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Quantitative PCR Assay Using Sputum Samples for Rapid Diagnosis of Pneumococcal Pneumonia in Adult Emergency Department Patients

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Accurate diagnosis of pneumococcal pneumonia in the acute-care setting remains a challenge due to the inadequate sensitivity of conventional diagnostic tests. Sputum cultures, which are likely to have the highest diagnostic yields of all specimen types, have been considered unreliable, due to their inability to differentiate colonization from infection. Our objective was to evaluate the potential clinical utility of a rapid quantitative real-time PCR assay using sputum samples for *Streptococcus pneumoniae* in adult patients with community-acquired pneumonia (CAP). A prospective clinical observational study of consecutively enrolled emergency department patients with CAP was performed; only those patients with excess good-quality sputum samples were included for evaluation. Sputum samples were tested for the presence of *S. pneumoniae* by using a quantitative PCR that targets the pneumolysin gene. PCR findings were compared with those of a composite reference standard comprising Gram staining of sputum samples and sputum/blood cultures. The area under the curve (AUC) and a log-transformed threshold, which provides the maximal sensitivity and specificity, were calculated. Of 487 subjects enrolled, 129 were evaluable. Receiver operating characteristic curve analysis demonstrated an AUC of 0.87. Sensitivity and specificity were 90.0 percent and 80.0 percent, respectively; positive and negative predictive values were 58.7 percent and 96.2 percent, respectively. We have demonstrated that a quantitative rapid pneumolysin PCR assay has favorable accuracy for diagnosis of pneumococcal pneumonia in adult patients with CAP; this assay may be a useful diagnostic adjunct for clinicians, particularly those practicing in the acute-care setting, where rapid pathogen identification may assist in selection of the most appropriate antibiotics.

Streptococcus pneumoniae, the leading cause of community-acquired pneumonia (CAP), accounts for roughly two-thirds of cases in which an etiology can be found (11). Early and accurate diagnosis of pneumococcal pneumonia in the acute-care setting remains difficult due to the limitations of conventional diagnostic methods. Blood and sputum cultures are time-consuming and lack sensitivity (18, 30), particularly for patients with antecedent antibiotic exposure. Sputum culture is also difficult to interpret due to oropharyngeal contamination, and sputum Gram stains are frequently unreliable (2, 10, 17); pleural fluid aspirates are rarely performed. Newer pneumococcal urinary antigen tests offer the promise of improved sensitivity (70 to 82%) but are reported to have variable specificity based on the standards of comparison chosen, the manner in which the test is performed and interpreted, and the particular population studied (6, 8, 14, 20, 26). In the absence of a reliable, rapid diagnostic tool, empirical use of broad-spectrum antibiotics is recommended due to the uncertainties of identifying the causative pathogen (2). This approach to patient care results in significant and potentially unnecessary costs, exposure of patients to potential adverse medication effects, and increases in the emergence of multidrug-resistant strains. A rapid, sensitive, and specific assay for pneumococcal pneumonia could lead to earlier etiologic diagnosis and better targeted therapy.

PCR is an attractive tool for identifying pneumonia pathogens, since timely and accurate detection does not depend on the microbiologic viability of the organism. Although several investigators have evaluated PCR for diagnosis of pneumococcal pneumonia, most have focused on blood samples as the specimen type for detection of pneumococcal bacteremia, with sensitivities ranging from 29 to 100% (7, 9, 19, 24, 25). Development and optimization of an assay that can be performed with noninvasively obtained sputum samples offers the potential for increased sensitivity, due to the known higher diagnostic yield from this sample type. To date, however, few studies have examined the utility of PCR detection using respiratory samples (4, 12, 22, 32). Those that have been done report difficulties in determining whether PCR-positive findings represent colonization or infection, limiting clinical applicability.

