Comparison of Polymerase Chain Reaction with Culture and Enzyme Immunoassay for Diagnosis of Pertussis

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A polymerase chain reaction (PCR) assay amplifying a segment of a repeated gene element of Bordetella pertussis was compared with culture and enzyme immunoassay (EIA) for the diagnosis of pertussis. The PCR assay was specific for B. pertussis in tests with a panel of other bacteria and with an extensive collection of specimen material from healthy persons and children with respiratory infections other than pertussis. The PCR assay was used in the analysis of 117 nasopharyngeal swabs collected from children at an elementary school at which a pertussis outbreak occurred. Fifty-six (48%) of the 117 swabs were positive, including those for all six culture-positive cases. The PCR method was then applied to analyze another pertussis outbreak. Of 40 nasopharyngeal aspirates taken from 37 clinically susceptible pertussis patients and from three asymptomatic contacts, the PCR identified 18 (45%), including all 3 culture-positive and 5 (35%) of the 14 seropositive patients. The most consistent and reliable diagnosis by positive PCR result was observed with those patients experiencing symptoms within 1 to 6 weeks of sample collection. We conclude that PCR is a rapid, sensitive, and specific means of diagnosing pertussis, especially during the first weeks of disease. The assay can be performed with both nasopharyngeal swabs and aspirates.

Early diagnosis of pertussis is important for effective therapy and prevention of transmission of the infection. Moreover, laboratory-defined diagnosis is necessary for epidemiologic surveys and for studies of the efficacy of pertussis vaccines. For diagnostic purposes, culture of Bordetella pertussis from nasopharyngeal specimens is the “gold standard,” but culture is not sensitive enough (17). While the enzyme immunoassay (EIA) as a serologic test has markedly increased detection capability (10, 11, 24), it remains problematic that culture and serology assays detect only a portion of clinically diagnosed cases. Clearly, a simple, more rapid, and more sensitive diagnostic test is needed.

The polymerase chain reaction (PCR) is revolutionizing the diagnosis of many infectious diseases, particularly those caused by organisms that are difficult to cultivate (3, 18). The PCR has been shown to have potential in pertussis diagnostics (5, 6). Glare et al. (5) have suggested that PCR amplification of the reiterated gene sequence of B. pertussis could potentially serve as an efficient diagnostic tool. We have investigated whether PCR amplification of this sequence could complement culture and serology assays in the diagnosis of pertussis. Special emphasis was devoted to comparing the diagnostic efficacy of the methods with the duration of disease symptoms.

MATERIALS AND METHODS

Patients and samples. The specimens were obtained from patients during two outbreaks of pertussis. During the first outbreak, in November 1990, nasopharyngeal swabs (NPSs) were taken from each of 117 children in six classes (age range, 7 to 9 years; male/female ratio, 1:1.2) at an elementary school in Turku in southwestern Finland. Of these children, 39 suffered from coughing, the duration of which ranged at the time of sampling from 2 to 50 days. The second outbreak occurred in Parkano in western Finland in December 1990. Nasopharyngeal aspirates (NPAs) and serum samples were collected from 40 persons (age range, 6 months to 76 years; mean age, 22 years; male/female ratio, 1:1.9). Of the 40 subjects tested, 37 had consistent coughing with vomiting or whooping or both (duration of symptoms at the time of sampling, 1 to 150 days; mean duration, 26 days) and 3 were asymptomatic contact persons of a culture-positive patient. For assessing the specificity of the PCR assay, NPAs were also taken from 75 healthy persons (60 from northern Finland and 15 from Turku) in nonepidemic conditions and from 29 children with respiratory infections other than pertussis in Turku.

