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Evaluation of a PCR Assay for Detection of *Streptococcus pneumoniae* in Respiratory and Nonrespiratory Samples from Adults with Community-Acquired Pneumonia

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***Streptococcus pneumoniae* is the most common cause of community-acquired pneumonia, but it is undoubtedly underdiagnosed. We used a nested PCR assay (targeting the pneumolysin gene) to detect *S. pneumoniae* DNA in multiple sample types from 474 adults with community-acquired pneumonia and 183 control patients who did not have pneumonia. Plasma or buffy coat samples were PCR positive in only 6 of the 21 patients with positive blood cultures for *S. pneumoniae* and in 12 other patients (4 of whom had no other laboratory evidence of *S. pneumoniae* infection). Buffy coat samples from two control patients (neither having evidence of *S. pneumoniae* infection), but no control plasma samples, were PCR positive. Although pneumococcal antigen was detected in the urine from 120 of 420 (29%) patients, only 4 of 227 (2%) urine samples tested were PCR positive. Overall, 256 of 318 (81%) patients had PCR-positive sputum samples, including 58 of 59 samples from which *S. pneumoniae* was cultured. Throat swab samples from 229 of 417 (55%) patients were PCR positive and, in those who produced sputum, 96% also had positive PCR results from sputum. Throat swabs from 73 of 126 (58%) control patients were also PCR positive. We conclude that the pneumolysin PCR assay adds little to existing diagnostic tests for *S. pneumoniae* and is unable to distinguish colonization from infection when respiratory samples are tested.**

Streptococcus pneumoniae has consistently been shown to be the most common cause of community-acquired pneumonia in both adults and children, accounting for about two-thirds of cases in which an etiologic diagnosis is made (8). However, a definitive diagnosis of pneumococcal pneumonia is difficult to establish using conventional diagnostic tests; isolation of *S. pneumoniae* from blood lacks sensitivity, isolation of *S. pneumoniae* from sputum may represent colonization, and invasive tests are rarely performed.

Alternative approaches intended to improve the diagnostic yield for patients with suspected pneumococcal pneumonia have produced variable results. Measurement of pneumococcal antibodies in serum or circulating immune complexes has not proved reliable for diagnosing pneumococcal pneumonia and may require testing of both acute- and convalescent-phase sera (21). A new, rapid immunochromatographic urinary antigen test has shown promise for adults with pneumonia (4, 19) but may be less useful for children (5). Several investigators have evaluated PCR as a tool for diagnosing pneumococcal pneumonia (2, 3, 6, 14, 16–18, 24, 25). In general, PCR appears to be more sensitive than culture, but most studies have tested blood samples from only a small number of selected patients. Moreover, few studies have compared different sample types from the same patients.

Therefore, we evaluated PCR for the detection of *S. pneumoniae* DNA in both respiratory and nonrespiratory samples from a large number of adults with community-acquired pneumonia and a control group of patients without pneumonia. Our main aim was to compare PCR with culture and antigen detection methods with a view to assessing whether PCR may be a useful tool for diagnosing pneumococcal pneumonia.

MATERIALS AND METHODS

Subjects. Between July 1999 and July 2000, 474 adults were enrolled in a prospective study of community-acquired pneumonia (15). All patients had clinical and radiographic evidence of pneumonia and were admitted to one of two large metropolitan hospitals (Christchurch Hospital, Christchurch, and Waikato Hospital, Hamilton, New Zealand). In addition, 183 patients who had no evidence of infection or active respiratory disease served as controls. These patients had been admitted to one of the study hospitals (Christchurch Hospital) at the same time as the patients with community-acquired pneumonia, with whom they were matched for age and sex.

Samples. Blood, sputum, urine, throat swab, and nasopharyngeal samples were collected from the pneumonia patients at the time of admission to the hospital, and convalescent-phase sera were collected from survivors 6 weeks after discharge. Blood, urine, and throat swab samples were collected from all control patients.

