

Real-time polymerase chain reaction assay for detection of *Streptococcus pneumoniae* in sputum samples from patients with community-acquired pneumonia

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Background and Purpose: A quantitative real-time polymerase chain reaction (PCR) method targeting the pneumolysin gene of *Streptococcus pneumoniae* in sputum specimens from patients with community-acquired pneumonia (CAP) was evaluated for the identification of pneumococci.

Methods: The applicability of the assay to clinical samples was evaluated by studying 140 sputum specimens from patients with CAP. Of the specimens, 96 (68.6%) were found to be positive by real-time PCR. The results were compared to culture findings.

Results: The sensitivity and specificity of the real-time PCR assay developed in this study as compared to those of the culture method were 97.2% and 60.9%, respectively.

Conclusion: Real-time PCR assay was found to be a rapid and sensitive method for the detection of pneumococci.

Key words: Pneumonia, polymerase chain reaction, sensitivity and specificity, sputum, *Streptococcus pneumoniae*

Introduction

Streptococcus pneumoniae is an important bacterial pathogen in humans that is recognized as a major cause of pneumonia, meningitis, sinusitis, and otitis media and as an uncommon cause of a variety of other infectious diseases [1]. It is also the major bacterial agent in community-acquired pneumonia (CAP) [2,3]. While traditional antimicrobial therapy has proven to be an effective treatment, the emergence of penicillin- and multidrug-resistant strains has resulted in an increase in the number of cases of illnesses and fatalities [4]. A timely diagnosis of pneumococcal disease based on highly sensitive and specific assays may reduce unnecessary broad-spectrum antibiotic therapy and may improve patient care [5].

S. pneumoniae strains are classically determined using bacterial culture combined with biochemical or immunochemical identification tests. However,

pneumococcal isolation and identification can be complicated by antimicrobial suppression of growth in culture and by contamination of the growth medium by alpha-hemolytic streptococci in the normal flora. The major limitation of the current culture techniques is that results are not available early enough to guide antibiotic management. Serologic tests such as latex particle agglutination are rapid but lack sensitivity [5].

Molecular diagnostic methods that are based on the replication of nucleic acids have been used for the diagnosis of pneumococci since 1987 [6]. Molecular assays are inherently valuable because detection can be achieved with enhanced sensitivity and specificity. Polymerase chain reaction (PCR)-based assays for the detection of *S. pneumoniae* with primers specific to genes encoding rRNA, autolysin, and pneumolysin have been used in several studies [7-12]. Pneumolysin is one of the most important virulence factors of *S. pneumoniae*. It is produced by all clinical isolates of pneumococci and has been shown to be well conserved [13].

Besides conventional PCR, real-time PCR, by which DNA/RNA in a clinical specimen could be quantitated,

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was used for the identification of *S. pneumoniae* [4,5, 14-17]. In real-time PCR, amplification and detection of products occur simultaneously, which makes it possible to monitor the phase of the reaction at a particular stage or continuously. Since the assay can be completed within a working day, it is considerably faster than culturing and subsequent identification.

In this study, we assessed rapid DNA isolation and quantitative real-time PCR methods for the identification of *S. pneumoniae* from sputum samples of patients with CAP. Samples were investigated using both the traditional culture method and the modern quantitative real-time PCR assay. Primers specific for the pneumolysin-encoding gene were used for the detection of *S. pneumoniae* in clinical samples. The sensitivity of the real-time PCR method in detecting pneumococci was compared with that of the culture method.

Methods

Collection of clinical samples

This study was performed using sputum specimens collected from adult patients with CAP who were admitted to 3 regional hospitals in Gaziantep, Turkey in 2004. Sputum specimens were obtained from patients with proven clinical and radiographic evidence of pneumonia. Before obtaining a sputum sample, food, gum, tobacco, and dentures were removed from the mouths of patients. An early morning and an induced sputum specimen were preferred. The sputa were collected in sterile specimen cups and were transferred immediately to the laboratory.

