Use of polymerase chain reaction to identify pneumococcal infection associated with hemorrhage and shock in two previously healthy young children

Trudy O. Messmer,* Cynthia G. Whitney, and Barry S. Fields

A PCR assay was developed for detection of *Streptococcus pneumoniae* in clinical specimens including blood and paraffinized tissues. We were able to detect one organism of purified DNA or 4.5 colony-forming units in blood. The primers did not cross-react with other upper respiratory tract streptococci or with pathogens commonly found in clinical specimens. This assay was used in an investigation of an outbreak of severe illness characterized by septic shock and hemorrhage in previously healthy children. PCR detected *S. pneumoniae* in cerebrospinal fluid and autopsy tissues of the two infants who died. The findings from this assay indicated that PCR offers increased specificity and sensitivity over latex agglutination and counterimmunoelectrophoresis and should prove useful in the identification of additional cases of severe illness caused by *S. pneumoniae*.

INDEXING TERMS: PCR • *Streptococcus pneumoniae* • clinical specimens • paraffinized tissues

*Streptococcus pneumoniae* is the most common cause of invasive bacterial disease in the US [1]. It is the leading cause of community-acquired pneumonia and a frequent cause of meningitis, sinusitis, bronchitis, and otitis media in both children and adults. Diagnosis of pneumococcal pneumonia relies, in part, on isolation and identification of the organism from a 24–48-h blood culture, but bacteremia is confirmed in <30% of cases [2, 3]. In addition, culture may be negative if a patient has been treated with antimicrobial agents. Because of carriage of *S. pneumoniae* in the respiratory tracts of normal hosts, isolation of *S. pneumoniae* from this site may not reliably indicate disease. Thus, accurate diagnosis requires a spectrum of clinical and laboratory test findings, including radiologic evidence.

There is a lack of rapid, sensitive, and specific tests for diagnosing pneumococcal infections. Historically, identification of *S. pneumoniae* sepsis was based on obtaining a positive blood culture if other signs and symptoms consistent with the diagnosis were present. The laboratory identification of *S. pneumoniae* is based on the hemolysis pattern when cultured on blood agar plates and by additional biochemical tests on the cultured organisms: optochin sensitivity, the Quellung reaction, and bile solubility [4]. Nonculture methods now available, such as counterimmunoelectrophoresis (CIE) and coagglutination, have failed to show uniform diagnostic sensitivity or specificity even when applied to specimens collected from patients with culture-confirmed bacteremic pneumococcal pneumonia [5, 6]. The lack of sensitive nonculture-based methods and the limitations of culture often result in only presumptive diagnoses of pneumococcal pneumonia [7]. Therefore therapy is frequently empiric and potentially suboptimal [7], and reliably identifying cases of pneumococcal infections for epidemiological studies is difficult.

We applied a new nested PCR for detection of *S. pneumoniae* from clinical specimens from an outbreak of invasive pneumococcal disease in a child care center [8]. In December 1993, two previously healthy infants in a New Mexico child care center developed a severe illness
characterized by septic shock and hemorrhage into the skin or internal organs and died. In this outbreak, cultures of blood and spinal fluid from the two patients were negative. Latex agglutination testing and CIE on cerebrospinal fluid (CSF) from the second infant affected suggested infection with \textit{S. pneumoniae} as the cause of death. Clinical specimens tested included buffy coat, CSF, and autopsy tissue.

This is the first PCR application for detection of pneumococcus in autopsy tissues. Identifying the cause of death in autopsy tissues is important to treat similar cases with appropriate antibiotics, and to determine the etiology of an illness. Several reports describe PCR assays from clinical specimens of blood and (or) buffy coat \cite{9–13}, CSF \cite{14, 15}, sputum \cite{16}, and ear fluids \cite{17, 18}. The assay was critical in identifying the etiology of the deaths in the outbreak.

\textbf{Materials and Methods}

\textbf{CELL CULTURES}

All 48 type strains for the capsular types of \textit{S. pneumoniae} were obtained from the Statens Serum Institute, Copenhagen, Denmark. The type strains for all the other streptococci were from the American Type Culture Collection, Rockville, MD. Streptococci were cultured overnight at 37 °C with 5% CO2 on tryptic soy agar plates with 50 mL/L defibrinated sheep blood (Becton Dickinson Microbiology Systems, Cockeysville, MD), or in Pediatric TSB (Microbiology Systems) by flooding the plate with 3 mL of normal human blood and processed in a like manner as \textit{S. pneumoniae} grown on SBA plates. There was no difference in sensitivity of detection of plate-grown vs broth-grown organisms.