One approach that may help differentiate bacterial colonization from infection in respiratory samples would be to quantify the target organism by using real-time PCR techniques. This method allows measurement of bacterial load in test samples by quantifying the starting template copy number. In addition, by coupling amplification with detection, this method obviates the laborious post-PCR processing steps required in conventional PCR, greatly enhancing throughput. Several recent studies have reported optimization and analytic validation

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of real-time PCR assays for detection and quantification of *S. pneumoniae* using clinical isolates and nasopharyngeal aspirates (13, 21, 29); however, no study to date has evaluated the clinical utility of real-time PCR using sputum samples for the detection of pneumococcal pneumonia in patients with CAP.

In this report, we modified a previously developed quantitative (TaqMan) PCR assay (13) that targets the pneumolysin gene and evaluated its ability to detect and quantify *S. pneumoniae* in sputum samples from adult patients with CAP.

MATERIALS AND METHODS

Study site and design. The Johns Hopkins Hospital is a large inner-city university tertiary teaching hospital. The Emergency Department (ED) has an annual census of 57,000; the prevalence of CAP is 2% (R. Rothman, unpublished data).

From 5 October 2001 through 14 May 2003, all ED patients who met the case definition for CAP (see below) were eligible for the study and were approached for enrollment. Patients who consented were assigned study numbers, which were used to code both clinical data (demographics and results of microbiology laboratory testing) and "excess" sputum specimens (beyond those needed for patient care). Sputa were tested after storage at −70°C for batched PCR analysis. Clinical data and results from batched PCR analysis were entered into an SPSS (Chicago, IL) database (SPSS 12.0 for Windows) in a de-identified manner. The study was approved by The Johns Hopkins University Institutional Review Board.

Enrollment and specimen collection. Blood cultures and sputum specimens for Gram staining and culture were collected at the discretion of the ED physician. An additional sputum sample was collected if the patient was able to expectorate within 24 h of ED presentation. Sputum Gram staining was performed on purulent portions of samples. Sputum specimens were judged to be of good quality if >1 polymorphonuclear cell per squamous epithelial cell was observed under low-power (×10) magnification. Only good-quality specimens were screened under high-power (×100) magnification for a predominant bacterial morphotype. A predominant morphotype was defined as >75% of the organisms showing a single morphology. Quellung testing was performed when gram-positive diplococci were visualized under high-power microscopy. Only good-quality sputa were cultured using blood sheep agar, chocolate agar, and MacConkey agar media.

Case definitions and diagnosis of pneumococcal pneumonia. A case of CAP was defined as previously described (24). Diagnosis of pneumococcal pneumonia was defined using Infectious Disease Society of America (IDSA) guidelines (2). For the purposes of the study, a patient was considered to have pneumococcal pneumonia if he or she met criteria for definitive or probable diagnosis. Definitive diagnosis required culture of *S. pneumoniae* from a specimen obtained from a normally sterile site (i.e., blood, pleural fluid, or a transthoracic aspirate). Diagnosis was considered probable when *S. pneumoniae* was detected (i.e., by positive culture or a positive Gram stain and quellung test) from an adequate respiratory specimen (i.e., good-quality expectorated sputum or a bronchoalveolar lavage specimen).

DNA extraction. All frozen excess sputum samples, regardless of quality, were thawed and mixed with freshly prepared dithiotreitol (Roche Applied Science) to a final concentration of 0.15% and incubated for 15 min at 37°C. DNA extraction of the liquefied sputum samples was performed with the MagNA Pure LC robot (Roche Molecular Diagnostics, Indianapolis, IN), based on the extraction technology described by Boom et al. (3). The DNA Isolation Kit III (Roche Applied Science) was used to purify bacterial DNA. The protocol followed the manufacturer's instructions in the kit, including manual steps for efficient bacterial lysis (prior to automated DNA extraction) in which 100 μl of each sputum sample was mixed with 130 μl of bacterial lysis buffer and 20 μl of proteinase K solution, provided with the kit. Samples were incubated at 65°C for 10 min and 95°C for 10 min before undergoing the automated DNA III Bacteria-Standard Protocol for DNA extraction. The final elution volume was 100 μl.

