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Detection of *Bordetella pertussis* in Clinical Specimens by PCR and a Microtiter Plate-Based DNA Hybridization Assay

S. NELSON,[1,2]* A. MATLOW,[1,2] C. MCDOWELL,[1] M. ROSCOE,[1] M. KARMALI,[1,2] L. PENN,[1,2] AND L. DYSTER[3]

Department of Pediatric Laboratory Medicine, Division of Microbiology, The Hospital for Sick Children,[1] *and Department of Microbiology,*[2] *University of Toronto, Toronto, Ontario, Canada, and Biolyne, Inc., Amherst, New York*[3]

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In order to improve detection of *Bordetella pertussis* in nasopharyngeal aspirates (NPAs) in our laboratory, a PCR-based assay was optimized, and a study was designed (i) to compare results obtained by PCR to those obtained by culture and (ii) to evaluate a novel microtiter plate-based DNA hybridization assay (PCR-plate) by comparing it to agarose gel electrophoresis (PCR-gel) for detection of the PCR product. DNA for the PCR was extracted with a guanidine thiocyanate buffer and used in a PCR mixture containing primers directed against a reiterated gene sequence in *B. pertussis* (Q. He, J. Mertsola, H. Soini, M. Skurnik, O. Ruuskanen, and M. K. Viljanen, J. Clin. Microbiol. 31:642–645, 1993). Of 96 NPAs submitted from a targeted study group, 23 were positive by culture, 27 were positive by PCR-gel, and 31 were positive by PCR-plate. All culture-positive specimens were also positive by PCR. Of nine patients with culture-negative–PCR-positive results, six had discharge diagnoses of pertussis. Thus, PCR with plate-based product detection is a sensitive method for the laboratory detection of *B. pertussis* in NPAs. Additional advantages of the plate assay include rapidity, objectivity in reading results, specificity, and the capability of being adapted to a high-volume, automated system.

Despite vaccination, pertussis remains a significant cause of morbidity in young children, particularly in infants less than 1 year of age. It is widely accepted that culture of *Bordetella pertussis* lacks sensitivity, and yield may be further compromised by prior antibiotic treatment, duration of illness prior to culture, immunization status, specimen transport time greater than 3 days, poor specimen quality, and lack of laboratory expertise. In addition, several days of incubation are often required to isolate the colonies. For this reason, PCR assays targeting several regions of the *B. pertussis* genome, including the pertussis toxin promoter (3, 10), a region upstream of the porin gene (7), and a repetitive element (IS*481* [9]), have been developed in various laboratories (2, 6, 11). Many of these studies have shown PCR to be a more sensitive method than culture for detection of *B. pertussis* in clinical specimens. Commonly, detection of the PCR product is achieved by ethidium bromide-stained agarose gel electrophoresis. However, this method has certain disadvantages, including handling time, safety hazards, risk of nonspecific banding patterns, and subjectivity in reading results. Thus, we sought to optimize and implement a PCR assay by using a novel microtiter plate-based DNA hybridization assay (Gene Quest; IMS, Lewiston, N.Y.) for the detection of the *B. pertussis* PCR product. This was done in the context of a prospective study, targeting our emergency ward, with the objectives (i) to directly compare the results obtained by PCR to those obtained by culture and (ii) to compare ethidium bromide-stained agarose gel electrophoresis to the microtiter plate-based system for detection of the PCR product.

* Corresponding author. Mailing address: Department of Pediatric Laboratory Medicine, Division of Microbiology, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5G 1X8. Phone: (416) 813-5625. Fax: (416) 813-5993. E-mail: suenelson@mailhub.sickkids.on.ca.

