that typically are found in NP specimens. Other *Bordetella* spp. (*B. bronchiseptica* and *B. parapertussis*) also showed no reactivity with IB₁₂ MAb in the assay (Table 1).

Recent studies by Gustafsson and Askelöf reported the results of using MAb against both *B. pertussis* filamentous hemagglutinin and lipopolysaccharide in a colony blot assay for detection of *B. pertussis* (15). MAb against filamentous hemagglutinin reacted in the assay with all three *Bordetella* spp., whereas MAb against lipopolysaccharide varied in their reactivity with the *B. bronchiseptica* and *B. parapertussis* strains tested. These results demonstrate that MAb to filamentous hemagglutinin or lipopolysaccharide of *B. pertussis* are cross-reactive with other *Bordetella* spp., whereas the present study suggests that MAb against PT is reactive only for *B. pertussis*.

To evaluate the ability of the dot blot assay to potentially diagnose cases of pertussis, initial preliminary studies were done with 25 NP specimens from patients with laboratory-confirmed cases of pertussis and 25 control NP specimens (Table 2). Of the control NP specimens, 88% showed negative reactivity in the study. Three control specimens showed positive results, a 12% level of false-positives in the assay. Clinical reports were not available on these control samples to determine whether these may have been possible adult pertussis cases. A recent study by Robertson et al. suggests that adult infection may be more common than was previously realized (33).

Overall, 64% of the pertussis NP specimens were positive by the dot blot assay. The results presented in Table 2 suggest that specimens of choice for the dot blot assay are NP aspirates, which gave 100% positive reactions in the assay. These specimens were obtained undiluted, whereas the NP swab and wash specimens were all received diluted in various volumes of transport medium. Of these NP specimens, 75% of the swabs and 47% of the washes gave positive reactions by the dot blot assay.

These studies are the first direct demonstration that PT is produced and secreted into NP secretions of pertussis pa-

tients at levels detectable by immunoassays. This technique can detect PT either free in the sample or in direct association with *B. pertussis* (Fig. 1; Table 1). Previous studies suggested the *in vivo* presence of PT during infection by demonstrating increases in anti-PT antibody titers following infection (4, 14). Patients with pertussis have clinical findings of lymphocytosis, hypoglycemia, and an impaired rise in glucose levels in serum in response to epinephrine; all these effects are known to be induced by PT (23).

These preliminary results on the dot blot assay are very promising. However, because of the small numbers of specimens initially tested, further clinical studies are needed before a definitive conclusion on the sensitivity, accuracy, and reliability of the method can be determined. These studies are currently in progress.

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