Samples for molecular testing were processed in the following manner and then stored at −80°C until testing was performed. Whole-blood samples collected in EDTA were centrifuged at 2,000 × *g* for 5 min and then separated into plasma and cellular fractions. After the deposited cellular material was incubated at 37°C for 5 to 10 min in red cell lysis buffer (0.8% NH_4Cl solution), white blood cells (buffy coat) were recovered by centrifugation, washed with molecular-grade phosphate-buffered saline (PBS), and reconstituted in PBS to 2.0-McFarland

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standard opacity. Sputum samples were digested with dithiothreitol (Sputosol; Oxoid) in a 1:1 ratio using sterile glass beads and washed twice with PBS buffer, and the wash pellet was reconstituted to 2.0 McFarland standard. Dry throat swabs were placed in 1.0 ml of molecular-grade PBS and vortexed for 30 s. Urine samples were aliquoted and stored without additional processing.

Microbiology. Blood cultures were incubated aerobically and anaerobically using the BacT/Alert Microbial Detection System (Organon Teknika, Durham, N.C.). Sputum was examined by Gram stain microscopy and cultured on sheep blood agar, chocolate agar, buffered charcoal yeast extract agar supplemented with α-ketoglutarate, and modified Wadowsky-Yee medium. Urine was concentrated 25-fold using Minicon-B15 concentrators (Millipore, Bedford, Mass.) and tested for antigens of *S. pneumoniae* and *Legionella pneumophila* serogroup 1 by the NOW *S. pneumoniae* and NOW *Legionella* urinary antigen tests (Binax, Portland, Maine). Cellular material from nasopharyngeal swabs in viral transport medium was directly examined by immunofluorescence and cultured for respiratory viruses. Paired serum samples were tested for antibodies to legionellae, *Mycoplasma pneumoniae*, *Chlamydia pneumoniae*, influenza A and B viruses, adenovirus, respiratory syncytial virus, and parainfluenza viruses.

PCR. Samples from patients with pneumonia (plasma, buffy coat, sputum, throat swabs, and urine) and from control patients (plasma, buffy coat, and throat swabs) were tested for *S. pneumoniae* DNA by PCR.

DNA was extracted using the QIAamp DNA minikit or QIAamp Viral RNA minikit (Qiagen, Hilden, Germany) spin column method following the manufacturer's protocol. Plasma and buffy coat samples (200 μl) were processed using the blood and body fluid spin protocol, throat swab and sputum samples (200 μl) were processed using the tissue protocol, and urine samples (140 μl) were processed using the QIAamp Viral RNA minikit.

The nested PCR assay used was a modification of the method used by Salo et al. (25); the first-round (outer) PCR primers Ia (5′-ATTTCTGTAACAGCTA CCAACGA-3′) and Ib (5′-GAATTCCCTGTCTTTTCAAAGTC-3′) amplify a 348-bp region, and the second-round (inner) PCR primers IIa* (5′-CCCACTC TTCTTGCGGTTGA-3′) and IIb (5′-TGAGCCGTTATTTTTTCATACTG-3′) amplify a 208-bp region of the pneumolysin gene. We used primer IIa* instead of primer IIa, used by Salo et al. (25), because the latter incorrectly includes an extra nucleotide not present on the published pneumolysin gene sequence (26). Five microliters of DNA was added to 20 μl of first-round PCR mixture to give a final volume of 25 μl. The reaction mixture contained 10× PCR buffer with 15 mM $MgCl_2$ (Qiagen), 100 μM each deoxynucleoside triphosphate, 0.2 μM each primer, and 1.25 U of *Taq* DNA polymerase (HotStart; Qiagen). Amplification was performed on the Mastercycler Gradient Thermocycler (Eppendorf AG, Hamburg, Germany) with the following parameters: 94°C for 15 min followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with the last cycle concluding with 72°C for 5 min and 4°C for 5 min. Nested PCR was carried out using the same PCR mixture conditions with primers IIa* and IIb, with 1 μl of amplicon transferred to the PCR tube containing 24 μl of PCR mixture. Amplification was carried out using the same cycling parameters as the first-round PCR but cycling only 20 times. The PCR mixtures were analyzed using electrophoresis on 2% agarose gels with visualization of the amplicon with ethidium bromide and UV illumination.