MATERIALS AND METHODS

Specimen collection and processing. Pertussis kits consisting of a vial with Casamino Acid transport media (4) (1 ml of sterile 1% Casamino Acids [Difco, Detroit, Mich.] in phosphate-buffered saline [Oxoid, Hampshire, United Kingdom]) and a clinical questionnaire were supplied to the emergency unit (targeted study group) for collection of nasopharyngeal aspirates (NPAs) from patients with suspected pertussis. Inoculated transport media were then transported directly to the laboratory or held at 4°C if taken outside laboratory hours (4). To allow for direct comparison between detection methodologies, collected specimens were vortexed, and equal aliquots (50 μl) were processed by culture techniques and by PCR. Aliquots for PCR were stored at −30°C and processed in batches of approximately 20 for the purpose of this study. Uninoculated Casamino Acid transport media, set up after every 5 to 10 specimens, were included as negative controls for the PCR process.

Culture. Specimen aliquots were placed on Bordet-Gengou medium (Difco) containing 15% horse blood and 2.5 μg of methicillin per ml as well as on charcoal agar (Oxoid) containing 10% horse blood and 40 μg of cephalexin per ml. Plates were incubated in a moist environment at 35°C for up to 7 days. Suspect colonies of *B. pertussis* were confirmed by Gram staining, fluorescent antibody staining (Difco), latex agglutination (Murex Diagnostics, Ltd., Dartford, United Kingdom), and standard biochemical tests (8).

PCR. (i) Specimen extraction. To 50-μl specimen aliquots, 4 volumes of extraction buffer (5.75 M guanidine thiocyanate, 50 mM Tris [pH 7.4], 50 μg of glycogen per ml, and 1% β-mercaptoethanol [added immediately prior to use]) were added. The mixture was then vortexed and incubated at room temperature for 10 min. After the addition of 250 μl of isopropanol, the tubes were vortexed thoroughly and centrifuged at 14,000 rpm for 30 min, and then the supernatants were decanted carefully. DNA pellets were washed with 80% ethanol, air dried, and resuspended in 25 μl of water (DNase, RNase, and protease free).

(ii) PCR amplification. Fifty-microliter reaction mixtures were set up, which consisted of 15 mM Tris (pH 8.6); 75 mM KCl; 2.5 mM $MgCl_2$; 200 μM (each) dGTP, dATP, and dCTP; 600 μM dUTP; 0.1% Tween 20; 1 U of uracil *N*-glycosylase (Amersham); 2.5 U of *Taq* DNA polymerase (Amplitaq; Perkin-Elmer); 10 μl of template; and 0.5 μM (each) primers BP1 (5-GATTCAATA GGTTGTATGCATGGTT-3) (2, 6) and BP2 (5-TTCAGGCACACAAACTTG ATGGGCG-3) (6) (synthesized by General Synthesis and Diagnostics, Toronto, Ontario, Canada), which amplify a 181-bp region (nucleotides 12 to 192) of the repeated gene element of *B. pertussis* (2, 6, 9). The reaction mixtures were covered with mineral oil and subjected to the following thermal cycling parameters in the Robocycler 40 (Stratagene): 1 cycle of 50°C for 5 min; 1 cycle of 95°C for 3 min; 58°C for 1 min, and 72°C for 1 min; 32 cycles of 95°C for 45 s, 58°C for 45 s, and 72°C for 30 s; and 1 cycle of 72°C for 3 min. Included with every run were Casamino Acid (10 to 20% of the reaction mixtures) and water (two

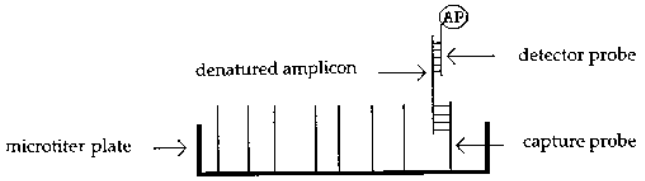

FIG. 1. DNA hybridization assay (Gene Quest; IMS) for detection of *B. pertussis* PCR products. Denatured PCR products were mixed with an alkaline phosphatase (AP)-conjugated detector probe, and the mixture was transfered to specific capture probe-coated microtiter wells. After incubation, washing, and substrate addition, OD_{405}s were recorded. Positive and negative controls were included with each assay.

reaction mixtures per run) blanks as negative controls and a 10-μl DNA aliquot from a known *B. pertussis*-positive specimen as a positive control.

