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Detection of *Streptococcus pneumoniae* in Sputum Samples by PCR

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A method for the detection of *Streptococcus pneumoniae* in sputum samples by PCR has been developed. The assay employs oligonucleotide primers specific for a portion of the autolysin gene *lytA* of *S. pneumoniae*. Other closely related streptococci, *Haemophilus influenzae*, and *Moraxella catarrhalis* do not give a positive result in the assay. The assay was capable of detecting between 10 and 100 CFU of *S. pneumoniae* in distilled water and 1.4 × 10^4 CFU/ml in simulated sputum samples. Sputum samples from 33 patients with acute pneumonia were collected and subjected to culture, PCR, and C-polysaccharide antigen detection by enzyme-linked immunosorbent assay (ELISA). A significant isolate of *S. pneumoniae* was isolated from 14 patients, of which 13 were positive by PCR and C-polysaccharide antigen ELISA. No positive results were obtained for the 19 patients in whom other pathogens or upper respiratory tract floras only were isolated. The sensitivity of the autolysin PCR is 92.8%, the specificity is 100%, the predictive value of a positive result is 100%, and the predictive value of a negative result is 95%. This suggests that autolysin PCR is suitable for the detection of *S. pneumoniae* in clinical samples.

Streptococcus pneumoniae possesses an autolysin *N*-acetylmuramyl-L-alanine amidase which is responsible for bacterial cell wall remodelling, is important in the process of cell division, and is thought to be responsible for the irreversible effects induced by beta-lactam antibiotics.

The availability of DNA clones of the *lytA* gene has enabled molecular diagnostic tests to be developed. A 0.65-kb fragment of the *lytA* gene has been used as a specific probe for *S. pneumoniae*. This consisted of a *Hin*dIII-*Hin*cII fragment in the amino-terminal region of the gene. This probe showed no cross-hybridization with the related species *Streptococcus oralis* NCTC 11427. In contrast, when a probe utilizing the entire *lytA* gene was used for hybridization, cross-reactivity between these organisms was apparent (6). This suggested that a PCR approach based on the domain coding for amidase activity could potentially be a specific method for detecting *S. pneumoniae* DNA. A PCR method based on amplification of nucleotides 107 to 660 of the autolysin gene with nested primers has been described recently (18). This was used to detect *S. pneumoniae* in the blood and buffy coat of 16 patients with culture-proven bacteremic *S. pneumoniae* infection. The best sensitivity was obtained by examining eight samples of buffy coat, of which five were positive (63%). A single false-positive result was obtained from 14 specimens of blood tested. We evaluated a PCR method based on the amplification of a 263-bp fragment of the *lytA* gene in 33 patients with community- and hospital-acquired pneumonia.

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MATERIALS AND METHODS

PCR protocol. Primers 25 nucleotides in length were chosen from the 5′ end of the *lytA* gene (nucleotides 191 to 454 of the prototype sequence 94813). The primers were 5′ GGA GTA GAA TAT GGA AAT TAA TGT and 5′ GCT GCA TAG GTC TCA GCA TTC CAA. These primers were supplied by British Biotechnology Products, high-pressure liquid chromatography pure in sterile distilled water at a concentration of 1.85 μg/μl. The optimal PCR buffer for amplification contained the following: 1 U of *Taq* polymerase per reaction mixture, 10 μl of *Taq* buffer, 10 μl of 50 mM $MgCl_2$ (Bioline, London, England), 5 μl of extracted sputum sample or 2 μl (4 ng per reaction mixture) of purified DNA, 3 μl of deoxynucleoside triphosphate (5 mM; Promega, Southampton, England), 10 μl of primers (9.25 ng/μl), and sterile distilled water (Baxter Healthcare, Norfolk, England) to a final volume of 100 μl. This was overlaid with mineral oil and processed on a Hybaid TR2 Combi thermoreactor. The PCR cycling conditions consisted of an initial denaturation of 95°C for 6 min followed by annealing at 60°C for 2 min and extension at 72°C for 2 min. This was followed by 32 cycles of 94°C for 75 s, 60°C for 75 s, and 72°C for 120 s. The final two cycles comprised 94°C for 75 s, 60°C for 75 s, and 72°C for 10 min. The products of the PCR were analyzed by electrophoresis through a 1.8% agarose gel by standard methods, using PGem molecular weight markers (Promega) (19).

