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# Detection of *Streptococcus pneumoniae* DNA in Blood Cultures by PCR

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**We have developed a PCR assay, with primers derived from the autolysin (*lyt*) gene, for the detection of *Streptococcus pneumoniae* DNA in blood cultures. The predicted fragment of 247 bp was detected in all strains of pneumococci, embracing 12 different serotypes that were tested. Although DNA extracted from four viridans streptococci spp. *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus sanguis*, and *Streptococcus parasanguis*) gave amplification products, these were quite different from the predicted fragment for *S. pneumoniae*. Application of the assay for diagnosis of septicemia caused by *S. pneumoniae* showed concordance between PCR and culture results. However, on four occasions PCR was positive in supernatants from both paired culture bottles while pneumococci were cultured from only one. Performing PCR on negative cultures in controlled studies such as vaccine trials may provide a sensitive tool for increasing case detection.**

Blood culture is used widely for the diagnosis of septicemia caused by organisms such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. However, in many clinical situations the yield from blood culture is low: for example, positive cultures are obtained from fewer than 30% of patients with pneumococcal pneumonia (7). The reason for this is not entirely clear. It is possible that some patients are not bacteremic at the time of blood collection for culture (7). Alternatively, the patient may have had prior antibiotic treatment, or the organisms may be present in protected sites such as polymorphoneutrophil leukocytes. An alternative to blood culture is antigen detection in body fluids such as serum and urine. However, currently available methods such as latex agglutination and counter immunoelectrophoresis have been plagued with problems of specificity or sensitivity (9).

In recent years, commercial DNA probes have become available for the diagnosis of a number of infectious diseases (13). Davis and Fuller (1) have used DNA probes for the identification of bacterial isolates including *S. pneumoniae* from BACTEC blood culture vials. However, this test, although having a 100% specificity, is based on a hybridization format that requires a fair number of organisms (2). Therefore, it is doubtful whether such assays will be sensitive enough to detect the causative organism in the large majority of cases in which blood culture results are negative. The development of PCR (8) and the recognition of the power of this technique to detect small numbers of organisms in clinical samples (4) present a unique opportunity to improve on the frequency of detection of organisms such as *S. pneumoniae* and *H. influenzae* in blood cultures.

The *lyt* gene codes for the major autolysin of *S. pneumoniae*, which is a choline-dependent *N*-acetylmuramic acid L-alanine amidase (6). Apart from in a few viridans streptococci (12), choline is found exclusively in the cell walls of pneumococci (14). In a previous study (10), the *lyt* gene was used as a DNA probe for the identification of *S. pneumoniae* isolates by dot blot hybridization. Although the probe was reactive against all isolates tested, including autolysis-deficient strains, its sensitivity was low and a large number of organisms were required to give a positive reaction. The sensitivity of the PCR technique offers a way of overcoming this problem, and one study (11) has already used the technique to detect *S. pneumoniae* in whole blood and buffy coat samples obtained from patients with pneumonia. In this study, a nested primer system was used to amplify sections of the autolysin and the pneumolysin genes. We have used primers derived from the published sequence of the *lyt* gene (3) to develop a single paired primer assay to amplify a section of the autolysin gene different from that used in the study described above for the detection of *S. pneumoniae* DNA in blood culture samples.

## MATERIALS AND METHODS

**Bacterial strains.** Clinical isolates of *S. pneumoniae* belonging to serotypes commonly encountered in The Gambia (serotypes 1, 3, 5, 6, 7, 9, 14, 19, 22, 23, 29, and 46) were used for the development of the PCR assay. The specificity of the assay was established with the following organisms: *Neisseria meningitidis* group A, *N. meningitidis* group C, *H. influenzae* type b, *Escherichia coli*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Streptococcus bovis*, *Streptococcus agalactiae*, *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus sanguis*, *Streptococcus parasanguis*, and *Plasmodium falciparum*. DNA was extracted from these organisms by the standard phenol-chloroform method followed by ethanol precipitation of DNA.