Using 10-fold serial dilutions of *S. pneumoniae* ATCC 49619, the analytical sensitivity of the PCR assay was 2×10^1 CFU/ml.

Controls. Each extraction run contained a positive control (*S. pneumoniae* ATCC 49619) and a negative control (molecular-grade PBS). All samples were also tested for the presence of the human β-globin gene. This gene is found in human cells, and inclusion of these primers served as an internal control in each PCR amplification. For this purpose, the first-round PCR step used the above parameters but replaced the primers Ia and Ib with the primers BGF (5′-GCC AGTGCCAGAAGAGCCAA-3′) and BGR (5′-TTAGGGTTGCCCATAACA GC-3′), which amplify a 500-bp region of the human β-globin gene. Samples that tested negative for the β-globin and pneumolysin genes were spiked with *S. pneumoniae* ATCC 49619 DNA and retested for the pneumolysin gene to check for the presence of PCR inhibitors. Multiple negative samples (molecular-grade PBS) were included in each second-round or nested step to detect possible amplicon contamination of the neighboring sample during the nested procedure. Universal contamination precautions were undertaken to ensure that no amplicon contamination of molecular reagents occurred. These precautions included the physical separation of the postamplification manipulation procedures from the extraction and reagent preparation, use of barrier-filtered pipette tips, and extensive surface cleaning using hypochlorite and UV irradiation.

Ethical approval. Informed consent was obtained from all subjects, and the study was approved by the Canterbury Ethics Committee.

TABLE 1. PCR results for different sample types

Sample	No. of patients[a]	No. of controls[a]
Plasma	11/463 (2)	0/177 (0)
Buffy coat	8/465 (2)	2/181 (1)
Throat swab	229/417 (55)	73/126 (58)
Sputum	256/318 (81)	
Urine	4/227 (2)	

[a] Values are numbers of positive results/total number tested. Values in parentheses are percentages.

RESULTS

The general characteristics of the pneumonia patients have been described in detail elsewhere (15). The mean age of the patients was 64 years (range, 18 to 99 years), 53% were male, and 130 patients (27%) had received antibiotics prior to admission. Sputum was cultured in 331 (70%) cases, blood was cultured in 443 (94%) cases, and urine was tested for *S. pneumoniae* antigen in 420 (89%) cases. Samples available for PCR testing included sputum from 318 (67%) patients, plasma from 463 (98%) patients, buffy coat from 465 (98%) patients, and throat swabs from 417 (88%) patients. Urine samples from a subgroup of 227 patients were tested by PCR. This subgroup included all patients with evidence of pneumococcal disease based on positive results from blood or sputum culture and/or positive tests for urinary antigen and another 88 patients with negative culture and urinary-antigen test results.

The mean age of the control patients was 65 years (range, 20 to 91 years), and 54% were male. Urine from 169 (92%) of these patients was tested for *S. pneumoniae* antigen, and samples available for PCR testing included plasma from 177 (97%) patients, buffy coat from 181 (99%) patients, and throat swabs from 126 (69%) patients. Urine samples from control patients were not tested by PCR because of the low yield from testing urine samples from pneumonia patients.

Twenty-two (5%) pneumonia patients had positive blood cultures for *S. pneumoniae*, and 62 (19%) had *S. pneumoniae* isolated from sputum samples. *S. pneumoniae* urinary-antigen tests were positive for 120 patients (29%), as described in detail elsewhere (19). All urine samples from control patients tested negative for *S. pneumoniae* antigen.