Bacteria and culture. To test the specificity of B. pertussis reactivity, a total of 130 bacterial strains of 21 species were examined. These included 22 B. pertussis, 16 Bordetella parapertussis, and 1 Bordetella bronchiseptica strain as well as 8 Branhamella catarrhalis, 2 Enterococcus faecalis, 5 Escherichia coli, 14 Haemophilus influenzae, 7 Klebsiella sp., 1 Proteus vulgaris, 5 Pseudomonas sp., 14 Staphylococcus sp., 1 Streptococcus pyogenes, 10 Streptococcus pneumoniae, and 24 Streptococcus sp. strains. The prototype strain of B. pertussis (no. 1008) was obtained from the Central Public Health Laboratory, Colindale, London, United Kingdom. Other bacterial strains were from the collection of clinical isolates of the National Public Health Institute, Department in Turku, and the Department of Medical Microbiology, University of Turku, Turku, Finland. B. pertussis was grown on charcoal agar plates (Oxoid CM 19), supplemented with 10% defibrinated horse blood and 40 mg of cephalaxin per liter, in a humid atmosphere at 35°C for 5 days (20). Other bacteria were cultivated on routinely used chocolate, blood, or lactose agar plates.

To assess the sensitivity of the PCR, the colonies of B. pertussis were harvested from the plates and suspended in sterile physiological saline. Serial 10-fold dilutions were
made from the suspension, and a colony count was made from each dilution. Aliquots from the dilutions were taken for PCR. After clean swabs (Calgiswab type 1; Spectrum Laboratories, Inc., Houston, Tex.) were placed in the bacterial dilutions for 1 min, the swabs were removed and stored in sterile tubes at −20°C for further treatment.

During the outbreaks, NPSs and NPsAs were immediately inoculated on charcoal agar plates. (After inoculation, these swabs and aspirates were stored at −20°C for PCR.) The plates were incubated as described above and inspected daily for 7 days to determine pertussis-like colony growth. Suspected colonies were Gram stained and studied by slide agglutination with antisera to *B. pertussis* and *B. parapertussis* (Wellcome, Beckenham, United Kingdom). The identity of *B. pertussis* strains was confirmed by gas-liquid chromatography (2).

**Extraction of DNA.** Bacteria were pelleted by centrifugation, and the pellet was resuspended in 300 μl of SDS solution containing 0.1 M NaOH, 2 M NaCl, and 0.5% sodium dodecyl sulfate. After incubation at 95°C for 15 min, 200 μl of 0.1 M Tris-HCl (pH 8) was added. The DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in 50 μl of water.

NPSs were placed in 300 μl of SDS solution, shaken vigorously for 5 min, and incubated at 95°C for 15 min. NPSs were liquefied by adding an equal volume of SDS solution and mixing until mucolysis was complete. The samples were then incubated at 95°C for 15 min. The extraction and precipitation of DNA from NPSs and NPsAs were as described above for bacteria.

**Selection and synthesis of primers.** The primers were selected from the repeated gene element of *B. pertussis* (5, 12) and synthesized on an automated DNA synthesizer (model 391; Applied Biosystems, Inc., Foster City, Calif.) on the basis of phosphoramidite chemistry. The sequence of primer BP1 is the same as that used by Glare et al. (5); primer BP2 was based on our selection. The sequence of primer BP1 was (12) 5'-GATTCAATAGTTGATGATGCTGTT-3' (36); that of primer BP2 was (192) 5'-TACAGGCCCACAAACACTTGGGCGG-3' (186) (numbers in parentheses indicate nucleotide number).

**PCR.** PCR was performed essentially as described by Saiki et al. (21). The various PCR parameters were optimized before application of the clinical samples. A DNA thermal reactor (HB-TR1, Hybaid Ltd., Middlesex, United Kingdom) and the GeneAmp kit with Taq polymerase (Perkin-Elmer Cetus, Emeryville, Calif.) were used. The reaction mixture of 100 μl contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl2, 0.01% (vol/vol) gelatin, 200 μM deoxyribonucleotides, 20 pmol of primers, 2.5 U of Taq polymerase, and 5 μl of aqueous DNA extracted from the bacteria. With the thermal reactor, a total of 40 cycles of denaturation at 94°C for 1 min and annealing and synthesis at 66°C for 3 min were carried out. The tubes were allowed to cool at ambient temperature, and the reaction products were stored at 4°C for electrophoresis.