Quantitative (TaqMan) pneumolysin PCR. The PCR primers and TaqMan probe used were designed based on the sequences published by Greiner et al. (13) targeting the pneumolysin gene of *S. pneumoniae* and were synthesized commercially by Applied Biosystems (Foster City, CA). The fluorescent reporter dye at the 5′ end of the TaqMan probe was 6-carboxyfluorescein, and the 3′ end contained a minor groove binding group. Quantitative PCR amplifications were performed in 50-μl reaction mixture volumes containing 2× TaqMan Universal Master Mix (Applied Biosystems), which includes dUTP and uracil-*N*-glycosylase; each primer was used at a final concentration of 400 nM. The TaqMan probe was used at a final concentration of 160 nM, and 10 μl of either DNA extract from sputum samples or positive controls was used. For no-template controls, a 10-μl volume of sterile PCR-grade H_2O was added to the PCR mixture in place of DNA extract. Internal controls were run; these consisted of 5 μl each of human β-actin primers and the probe from the TaqMan β-actin control reagent kit (Applied Biosystems) added to the PCR mix in place of the pneumolysin primers and probes. Amplification failure was defined as a cycle threshold (C_T) of >34 for both the pneumolysin gene and the internal control. All amplifications were performed with the ABI PRISM 7900 sequence detection system, and the parameters used were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All PCRs were performed in triplicate, and the C_Ts in each triplicate were averaged. Amplification data were analyzed by SDS software (Applied Biosystems). Quantification of *S. pneumoniae* organisms present in each sample was based on a standard curve generated by plotting the C_T value against known genomic equivalents (see below).

Standard curves for quantifying target organisms. Known quantities of *S. pneumoniae* (ATCC 49619) DNA obtained from Infectio Diagnostic (IDI), Inc. (Quebec, Canada), were serially diluted, and the number of genomic equivalents was calculated based on 2.4 femtograms of DNA per *S. pneumoniae* genome (5).

Statistical analysis. Using STATA version 7.0 (Stata Corp., College Station, TX), the data were summarized in a receiver operating characteristic (ROC) curve to determine the diagnostic efficacy of the quantitative pneumolysin PCR. For this curve, the sensitivity (true positives) was plotted against 1 − specificity (false positives). Using all observed C_Ts as cutoff values, the area under the curve (AUC) and its 95% confidence interval (95% CI) were calculated as a measure for the discriminative efficacy of the tested marker.

RESULTS

A total of 487 patients with CAP were enrolled; 131 patients had excess sputum samples available for analysis. Two patients had neither of the standard reference tests (blood culture and sputum Gram stain/culture) reported and were thus excluded. Among the remaining 129 patients, 112 patients (87%) had blood cultures and 114 (88%) had Gram stain and culture of good-quality sputum samples. There were no significant differences in age, gender, or ethnicity; however, there was a significantly greater frequency of definite diagnoses of pneumococcal pneumonia among the patients with excess sputum than among those without.

As shown in Table 1, 30/129 (23%) patients received a diagnosis of definite or probable pneumococcal pneumonia, based on recovery of *S. pneumoniae* from blood cultures (11 patients [9%]) or sputum tests (19 patients [14%]) alone, respectively. Only 2/11 (18%) patients with blood cultures positive for pneumococci also had positive sputum cultures. Gram stains showing both predominant gram-positive diplococci and positive Quellung reactions were found in 15/21 (71%) sputum specimens subsequently found to be positive by culture.

Among the 99/129 (77%) patients who did not meet criteria for definite or probable pneumococcal pneumonia, blood culture results were available for 86 patients (87%) and good-quality sputum specimens from a different but overlapping set of 86 patients (87%). Seventy-seven out of the 99 (78%) had both blood culture and good-quality sputum cultures. One or more etiologies were identified for 43 (43%) patients (Table 1); 56 (56%) had no etiology found.