The heat detergent method (HD) of Dawson et al. \cite{19} was used to prepare blood samples for PCR. Briefly, the red cells were lysed by hypotonic treatment with ammonium chloride followed by addition of sterile water. The remaining cells were collected by centrifugation, resuspended in 100 \(\mu\)L of detergent buffer (Nonidet P40 and Tween 20), and boiled in a boiling water bath for 30 min. Five to 10 \(\mu\)L were used in a final PCR reaction volume of 50 \(\mu\)L.

\textbf{DNA PREPARATION OF STREPTOCOCCI AND OTHER BACTERIA FOR PCR}

After overnight growth on blood agar plates, the cultures of streptococci or other bacteria were harvested by flooding each plate with 3 mL of isotonic saline and dislodging the colonies with a sterile inoculation loop. The suspension was removed to a sterile 1.5-mL Eppendorf tube and centrifuged at 3000g for 10 min. The supernatant was removed and the pellet was treated with 50 \(\mu\)L of 0.5 mol/L potassium hydroxide for 30 min at 37 °C. Fifty microliters of 0.5 mol/L Tris, pH 8, was added, and the tubes were boiled in a boiling water bath for 20 min. The tubes were centrifuged at 16 000g in an IEC MicroMax microcentrifuge (International Equipment Co., Needham Heights, MA). The supernatants were diluted at least 1:10 for PCR.

Quantified DNA was prepared by reading the absorbance at 260 nm after extraction on an ABI model 340-A DNA extractor (Perkin-Elmer, Applied Biosystems Division, Foster City, CA). This DNA was used to measure sensitivity of the PCR.

\textbf{PCR AMPLIFICATION}

Samples for PCR were prepared in a class II laminar flow hood, and amplification and analysis of PCR products were each performed in separate locations. Reaction volumes of 50 \(\mu\)L containing 10 mmol/L Tris-HCl, pH 8.8, 75 mmol/L KCl, 2.8 mmol/L MgCl2, 200 \(\mu\)mol/L of each deoxynucleoside triphosphate, 0.1 g/L bovine serum albumin (Sigma Chemical Co., St. Louis, MO), 1.25 U of Taq polymerase (Boehringer Mannheim, Indianapolis, IN), 0.2 \(\mu\)mol/L of each outer primer, and 5 \(\mu\)L of DNA sample were overlaid with one drop of mineral oil and placed in a Perkin-Elmer Thermal Cycler Model 480 (Perkin-Elmer, Norwalk, CT) for 1 cycle of 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 62 °C for 30 s, and 72 °C for 1 min. A final 5-min extension at 72 °C concluded the amplification. The nested or inner PCR reaction mixture was similar to the first except that it contained 0.1 \(\mu\)L of the product of the outer PCR and 0.2 \(\mu\)mol/L of each inner primer. The cycling conditions were identical.

The primers target the pneumococcal autolysin gene sequenced by Garcia et al. \cite{20}, which is unique and characteristic of \textit{S. pneumoniae} \cite{21}. A computation at the National Center for Biotechnology Information with the BLAST network service confirmed the uniqueness of the target sequence: The only matches were with the phage HB-3 of \textit{S. pneumoniae}. There was no human sequence homology.

A750 Outer sense 5’-GGCTACTGGTACGTACATTC-3’
A1145 Outer antisense 5'-AATCAAGCCATCTGGCTATA-3'  
A781 Inner sense 5'-ATCCAAAAAGACAAATTTTGGAA-3'  
A1055 Inner antisense 5'-CTGGATAAAAGGCATTAGTAC-3'  
The outer PCR product is 395 bp and the inner product is 274 bp.

Amplification products were separated by electrophoresis through 2.5% agarose gels [1.5% Nusieve GTG agarose (FMC Bioproducts, Rockland, ME) and 1.0% agarose (Bio-Rad, Richmond, CA)] in Tris–borate–EDTA buffer volume decreased.

Table 1. Microorganisms tested for cross-reactivity in the PCR assay with both the first- and second-step primers.

<table>
<thead>
<tr>
<th>Streptococci</th>
<th>S. crista</th>
<th>S. gordonii</th>
<th>S. mitis</th>
<th>S. oralis</th>
<th>S. parasanguis</th>
<th>S. pneumoniae type strains 1–48</th>
<th>S. sanguis</th>
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<tr>
<td>Corynebacterium diphtheriae</td>
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<td>C. coryneform E6756, E378</td>
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<td>C. maruchotis B1 G5048, BC F124</td>
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<td>C. striatum D9110(A), E 4684</td>
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<td>Ehrlichia chaffensis</td>
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<td>Flavobacterium meningosepticum</td>
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<td>Haemophilus influenzae strains KC 818A, KC 1050B, KC 1051C,</td>
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<td>KC 819D, KC 528E, KC 529F</td>
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<td>Kingella kingae</td>
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<td>Legionella pneumophila serogroup 1</td>
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<td>Mycobacterium tuberculosis</td>
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<td>Proteus mirabilis</td>
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<td>Pseudomonas aeruginosa</td>
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<td>Staphylococcus aureus</td>
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Results

The nested PCR was both sensitive and specific. Neither the outer nor inner primer sets cross-reacted with other streptococci or with other respiratory pathogens. Table 1 lists the microorganisms tested for specificity. Fifty femtograms of S. pneumoniae DNA was included in all the tests on other bacteria to confirm that the PCR was able to amplify target tissues had they been present.