**Blood cultures.** A total of 1 to 2 ml of blood was obtained from 25 selected patients with suspected pneumonia by venipuncture and inoculated into 20 ml of either tryptone soya broth or thioglycolate broth for aerobic and anaerobic culture of organisms. The cultures were incubated at 35°C and were inspected visually every morning. Subcultures were performed 14 to 18 h after inoculation. Samples were removed from each culture bottle aseptically with a sterile 1-ml syringe, and one drop was subcultured on duplicate blood agar, chocolate agar, and MacConkey agar plates which were then incubated at 35°C. One blood agar plate, along with the chocolate agar and MacConkey agar plates, was incubated in 5% $CO_2$. DNA for PCR analysis was extracted from blood culture bottles either 48 h after inoculation, if positive growth was recorded, or after

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7 days, when culture bottles were discarded. The following procedure was adopted prior to DNA extraction. A total of 9 ml of the supernatant fluid, including any buffy coat, was removed carefully into 10-ml oak-ridged tubes (Nalgene), ensuring that the red cell pellet was not dislodged. The supernatant was then subjected to high-speed centrifugation to pellet bacteria (26,500 × *g* for 10 min at 4°C) with a Beckman J2-21 centrifuge. The pellet was later used for DNA extraction. A further 2 ml of fluid containing erythrocytes was then removed from each culture bottle and mixed with an equal volume of 3.5% Dextran 500 (Pharmacia) and incubated at 37°C for 1 hr to sediment erythrocytes. The supernatant was removed and washed three times in 0.01 M sodium phosphate (pH 7.0) containing 0.1 M sodium chloride. The pellet was used for DNA extraction.

**Extraction of DNA from blood cultures.** Blood culture samples from the first 10 patients with suspected pneumonia were lysed with 0.1% sodium deoxycholate followed by standard phenol-chloroform extraction of DNA. None of these samples gave the usual threadlike material characteristic of DNA after the addition of ethanol and incubation of the samples at −20°C. However, after centrifugation for 3 min in an Eppendorf centrifuge, pellets were visible, ones which were much larger than expected. It was felt that materials other than DNA, possibly polysaccharides, were also being pelleted. The pellets were dried under vacuum and reconstituted in 25 μl of distilled water. Spotting 1 μl of these samples onto agarose containing ethidium bromide gave the characteristic fluorescence of DNA under UV illumination, which ranged in intensity from strong (+) to weak (±). DNA was extracted from blood cultures set up for the remaining 15 patients with suspected pneumonia with a commercial DNA extraction and purification kit according to the instructions of the manufacturer (Micro Tubergen Genomic DNA extraction kit, Invitrogen Corporation). DNA pellets obtained with this kit were much smaller and after drying were reconstituted in 10 to 15 μl of distilled water.

PCR was performed in a final reaction volume of 50 μl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2 mM $MgCl_2$, 0.01% gelatin, 200 μM (each) deoxynucleotide triphosphate, 2.5 U of *Taq* polymerase (Perkin-Elmer Cetus), 40 pmol of primers, and 2.5 μl of template DNA diluted 1:100 in sterile distilled water for DNA extracted from isolates and neat for DNA extracted from blood cultures. Amplification in an automated thermal cycler (Perkin-Elmer Cetus) consisted of 35 cycles with the following parameters: (i) denaturation for 2 min at 94°C, (ii) annealing for 2 min at 55°C, and (iii) extension for 2 min at 72°C. After 35 cycles, samples were incubated for a further 6 min at 72°C and then stored at 4°C until analyzed. The sequences of the primers and probe used in the study are as follows: SPI, ATG-GAA-ATT-AAT-GTG-AGT-A (nucleotides 200 to 219 on the plus strand); SPII, AGG-TCT-CAG-CAT-TCC-A (nucleotides 431 to 447 on the minus strand); and SPIII, TTA-CTT-CGC-CTA-ATA-GTG-ACC (nucleotides 308 to 329 on the plus strand). They were obtained commercially from Research Genetics, Huntsville, Ala. Samples for PCR were prepared under a laminar flow hood (Gelaire, Flow Laboratories) in a PCR-dedicated area to minimize the risk of contamination. The specificity of the PCR assay was determined with DNA from 14 different organisms as templates for amplification, while the sensitivity limits of the assay were established with serial dilutions of DNA, extracted from an *S. pneumoniae* isolate (serotype 7) and a bacterial suspension of the same isolate.

