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Comparison of Sputum and Nasopharyngeal Aspirate Samples and of the PCR Gene Targets *lytA* and Spn9802 for Quantitative PCR for Rapid Detection of Pneumococcal Pneumonia

Kristoffer Strålin,[a,b] Björn Herrmann,[c] Guma Abdeldaim,[c,d,e] Per Olcén,[f] Hans Holmberg,[b] Paula Mölling[f]

Department of Infectious Diseases, Karolinska University Hospital, Stockholm, Sweden[a]; Department of Infectious Diseases, Örebro University Hospital, Örebro, Sweden[b]; Section of Clinical Bacteriology, Department of Medical Sciences, Uppsala University, Uppsala, Sweden[c]; Department of Microbiology, Faculty of Medicine, Benghazi University, Benghazi, Libya[d]; Department of Clinical Mycobacteriology, National Center for Diseases Control, Benghazi, Libya[e]; Department of Laboratory Medicine/Clinical Microbiology, Örebro University Hospital, Örebro, Sweden[f]

We aimed to compare sputum and nasopharyngeal aspirate (NpA) samples and the PCR gene targets *lytA* and Spn9802 in quantitative PCR (qPCR) assays for rapid detection of pneumococcal etiology in community-acquired pneumonia (CAP). Seventy-eight adult patients hospitalized for radiologically confirmed CAP had both good-quality sputum and NpA specimens collected at admission. These samples were subjected to *lytA* qPCR and Spn9802 qPCR assays with analytical times of <3 h. Thirty-two patients had CAP with a pneumococcal etiology, according to conventional diagnostic criteria. The following qPCR positivity rates were noted in CAP cases with and without pneumococcal etiology: 96% and 15% (sputum *lytA* assay), 96% and 17% (sputum Spn9802 assay), 81% and 11% (NpA *lytA* assay), and 81% and 20% (NpA Spn9802 assay), respectively. The mean *lytA* and Spn9802 DNA levels were significantly higher in qPCR-positive sputum samples from cases with pneumococcal etiology than in qPCR-positive sputum samples from CAP cases without pneumococcal etiology or qPCR-positive NpA samples from cases with pneumococcal etiology ($P < 0.02$ for all comparisons). For detection of pneumococcal etiology, receiver operating characteristic curve analysis showed that sputum specimens were superior to NpA specimens as the sample type ($P < 0.02$ for both gene targets) and *lytA* tended to be superior to Spn9802 as the gene target. The best-performing test, the sputum *lytA* qPCR assay, showed high sensitivity (94%) and specificity (96%) with a cutoff value of 10^5 DNA copies/ml. In CAP patients with good sputum production, this test has great potential to be used for the rapid detection of pneumococcal etiology and to target penicillin therapy.

In order to handle the global emergence of antibiotic resistance, interventions for more appropriate antibiotic use have been identified (1, 2). These interventions include action for development of rapid microbiological tests (1, 2). Accordingly, it has been proposed that antibiotic use in community-acquired pneumonia (CAP) should be changed from empirical broad-spectrum therapy to pathogen-directed (narrow-spectrum) therapy (3, 4).

Streptococcus pneumoniae is the most common microbiological cause of CAP (5, 6). In a recently published meta-analysis (7), this bacterium was identified by blood culture, sputum culture, and/or urinary antigen test to be the microbiological cause in 27.3% of CAP cases. The drug of choice to treat pneumococcal pneumonia in most parts of the world is the narrow-spectrum antibiotic penicillin (8). Penicillin therapy can be targeted in CAP if the pneumococcal etiology can be detected rapidly and accurately (9).

The Binax NOW urinary antigen test is useful for the rapid detection of pneumococcal pneumonia (7, 10) and for targeting penicillin therapy (9). However, as this test fails to detect *S. pneumoniae* in a substantial proportion of patients with pneumococcal pneumonia (6, 7, 9, 10), additional rapid diagnostic tests would be useful.

The PCR technique is improving in terms of speed, hands-on time, and diagnostic performance. Although quantitative PCR (qPCR) for *S. pneumoniae* has shown a lack of sensitivity when applied to blood samples (11, 12), it has shown promising results when applied to respiratory secretions. Albrich et al. (13) found that qPCR for the pneumococcal gene *lytA*, when applied to nasopharyngeal secretions, could discriminate between pneumococcal infection and colonization. Accordingly, we found that results of qPCR for the pneumococcal gene fragment Spn9802 were correlated with culture results, when both tests were applied to nasopharyngeal secretions (14). The *lytA* and Spn9802 gene targets have been found to be specific for *S. pneumoniae*, although the previously popular PCR gene target *ply* has been found to be nonspecific (15–17). Although qPCR for *ply* has been evaluated with sputum specimens (18, 19), studies of the diagnostic usefulness of sputum *lytA* qPCR and sputum Spn9802 qPCR assays are lacking. To our knowledge, sputum *lytA* qPCR testing has been evaluated in only one study, in which the results were found to correlate to sputum culture positivity (20). It has not been clarified whether sputum specimens are preferable to nasopharyngeal secretion specimens for *S. pneumoniae* qPCR analysis.

The aim of the present study was to evaluate and to compare the usefulness of rapid (<3-h) qPCR assays for *lytA* and Spn9802, applied to sputum and nasopharyngeal aspirate (NpA) samples,

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Address correspondence to Kristoffer Strålin, kristoffer.stralin@karolinska.se.

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for rapid detection of pneumococcal etiology in CAP. For this study, we chose to use prospective patient material, which has been used for evaluation of NpA Spn9802 qPCR assays (14) but not for evaluation of sputum Spn9802 qPCR, sputum *lytA* qPCR, or NpA *lytA* qPCR assays.

(Data from this study were presented at the 8th International Symposium on Pneumococci and Pneumococcal Diseases, Iguacu Falls, Brazil, 11 to 15 March 2012 [21].)