Specificity of the assay. Representative strains of the currently recognized species of alpha-hemolytic streptococci colonizing humans were studied. These organisms had previously been identified to species level by DNA-DNA hybridization and phenotypic testing as previously described (2, 8, 21). The *Streptococcus* species studied were *S. mutans* (KPSK2, 161, and B48), *S. sobrinus* (B 542, OMZ 176, SL-1^T, OMZ 65, TH 21, and TH 62), *S. salivarius* (NCTC 8618, A385, NCTC 8606, and H53), *S. vestibularis* (JW 3, LV 71, and MM1^T), *S. sanguis* (NCTC 7863^T and KPE 2), *S. parasanguis* (85-81, UC 4989, MGH 143, SS 897, SS 895, and FW 213), *S. gordonii* (NCTC

7868, HF 9017, and M 5), *S. crista* (CR 311, CR 3, and AK 1), *S. oralis* (NCTC 7864, LGV 1 PC 1467, and OPA 1), and *S. mitis* (K 208 and NCTC 10712). In addition, DNA was extracted from 10 strains of *Haemophilus influenzae*, 10 strains of *Moraxella catarrhalis*, and 6 strains each of *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus pyogenes* and assayed. A total of 11 clinical isolates of *S. pneumoniae* (two isolates of type 6, 14, and 16 and single isolates of types 4, 6, 7, 8, and 11) were studied.

Bacterial isolates were grown for 18 h in 20 ml of brain heart infusion broth. Bacteria were harvested by centrifugation for 10 min at 1,500 × *g*, and the supernatant was discarded. The cells were resuspended in 1 ml of phosphate-buffered saline (PBS), vortexed, and washed twice by centrifugation in PBS. The pellets were resuspended in 250 μl of cracking buffer (10 mM Tris, 0.14 M NaCl, 0.1 M sodium citrate, 10 mM EDTA) and heated for 10 min at 80°C in a water bath. DNA was purified from the bacteria listed above by the standard phenol-chloroform extraction method (19).

Sensitivity of the PCR. An isolate of *S. pneumoniae* was grown overnight in 20 ml of brain heart infusion broth and a viable count was performed by a previously described method (16). The bacterial cells were washed twice with sterile distilled water (Baxter Healthcare), and an initial dilution of 1:1,000 was made by adding 10 μl to 10 ml of sterile distilled water. Further 1:10 dilutions were made. For each dilution, five samples of 10 μl were taken and analyzed in the PCR system described above.

A specimen known to be negative by PCR was taken, and known concentrations of *S. pneumoniae* were added. DNA was extracted from this specimen and assayed by PCR by the methods set out below.

Patients and samples. Patients with lobar pneumonia with and without bacteremia or exacerbations of obstructive airway disease were selected. Specimens of sputum were cultured immediately and then stored at −70°C until tested.

A Gram stain was performed on a purulent portion of each sputum specimen. The slide was evaluated for quality by light microscopy under low power (10× objective). Salivary contamination was detected by noting the presence of buccal squamous cells on a scale of 0 to 3, and purulence was determined by noting the presence of pus cells on a scale of 0 to 3. Specimens with a purulence score of 1 or less and a contamination score of 1 or more were not included in the study. All specimens chosen for bacterial culture were mixed 1:5 in 2.5% (wt/vol) *N*-acetyl-cysteine and homogenized at room temperature for 15 min. An aliquot of 100 μl was taken and diluted in 9.9 ml of Ringer's solution, and a second dilution was made (100 μl in 9.9 ml). An aliquot of 100 μl was inoculated onto blood agar, and a second aliquot was inoculated onto chocolate agar. Both plates were incubated at 37°C in an atmosphere of 5 to 10% CO_2 and increased humidity. The number of CFU for each morphological type was recorded. Isolates were identified to species level by recognized methods (1). Isolation of a respiratory pathogen was considered significant if the isolate was present at a concentration of $>10^7$ CFU/ml.