The amplified product produced by PCR was detected by agarose gel electrophoresis. Ten microliters of the amplified product was electrophoresed on a 1.8% agarose gel containing TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA). Gels were stained with ethidium bromide and photographed under UV illumination. Confirmation of PCR results was carried out by Southern blot analysis. DNA was transferred overnight from agarose gel to a GeneScreen Plus membrane (NEN Research Products) with a 10× SSC buffer (1.5 M sodium chloride–0.15 M sodium citrate). The probe was a 21-bp DNA located within the amplified PCR product labelled at the 5′ end with (γ-$^{32}$P)ATP (3,000 Ci/mmol; Amersham) with polynucleotide kinase (Pharmacia LKB Biotechnology). Hybridization was carried out overnight at 55°C in 5× SSC–0.5% sodium dodecyl sulfate (SDS)–5× Denhardt's solution–100 μg of sheared denatured salmon sperm DNA per ml–50 pmol of $^{32}$P-labelled probe. After hybridization, 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 XAR-5 X-ray film for 24 to 48 h.

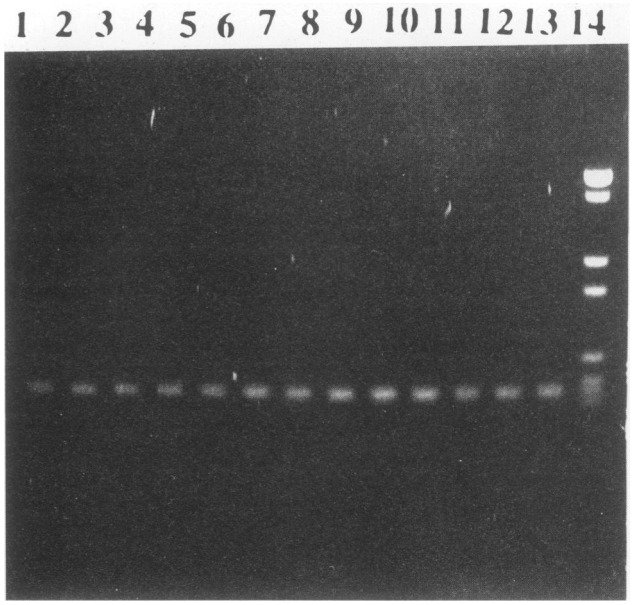


FIG. 1. Amplification of DNA from various serotypes of *S. pneumoniae* clinical isolates by PCR for the *lyt* gene with primers SPI and SPII. PCR products were analyzed by electrophoresis on a 1.8% agarose gel. Lanes 1 to 14, serotypes 1, 3, 5, 6, 7, 9, 14, 19, 22, 23, 29, and 46 plus control and molecular weight markers, respectively. The sizes of the markers are 1,746, 1,434, 800, 634, 303, 279, 249, and 222 bp.

## RESULTS

The results of PCR experiments to determine the presence of the expected fragment size of 247 bp in isolates of *S. pneumoniae* belonging to 12 different serotypes are shown in Fig. 1. Varification of the specificity of the assay showing that the primers were specific for *S. pneumoniae* is shown in Fig. 2a. A positive PCR result was obtained only with *S. pneumoniae* isolates; all other streptococcal strains tested were negative for the expected fragment of 247 bp. Four of the viridans streptococci spp., *S. oralis*, *S. mitis*, *S. sanguis*, and *S. parasanguis*, gave amplification products on PCR which were quite different from the specific fragment for *S. pneumoniae* (data not shown). Confirmation of these results by Southern blot hybridization with five streptococcal species is shown in Fig. 2b, with only