MATERIALS AND METHODS

Patients and samples. In a previously described prospective study of 235 patients hospitalized for radiologically confirmed CAP at Örebro University Hospital (Örebro, Sweden) (22), samples were collected at admission for studies of CAP causes. The study was approved by the local ethics committee. Representative sputum samples (>5 leukocytes per epithelial cell) were collected in 112 cases and were preserved at −70°C in 78 cases, all of which also had NpA samples preserved. These 78 patients (median age, 71 years [range, 18 to 96 years]) were included in the present study. Thirty-five patients (45%) were female, and 21 patients (27%) were smokers. The median Pneumonia Severity Index (PSI) score was 89 (range, 18 to 148), and 36 patients (46%) belonged to PSI risk class IV or V.

The following conventional microbiological tests were performed for all 78 study patients: blood culture (with a Bactec nonradiometric system), culture of both representative sputum and NpA specimens (blood agar and hematin agar, with incubation in carbon dioxide for 24 to 28 h), and PCR assays for *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* with sputum and NpA specimens, as described previously (22). In the cultures, bacterial pathogens were identified according to standard microbiological methods (23). For sputum cultures, the method described by Kalin et al. (24) was used. The detection limit for a positive sputum culture was 10^5 CFU/ml. Apart from the detection limit, no quantification of growth in sputum cultures was performed. NpA samples were collected by aspiration in the nasopharynx with a catheter connected to an electronic suction device. After aspiration, 0.5 to 1 ml of NaCl (0.85%) was aspirated to collect the secretions within the catheter. For 73 patients, urinary antigen tests for *S. pneumoniae* and *Legionella pneumophila* (Binax NOW) were performed on nonconcentrated urine (22).

The study population was used previously to evaluate the performance of the NpA Spn9802 qPCR assay (14). However, in that study, a manual DNA extraction method was used, and the qPCR results were compared with the results of NpA culture only. The study population has not been used in any evaluation of sputum Spn9802 qPCR, sputum *lytA* qPCR, or NpA *lytA* qPCR assays.

DNA extraction. Previously frozen (−70°C) sputum samples and NpA specimens (200 μl) were subjected to DNA extraction at the Department of Laboratory Medicine, Örebro University Hospital, using a MagNAPure Compact Nucleic Acid Isolation Kit I with a MagNAPure Compact robot (Roche Diagnostics, Mannheim, Germany), with approximately 5 min of hands-on time per sample (25). Sputum samples were pretreated as follows: 1 ml sputum was centrifuged at 5,000 × *g* for 10 min, the supernatant was removed, and 100 μl of the remaining sample was mixed with 100 μl Mucolyse (1 mg/ml Mucolyse; Pro-Lab Diagnostics), vortex-mixed, and shaken at 1,000 rpm for 30 min in 37°C.

***lytA* PCR and Spn9802 PCR assays.** Extracted DNA was divided into two aliquots. One aliquot was subjected to *lytA* qPCR at Örebro University Hospital and one aliquot was sent immediately to the Department of Clinical Microbiology, Uppsala University Hospital (Uppsala, Sweden), for Spn9802 qPCR. The qPCR analyses were performed shortly after DNA extraction. Extracted DNA was not subjected to freezing prior to PCR analysis.

The *lytA* gene was amplified using a LightCycler 2.0 system with LightCycler software version 4.1 (Roche Diagnostics), as described previously (12). Briefly, the real-time PCR amplification was performed using the *lytA* forward (CAGCGGTTGAACTGATTGA) and *lytA* reverse (TGGTTGGTTATTCGTGCAA) primers and the P1 (GAAAACGCTTGATACAGGGAGTT-FL) and P2 (LC Red 640-AGCTGGAATTAAAACGCACGAG-PH) probes, with LightCycler FastStart reaction mix hybridization probes (Roche Diagnostics).

The real-time PCR assay based on the Spn9802 fragment (15) was performed in a Rotor-Gene 3000 instrument (Qiagen, Hilden, Germany), as described previously (14). The primers used were Spn9802-F (5′-AGTCGTTCCAAGGTAACAAGTCT-3′) and Spn9802-R (5′-ACCAACTCGACCACCTCTTT-3′). For detection, the Spn9802-FAM probe was designed as 5′-FAM-aTcAGaTTgAAGCTgAtaAAaCgA-black hole quencher 1 (BHQ1)-3′ (incorrectly described probe sequence in our previous publication [14]), with lowercase letters indicating locked nucleic acids.

The *lytA* qPCR and Spn9802 qPCR assays are currently used in routine practice at Örebro University Hospital and Uppsala University Hospital, respectively. Both assays have total analysis times from original sample preparation to result of 2 h for NpA samples and 2 h 40 min for sputum samples, i.e., 40 min for pretreatment of sputum samples, 60 min for DNA extraction (including hands-on time), and 60 min for real-time PCR.

Quantification of DNA. After *lytA* and Spn9802 PCR assays, the PCR cycle threshold values were transformed to DNA copies/ml, using standards with known DNA concentrations. Different but very similar standards were used for *lytA* qPCR and Spn9802 qPCR assays.

Criteria for pneumonia etiology and pneumococcal carriage. Patients were considered to have (i) pneumococcal etiology if *S. pneumoniae* was detected by at least one of the three reference tests, i.e., blood culture, culture of representative sputum specimen, or *S. pneumoniae* urinary antigen test; (ii) pneumococcal carriage if *S. pneumoniae* was isolated in NpA cultures and no reference test was positive for *S. pneumoniae*; (iii) other etiology if a nonpneumococcal bacterial pathogen was identified by blood culture, culture of representative sputum sample, urinary antigen test (*L. pneumophila*), or PCR (*M. pneumoniae* and *C. pneumoniae*) and no reference test or NpA culture was positive for *S. pneumoniae*; or (iv) no identified etiology if no conventional microbiological test (culture, urinary antigen test, or PCR assays for *M. pneumoniae* and *C. pneumoniae*) was positive. In the performance analyses, cases with pneumococcal carriage, other etiology, or no identified etiology were considered not to have pneumococcal etiology.