The DNA extracted from the prototype strain of *B. pertussis* at a concentration of 5 ng/μl was used as the positive control and to test for any possible inhibitory activity of the specimens. All reagents, devoid of added DNA, were included in the negative control tube in each PCR run. A 15-μl amount of the reaction products was run in a 2.0% agarose gel (Seakem ME agarose; FMC Bioproducts, Rockland, Maine). After staining with ethidium bromide and destaining with water, PCR products were visualized and photographed under UV light.

![FIG. 1. Amplification of an 181-bp segment of *B. pertussis* DNA.](http://jcm.asm.org/)

**EIA.** Details of the EIA used for detection of immunoglobulin M, immunoglobulin A, and immunoglobulin G antibodies against *B. pertussis* antigens have been described earlier (15). The purified antigens used were pertussis toxin, filamentous hemagglutinin, and 69-kDa outer membrane protein, kindly provided by Carine Capiu, SmithKline Beecham Pharmaceuticals. The coupling concentrations of the antigens were 1, 5, and 5 μg/ml, respectively. Seropositivity was determined by comparing the antibody results from patient serum samples with those of samples from 80 healthy controls similar in age to the patients. Patients with results exceeding the means + 3 standard deviations of those of the controls for two of the three antigens were considered seropositive. To define significant seroconversion, three serum samples were collected from 10 healthy individuals at 7- and 28-day intervals. The lowest antibody value of each follow-up control was subtracted from the highest value. The result was considered to represent the biological normal variation in antibodies. The means + 3 standard deviations of the individual change values served as the limit for positive seroconversion.

**RESULTS**

The sensitivity of the PCR assay was 25 bacteria per reaction tube. With immersed swabs, the lowest concentration of bacteria yielding a positive result was 2.5 × 104 cells per ml (Fig. 1). All 22 *B. pertussis* strains gave positive results, whereas none of the other bacterial species was positive. Likewise, none of the NPSs from healthy persons or from children with other respiratory infections proved to be positive by the PCR assay. No inhibitory phenomenon was found in specimens examined (data not shown).

Of the 117 NPSs, 6 (5%) were pertussis culture positive while 56 (48%) were PCR positive (Table 1). All culture-positive samples were positive by the PCR assay. Of the 39 samples from patients with coughing, 22 (56%) were positive by PCR. Of the 78 samples from asymptomatic classmates, 34 (44%) were PCR positive. Only one of these cases yielded *B. pertussis* in culture. PCR positivity among the asympt-
The positivity rate was significantly higher by PCR than by culture ($P < 0.001$, chi-square test).

The overall PCR operation can be handled within 24 h. The rapid nature of this assay further depicts the advantage of PCR over culture and EIA, which frequently needs paired sera. On the other hand, present-day PCR technology demands rather sophisticated laboratory regimens to avoid contamination problems (9). In order for PCR to become a method of conventional microbiological laboratories, technological innovations are needed to overcome this obstacle.

In Finland, about 97% of children have been vaccinated against pertussis (22). Pertussis occurs among this vaccinated population mainly in schoolchildren and adults (45% of the patients were adults in the second outbreak), with many of them experiencing only mild and atypical symptoms. During the last 14 years in Finland, EIA has been very confirmed advantages of this method for diagnosis during early stages of disease.

### DISCUSSION

Our investigation of two pertussis outbreaks shows that the PCR significantly complements culture and serology for the diagnosis of pertussis. Particularly noteworthy is the

<table>
<thead>
<tr>
<th>Class</th>
<th>Duration of cough, days (range)</th>
<th>No. culture positive/total</th>
<th>No. PCR positive</th>
<th>No. of laboratory defined cases (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A</td>
<td>15 (4–50)</td>
<td>1/22 (5)</td>
<td>14</td>
<td>Laboratory positive, cough positive</td>
</tr>
<tr>
<td>1B</td>
<td>19 (3–49)</td>
<td>1/24 (4)</td>
<td>11</td>
<td>Laboratory positive, cough positive</td>
</tr>
<tr>
<td>2A</td>
<td>5</td>
<td>0/18 (0)</td>
<td>1</td>
<td>Total (46%)</td>
</tr>
<tr>
<td>2B</td>
<td>15 (2–27)</td>
<td>1/18 (6)</td>
<td>4</td>
<td>Laboratory positive, cough positive</td>
</tr>
<tr>
<td>3A</td>
<td>5 (2–9)</td>
<td>2/21 (10)</td>
<td>18</td>
<td>Laboratory positive, cough positive</td>
</tr>
<tr>
<td>3B</td>
<td>8 (5–14)</td>
<td>1/14 (7)</td>
<td>8</td>
<td>Total (56%)</td>
</tr>
<tr>
<td>Total</td>
<td>6/117 (5%)</td>
<td>56</td>
<td>22/29 (56%)</td>
<td>Total (56%)</td>
</tr>
</tbody>
</table>