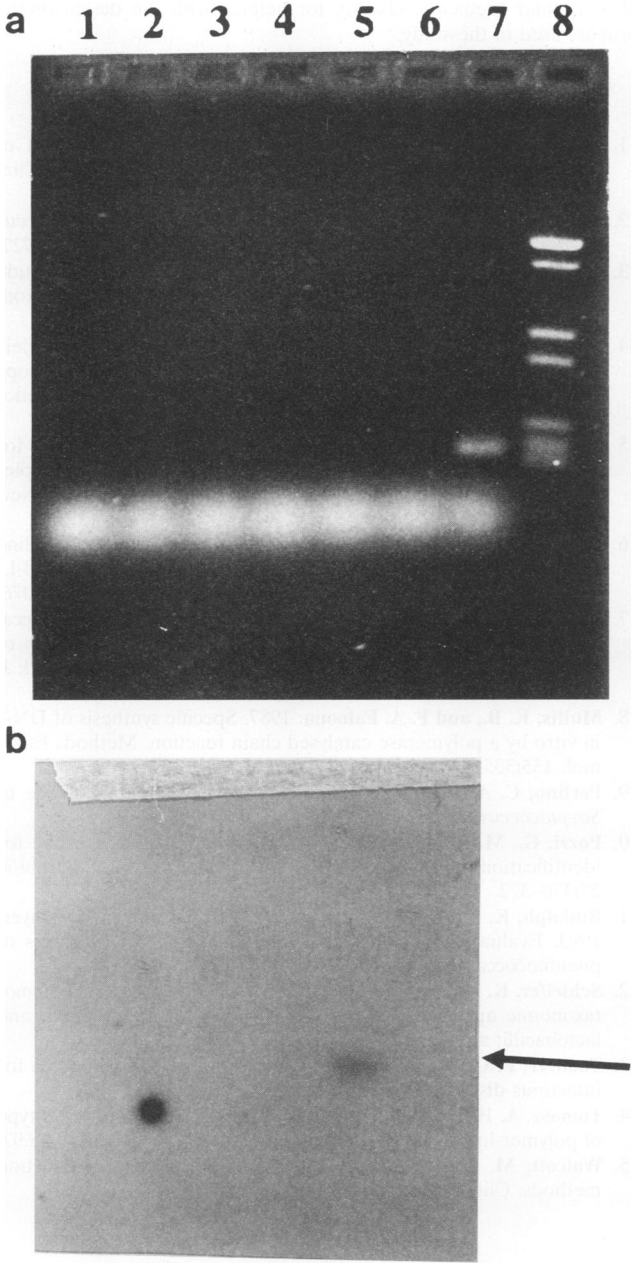


FIG. 2. (a) Specificity of the PCR assay with DNA from various species of streptococci, analyzed by electrophoresis on a 1.8% agarose gel. Lanes 1 to 8, *S. pyogenes*, *Streptococcus faecalis*, *S. mutans*, *S. bovis*, *Streptococcus faecum*, *S. agalactiae*, *S. pneumoniae*, and molecular weight markers, respectively. (b) Autoradiography of panel a showing one positive signal with DNA from *S. pneumoniae* after hybridization with a 21-bp probe derived from sequences within the predicted 247-bp fragment. The probe was labelled at the 5′ end with ($\gamma$-$^{32}$P)ATP. Autoradiography was carried out at −70°C on a Kodak XAR-5 X-ray film for 48 h.

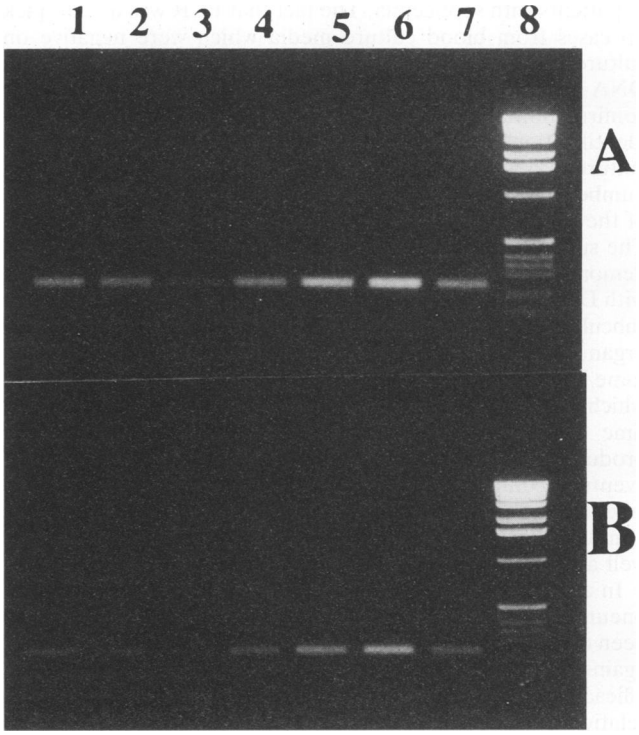


FIG. 3. Amplification of the predicted 247-bp fragment by PCR from DNA extracted from seven blood cultures. (A) DNA extracted from the supernatant fraction. (B) DNA extracted from the deposite fraction. Lanes 1 to 8, samples 041, 178, 198, 219, 250, 252, and 314, and molecular weight markers, respectively.