The PCR results are summarized in Table 1. PCR inhibitors were not detected in any sample. Overall, 18 patients had blood samples that tested positive by PCR: 10 were plasma only, 7 were buffy coat only, and one was both plasma and buffy coat. Six of these patients had positive blood cultures for *S. pneumoniae*, and 15 patients with pneumococcal bacteremia had negative PCR results from blood samples. Only 4 of the 18 patients with positive PCR results from blood samples had no other evidence of pneumococcal disease (negative results for sputum or blood culture and urinary antigen), and 2 of these had a predominant growth of *Haemophilus influenzae* in sputum samples. Both control patients with positive results from buffy coat samples had no evidence of pneumococcal infection.

All four patients with PCR-positive urine samples had positive urinary-antigen tests, one had positive blood cultures, and two others had positive sputum cultures for *S. pneumoniae*.

Included among the 256 sputum samples that were PCR positive were 58 of 59 samples from which *S. pneumoniae* was

TABLE 2. Comparison of blood and sputum culture, urinary-antigen detection, and PCR for diagnosing pneumococcal pneumonia

Test for *S. pneumoniae*	Results with reference gold standards for *S. pneumoniae*[a]		
	Positive blood culture (n = 22)	Positive sputum culture (n = 62)	Positive urinary-antigen test (n = 120)
Blood culture		2/55 (4)	16/112 (14)
Sputum culture	2/14 (14)		28/86 (33)
Urinary-antigen test	16/20 (80)	27/53 (51)	
PCR			
Buffy coat	2/21 (10)	1/61 (2)	6/117 (5)
Plasma	5/21 (24)	2/61 (3)	9/117 (8)
Sputum	14/15 (93)	58/59 (98)	78/81 (96)
Throat swab	17/20 (85)	41/58 (71)	88/111 (79)
Urine	1/17 (6)	2/50 (2)	4/109 (4)

[a] Values are numbers of positive results/total number tested. Values in parentheses are percentages.

cultured. Of the patients with positive sputum samples by PCR, 105 (41%) had positive culture and/or urinary-antigen test results for *S. pneumoniae* and 73 (29%) had evidence of infection with microorganisms other than *S. pneumoniae* (*H. influenzae*, 26; *Legionella* spp., 7; *M. pneumoniae*, 7; *Moraxella catarrhalis*, 4; *Staphylococcus aureus*, 3; respiratory viruses, 26; and mixed infections, 10). Of the 168 patients with PCR-positive throat swab samples who produced sputum, 161 (96%) also had positive PCR results from sputum.

Table 2 compares blood culture, sputum culture, urinary-antigen testing and PCR for diagnosing pneumococcal pneumonia when positive blood cultures, positive sputum cultures, and positive urinary-antigen tests are used as "gold standards."

DISCUSSION

To our knowledge, this is the first study to evaluate PCR for the diagnosis of pneumococcal pneumonia in adults by testing multiple respiratory and nonrespiratory samples. We recorded a lower PCR positivity rate in blood samples than reported by other investigators, most of whom also have used assays that have targeted the pneumolysin gene. Using positive blood culture as the gold standard, the reported sensitivities of PCR for detecting *S. pneumoniae* in blood samples have ranged from 35 to 100% in adults (3, 6, 16, 17, 24, 25) and from 57 to 100% in children (2, 14, 18, 30). When the definition of pneumococcal pneumonia also includes patients with positive sputum cultures, the reported sensitivities range from 26 to 88% (3, 6,16). Positive PCR results have also been recorded by some investigators for blood samples from control subjects (2, 14, 16, 24, 25). These presumed false-positive results may be due to colonization with *S. pneumoniae*, but they have been recorded in populations (e.g., healthy elderly persons) in whom the carriage rates of *S. pneumoniae* are generally low (25).