Statistics. SPSS statistical software (version 14.0.1) was used for comparisons of proportions (Pearson's chi-square test), comparisons of means (Student's *t* test), and calculations of correlation coefficients. When quantitative DNA data sets were compared, $\log_{10}$ copies/ml values were used, in order to enable statistical calculations. MedCalc statistical software (version 12.6.1) was used for receiver operating characteristic (ROC) curve analyses and comparisons between ROC curves. *P* values below 0.05 were regarded as significant.

RESULTS

Results of conventional microbiological tests. Thirty-two CAP patients were considered to have pneumococcal etiology, with positive results for *S. pneumoniae* in blood cultures ($n = 9$), cultures of representative sputum samples ($n = 28$), and/or urinary antigen tests ($n = 16$). NpA cultures were positive for *S. pneumoniae* for 28 patients, including 24 patients with pneumococcal etiology. Thus, four patients were pneumococcal carriers. For 25 patients without pneumococcal etiology or carriage, other etiologies were identified, i.e., *Haemophilus influenzae* ($n = 11$), *M. pneumoniae* ($n = 9$), *Staphylococcus aureus* ($n = 2$), *C. pneumoniae* ($n = 1$), *H. influenzae* and *M. pneumoniae* ($n = 1$), and *H. influenzae* and group G streptococci ($n = 1$). Seventeen patients had no etiology identified.

The *S. pneumoniae* urinary antigen test was not performed for 5 patients, including 2 patients with pneumococcal etiology, one patient with *S. aureus* etiology, and 2 patients with no identified etiology. The three patients without pneumococcal etiology were negative with the *lytA* and Spn9802 qPCR tests.

TABLE 1 Different result combinations for *lytA* and Spn9802 qPCR tests for 78 patients with community-acquired pneumonia

Reference result for pneumococcal etiology[a]	qPCR result for indicated test[b]:				No. of patients with result combination
	Sputum *lytA*	Sputum Spn9802	NpA *lytA*	NpA Spn9802	
+	+	+	+	+	24
+	+	+	+	−	2
+	+	+	−	+	2
+	+	+	−	−	3
+[c]	−	−	−	−	1
−	+	+	+	+	4
−	+	+	−	−	2
−	+[d]	−	−	−	1
−	−	+	−	+	2
−	−	−	+	+	1
−	−	−	−	+[e]	2
−	−	−	−	−	34

[a] Positive if at least one reference test (blood culture, culture of representative sputum specimen, or urinary antigen test) was positive for *Streptococcus pneumoniae*.
[b] NpA, nasopharyngeal aspirate.
[c] Positive urinary antigen test, negative blood culture, and negative sputum culture results.
[d] PCR positive at 1.1×10^2 DNA copies/ml (no identified etiology).
[e] PCR positive at 7.7×10^4 DNA copies/ml (other etiology) and 7.5×10^4 DNA copies/ml (no identified etiology).

Results of *lytA* and Spn9802 PCR assays. Positive *lytA* and/or Spn9802 qPCR test results were noted for 43 patients, as sputum *lytA* qPCR results were positive in 38 cases, sputum Spn9802 qPCR results in 39 cases, NpA *lytA* qPCR results in 31 cases, and NpA Spn9802 qPCR results in 35 cases. The different result combinations of the qPCR tests are presented in Table 1. Forty of 43 qPCR-positive cases were positive with at least two qPCR tests. The following qPCR positivity rates were noted for 32 patients with and 46 patients without established pneumococcal etiology: sputum *lytA* qPCR, 96% ($n = 31$) and 15% ($n = 7$); sputum Spn9802 qPCR, 96% ($n = 31$) and 17% ($n = 8$); NpA *lytA* qPCR, 81% ($n = 26$) and 11% ($n = 5$); NpA Spn9802 qPCR, 81% ($n = 26$) and 20% ($n = 9$), respectively. Quantitative results of *lytA* qPCR and Spn9802 qPCR tests applied to sputum and NpA specimens in the different patient categories are shown in Fig. 1A to D.

In Table 2, mean DNA levels in CAP patients with and without pneumococcal etiology are compared. When both qPCR-positive and qPCR-negative results were included in the comparison, highly significant mean differences were noted for all qPCR tests. A major reason for this was that most cases with pneumococcal etiology were qPCR positive, although the majority of cases without pneumococcal etiology were qPCR negative. When only qPCR-positive cases were included in the comparison, sputum DNA levels were significantly higher in cases with versus without