Processing samples for PCR. Specimens were mixed with 2 ml of 50 mM Tris-buffered saline (pH 8.3) and then divided into four aliquots in glass 7-ml bottles and processed separately. The specimens were heat inactivated at 80°C for 20 min. A portion of each specimen (0.5 ml) was transferred to a microcentrifuge tube and centrifuged at 18,000 × *g* for 5 min. The supernatant was discarded. An aliquot of 50 μl of chloroform (BDH Analar, London, England) was added, and the solution was mixed for 15 s with a vortex mixer. To this, 50 μl of sterile distilled water was added, and the solution was mixed again with the vortex mixer. The specimen was centrifuged at 18,000 × *g* for 2 min. The aqueous phase contained the extracted DNA, and this was used in the PCR immediately or stored at −20°C for testing later. All samples were tested in duplicate, with discrepant results being repeated. Sputum samples were also tested in parallel for the presence of C-polysaccharide (PnC) antigen as previously described (7).

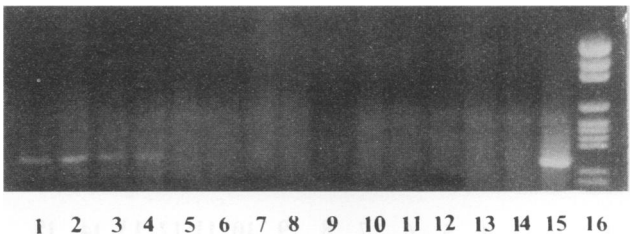

FIG. 1. Results of dilution study to determine the sensitivity of autolysin PCR. Lanes contained the following CFU per milliliter: lanes 1 and 2, 1.4×10^6; lanes 3 and 4, 1.4×10^5; lanes 5 and 6, 1.4×10^4; lanes 7 and 8, 1.4×10^3; lanes 9 and 10, 1.4×10^2; lanes 11 and 12, 14. Lanes 13 and 14, negative controls; lane 15, isolated *S. pneumoniae* DNA (4 ng per reaction mixture); lane 16, PGem molecular weight markers.

RESULTS

Specificity of the assay. The results of autolysin PCR for 36 isolates of oral streptococci obtained were negative. Negative reactions also occurred for all isolates of *H. influenzae*, *M. catarrhalis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *S. pyogenes*. All isolates of *S. pneumoniae* of different serotypes gave a positive result.

Sensitivity of the PCR. The viable count of the *S. pneumoniae* culture was 3×10^8 CFU/ml. The first specimens for PCR contained approximately 1.2×10^3 CFU. The results showed that all samples with 10^3 CFU and three samples with 10^2 CFU but only one of the five samples which contained only approximately 10 CFU were positive, giving an approximate lower level of detection of 10^4 CFU/ml in sterile water. When *S. pneumoniae* was added to a known negative sample and the DNA was extracted, a lower limit of detection of 1.4×10^5 CFU/ml was obtained (Fig. 1).

Clinical evaluation. A total of 33 patients with suspected lower respiratory tract infection were tested. There were 9 females and 24 males, with a mean age of 61 (range, 22 to 88 years). An infective exacerbation of chronic obstructive airway disease was the diagnosis in 14 cases, and acute community-acquired pneumonia was diagnosed in 16 cases. Two patients had postoperative pneumonia and one patient suffered from bronchiectasis. A history of preadmission antibiotic therapy was obtained in 7 cases, and no antibiotics were given before admission in 15 cases. In 10 cases, the prescription of antibiotics before admission could not be determined.

All specimens were purulent, with a mean purulence score of 2.33 and a mean contamination score of 0.72. There were 14 patients with a significant culture of *S. pneumoniae*, 7 with *H. influenzae*, and 2 with *M. catarrhalis*, and no unequivocal bacterial pathogens were isolated from 10 patients. Results of a representative assay are illustrated in Fig. 2. All but one of the patients from whom *S. pneumoniae* was isolated were positive by autolysin PCR, and the same specimens were negative as determined by PnC antigen detection enzyme immunoassay. None of the culture-negative specimens was