Real-time pneumolysin PCR was performed in triplicate on each sputum sample. The overall assay time, including sample processing and PCR, was approximately 1 h, 40 min. Standard curves based on genomic equivalents of *S. pneumoniae* were generated with each PCR run to quantify the bacterial load in

TABLE 1. Results of testing of CAP patients for *S. pneumoniae*

Test for *S. pneumoniae*	No. (%) of patients with community-acquired pneumonia (n = 129) with the indicated result											
	Pneumococcal pneumonia (n = 30)						Other pneumonia (n = 99)					
	Definitive (n = 11)			Probable (n = 19)			Etiologic diag. (n = 43)[a]			No etiologic diag. (n = 56)		
	Positive	Negative	Not available	Positive	Negative	Not available	Positive	Negative	Not available	Positive	Negative	Not available
Blood culture (n = 111)	11 (100)	0	0	0	15 (79)	4 (21)	0	38 (88)	5 (12)	0	48 (86)	8 (14)
Sputum culture (n = 114)	2 (18)	7 (64)	2 (18)	19 (100)	0	0	0	39 (91)	4 (9)	0	47 (84)	9 (16)
Sputum Gram stain (n = 114)	2 (18)	7 (64)	2 (18)	13 (68)	6 (32)	0	1 (2)	38 (88)	4 (9)	2 (4)	45 (80)	9 (16)
Quellung test	2 (18)	0	9 (82)	13 (68)	0	6 (32)	1 (2)	0	42 (98)	2 (4)	0	54 (96)

[a] diag., diagnosis. Etiologies identified included aspiration pneumonia (n = 8), *Haemophilus influenzae* (n = 10), *Pneumocystis carinii* (n = 5), *Staphylococcus aureus* (n = 5), *Legionella pneumoniae* (n = 3), influenza A virus (n = 2), *Pseudomonas aeruginosa* (n = 2), group B streptococcus (n = 2), group A streptococcus (n = 1), group C streptococcus (n = 1), *Klebsiella pneumoniae* (n = 1), *Moraxella catarrhalis* (n = 1), *Burkholderia cepacia* (n = 1), *Mycoplasma pneumoniae* (n = 1), varicella zoster virus (n = 1), and a miscellaneous fungus (n = 1).

each sputum specimen. Amplification failures were observed for four sputum samples.

ROC curve analysis was performed to assess the diagnostic accuracy of the assay for pneumococcal pneumonia using PCR results from the remaining 125 sputum samples. For purposes of the analysis, the 30 patients with definite or probable pneumococcal pneumonia by blood or sputum cultures were classified as cases, and the remaining 95 patients were classified as controls. The calculated AUC was 0.87 (95% CI, 0.79 to 0.95) (Fig. 1). Since the reference test results used for case definition were not uniformly available for all patients, patients missing one of the reference test results may have been misclassified as controls. To eliminate this possibility as a potential source of bias, we repeated the ROC curve analysis using the same case definition but considering only patients with both negative blood and sputum cultures (n = 73) as controls. The calculated AUC remained the same, at 0.87 (95% CI, 0.78 to 0.94). We have also considered only patients with etiologic diagnoses other than *S. pneumoniae* as controls (n = 41), and the resulting AUC was calculated to be 0.85 (95% CI, 0.76 to 0.94). In case the quality of the sputum used for culturing was suboptimal due to contamination with normal flora, we performed a subanalysis including only patients with both negative high-quality sputum samples (with many polymorphonuclear cells and <1 squamous cell per low-power field) and blood cultures (n = 68) as controls. The resulting AUC improved only slightly, to 0.90 (95% CI, 0.82 to 0.98).

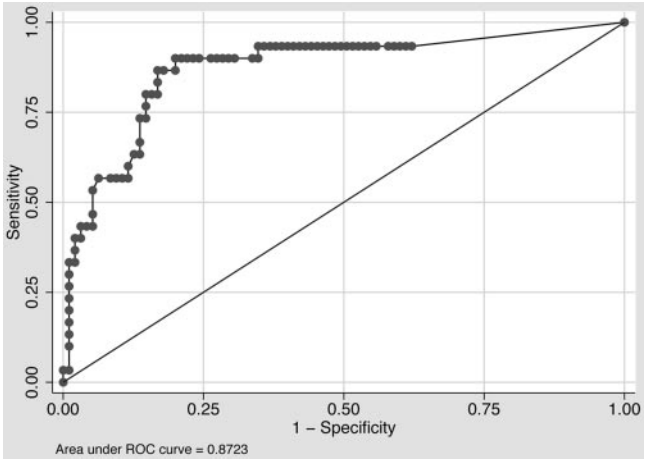

FIG. 1. ROC curve analysis. Plot of true versus false positives at all possible cutoffs. The probabilities were generated by fitting a logistic regression of the diagnosis on the starting template quantity (derived from C_Ts).