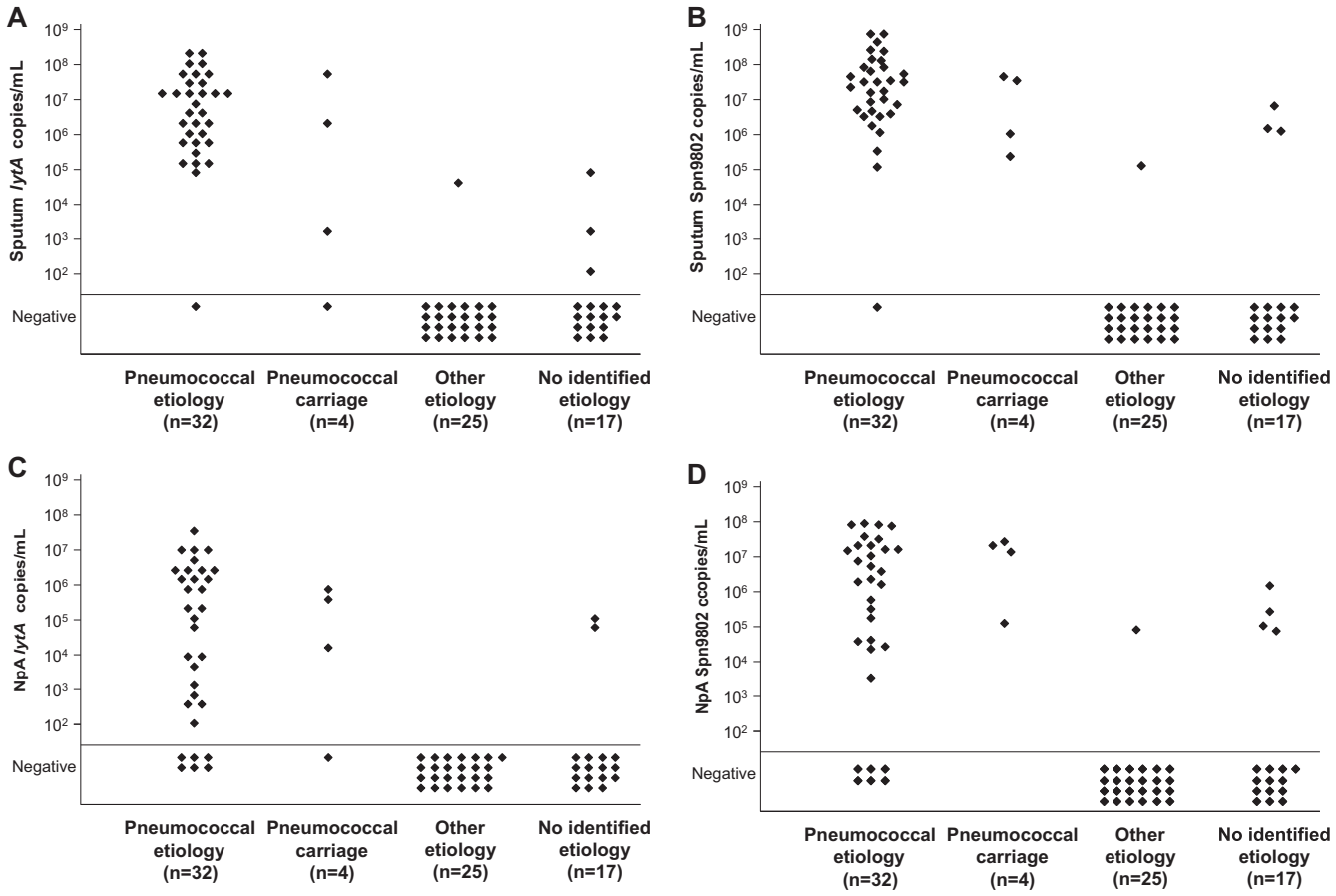

FIG 1 Results of quantitative PCR (qPCR) applied to sputum and nasopharyngeal aspirate (NpA) specimens from 78 patients with community-acquired pneumonia. (A) Sputum *lytA* qPCR results; (B) sputum Spn9802 qPCR results; (C) NpA *lytA* qPCR results; (D) NpA Spn9802 qPCR results. All cases with "other etiology" or "no identified etiology" had NpA cultures negative for *Streptococcus pneumoniae*.

TABLE 2 Quantitative results of *lytA* and Spn9802 qPCR assays applied to sputum and NpA specimens from pneumonia patients with or without pneumococcal etiology

Assay	PCR results (mean ± SD) (log_{10} copies/ml)[a]		
	Pneumococcal etiology[b]	Not pneumococcal etiology[c]	*P*
Both PCR-positive and PCR-negative cases included			
Sputum *lytA* qPCR	6.50 ± 1.55	0.69 ± 1.81	<0.00001
Sputum Spn9802 qPCR	7.01 ± 1.57	1.09 ± 2.43	<0.00001
NpA *lytA* qPCR	4.35 ± 2.61	0.57 ± 1.65	<0.00001
NpA Spn9802 qPCR	5.18 ± 2.76	1.15 ± 2.40	<0.00001
Only PCR-positive cases included			
Sputum *lytA* qPCR	6.71 ± 1.01	4.56 ± 1.98	0.00017
Sputum Spn9802 qPCR	7.24 ± 0.92	6.27 ± 0.92	0.012
NpA *lytA* qPCR	5.35 ± 1.69	5.21 ± 0.68	0.86
NpA Spn9802 qPCR	6.37 ± 1.25	5.89 ± 1.07	0.31

[a] SD, standard deviation.
[b] Pneumonia patients with *Streptococcus pneumoniae* detected by blood culture, culture of representative sputum specimens, and/or urinary antigen test.
[c] Pneumonia patients with pneumococcal carriage, other etiology, or no identified etiology.

pneumococcal etiology, although NpA DNA levels were the same in cases with and without pneumococcal etiology. In cases with pneumococcal etiology, mean DNA levels of qPCR-positive cases (Table 2) were significantly higher for sputum *lytA* qPCR than for NpA *lytA* qPCR ($P = 0.0004$) and for sputum Spn9802 qPCR than for NpA Spn9802 qPCR ($P = 0.0038$).

As noted in Fig. 2A, a correlation in quantitative data for sputum *lytA* qPCR and sputum Spn9802 qPCR assays could be demonstrated ($r = 0.55$; $P < 0.001$). Correlations were also noted for quantitative data for NpA *lytA* qPCR and NpA Spn9802 qPCR assays ($r = 0.27$; $P = 0.018$) and for sputum Spn9802 qPCR and NpA Spn9802 qPCR assays ($r = 0.26$; $P = 0.022$) but not for quantitative data for sputum *lytA* qPCR and NpA *lytA* qPCR assays ($r = 0.049$; $P = 0.67$) (Fig. 2B).

It can be noted that, among 19 cases with pneumococcal etiology with positive blood culture and/or *S. pneumoniae* urinary antigen test results, positive qPCR results were noted in sputum *lytA* qPCR assays in 18 cases at $\geq 10^4$ DNA copies/ml, in sputum Spn9802 qPCR assays in 18 cases at $\geq 10^5$ DNA copies/ml, in NpA *lytA* qPCR assays in 16 cases at $\geq 10^2$ DNA copies/ml, and in NpA Spn9802 qPCR assays in 15 cases at $\geq 10^3$ DNA copies/ml.