The reason for our low PCR positivity rate in blood samples is unclear. We used an extraction method and a PCR assay similar to those used by other investigators, no PCR inhibitors were detected, and the assay was able to detect low levels of DNA. It is possible that rapid clearance of *S. pneumoniae* from the bloodstream due to prior antibiotic use may reduce the ability to detect *S. pneumoniae* DNA in blood samples. Twenty-seven percent of our patients had received antibiotics prior to admission to the hospital, and despite efforts to obtain samples prior to antibiotic administration in the hospital, some samples were collected after administration. However, higher levels of prior antibiotic use have been documented by other studies that recorded higher PCR positivity rates (16, 17), and when specifically examined, the results of PCR testing of whole blood was not affected by antibiotic administration prior to sample collection (29).

The results of the PCR testing of blood samples greatly contrast with those of respiratory samples. Among the pneumonia patients, 81% of sputum samples and 55% of throat swab samples tested positive. The sputum PCR positivity rate is similar to that reported by three other relatively small studies (83 to 100%) (10, 22, 28). For several reasons, we believe that PCR testing of these respiratory samples is not a reliable tool for diagnosing pneumococcal pneumonia and that this approach is unable to distinguish colonization from infection. First, the throat swab PCR positivity rates were the same for patients with pneumonia and control patients, suggesting that the positive results are more likely to represent colonization rather than infection. Furthermore, for patients who produced sputum, almost all of those with positive throat swab samples also had positive sputum samples. By extrapolation, these findings imply that many of the positive PCR results for sputum samples are detecting colonizing organisms rather than pathogens. It is possible that quantitative PCR may be useful in this setting and that infection can be distinguished from colonization by a higher bacterial burden in the former (11), but this has yet to be systematically examined using nucleic acid amplification methods. Another reason to question the specificity of the PCR results from respiratory samples is that 29% of the patients with PCR-positive sputum samples had evidence of infection with pathogens other than *S. pneumoniae*. While mixed infections do occur, this proportion is higher than generally reported (1, 7, 23), and it implies that some of these PCR results are falsely positive.

The throat swab PCR positivity rates for both patients and controls are higher than reported carriage rates for *S. pneumoniae*. About 10 to 20% of adults are found to be colonized with *S. pneumoniae* at any one time when assessed by culture-based methods (9, 13). Given that PCR is more sensitive than culture, it is not surprising that PCR will detect additional cases. It is also possible that our PCR assay detected oropharyngeal colonizing bacteria other than *S. pneumoniae*, resulting in false-positive results. Although generally regarded as specific to *S. pneumoniae*, genes encoding pneumolysin and autolysin have recently been reported in other streptococci that most closely resemble *Streptococcus mitis* (27). A preliminary investigation in one of our laboratories showed that some *S. mitis* isolates cultured from sputum samples could be amplified by our pneumolysin gene PCR assay (T. Anderson, unpublished findings). These findings indicate the need for more specific PCR targets for *S. pneumoniae*, especially given that the most commonly used targets in diagnostic PCR assays are the pneumolysin and autolysin genes.

Our study is the first to test for *S. pneumoniae* DNA in urine

samples from adults with pneumonia. Only four patients had samples that tested positive by PCR, and all had other laboratory evidence of pneumococcal infection. Of particular note, all four urine samples tested positive for *S. pneumoniae* antigen. Testing urine samples for bacterial DNA has been a useful approach for diagnosing legionella pneumonia (12, 20), but it does not appear to have a role in the diagnosis of pneumococcal pneumonia. On the other hand, the new generation of *S. pneumoniae* urinary-antigen tests appear to have high sensitivity and specificity for diagnosing pneumococcal pneumonia in adults and have the added advantage of being very simple to use (4, 19).

We conclude that the pneumolysin PCR assay adds little to existing tests for diagnosing pneumococcal pneumonia in adults and is unable to distinguish colonization from infection when respiratory samples are tested. Based on our results, detection of *S. pneumoniae* antigen in urine by the NOW *S. pneumoniae* urinary-antigen test is a superior method for the diagnosis of nonbacteremic pneumococcal pneumonia. Further research should focus on the comparative specificities of different pneumococcal PCR assays, the role of quantitative PCR, and the influence of antimicrobial therapy on PCR testing of blood samples.

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