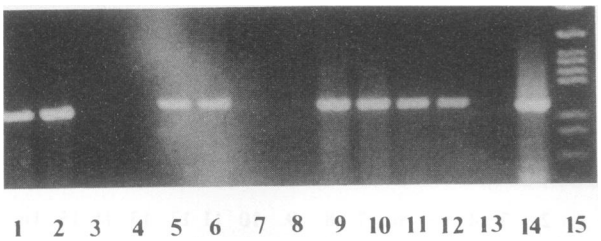

FIG. 2. Results of a representative autolysin PCR of sputum samples from patients with suspected *S. pneumoniae* pneumonia. Lanes 1 and 2 and 5 and 6, positive specimens; lanes 3 and 4, 7 and 8, and 9 through 12, negative specimens; lane 13, negative control; lane 14, *S. pneumoniae* DNA (4 ng per reaction mixture); lane 15, PGem molecular weight markers.

positive by PCR. For two specimens which were negative by both culture and PCR, a positive result was obtained with PnC antigen testing.

Although the numbers of samples analyzed are relatively small, the sensitivity of the autolysin PCR was 92.8%, the specificity was 100%, the predictive value of a positive result was 100%, and the predictive value of a negative result was 95%.

DISCUSSION

PCR has already been applied to the diagnosis of a diverse range of respiratory pathogens (4, 9, 11–13, 15, 17, 20). If PCR is to be widely applied, a full range of respiratory pathogens must be available.

Isolation of *S. pneumoniae* is difficult once antibiotic therapy has commenced. There is no evidence that antibiotics will interfere with results of the PCR reported here, at least in the initial stages. At least three of the patients studied had a history of a prescription of antibiotics before hospital admission, but this did not affect the results of the autolysin PCR.

The method developed in this work uses primers to sequences in the *S. pneumoniae* autolysin gene (*lytA*). These primers yield an amplified fragment 263 bp in length. Unlike some other reported PCR methods, the target is not known to be present in a high copy number. This contrasts with PCR methods for *Mycobacterium tuberculosis*, which detect the IS*6110* insertion sequence which may be present in multiple copies in the *Mycobacterium tuberculosis* genome (22). By using primers for a sequence present in multiple copies, the sensitivity of the test is enhanced. In view of the potential difficulties of differentiating carriage of low numbers of organisms from infection with high numbers of organisms, extreme sensitivity is not necessarily desirable. When *Mycobacterium tuberculosis* is the target, a single isolation or PCR detection is enough to require the initiation of therapy. It is important to realize that the detection of *S. pneumoniae* alone does not have the same clinical significance.

The method described here is able to detect *S. pneumoniae*-positive results in specimens containing more than 1.4×10^4 CFU/ml. This is sufficiently sensitive for clinical purposes.

In the clinical evaluation of the autolysin PCR in a small number of clinical specimens, there were no false-positive results and only one false-negative result. These results compare favorably with PnC- and capsular polysaccharide (CPS)-based antigen detection systems (3, 10, 14). The sensitivity of the test could, of course, be improved by Southern blotting (5), but it might be argued that, in this clinical situation, additional sensitivity might sacrifice specificity when small numbers of isolates of *S. pneumoniae* are detected in healthy patients. The need for a quantitative PCR method based on the primers detailed here is manifest, and this may provide further insight into the pathogenesis of *S. pneumoniae* infection.

Cross-reactions are the single most important drawback of specific diagnosis using PnC antigens, and it is possible that there are many similar cross-reactions for the multiplicity of *S. pneumoniae* capsular antigens in CPS-based antigen detection methods. The results suggest that false-positive reactions are not likely to be a problem. There were no positive reactions in 36 closely related strains of oral streptococci, *S. pyogenes*, *H. influenzae*, *M. catarrhalis*, or staphylococci tested. Additional evidence comes from the clinical studies, in which no false-positive results were found for 33 clinical specimens examined. These specimens not only contained DNA from other respiratory pathogens (*H. influenzae* and *M. catarrhalis*) but they inevitably also contained DNA from the multiplicity of other commensal organisms present.

The current method of PCR product detection is cumbersome and is not suitable for the degree of automation required in routine diagnosis. Methods for colorimetric detection of PCR products based on biotin- and digoxigenin-labelled primers have been developed (22), and application of these methods may facilitate the use of this PCR in the clinical laboratory.

The autolysin PCR has been shown to be of sufficient sensitivity and specificity to merit further clinical development. In the future, it may form the basis, with other tests, of a diagnostic battery for acute lower respiratory tract infection. These developments are under way in our laboratory.

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