Diagnostic performance of PCR tests. Figure 3 presents ROC curves for the qPCR tests for detection of pneumococcal etiology in CAP patients. The area under the curve (AUC) values were significantly greater for the sputum qPCR tests than the NpA qPCR tests, with AUC differences of 0.0951 (95% confidence interval [CI], 0.0200 to 0.170; $P = 0.013$) between sputum *lytA* qPCR and NpA *lytA* qPCR results and 0.114 (95% CI, 0.0309 to 0.197; $P = 0.0072$) between sputum Spn9802 qPCR and NpA Spn9802 qPCR results. The AUC values tended to be greater for *lytA* qPCR tests than for Spn9802 qPCR tests, i.e., AUC differences of 0.0143 (95% CI, −0.00917 to 0.0377; $P = 0.23$) between sputum *lytA* qPCR and sputum Spn9802 qPCR results and 0.0333 (95% CI, −0.0381 to 0.105; $P = 0.36$) between NpA *lytA* qPCR and NpA Spn9802 qPCR results.

Table 3 presents sensitivities, specificities, and predictive values for the qPCR tests. The best-performing test according to the ROC curve analysis, the sputum *lytA* qPCR test, showed a sensitivity of 94%, a specificity of 96%, a positive predictive value of 94%, and a negative predictive value of 96%, at a cutoff value of 10^5 DNA copies/ml.

If the four patients with pneumococcal carriage would be classified as pneumococcal pneumonia cases, then the positive predictive values would increase to 100% (32/32 cases) for the sputum *lytA* qPCR test (with a cutoff value of 10^5 DNA copies/ml), to 90% (35/39 cases) for the sputum Spn9802 qPCR test (with a cutoff value of 10^5 DNA copies/ml), to 94% (29/31 cases) for the NpA *lytA* qPCR test (with a cutoff value of 10^2 DNA copies/ml), and to 86% (30/35 cases) for the NpA Spn9802 qPCR test (with a

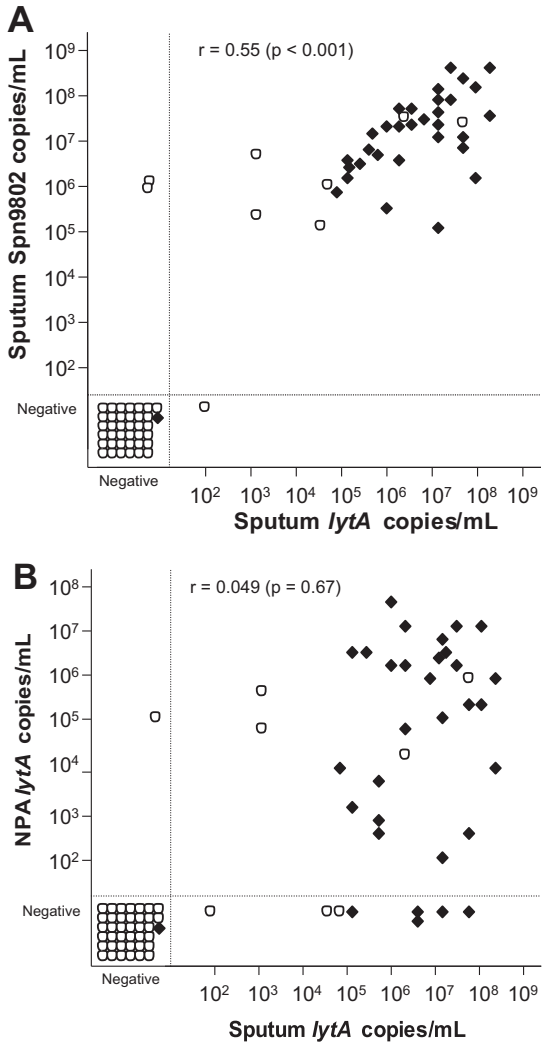

FIG 2 Correlations of quantitative PCR (qPCR) data for individual patients with community-acquired pneumonia. ◆, cases with pneumococcal etiology; ○, cases without pneumococcal etiology. (A) Sputum *lytA* qPCR results correlated with sputum Spn9802 qPCR results; (B) sputum *lytA* qPCR results correlated with nasopharyngeal aspirate (NpA) *lytA* qPCR results.

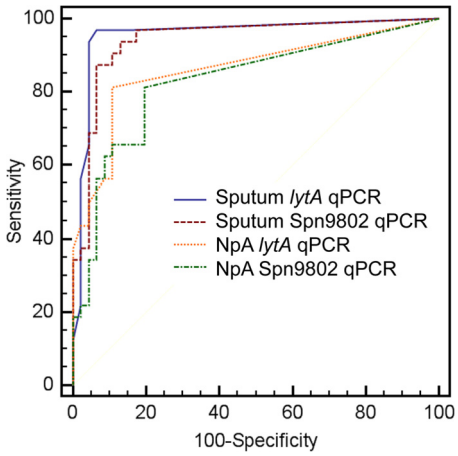

FIG 3 Receiver operating characteristic (ROC) curves for quantitative PCR (qPCR) assays (*lytA* and Spn9802) applied to sputum and nasopharyngeal aspirate (NpA) specimens for detection of pneumococcal pneumonia. The area under the ROC curve values were 0.957 (95% confidence interval [CI], 0.885 to 0.990) for the sputum *lytA* qPCR test, 0.942 (95% CI, 0.865 to 0.982) for the sputum Spn9802 qPCR test, 0.861 (95% CI, 0.765 to 0.929) for the NpA *lytA* qPCR test, and 0.828 (95% CI, 0.726 to 0.904) for the NpA Spn9802 qPCR test.

cutoff value of 10^2 DNA copies/ml). At these cutoff values, the negative predictive values would decrease to 91% (42/46 cases) for the sputum *lytA* qPCR test and to 85% (40/47 cases) for the NpA *lytA* qPCR test, although they would be unchanged for the sputum Spn9802 qPCR (97%) and NpA Spn9802 qPCR (86%) tests.

PCR results in pneumococcal pneumonia cases with negative urinary antigen test results. The *S. pneumoniae* urinary antigen test results were negative in 14 cases with established pneumococcal etiology that were positive for *S. pneumoniae* in blood cultures ($n = 2$) and/or cultures of representative sputum samples ($n = 13$). These cases were positive by sputum *lytA* qPCR in all 14 cases (at $>10^5$ DNA copies/ml), sputum Spn9802 qPCR in all 14 cases (at $>10^5$ DNA copies/ml), NpA *lytA* qPCR in 12 cases (all cases at $>10^2$ DNA copies/ml), and NpA Spn9802 qPCR in 11 cases (all cases at $>10^4$ DNA copies/ml).