(iii) PCR product detection. After thermal cycling, samples (15 μl) were immediately electrophoresed through a 1.5% agarose gel (0.3 μg of ethidium bromide per ml) in Tris-borate-EDTA at 80 V for 1.5 h or frozen at −20°C for later analysis. A DNA product band with a size of 181 bp, as visualized by UV illumination, was considered positive.

The *B. pertussis* DNA hybridization assay (Gene Quest; IMS) for PCR product detection utilizes a probe-specific product capture technique with a solution-phase alkaline phosphatase-conjugated detector probe (Fig. 1). This assay is commercially available from Biolyne, Inc. (Amherst, N.Y.) for research purposes only. The manufacturer's protocol was followed, which, in brief, included heat denaturation of the samples, snap cooling on ice, the addition of detector probe in hybridization buffer, and transfer of the mixture to the capture probe-coated microtiter wells. After incubation at 37°C, the wells were washed and substrate was added. Optical density at 405 nm (OD_{405}) determinations were made after further incubation at 37°C for 90 min. Positive (5×10^9 copies of a synthetic DNA target to represent postamplification levels) and negative (Tris-EDTA buffer) controls supplied with the detection kit were included in each plate detection assay as controls for the kit reagents. The manufacturer's recommendations for determining the assay cutoff value were to empirically test a number of negative controls, calculate the mean OD and standard deviation, and use the mean plus 5 standard deviations as the cutoff point. This was achieved with 30 negative controls consisting of Casamino Acid, saline, and water extraction blanks and the negative control supplied with the detection kit.

Stringent contamination control procedures were adhered to, including the physical separation of the reagent preparation, specimen extraction, amplification, and product detection areas. The reagents used for PCR were of molecular grade and were purchased from Sigma or Boehringer Mannheim unless otherwise stated.

(iv) Level of inhibition in PCR-negative samples. In order to determine whether inhibition of the PCR was a factor in specimens yielding negative results, an internal control plasmid was constructed, and PCRs were set up with DNA extracted from the 64 negative study specimens. The reaction mixtures were spiked with 500 copies of the internal control plasmid, and detection of the product was performed by ethidium bromide-stained agarose gel electrophoresis. The control consisted of a plasmid containing the *B. pertussis* IS*481* (9) primer binding site (2, 6), a unique internal sequence with a GC content similar to that of the wild-type amplicon, and restriction enzyme sites for cloning. The insert was constructed with synthetic oligonucleotides (104 bases long) that were complementary at their 3′ ends for 22 bases. After hybridization, the Klenow fragment was utilized to synthesize a double-stranded molecule, and sticky ends were generated by *Bam*HI/*Hin*dIII digestion. The fragment was cloned into pGEM3Z (Promega) and transformed into *Escherichia coli* TB1. Transformants were selected on media containing ampicillin, and clones were screened by restriction digest analysis on ethidium bromide-stained agarose gels.

(v) Determination of limits of detection. Two strains of *B. pertussis* (ATCC 9340 and a clinical isolate) were grown on charcoal-cephalexin medium and used to make separate suspensions in saline equivalent to approximately 10^9 organisms/ml based on McFarland standards. DNA from 100-μl aliquots of dilutions of the medium described above, equivalent to 100, 10, 5, or 1 organism per reaction, was extracted and used in PCRs as described above. Detection was performed by both the agarose gel electrophoresis and plate hybridization methodologies. Reactions were performed in duplicate, and the experiment was repeated once.

(vi) Repeat testing of discrepant results. For specimens positive by PCR (with either detection method) but negative by culture, the PCR from the original specimen was repeated, and the product was analyzed with both the gel and plate assays.

Correlation of results with clinical diagnosis. The final clinical discharge diagnoses were obtained and recorded for all study patients.

Statistical analysis. For the microtiter plate assay, Student's *t* test was used to compare the mean OD_{405} of the culture-negative–gel-negative group of specimens with the mean OD_{405}s of the culture-negative–gel-positive and culture-positive–gel-positive groups.

