Detection of *Haemophilus influenzae* and *Streptococcus pneumoniae* DNA in Blood Culture by a Single PCR Assay

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A multiplex PCR assay was developed to screen blood cultures from children in The Gambia with suspected pneumonia for the simultaneous detection of *Haemophilus influenzae* type b and *Streptococcus pneumoniae* isolates. Analysis of 295 blood cultures showed that PCR detected the organisms in all samples positive by culture in two samples infected with *H. influenzae* type b and four samples infected with *S. pneumoniae* that were culture negative, indicating that this method is sensitive for detecting these organisms in blood cultures.

An *Haemophilus influenzae* type b conjugate vaccine trial was recently concluded in The Gambia. One of the main endpoints of the trial was the incidence of *H. influenzae* type b pneumonia in children enrolled in the study. Children were assessed for pneumonia by using the World Health Organization criteria: cough or difficult breathing and a respiratory rate of 50 breaths per minute or more in children ages 2 to 11 months or 40 breaths per minute or more in children ages 12 to 59 months. However, the children enrolled in the study were seen by physicians whenever they were ill, and blood was cultured for conditions other than pneumonia. Therefore, the blood cultures tested in the present study were not exclusively for suspected cases of pneumonia.

PCR was introduced into the study as a sensitive means of detection of *H. influenzae* type b. It was envisaged that the technique would be able to detect a few more cases of *H. influenzae* type b infection than culture, thereby increasing the power of the detection method in the trial for the pneumonia endpoint. Previous studies in The Gambia have shown that *H. influenzae* type b and *Streptococcus pneumoniae* are the two organisms most likely to be isolated from clinical samples from children with suspected pneumonias (2, 3, 5). It was therefore decided to screen blood cultures from the trial for both *H. influenzae* type b and *S. pneumoniae*. In this way it was reasoned that the vaccination trial would provide a forum for testing not only the sensitivity of PCR but also the robustness of the technique in this environment.

It was initially intended that buffy coat DNA obtained from EDTA-treated samples would be used for PCR analysis in the vaccination trial study. The feasibility of this approach was first investigated by comparing the PCR results obtained by using DNA extracted from EDTA-treated blood and blood culture samples obtained from 8 patients whose culture results were positive for *H. influenzae* type b and from 15 patients whose culture results were positive for *S. pneumoniae*. Prior to DNA extraction, erythrocytes were removed from samples by dextran sedimentation to eliminate a possible inhibitory effect by heme compounds on the PCR. Pellets obtained from supernatants devoid of erythrocytes were used for DNA extraction (4). Separate PCR assays were used to detect *H. influenzae* type b (primers Hib1 [GCG-AAA-GTG-ACC-TCT-TAT-CTC-TC] and Hib2 [GCT-TAC-GCT-TCT-ATC-TGC-GTG-AA]) (1) and *S. pneumoniae* (primers SP1 [ATC-GAA-ATT-AAT-GTG-AGT-A] and SP2 [AGC-TCT-CAG-CAT-TCC-A]) (4). Any residual inhibitory products in the DNA samples were first removed by incubating 3.5 μl of each sample with 10 μl of the capture resin Gene Releaser (Bioventures Inc.) in an automated thermal cycler (Perkin-Elmer Cetus) by following the protocol recommended by the manufacturer. PCR was performed in a final volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM MgCl2, 0.001% gelatin, 200 μM (each) deoxynucleotide triphosphate, 2.5 U of Taq polymerase (Perkin-Elmer Cetus), and 40 pmol of primers, which were obtained from Eurosequence, Amsterdam, The Netherlands. Amplification in an automated thermal cycler consisted of 35 cycles with the following parameters: (i) denaturation for 2 min at 95°C, (ii) annealing for 2 min at 55°C, and (iii) extension for 2 min at 72°C. After 35 cycles the samples were incubated for a further 6 min at 72°C and were then stored at 4°C until they were analyzed. Samples for PCR were prepared in a laminar flow hood (Gelair Flow Laboratory) in an area dedicated to PCR to minimize the risk of contamination. Amplified PCR products of 480 bp for *H. influenzae* type b and 247 bp for *S. pneumoniae* were detected by agarose gel electrophoresis.