Microbiological methods

Sputum samples were evaluated by gross appearance and subjectively categorized as purulent (containing pus with or without mucus, saliva, or blood) or non-purulent (no pus present). Gram-stained samples were prepared according to standard procedures, such that only the purulent-appearing portions of the sputa were sampled. Each Gram-stained specimen was read twice in a blinded manner by 2 medical microbiologists, and scored for polymorphonuclear leukocytes and squamous epithelial cells. The quality of the sample was rated by Q score — assigning a value to the number of neutrophils and squamous epithelial cells per low-powered field (10× lens objective) and summing the results [18]. Contamination of the oral flora was detected by noting the presence of squamous cells on a scale of 0 to 3, and purulence was determined by noting the presence

of neutrophils on a scale of 3 to 0. Specimens with a purulence score of 1 or less and a contamination score of 1 or more were not included in the study.

Culture and classical identification of the specimens

Sputum specimens chosen for bacterial culture were mixed (1:5) in 2.5% (w/v) N-acetyl-L-cysteine and homogenized at room temperature for 15 min. An aliquot of 100 µL was taken and diluted in 9.9 mL Ringer's solution; it was further diluted a second time (100 µL in 9.9 mL). An aliquot of 100 µL was inoculated onto 5% blood agar plates (Oxoid, Hampshire, UK) and incubated at 37°C in 5% CO₂ for 24-48 h. All the bacterial cultures were made as duplicates. The number of colony forming units (CFU) in each culture was recorded after 24 and 48 h. Isolation of an alpha-hemolytic bacteria was considered significant if the isolate was present at a concentration of >10⁷ CFU/mL. Identification was confirmed in all isolates by optochin susceptibility and bile solubility tests.

Optochin susceptibility test

Susceptibility to optochin was determined using 5-mg optochin disks (Bioanalyse, Ankara, Turkey). Bacteria were cultured with an optochin disk on plates containing 5% sheep blood agar and incubated overnight at 37°C in 5% CO₂. An inhibition zone of 14 mm and greater around the optochin disk indicated susceptibility to optochin.

Bile solubility test

Fresh cultures of *S. pneumoniae* were prepared using a suspension of McFarland 0.5 standard in 1 mL saline. The suspension was divided in half in 2 glass tubes, and 0.5 mL saline was added to 1 tube and 0.5 mL 10% deoxycholate (Becton Dickinson, Sparks, MD, USA) was added to the other. The tubes were incubated for up to 30 min at 35°C and evaluated visually. Bile solubility of the strain was assessed by bacterial lysis with 10% deoxycholate.

Controls

Four different types of alpha-hemolytic streptococci were selected as the control bacteria in order to test the specificity of real-time PCR assay. The control bacteria included *Streptococcus mitis* strain American Type Culture Collection (ATCC; Rockville, MD, USA) 15914, *Streptococcus oralis* strain ATCC 10557, *Streptococcus gordonii* strain ATCC 12396, and

Streptococcus sanguinis strain ATCC 10556. *S. pneumoniae* strain ATCC 49619 was used as a reference strain in the study. All isolates were obtained from the ATCC. In each series with clinical specimens, 1 positive control (pneumococcal DNA preparation of the reference strain) and 1 negative control (sterile distilled water) were included for each set of 10 clinical specimens.

DNA extraction from specimens

Specimens were mixed with 2 mL of 50 mM Tris-buffered saline (pH 8.3) and heated 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, 50 µL of sterile distilled water was added to each tube, and the solution was mixed for 15 sec using a vortex. DNA from the specimen was extracted using the QIAamp DNA minikit (Qiagen, Hilden, Germany) spin column method following the manufacturer's body fluid and tissue protocols. The extracted DNAs were used in the PCR immediately or stored at -20°C for testing later.

DNA extraction from control bacteria

Bacterial isolates were grown for 18 h in 20 mL of brain heart infusion broth (Oxoid, Basingstoke, UK). Broth cultures were harvested by centrifugation for 10 min at 1500 *g*, and the supernatant was discharged. 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 cracking buffer (10 mM Tris, 0.14 M sodium chloride, 0.1 M sodium citrate, 10 mM ethylenediamine tetra-acetic acid) and heated for 10 min at 80°C in a water bath. DNA was purified from the bacteria using the standard phenol-chloroform extraction method.