Based on the ROC curve analysis, the log-transformed C_T that gave the maximal sensitivity and specificity for the PCR assay was 28.96 (corresponding to a predicted probability of 0.173 via the logistic regression fit), which is equivalent to approximately 3.7×10^4 genomic equivalents of *S. pneumoniae* per ml of sputum (Fig. 2). The C_Ts for cases and controls relative to the C_T cutoff are depicted in Fig. 3. At this threshold, the assay sensitivity was 90.0 percent, the specificity was 80.0 percent, the positive predictive value was 58.7 percent, and the negative predictive value was 96.2 percent.

DISCUSSION

This is the first prospective study evaluating the clinical utility of a quantitative PCR assay on sputum for the diagnosis of pneumococcal pneumonia in adults. Based on our ROC curve analysis, the assay has favorable diagnostic accuracy.

A major challenge in evaluating the diagnostic accuracy of a new test for pneumococcal pneumonia is the lack of a perfect "gold standard" test for comparison. Choosing a single existing reference test as the gold standard can bias estimates of the assay's accuracy, since the reference tests themselves are known to have a relatively low sensitivity for pneumococcal pneumonia (18, 30). We employed the IDSA diagnostic guidelines for pneumococcal pneumonia as our gold standard for comparison; these guidelines combine the results of multiple imperfect reference tests (blood culture and sputum Gram stain/culture) to define a composite standard that has increased sensitivity and specificity relative to those of any individual test alone (1, 2). The Binax NOW urinary antigen test may ultimately augment the diagnostic standards for pneumococcal pneumonia (16). However, its specificity remains questionable for younger patients and those with human immunodeficiency virus infection (4, 8, 20). Because patients with CAP at our institution tend to be younger (mean age, 47.8 years) and nearly 50% are infected with human immunodeficiency

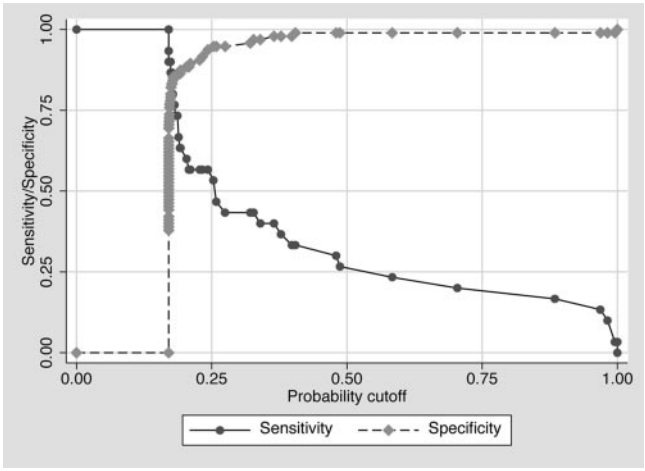

FIG. 2. Plot of sensitivity and specificity versus probability of being a case. The values were derived from the same fit used to generate Fig. 1.

virus (23), we did not include the urinary antigen test as part of our composite reference standard. Other approaches used to evaluate a new diagnostic test in the absence of a perfect gold standard include discrepant resolution and latent class analysis. Discrepant resolution has been used extensively, particularly in evaluation of new tests for *Chlamydia trachomatis* infection. However, concerns regarding inherent biases, which can result in overestimation of the sensitivity and specificity (15, 16), have been raised with this approach and led us not to choose this method for analysis. We also chose not to utilize latent class analysis, because its reference standard is not explicitly defined (4, 1).