Antibiotic treatment prior to admission. Ten patients had received antibiotics prior to admission. None of them was positive for *S. pneumoniae* by any etiological test or qPCR analysis. However, 8 patients were positive for other etiologies, i.e., *M. pneumoniae* ($n = 4$), *H. influenzae* ($n = 3$), and *C. pneumoniae* ($n = 1$). Two patients had no etiology identified.

DISCUSSION

Using quantitative molecular methods, the present study showed that CAP patients with pneumococcal etiology were most often positive for *lytA* and Spn9802 DNA at high levels in sputum samples and at moderate levels in NpA samples, although CAP patients without pneumococcal etiology most often were DNA negative or were positive for DNA at low to moderate levels. Accordingly, the qPCR methods that were used have great potential to be used for detection of pneumococcal etiology in CAP.

In the performance analyses of the qPCR tests, we found that sputum specimens were superior to NpA specimens as the sample type and that *lytA* tended to be superior to Spn9802 as the gene target. Thus, sputum *lytA* qPCR was found to be the most useful test, with high sensitivity and specificity, at a cutoff value of 10^5 DNA copies/ml. A correlation was found between quantitative data from sputum *lytA* qPCR and sputum Spn9802 qPCR assays

TABLE 3 Performance of *lytA* and Spn9802 qPCR assays applied to sputum and NpA specimens for detection of pneumococcal etiology in community-acquired pneumonia

qPCR test	Cutoff value (DNA copies/ml)	Sensitivity (% [no. of true-positive cases/ all cases with pneumococcal etiology])	Specificity (% [no. of true-negative cases/ all cases without pneumococcal etiology])	Positive predictive value (% [no. of true-positive cases/all cases with positive PCR test results])	Negative predictive value (% [no. of true-negative cases/all cases with negative PCR test results])
Sputum *lytA*	10^2	97 (31/32)	85 (39/46)	82 (31/38)	98 (39/40)
	10^3	97 (31/32)	87 (40/46)	84 (31/37)	98 (40/41)
	10^4	97 (31/32)	91 (42/46)	89 (31/39)	98 (42/43)
	10^5	94 (30/32)	96 (44/46)	94 (30/32)	96 (44/46)
	10^6	72 (23/32)	96 (44/46)	92 (23/25)	83 (44/53)
Sputum Spn9802	10^2	97 (31/32)	83 (38/46)	79 (31/39)	97 (38/39)
	10^3	97 (31/32)	83 (38/46)	79 (31/39)	97 (38/39)
	10^4	97 (31/32)	83 (38/46)	79 (31/39)	97 (38/39)
	10^5	97 (31/32)	83 (38/46)	79 (31/39)	97 (38/39)
	10^6	88 (28/32)	89 (41/46)	85 (28/33)	91 (41/45)
	10^7	59 (19/32)	96 (44/46)	90 (19/21)	77 (44/57)
NpA *lytA*	10^2	81 (26/32)	89 (41/46)	84 (26/31)	87 (41/47)
	10^3	69 (22/32)	89 (41/46)	81 (22/27)	80 (41/51)
	10^4	62 (20/32)	89 (41/46)	80 (20/25)	77 (41/53)
	10^5	53 (17/32)	93 (43/46)	85 (17/20)	74 (43/58)
NpA Spn9802	10^2	81 (26/32)	80 (37/46)	74 (26/35)	86 (37/43)
	10^3	81 (26/32)	80 (37/46)	74 (26/35)	86 (37/43)
	10^4	78 (25/32)	80 (37/46)	74 (25/34)	84 (37/44)
	10^5	66 (21/32)	87 (40/46)	78 (21/27)	78 (40/51)

(Fig. 2A). The sputum Spn9802 qPCR test was highly sensitive, although the specificity was lower than that of the sputum *lytA* qPCR test (Table 3). It is possible than some false-positive Spn9802 qPCR results were caused by *Streptococcus pseudopneumoniae*, as this species has been found to harbor the Spn9802 gene fragment (26, 27). The frequency of *S. pseudopneumoniae* in the normal respiratory flora is poorly studied (26).

The levels of pneumococcal DNA in sputum samples from patients with pneumococcal etiology in the present study were similar to those recently reported by Werno et al. (20). With a quantitative *lytA* PCR assay, that group found sputum bacterial loads of 10^5 to 10^8 CFU/ml in CAP patients with *S. pneumoniae* isolated in sputum cultures. It could be argued that the concentration of pneumococcal DNA in sputum is high because a high concentration of growing bacteria is required for a positive sputum culture (10^5 CFU/ml in the present study). However, among 19 patients with pneumococcal etiology with positive blood culture and/or *S. pneumoniae* urinary antigen test results in the present study, 18 patients had high concentrations of *lytA* DNA and Spn9802 DNA in sputum specimens. This indicates that the levels of pneumococcal DNA in sputum are generally high in pneumococcal pneumonia.

Compared with sputum qPCR results, NpA qPCR results showed lower DNA levels and more-scattered patterns of quantitative results in cases with pneumococcal etiology (Fig. 1). Thus, no correlation between quantitative results of sputum qPCR and NpA qPCR assays could be noted (Fig. 2B). Accordingly, with a quantitative *lytA* PCR test applied to nasopharyngeal secretions, Albrich et al. (13) found varying nasopharyngeal pneumococcal colonization densities in CAP patients with *S. pneumoniae* isolated in nasopharyngeal swab cultures. In a recent presentation (28), that group suggested that the nasopharyngeal pneumococcal colonization density was correlated with disease severity in pneumococcal pneumonia. This interesting hypothesis should be further studied. However, the wide range of quantitative results, combined with DNA copy number means similar for qPCR-positive cases with and without pneumococcal etiology, made it difficult to find suitable cutoff values for NpA qPCR assays. As the positive predictive values for the NpA *lytA* qPCR and NpA Spn9802 qPCR assays were as low as 84% and 74%, respectively, the present study does not support use of the NpA qPCR assay for detection of pneumococcal pneumonia. However, the positive predictive value would increase to 94% for the NpA *lytA* qPCR assay if cases of pneumococcal carriage were classified as pneumococcal pneumonia cases. This result, the promising results of Albrich et al. (13), and our previous study (14) support additional studies of the usefulness of qPCR assays with nasopharyngeal secretion specimens.