Oligonucleotides used in the study

The primers and fluorogenic probe used for real-time PCR were designed by Primer Express Software (Perkin Elmer, Applied Biosystems, Foster City, CA, USA) to target a 206-bp fragment of the single copy pneumolysin gene of *S. pneumoniae*, and were based on a published sequence [6]. The forward primer oligonucleotide sequence was 5'-AGCGATAGCTTTCTCCAAGTGG-3' (positions 531 to 552), the reverse primer sequence was 5'-CTTAGCCAACAAATCGTTTACCG-3' (positions 605 to 583), and the probe sequence was 5'-ACCCAGCAATTCAAGTGTTTCGCG-3' (positions 556 to 580).

Real-time quantitative PCR

Amplification of a 206-bp fragment of the pneumolysin-encoding gene was performed using the ABI 7000 Instrument (Perkin Elmer, Applied Biosystems, Foster City, CA, USA). For the detection of the pneumococcal DNA, a reaction mixture was prepared. The 20 µL reaction mixture contained 5 µL of TaqMan buffer, 25 mM magnesium chloride 10 mM of each deoxyribonucleotide (dATP, dCTP, dGTP, dUTP), 0.5 U Amp Erase uracil N-glycosylase, 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Warrington, UK), 10 pmol of each forward and reverse primers, and 5 pmol of TaqMan probe. Five µL of the template DNA was added to the reaction mixture. The PCR protocol consisted of a holding step at 50°C for 2 min, an initial denaturation step at 95°C for 10 min, 50 cycles of amplification, each consisting of 15 sec of denaturation at 95°C, and 1 min of annealing at 60°C. The fluorescence was measured once after each annealing step.

Evaluation of real-time PCR results

Real-time PCR results were based on fluorescence readings, which are used to calculate a baseline reading for each reaction. The fluorescent reporter dye at the 5' end of the probe was 6-carboxyfluorescein; the quencher at the 3' end was N,N,N',N'-tetramethyl-6-carboxyrhodamine. The cycle threshold value is the PCR cycle number at which the measured fluorescent signal exceeds a calculated background threshold, identifying amplification of the target sequence. If no increase in fluorescent signal is observed after 50 cycles, the sample is assumed to be negative. Each sputum sample was tested in duplicate by real-time PCR, with discrepant results being repeated.

Statistical analysis

The sensitivities of the real-time PCR and bacterial culture methods were compared using McNemar's test. Spearman's correlation was used to evaluate the correlation between the real-time PCR and culture results. A *p* value of <0.05 was considered statistically significant.

Results

A total of 140 sputum samples from patients with CAP were assessed. Of the patients, 46.7% were men. The mean age of the patients was 42 years (range, 24 to 82 years). Using the real-time PCR assay, 96 (68.6%) of 140 sputum samples were positive for *S. pneumoniae*.

Table 1. Comparison of results obtained by real-time polymerase chain reaction (PCR) and culture of the sputum samples

	Total No. (%)	Culture results	
		Positive No. (%)	Negative No. (%)
Real-time PCR (+)	96 (68.6)	69 (49.3)	27 (19.3)
Real-time PCR (-)	44 (31.4)	2 (1.4)	42 (30.0)
Total	140 (100)	71 (50.7)	69 (49.3)

The culture findings revealed that 71 (50.7%) of the 140 sputum samples were positive for *S. pneumoniae*. The real-time PCR assay applied to these 71 culture-positive samples yielded 69 positive results. In all, 69 (49.3%) sputum samples were negative in culture and 27 of them were PCR-positive. Two culture-positive samples remained negative by PCR. Thus, PCR was positive in 97.2% of pneumococcal culture-positive samples and in 39.1% of the sputum samples from which no pneumococci could be cultured (Table 1).