* The age distribution of schoolchildren was 7 to 9 years.
* The laboratory defined cases (laboratory positive) of pertussis were confirmed by either culture or PCR or both.

### TABLE 2. Detection of B. pertussis in NPAs by PCR compared with culture and EIA during the second outbreak

<table>
<thead>
<tr>
<th>PCR result</th>
<th>No. culture positive</th>
<th>No. culture negative</th>
<th>Total</th>
<th>EIA positive</th>
<th>EIA negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>3</td>
<td>2</td>
<td>13*</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>3</td>
<td>9</td>
<td>22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6</td>
<td>11</td>
<td>40</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Three asymptomatic contacts of a culture-positive patient were among 13 PCR-positive patients.

### TABLE 3. Relationship between duration of coughing and results of the three diagnostic tests in the second outbreak

<table>
<thead>
<tr>
<th>Duration of coughing (wk)</th>
<th>Symptomatic cases*</th>
<th>No. with positive result by:</th>
<th>Total no. with positive result (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total no.</td>
<td>P</td>
<td>V</td>
</tr>
<tr>
<td>0–1</td>
<td>11</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2–3</td>
<td>11</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>4–6</td>
<td>7</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>≥9</td>
<td>8</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>37</td>
<td>26</td>
<td>12</td>
</tr>
</tbody>
</table>

* Three asymptomatic laboratory-defined cases were not included. Abbreviations: P, paroxysms; V, vomiting; W, whooping.
helpful for the diagnosis of pertussis in these patients (15, 16, 24). Our results in this study firmly support the concept that thus far antibody detection is the only way to diagnose late pertussis.

In contrast, more powerful tools are definitely needed for the early diagnosis of childhood pertussis, especially in vaccinated populations. Vaccination has been shown to lower the diagnostic efficacy of culture (1, 19). This was apparent in the present study: the sensitivity of culture was very low even in early disease. With respect to this problem, our results suggest that PCR may serve as the desired diagnostic tool. PCR positivity was clearly most pronounced in the early stage of illness. This is in agreement with earlier studies showing that detection of the B. pertussis organism in the nasopharynx of patients is dependent on the duration of the infection (7, 8). The lower positivity rate of PCR and EIA during the first week may be caused by respiratory infections other than pertussis.

PCR yielded positive results in almost half of the asymptomatic persons in the outbreak populations. In the first outbreak, the positivity rate of the asymptomatic classmate seemed to be inversely related to the duration of symptoms in the class. In classes with a recent onset of symptoms, a vast majority (88%) of asymptomatic pupils were PCR positive and evidently colonized by B. pertussis. This finding is supported by earlier observations of the efficient spread of pertussis among classmates (13, 16). Indeed, this concept is substantiated by our earlier study, in which EIA results indicated that silent transmission of pertussis in the contacts of culture-positive patients is common (11, 14). Observations in the present study show that PCR can serve as an efficient tool for tracing the transmission and spread of pertussis.

The difficulties associated with diagnosis of asymptomatic and atypical pertussis cases are important in that some studies have suggested that vaccination provides more protection against disease than against colonization or infection (4, 23). When, in fact, the target of pertussis vaccination is eradication of pertussis, an efficient vaccine should not only prevent the disease but also prevent colonization by B. pertussis. PCR seems to open totally new perspectives in monitoring colonization of B. pertussis. This makes it a valuable tool for the future evaluation of vaccine efficacy.

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REFERENCES