In the present study, sputum *lytA* qPCR results (cutoff value of 10^5 DNA copies/ml) were positive in 30 of 32 cases with pneumococcal etiology, including 14 cases with negative *S. pneumoniae* urinary antigen test results. These results indicate that the addition of the sputum *lytA* qPCR test to the Binax NOW urinary antigen test in the emergency room could increase the number of patients with rapidly diagnosed pneumococcal pneumonia, who could receive targeted penicillin therapy. As CAP is a common infectious disease, increased use of targeted narrow-spectrum penicillin therapy could be valuable as part of the effort to slow the emergence of antibiotic resistance.

The present study has some limitations. First, the fact that different (although very similar) standards with known DNA concentrations were used for the *lytA* qPCR and Spn9802 qPCR assays limits direct comparison of exact DNA copy numbers between the two methods. However, it does not limit comparisons of sensitivities, specificities, predictive values, and ROC curve analyses between the methods, which were major aims of the study. Second, five patients were not subjected to urinary antigen testing, although all other microbiological tests were performed for the patients. Three of them did not have pneumococcal etiology. As these patients were negative for *S. pneumoniae* in cultures of blood, sputum, and NpA samples, as well as the qPCR tests, most likely they did not have pneumococcal pneumonia. Third, the study was a single-site study with a limited number of study subjects. Additional studies are needed to validate and to establish the clinical usefulness of qPCR for *S. pneumoniae*.

In conclusion, the study showed that sputum specimens were superior to NpA specimens as the sample type and *lytA* tended to be superior to Spn9802 as the gene target for qPCR detection of pneumococcal etiology in CAP. The sputum *lytA* qPCR assay was identified as a useful diagnostic method that has great potential to be used in combination with the Binax NOW urinary antigen test to target penicillin therapy for CAP in patients who have good sputum production.

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REFERENCES

1. **Leung E, Weil DE, Raviglione M, Nakatani H, World Health Organization World Health Day Antimicrobial Resistance Technical Working Group.** 2011. The WHO policy package to combat antimicrobial resistance. Bull. World Health Organ. **89:**390–392. http://dx.doi.org/10.2471/BLT.11.088435.
2. **Spellberg B, Bartlett JG, Gilbert DN.** 2013. The future of antibiotics and resistance. N. Engl. J. Med. **368:**299–300. http://dx.doi.org/10.1056/NEJMp1215093.
3. **Yu VL.** 2011. A clinical solution to antimicrobial resistance in community-acquired pneumonia: narrowing the spectrum of antimicrobial therapy. Arch. Intern. Med. **171:**172–173. http://dx.doi.org/10.1001/archinternmed.2010.474.
4. **Spindler C, Strålin K, Eriksson L, Hjerdt-Goscinski G, Holmberg H, Lidman C, Nilsson A, Örtqvist Å, Hedlund J.** 2012. Swedish guidelines on the management of community-acquired pneumonia in immunocompetent adults: Swedish Society of Infectious Diseases 2012. Scand. J. Infect. Dis. **44:**885–902. http://dx.doi.org/10.3109/00365548.2012.700120.
5. **Woodhead M.** 2002. Community-acquired pneumonia in Europe: causative pathogens and resistance patterns. Eur. Respir. J. Suppl. **36:**S20–S27. http://dx.doi.org/10.1183/09031936.02.00702002.
6. **Johansson N, Kalin M, Tiveljung-Lindell A, Giske CG, Hedlund J.** 2010. Etiology of community-acquired pneumonia: increased microbiological yield with new diagnostic methods. Clin. Infect. Dis. **50:**202–209. http://dx.doi.org/10.1086/648678.
7. **Said MA, Johnson HL, Nonyane BAS, Deloria-Knoll M, O'Brien KL, Adult Pneumococcal Burden Study Team.** 2013. Estimating the burden of pneumococcal pneumonia among adults: a systematic review and meta-analysis of diagnostic techniques. PLoS One **8:**e60273. http://dx.doi.org/10.1371/journal.pone.0060273.
8. **Chiou CC.** 2006. Does penicillin remain the drug of choice for pneumococcal pneumonia in view of emerging in vitro resistance? Clin. Infect. Dis. **42:**234–237. http://dx.doi.org/10.1086/499596.
9. **Guchev IA, Yu VL, Sinopalnikov A, Klochkov OI, Kozlov RS, Stratchounski LS.** 2005. Management of nonsevere pneumonia in military trainees with the urinary antigen test for *Streptococcus pneumoniae*: an innovative approach to targeted therapy. Clin. Infect. Dis. **40:**1608–1616. http://dx.doi.org/10.1086/429919.