A poor correlation was observed between the results of PCR with DNA from EDTA-treated samples and culture results. Of the 8 cultures positive for *H. influenzae* type b, only 2 were positive by PCR, and of 15 cultures positive for *S. pneumoniae*, only 5 were positive by PCR. In contrast, results of PCR with DNA extracted from blood culture samples showed complete concordance with culture results for both organisms. One drawback with the EDTA-treated samples was that only a small amount of blood was available for testing: in most cases, less than 0.5 ml compared with the 1 to 2 ml that was inoculated into blood culture bottles (20 ml each of tryptic soy broth or brain heart infusion broth [Becton Dickinson]). These were in turn incubated at 37°C for up to 7 days before they were made available for DNA extraction and subsequent PCR analysis. It is therefore possible that the majority of the EDTA-treated samples had no bacterial organisms to start with. There was considerable limitation to the amount of blood that could be obtained from children who were the subjects involved in the study. Consequently, it was decided that blood culture samples would provide the most appropriate choice for DNA extraction in the PCR study.

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periods ranging from 1 to 6 months were also analyzed by the multiplex PCR assay. PCR results were compared with those of culture for 16 samples (6 infected with *H. influenzae* type b and 10 infected with *S. pneumoniae*) stored at −20°C for 1 to 3 months. Surprising results were obtained, however, for the remaining 14 culture-positive samples (7 each infected with *H. influenzae* type b and *S. pneumoniae*) stored at −20°C for 3 to 6 months. Only five of these samples (two infected with *H. influenzae*, three infected with *S. pneumoniae*) were positive by PCR. The reason for this is not clear, but it would appear that prolonged storage of blood culture samples can lead to a deterioration in the quality of DNA required for PCR analysis. Screening of these stored samples also revealed that a patient who had been positive for *S. pneumoniae* by culture was dually infected with both *H. influenzae* type b and *S. pneumoniae* organisms. This result (Fig. 1) as well as all positive PCR results were confirmed by Southern blot hybridization. The probe for confirmation of *H. influenzae* type b infection was generated by PCR with DNA extracted from an *H. influenzae* type b isolate. The amplified 480-bp fragment was excised from a low-melting-temperature agarose gel, and the DNA was recovered by treatment with Gelase (Epicenter Technology). A total of 25 ng of the DNA was labelled with [α-32P]dCTP by using the megaprime labelling system (Amersham). The probe for confirmation of *S. pneumoniae* infection was a 21-bp DNA fragment (TTA-CTT-CAG-CTA-ATA-GTG-ACC) located with the respective labelled probe for 14 h at 55°C in 5× SSC–0.5% sodium dodecyl sulphate (SDS)–5× Denhardt’s solution–100 μg of sheared denatured salmon sperm DNA per ml–50 pmol of 32P-labelled probe. After hybridization the membranes were washed twice in 2× SSC–0.1% SDS at room temperature for 10 min and twice in 2× SSC–0.5% SDS at 55°C for 15 min. Autoradiography was performed at −70°C on Kodak XAR5 X-ray film for 24 to 48 h.

The multiplex PCR assay has proved to be as sensitive as culture, if not more so, for the detection of *H. influenzae* type b and *S. pneumoniae* DNAs in blood culture samples. PCR-positive, culture-negative results were obtained on a number of occasions. While the possibility that these were false-positive results cannot be completely excluded, this would seem unlikely because all PCR results were confirmed by Southern blot hybridization. Furthermore, on one occasion in which a PCR-positive, culture-negative result was obtained for blood culture samples, a diagnosis of *S. pneumoniae* was established in this patient by culture of a lung aspirate sample. This probably reflects the findings from a number of studies which have shown that the yields for both *H. influenzae* type b and *S. pneumoniae* are higher from culture of lung aspirates than cultures of blood (7, 8). The PCR result could therefore represent a clear manifestation of the exquisite sensitivity of the technique. The multiplex approach could also be useful for diagnosing cases of meningitis caused by either *H. influenzae* type b or *S. pneumoniae* (6). Cerebrospinal fluid samples should be much easier to process than blood samples.

In summary, we have used a multiplex PCR assay to screen blood culture samples for the simultaneous detection of *H. influenzae* type b and *S. pneumoniae* DNAs. Multiplexing did not compromise the sensitivity of the technique, and our intention of reducing the number of assays per patient in the study was realized.
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REFERENCES