The detection limit of the nested PCR was 1.25 fg of purified S. pneumoniae DNA (less than one genome equivalent). An example of the sensitivity and specificity of the nested reaction is shown in Fig. 1. The figure illustrates the dilution effect of detecting very low concentrations of DNA in a sample. Five femtograms and 1.25 fg amplify, whereas 2.5 fg do not. This is a concentration effect at limiting dilution of target in the sample. Five femtograms represent ~2 genome equivalents. At a concentration of 1 fg/μL, a random 5-μL aliquot may not contain any genome equivalent, whereas another 5-μL aliquot may contain more than two genome equivalents.

Figure 2 shows the detection of S. pneumoniae by nested PCR in human blood. Four and one-half colony-forming units (CFU) of S. pneumoniae were detectable. Counted S. pneumoniae were added to 200 μL of human blood, and the blood was prepared for PCR with the HD method described in Materials and Methods. The figure shows the results of varying the volume of the HD buffer used to boil the final cell pellet on detection by PCR. A high (too numerous to count) and a low number (1 CFU per μL) of pneumococci were added to the blood before isolation. There was no effect on detection by PCR if the final HD buffer volume decreased.

Cultures of CSF, blood, and tissues from patients in this study were negative for S. pneumoniae. Gram-positive cocci were detected in blood buffy coat of one patient, and a latex agglutination test of CSF indicated infection with S. pneumoniae. CIE of the CSF specimen was positive for S. pneumoniae (personal communication of D.M. Musher, VA Medical Center, Houston, TX).
The specificity of the PCR is demonstrated by the failure of the primers to amplify even microgram quantities of other streptococcal DNA, examples of which are listed on the figure. Samples were tested at least three different times and none of the other upper respiratory tract streptococci amplified. At least one of the positive controls always amplified, although the concentration (5, 2.5, or 1.25 fg) of the other streptococci found in respiratory airways that could cross-react are present in clinical specimens of interest.

Discussion

The lack of sensitive and specific tests for pneumococcal pneumonia make it difficult to identify etiology of disease when results with traditional methods are negative or samples and specimens are scarce. Because CSF, blood, and tissue cultures of patients were negative in this outbreak, determining the etiology of the cases in New Mexico required use of alternative diagnostic methods.

We designed a nested PCR to detect pneumococci in respiratory infection. The target sequence was from the autolysin gene common to all S. pneumoniae. A nested strategy was used to increase specificity and sensitivity of detection of target in clinical specimens. The nested reaction is a two-step PCR. One percent or less of the first-step product is amplified in the second-step PCR, and the second-step primers target a sequence internal to the first-step sequence. Clinical specimens often contain inhibitors to PCR even after purification steps such as chloroform\phenol extraction. The two-step PCR often circumvents the effects of inhibitors in clinical specimens because while the first-step product may be too small for detection by ethidium bromide staining, enough product is synthesized for amplification and detection in the second step or nested reaction.

The detection limit of the nested PCR was equivalent to the amount of target contained in one bacterium as determined by using purified DNA or 4.5 CFU in blood. All of the type strains of S. pneumoniae were amplifiable with the expected size products. The most common forms of streptococci found in respiratory airways that could cross-react are S. viridans and group C; however, PCR products were not detected with any of these organisms tested, nor with other respiratory pathogens that might be present in clinical specimens of interest.

The PCR we developed was used to identify the etiology (S. pneumoniae) of an unknown illness causing the
deaths of two children in an Albuquerque child care center. One of the two infant deaths occurred 6 weeks before the investigation, and autopsy tissues were the only specimens remaining for one of the infants. This method was crucial to linking the two deaths to one etiology.

Because this study involved only two cases, the general use of PCR to retrospectively diagnose pneumococcal disease from paraffinized tissues must be interpreted with caution. Asymptomatic carriage of S. pneumoniae in up to 20% of adults requires the exclusion of detection of pneumococcus in control tissues. A prospective study of detection in control tissues removed and handled steriley at autopsy is required to establish the efficacy of this test as a general method.

We thank the members of the New Mexico State Health Department of Pathology for their collection and analysis of the specimens used in this study. We also thank both the Bacterial Diseases Pathology Laboratory of Leo Gorelik and Jeanine Bartlett and the Viral Diseases Pathology Laboratory of Sherif Zaki for shaving the tissue blocks and digesting the tissues for PCR.

References

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