The results of real-time PCR for 4 isolates of alpha-hemolytic streptococci (*S. mitis*, *S. oralis*, *S. gordonii*, and *S. sanguinis*) obtained were negative. The control strain of *S. pneumoniae* gave a positive result in each PCR run. The sensitivity of the real-time PCR for detection of *S. pneumoniae* in sputum samples was documented by serial 10-fold dilutions of the reference strain *S. pneumoniae* ATCC 49619. The results showed that all of the diluted samples within the range 10^2 - 10^5 CFU/mL were positive by PCR.

The sensitivity and specificity of the real-time PCR assay as compared to those of the culture method were 97.2% and 60.9%, respectively. The positive predictive value was 71.9% and the negative predictive value was 95.5%. There was a statistically significant difference between the real-time PCR and culture methods in the identification of pneumococci from sputum samples.

Discussion

The identification of pneumococci is based primarily on the detection of pneumococci in culture, detection of specific antigens, biochemical tests, and determination of target DNA/RNA by molecular methods in blood or other specimens. Although blood culture is currently the most accurate tool in the diagnosis of CAP, only approximately 30% of blood specimens test positive with this method [19]. Conventional serologic methods lack uniform diagnostic sensitivity and specificity and are time-consuming because they require reagents in which all serotypes are presented. Although the

sensitivities and specificities of biochemical tests such as optochin susceptibility and bile solubility tests vary between 80% and 100%, these methods are time-consuming and require a lot of laboratory work [20].

The real-time PCR system with sequence-specific primers and fluorescent TaqMan probe enables the simultaneous amplification and detection of bacterial DNA in a closed-tube system, which allows rapid, dynamic assessment of PCR products and virtually eliminates the possibility of contamination by extraneous nucleic acid. The combination of rapid nucleic acid extraction, amplification, and detection methods has considerably improved turnaround time for microbiology results, especially for infectious disease agents that require culture-based methods for identification [21].

Several investigators have evaluated PCR as a tool for diagnosing *S. pneumoniae* in respiratory samples such as sputum, bronchoalveolar lavage, and trans-tracheal aspiration [22-24]. The PCR positivity rates of sputum samples among pneumonia patients have been reported to be between 81% and 100% in several studies [10,12]. In the present study, 97.2% of culture samples were found to be positive by real-time PCR. The possible explanation for the 2 culture-positive and PCR-negative results in the present study could be the inefficiency of the DNA purification method that may have inhibited DNA polymerase activity during the PCR.

In general, real-time PCR assay appears to be more sensitive than culture method in several studies. Kearns et al [9] identified all the 12 culture-positive pneumococci from cerebrospinal fluid by real-time PCR. Corless et al [25] reported a 91.8% sensitivity of real-time PCR in culture-positive cerebrospinal fluid, serum, and whole blood specimens. When the assay was applied to nasopharyngeal secretions, Greiner et al [2] found a sensitivity of 100% as compared to the culture results.

Microorganisms closely related to *S. mitis* and harboring genes encoding the virulence determinants pneumolysin and autolysin classically associated with *S. pneumoniae* have been reported previously [22]. However, positive results were not obtained for the 4 closely related organisms from the mitis group of Streptococci — *S. mitis*, *S. oralis*, *S. gordonii*, and *S. sanguinis* — that were tested for cross-reactivity in this study.

In our study, 28.1% of PCR-positive samples remained negative in culture. This result suggests that some positive results in culture can be obscured by

factors such as prior antimicrobial therapy, small amounts of bacteria present in samples, or autolysis of pneumococci in culture media [26]. On the other hand, it is noteworthy that when identification was performed directly from clinical specimens, dead bacterial remnants or bacteria with impaired viability may be detected by PCR, which may cause discrepancies between the PCR and culture results [15].

As a result, it was observed that real-time PCR assay was more sensitive than the classical culture method in the diagnosis of *S. pneumoniae*. Real-time PCR can be used as a diagnostic tool in CAP due to its high sensitivity. The lack of an early and specific etiologic diagnosis creates the need for use of broad antimicrobial regimens, thus increasing the cost and potential for adverse events.

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