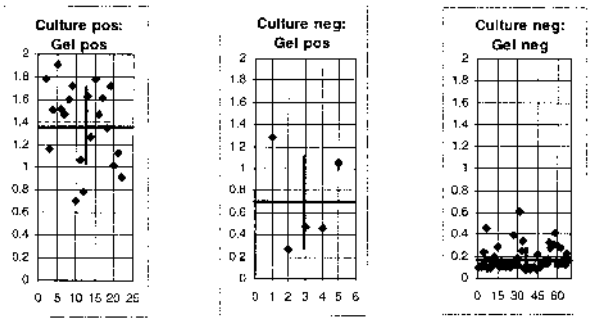

FIG. 2. Microtiter plate OD_{405}s of pertussis study specimens. OD_{405}s for specimens positive (pos) by culture and by PCR-gel, negative (neg) by culture and positive by PCR-gel, and negative by culture and PCR-gel are shown. The number of specimens (*n*) and OD_{405}s (mean ± standard deviation) for each group are as follows: culture positive-gel positive, $n = 22$; OD_{405}, 1.38 ± 0.34; culture negative-gel positive, $n = 5$; OD_{405}, 0.71 ± 0.44; culture negative-gel negative, $n = 68$; OD_{405}, 0.17 ± 0.10.

RESULTS

Microtiter plate OD_{405}s. The OD_{405} (mean ± standard deviation) obtained for the positive control included with each assay was 0.83 ± 0.17. This was 4 standard deviations above the mean of the negative controls (0.15 ± 0.042). The OD_{405} for specimens negative by both culture and PCR-gel methodologies ($n = 68$) was 0.17 ± 0.10 (Fig. 2). For specimens positive by both techniques ($n = 22$), this value was 1.38 ± 0.34 ($P < 0.001$). For specimens positive by PCR-gel but not culture ($n = 5$), the mean OD was lower but still significantly above the mean of those specimens negative by both methods (0.71 ± 0.44 [$P < 0.001$]). The numbers of standard deviations between the mean OD_{405} of negative specimens and the mean value of PCR-gel-positive–culture-negative specimens and PCR-gel-positive–culture-positive specimens were 5 and 12, respectively. The OD_{405} cutoff for the pertussis detection plate, calculated as described in Materials and Methods, was 0.36. Specimens with readings at or above these values were considered positive.

PCR detection limits. The limits of PCR detection by agarose gel electrophoresis were estimated to be 10 to 100 organisms. The limit of the plate assay was 100 organisms per reaction, with an OD_{405} of 0.46 ± 0.26.

Detection of *B. pertussis* in NPAs. From 5 April 1995 to 3 February 1996, 96 NPAs from 96 patients were processed for *B. pertussis* from the targeted study group. Of these, 32 (33%) were considered positive for *B. pertussis* by one or more detection methods, with 23 positive by culture, 27 positive by PCR with gel detection, and 31 positive by PCR with the plate detection assay (Table 1). The sensitivity and specificity of the plate detection methodology were 96% and 92%, respectively compared with those of the gel detection system (Table 2). Of 64 specimens negative by all techniques, no inhibition of the PCR process was noted as ascertained by product detection with the spiked internal control plasmid. Repeat PCR testing of the nine specimens (Table 1) negative by culture but positive by PCR with either detection method yielded eight repeat positives. One of four specimens initially positive only by PCR with plate detection was negative upon retesting. The remaining eight specimens were positive by both gel and plate detection methodologies when retested.

TABLE 1. Concordance of results obtained by culture, PCR-gel, and PCR-plate product detection methods

Result by:			No. of specimens	No. of specimens with pertussis as final diagnosis	Final diagnoses other than pertussis[a]
Culture	PCR-gel	PCR-plate			
+	+	+	22	15	Bronchitis, asthma (n = 2), influenza, parainfluenza, URTI (1 N/A)
−	−	−	64	13	Not listed
−	+	+	4	4	
−	−	+	4	1	Bronchitis, pneumonia, influenza
−	+	−	1	1	
+	−	+	1	1	

[a] For patients with specimens positive by any technique and whose final diagnosis was not pertussis, the discharge diagnoses are listed. URTI, upper respiratory tract infection; N/A, not available.