Previous studies have suggested that conventional PCR testing, which has dichotomous outcome measures and high positivity rates in sputum specimens, is an unreliable diagnostic tool for pneumococcal pneumonia because it cannot discriminate between oropharyngeal colonization and lower respiratory tract infection (25). The quantitative capacity of our PCR assay allowed us to distinguish between contamination and true infection. Our results suggest that clinical infection correlates with increased pneumococcal load. More importantly,

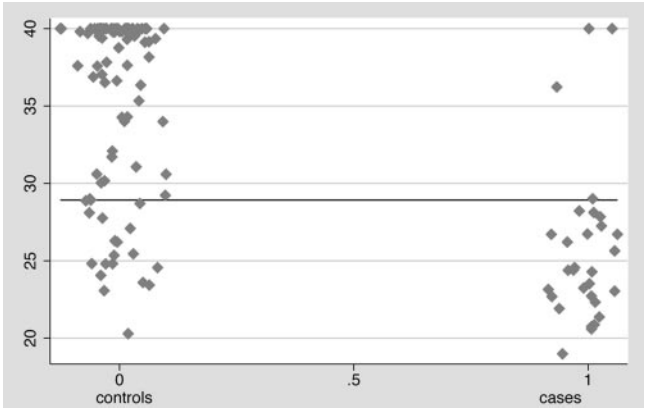

FIG. 3. C_Ts for cases and controls (diamonds). The C_T cutoff giving the greatest sensitivity and specificity is depicted as well (horizontal line).

this differential in bacterial burden was observed regardless of the quality of the sputum specimen tested; high quantities of *S. pneumoniae* were found in poor-quality sputum samples collected from two patients with pneumococcal bacteremia (data not shown).

In this study, the quantitative pneumolysin PCR is associated with a high negative predictive value of 96.2%, based on the cutoff point derived from ROC analysis. A larger study will need to be conducted to better define this threshold value. Nonetheless, the assay's ability to quickly exclude *S. pneumoniae* as the etiologic agent for pneumonia may have important clinical implications. A more focused selection of antimicrobial therapy (e.g., antimicrobials directed against the causes of atypical, nonpneumococcal pneumonia) may ultimately reduce the emergence of multidrug-resistant *S. pneumoniae* and decrease the incidence of adverse drug effects with the use of multiple agents.

Another major advantage of quantitative pneumolysin PCR over conventional diagnostic methods for pneumococcal pneumonia is its rapidity and relative simplicity. Both DNA extraction and PCR are automated, and unlike conventional PCR, which requires laborious post-PCR processing, detection of target amplification can occur in real time. By employing more-advanced high-speed thermocyclers, the complete process (from specimen collection to target detection) can potentially be accomplished in 1 to 2 h, well within the current Medicare rule which recommends antibiotic administration within 4 h of patient registration in the ED. Further refinement of instrumentation and optimization of throughput capacity may ultimately allow integration of this diagnostic platform into the acute-care setting, allowing for routine point-of-care testing.

The prevalence of pneumococcal pneumonia in our study was 23%, somewhat lower than that reported by others (4, 22, 25) but similar to that reported at our center in a previous study (23). Even among patients with pneumococcal bacteremia, the rates of positivity by sputum Gram staining (18%) and culture (18%) in our study were lower than those reported by Musher et al. (27). Possible explanations for the relatively low recovery rate of *S. pneumoniae* from blood and sputum specimens found here include delays between specimen collection and performance of microbiological studies, as well as administration of antibiotics prior to specimen collection; we were unable to formally evaluate this, however, because these data were not collected. Because a subset of patients with negative blood and sputum cultures may still have been infected or colonized with *S. pneumoniae*, the specificity of our PCR assay may have been underestimated. Alternative methods to evaluate specificity have been used in other studies, but each has its own inherent limitations as well. Several studies have used healthy adults or patients with diagnoses other than a respiratory or infectious disease as controls in determining test specificity (26); however, it is highly unlikely that the test would ever be used in such a context, making such controls less clinically meaningful. In addition, it would be difficult to procure sputum for analysis from asymptomatic subjects who normally do not produce sputum. Other groups have deliberately selected clinically relevant controls (i.e., patients with pneumonia caused by other etiologic agents) (14). Nonetheless, the possibility of coinfection of these patients with *S. pneumoniae*