one positive result from the *S. pneumoniae* isolate. The sensitivity limit of the assay with serial dilutions of DNA from an *S. pneumoniae* isolate was about 0.05 pg while the lowest number of bacterial cells that gave a positive reaction, determined by serial dilutions and plating of samples onto blood agar plates, was 3 CFU. The initial blood culture samples from which DNA was extracted by the phenol-chloroform method were all negative by PCR, even though five of these cultures yielded positive growth for *S. pneumoniae*. Spiking a control lambda DNA preparation into reaction mixtures containing these DNA samples and using primers supplied by the manufacturer (Perkin-Elmer Cetus) failed to produce the expected amplification product of 500 bp, which indicates strongly that these samples contained substances inhibitory to PCR (5). This negative result prompted us to use the Micro Tubergen DNA extraction and purification kit. PCR results with the commercial kit were concordant with those of culture for the diagnosis of *S. pneumoniae* in patients. During the period of the study, *S. pneumoniae* was isolated from 12 patients with suspected pneumonia, and no organisms were isolated from 3 patients. In the majority of cases, positive PCR results were obtained with DNA extracted from both the supernatant and the deposit fractions of the original blood cultures (Fig. 3). In spite of the similarity in the results obtained with PCR and culture for the diagnosis of *S. pneumoniae*, there was, nevertheless, a discernible difference in the interpretation of the results obtained from the two methods. Whereas growth was obtained from only one of the two culture bottles for 9 of 12 patients, positive PCR results were obtained with DNA extracted from both culture bottles for 7 of the 12 patients. Thus, on four of nine occasions in which there was no growth from culture, a positive PCR result was obtained.

## DISCUSSION

The objective of this study was to develop a PCR assay that would improve on the frequency of detection of *S. pneumoniae*

in patients with septicemia. The fact that PCR was able to pick up cases from blood culture media which were negative on culture shows that the technique may be more sensitive than DNA probe tests, which have largely been used as culture confirmation assays (2). The use of this technology for direct identification of organisms such as *S. pneumoniae* in blood cultures (1) would still require the presence of a considerable number of organisms, and it is debatable whether subculturing of the same inoculum would have produced a negative result. The superiority of the PCR assay to such DNA probe tests is demonstrated by the fact that on four occasions PCR results with DNA extracted from culture bottles were positive whereas subculturing from the same bottles yielded no growth of organisms. Our PCR assay, with a single primer system, has the same level of sensitivity as that described in a previous study in which a nested primer system was used (11) while at the same time avoiding the need for open transfer of amplification products from tube to tube, thereby minimizing the risk of eventual contamination. Admittedly, we have used a $^{32}P$-labelled probe for confirmation of our results. However, a number of nonisotopic labelling systems which work equally well are currently available (15).

In an attempt to redress the high mortality associated with pneumonia in young children, new conjugate vaccines have been developed to replace the original polysaccharide vaccines against *S. pneumoniae* and *H. influenzae*. Assessment of the efficacy of these vaccines will require large trials because of the relative infrequency of systemic infections with the pneumococcus or *H. influenzae*, even in high-risk communities. The need for very large trials and their subsequent cost could be reduced substantially if a rate of diagnosis could be achieved in patients with pneumonia that is higher than that which is presently possible by conventional blood culture techniques. Our preliminary results with a pneumococcal PCR assay are encouraging and suggest that this type of approach should be explored further. We do not advocate the use of PCR for routine clinical diagnosis of *S. pneumoniae* but as a research tool in controlled studies such as those relating to vaccine trials. The screening of negative blood cultures by this technique could provide a very sensitive means for improving on case detection methods.

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