Correlation with clinical diagnosis. Of 23 patients with culture-positive specimens, 70% had a discharge diagnosis of pertussis, compared with 20% of 64 patients with specimens negative by all methods (Table 1). Of nine patients with specimens positive by PCR alone (by either detection method), six had final clinical diagnoses of pertussis (Table 1).

DISCUSSION

The use of PCR for the laboratory detection of *B. pertussis* has been demonstrated by several groups (1–3, 5, 6, 10, 11), as well as in the present study. Most groups have found PCR to be more sensitive than culture, likely in part because of the fastidious nature of the organism. Furthermore, because culturing often requires 3 to 7 days, PCR methodology lends itself to a decreased time until reporting. For example, in our laboratory, we offer the test twice weekly, which provides a turnaround time of between 1 and 4 days.

PCR products are commonly detected by agarose gel electrophoresis, which employs hazardous chemicals, may be prone to nonspecific banding products, requires subjectivity in reading results, and is fairly laborious. The sensitivity and specificity of product detection may be enhanced by using Southern or solution hybridization techniques employing ^{32}P-labelled probes. However, this is not a desirable approach in a clinical laboratory, because radioactive chemicals are employed and the procedures are quite labor intensive and lengthy, which increases turnaround-times as well as cost. In this study, we have demonstrated the utility of using PCR with a novel, commercially available microtiter plate-based DNA hybridization product detection system for *B. pertussis*. With this system, hazardous chemicals are avoided, the establishment of OD cutoffs removes subjectivity in reading results, the specificity of the amplification product is confirmed by reactivity with both the internal detector and capture probe sequences, and the method is amenable to automation. A disadvantage of the assay is its increased cost compared to gel detection; however, this may be offset by future automation.

In our study, the plate assay showed a high degree of concordance with the gel results (Tables 1 and 2), with a sensitivity and specificity of 96% and 92%, respectively. The difference between the mean OD_{405}s for the culture-positive–PCR-gel-positive and culture-negative–PCR-gel-negative groups was highly significant ($P < 0.001$) (Fig. 2). The difference in the mean ODs for the culture-negative–PCR-gel-positive and culture-negative–PCR-gel-negative groups was smaller, although still significant ($P < 0.001$), perhaps indicating a lower organism burden in these specimens.

TABLE 2. Comparison of PCR product detection methodologies

Result	No. of specimens with microtiter plate result:		Total no. of specimens
	Positive	Negative	
Gel positive	26	1	27
Gel negative	5	64	69
Total	31	65	96

With regards to the reproducibility of the assay, the readings for the positive and negative controls were quite consistent between runs (0.83 ± 0.17 and 0.15 ± 0.042, respectively). The background remained quite low, because specimens negative by both culture and by PCR with gel detection had OD_{405}s (0.173 ± 0.101) similar to those of the negative buffer controls.

Of the nine patients with culture-negative–PCR-positive specimens, six had a very high likelihood of having pertussis based on clinical diagnosis and reproducibility of a positive PCR test. There were four specimens from four patients that were considered positive only by the plate method. These patients had discharge diagnoses of pertussis, bronchitis, pneumonia, and influenza, respectively. Upon retesting, the first three specimens were found to be positive by both the plate and gel detection systems. Because no other respiratory agents were detected in these three specimens, and the patient diagnoses have been associated with *B. pertussis*, these specimens may have been true positives. The reason why these three were detected by PCR with one detection system the first time and by both upon retesting may be attributed to low organism burden, in which sampling of the specimen becomes an issue.

In summary, it is clear that despite vaccination efforts, *B. pertussis* continues to cause significant morbidity in children. Detection of the organism in the laboratory by PCR and with a microtiter plate DNA hybridization assay is a useful tool in the diagnosis of *B. pertussis* infection and may be implemented in the routine laboratory with relative ease.

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