10. **Sordé R, Falcó V, Lowak M, Domingo E, Ferrer A, Burgos J, Puig M, Cabral E, Len O, Pahissa A.** 2011. Current and potential usefulness of pneumococcal urinary antigen detection in hospitalized patients with community-acquired pneumonia to guide antimicrobial therapy. Arch. Intern. Med. **171:**166–172. http://dx.doi.org/10.1001/archinternmed.2010.347.
11. **Avni T, Mansur N, Leibovici L, Paul M.** 2010. PCR using blood for diagnosis of invasive pneumococcal disease: systematic review and meta-analysis. J. Clin. Microbiol. **48:**489–496. http://dx.doi.org/10.1128/JCM.01636-09.
12. **Abdeldaim G, Herrmann B, Mölling P, Holmberg H, Blomberg J, Olcén P, Strålin K.** 2010. Usefulness of real-time PCR for *lytA*, *ply*, and Spn9802 on plasma samples for the diagnosis of pneumococcal pneumonia. Clin. Microbiol. Infect. **16:**1135–1141. http://dx.doi.org/10.1111/j.1469-0691.2009.03069.x.
13. **Albrich WC, Madhi SA, Adrian PV, van Niekerk N, Mareletsi T, Cutland C, Wong M, Khoosal M, Karstaedt A, Zhao P, Deatly A, Sidhu M, Jansen KU, Klugman KP.** 2012. Use of a rapid test of pneumococcal colonization density to diagnose pneumococcal pneumonia. Clin. Infect. Dis. **54:**601–609. http://dx.doi.org/10.1093/cid/cir859.
14. **Abdeldaim GM, Strålin K, Olcén P, Blomberg J, Herrmann B.** 2008. Toward a quantitative DNA-based definition of pneumococcal pneumonia: a comparison of *Streptococcus pneumoniae* target genes, with special reference to the Spn9802 fragment. Diagn. Microbiol. Infect. Dis. **60:**143–150. http://dx.doi.org/10.1016/j.diagmicrobio.2007.08.010.
15. **Suzuki N, Seki M, Nakano Y, Kiyoura Y, Maeno M, Yamashita Y.** 2005. Discrimination of *Streptococcus pneumoniae* from viridans group streptococci by genomic subtractive hybridization. J. Clin. Microbiol. **43:**4528–4534. http://dx.doi.org/10.1128/JCM.43.9.4528-4534.2005.
16. **Abdeldaim G, Herrmann B, Korsgaard J, Olcén P, Blomberg J, Strålin K.** 2009. Is quantitative PCR for the pneumolysin (*ply*) gene useful for detection of pneumococcal lower respiratory tract infection? Clin. Microbiol. Infect. **15:**565–570. http://dx.doi.org/10.1111/j.1469-0691.2009.02714.x.
17. **Carvalho MG, Tondella ML, McCaustland K, Weidlich L, McGee L, Mayer LW, Steigerwalt A, Whaley M, Facklam RR, Fields B, Carlone G, Ades EW, Dagan R, Sampson JS.** 2007. Evaluation and improvement of real time PCR assays targeting *lytA*, *ply*, and *psaA* genes for detection of pneumococcal DNA. J. Clin. Microbiol. **45:**2460–2466. http://dx.doi.org/10.1128/JCM.02498-06.
18. **Yang S, Lin S, Khalil A, Gaydos C, Nuemberger E, Juan G, Hardick J, Bartlett JG, Auwaerter PG, Rothman RE.** 2005. Quantitative PCR assay using sputum samples for rapid diagnosis of pneumococcal pneumonia in adult emergency department patients. J. Clin. Microbiol. **43:**3221–3226. http://dx.doi.org/10.1128/JCM.43.7.3221-3226.2005.
19. **Kais M, Spindler C, Kalin M, Örtqvist Å, Giske C.** 2006. Quantitative detection of *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* in lower respiratory tract samples by real-time PCR. Diagn. Microbiol. Infect. Dis. **55:**169–178. http://dx.doi.org/10.1016/j.diagmicrobio.2006.01.007.
20. **Werno AM, Anderson TP, Murdoch DR.** 2012. Association between pneumococcal load and disease severity in adults with pneumonia. J. Med. Microbiol. **61:**1129–1135. http://dx.doi.org/10.1099/jmm.0.044107-0.
21. **Strålin K, Herrmann B, Abdeldaim G, Olcén P, Mölling P.** 2012. *lytA* PCR and Spn9802 PCR applied to sputum for rapid detection of pneumococcal pneumonia, abstr P-69. Abstr. 8th Int. Symp. Pneumococci Pneumococcal Dis., Iguacu Falls, Brazil.
22. **Strålin K, Olcén P, Törnqvist E, Holmberg H.** 2010. Definite, probable, and possible bacterial aetiologies of community-acquired pneumonia at different CRB-65 scores. Scand. J. Infect. Dis. **42:**426–434. http://dx.doi.org/10.3109/00365540903552353.
23. **Baron EJ, Murray PR, Doern GV, Ferraro MJ, Gilligan PH, Janda JM, von Graevenitz A.** 1999. Bacteriology, p 246–832. *In* Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH (ed), Manual of clinical microbiology, 7th ed. ASM Press, Washington, DC.
24. **Kalin M, Lindberg A, Tunevall G.** 1983. Etiological diagnosis of bacterial pneumonia by Gram stain and quantitative culture of expectorates: leukocytes or alveolar macrophages as indicators of sample representativity. Scand. J. Infect. Dis. **15:**153–160.
25. **Tang YW, Sefers SE, Li H, Kohn DJ, Procop GW.** 2005. Comparative evaluation of three commercial systems for nucleic acid extraction from urine specimens. J. Clin. Microbiol. **43:**4830–4833. http://dx.doi.org/10.1128/JCM.43.9.4830-4833.2005.
26. **Rolo D, Simões SA, Domenech A, Fenoll A, Liñares J, de Lencastre H, Ardanuy C, Sá-Leão R.** 2013. Disease isolates of *Streptococcus pseudopneumoniae* and non-typeable *S. pneumoniae* presumptively identified as atypical *S. pneumoniae* in Spain. PLoS One **8:**e57047. http://dx.doi.org/10.1371/journal.pone.0057047.
27. **Wessels E, Schelfaut JJ, Bernards AT, Claas EC.** 2012. Evaluation of several biochemical and molecular techniques for identification of *Streptococcus pneumoniae* and *Streptococcus pseudopneumoniae* and their detection in respiratory samples. J. Clin. Microbiol. **50:**1171–1177. http://dx.doi.org/10.1128/JCM.06609-11.
28. **Albrich W, Madhi SA, Adrian PV, Sidhu M, van Niekerk N, Dollet S, Pride M, Telles JN, Ebrahim N, Messaoudi M, Zhao P, Deatly A, Paranhos-Baccala G, Giersdorf S, Vernet G, Mueller B, Klugman KP.** 2012. Nasopharyngeal pneumococcal colonization density as a marker for disease severity in adult pneumonia, abstr P-137. Abstr. 8th Int. Symp. Pneumococci Pneumococcal Dis., Iguacu Falls, Brazil.