cannot be excluded, because potential growth suppression by the predominant organism in the culture may contribute to the underdetection of other, coinfecting agents. Importantly, however, analysis of our data using patients with nonpneumococcal etiologic diagnoses as controls did not change the diagnostic accuracy of our assay.

Based on the cutoff value used in our study, 12 of 73 (16%) patients with both negative blood culture and negative sputum Gram stain/culture for pneumococcal pneumonia had positive PCR findings. Among these 12 patients, 8 had an alternative etiologic diagnosis and one with sputum culture positive for group C streptococcus also had a positive quellung test. Possible explanations for the discrepant results include the superior sensitivity of PCR compared to the reference tests or false-positive detection by PCR due to contaminants. False-positive detection of atypical *Streptococcus mitis* or *Streptococcus oralis*, both of which harbor the pneumolysin gene classically associated with *S. pneumoniae*, has been reported previously (31) and may partly account for these discrepant results. Based on the same primer and probe sequences, the assay developed by Greiner et al. had a reported specificity of 90% using culture-negative nasopharyngeal specimens, with specificity improved to 96% by increasing the annealing extension temperature from 60°C to 65°C (13). In our study, increasing the annealing temperature resulted only in decreased PCR efficiency, without any improvement in specificity (data not shown). Laboratory contamination from carryover DNA is an unlikely explanation for the discrepant results, given the stringent adherence to standard precautionary measures. Although we cannot exclude some false-positive results, the relatively high pneumococcal load detected in these 12 patients' sputa leads us to suggest that they mainly constitute true-positive cases. The use of an additional "resolver" test (e.g., PCR targeting an alternative pneumococcus-specific gene) may help adjudicate these discrepant cases; however, even with this approach, issues regarding introduction of potential bias remain (15).

Although the quantitative pneumolysin PCR may detect cases of pneumococcal pneumonia not diagnosed using traditional microbiological procedures, there were also 4 out of 30 patients (13%) with unequivocal pneumococcal pneumonia that was missed by our assay (false negatives). Three of these patients had pneumococcal bacteremia, and one had a sputum culture positive for *S. pneumoniae*. None of these specimens showed evidence of PCR inhibition, based on efficient amplification of the internal positive control. Thus, the definitive explanation for these false-negative findings remains unclear. One potential cause may be degradation of pneumococcal DNA from delayed processing of specimens. Alternative explanations for these false-negative results are similar to those described in previous studies (12, 25, 32).

In conclusion, we have demonstrated that a quantitative pneumolysin PCR assay can accurately diagnose pneumococcal pneumonia. As with all quantitative diagnostic assays, the ideal cutoff value for our PCR assay can be adjusted based on specific objectives. Integration of the assay into clinical-practice settings (e.g., intensive-care units) where definitive etiologic identification is critical will generally require choosing a high cutoff for optimization of specificity (Fig. 2). Here, the accuracy of this assay should increase the yield of identified pathogens for CAP and allow administration of earlier, more-focused therapy directed against *S. pneumoniae*. On the other hand, adjusting to a lower cutoff to increase the sensitivity of the PCR assay may be more useful for epidemiologic prevalence studies, especially given that conventional detection methods likely underestimate the true prevalence of pneumococcal pneumonia (28). The rapidity of this test may be particularly helpful in the acute-care setting for patients receiving antimicrobial therapy at the time of evaluation. Further studies of real-time testing are required for validation purposes and for evaluation of potential utility in clinical practice with regard to feasibility, cost, and turnaround time. We envision that this PCR assay will ultimately serve as an adjunct to, rather than a replacement for, conventional culture methodologies, which will still be required for confirmation and susceptibility testing.

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