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Evaluation and Improvement of Real-Time PCR Assays Targeting *lytA*, *ply*, and *psaA* Genes for Detection of Pneumococcal DNA▽

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The accurate diagnosis of pneumococcal disease has frequently been hampered not only by the difficulties in obtaining isolates of the organism from patient specimens but also by the misidentification of pneumococcus-like viridans group streptococci (P-LVS) as *Streptococcus pneumoniae*. This is especially critical when the specimen comes from the respiratory tract. In this study, three novel real-time PCR assays designed for the detection of specific sequence regions of the *lytA*, *ply*, and *psaA* genes were developed (*lytA*-CDC, *ply*-CDC, and *psaA*, respectively). These assays showed high sensitivity (<10 copies for *lytA*-CDC and *ply*-CDC and an approximately twofold less sensitivity for *psaA*). Two additional real-time PCR assays for *lytA* and *ply* described previously for pneumococcal DNA detection were also evaluated. A panel of isolates consisting of 67 *S. pneumoniae* isolates (44 different serotypes and 3 nonencapsulated *S. pneumoniae* isolates from conjunctivitis outbreaks) and 104 nonpneumococcal isolates was used. The 67 *S. pneumoniae* isolates were reactive in all five assays. The new real-time detection assays targeting the *lytA* and *psaA* genes were the most specific for the detection of isolates confirmed to be *S. pneumoniae*, with *lytA*-CDC showing the greatest specificity. Both *ply* PCRs were positive for all isolates of *S. pseudopneumoniae*, along with 13 other isolates of other P-LVS isolates confirmed to be non-*S. pneumoniae* by DNA-DNA reassociation. Thus, the use of the *ply* gene for the detection of pneumococci can lead to false-positive reactions in the presence of P-LVS. The five assays were applied to 15 culture-positive cerebrospinal fluid specimens with 100% sensitivity; and serum and ear fluid specimens were also evaluated. Both the *lytA*-CDC and *psaA* assays, particularly the *lytA*-CDC assay, have improved specificities compared with those of currently available assays and should therefore be considered the assays of choice for the detection of pneumococcal DNA, particularly when upper respiratory P-LVS might be present in the clinical specimen.

Streptococcus pneumoniae continues to be a serious etiologic agent of disease throughout the world, causing a range of illnesses which include otitis media, sinusitis, pneumonia, bacteremia, and meningitis (1, 6). The limitations of culture-based, conventional tests for the detection of *S. pneumoniae* make the establishment of a definitive diagnosis difficult. Isolation of *S. pneumoniae* from blood, the recognized "gold standard," occurs in only 20 to 30% of adult cases of pneumococcal pneumonia and less than 10% of cases among children (18, 24), and serologic assays for both antibody and antigen detection lack specificity and sensitivity (16, 30). Although the recently introduced Binax NOW urine antigen test has been shown to be sensitive and specific for the detection of the organism in adults in some studies (22, 23), it is unable to distinguish between carriage and disease in children (11). Compounding this problem, the misidentification of pneumococcus-like viridans group streptococci (P-LVS) as *S. pneumoniae* presents additional opportunities for the misidentification of the causative agents of infections (3), especially when identification is attempted with specimens from nonsterile sites, such as sputum. The identification of *S. pneumoniae* has classically been based on bile solubility, optochin sensitivity, and the GenProbe AccuProbe Pneumococcus identification test (14); but increasingly, there have been reports in the literature of the isolation of P-LVS from clinical specimens, which may give positive or variable reactions by one or more of these standard tests for the detection of pneumococci (3). Moreover, among a subset of reported isolates of P-LVS, a newly recognized species classified as *Streptococcus pseudopneumoniae* has been described and characterized (3). *S. pseudopneumoniae* organisms are bile solubility negative and are resistant to optochin in the presence of 5% CO_2, but they are AccuProbe assay positive (3). The appearance of these pneumococcus-like organisms has complicated identification and diagnosis even further, especially when specimens from nonsterile respiratory sites are used for organism identification. Therefore, special care must be taken to monitor and correctly identify isolates confirmed to be pneumococci in the clinical setting, and assays in development will need to include *S. pseudopneumoniae* and other P-LVS in specificity determinations for a more precise diagnosis. Previous studies in our laboratories have shown that DNA-DNA hybridization or the PCR assay based on the au-

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tolysin (*lytA*) and pneumococcal surface adhesion (*psaA*) gene sequences can reliably distinguish *S. pneumoniae* from *S. pseudopneumoniae* (21). In a recent publication by Llull et al. (19), *lytA* sequences from *S. pneumoniae* and *S. pseudopneumoniae* were carefully analyzed and pneumococcus-specific alleles were identified. For the differentiation of isolates confirmed to be pneumococci, they developed a standard PCR based on these sequences, followed by an enzyme digestion step. Although these techniques are very suitable for organism identification, they may not be the most ideal for rapid diagnostics. The development of simple, sensitive, rapid, and more accurate assays for the detection of *S. pneumoniae* has been encouraged and is of the utmost importance in making exact estimates of disease burden, tracking changes in the epidemiology of *S. pneumoniae* disease, and evaluating the effectiveness of currently used vaccines.

To this end, researchers are exploring the use of newer nucleic acid-based techniques such as real-time PCR to improve pneumococcal disease diagnosis. The advantages of real-time PCR over conventional assays are its speed; elimination of the need for postprocessing steps which could contribute to contamination; and its wider dynamic range, which allows detection over much larger variations in concentrations of the target. The most important advantage is the lower limit of detection. Thus, the real-time PCR technology shows great sensitivity and is the only means of detection of some of the respiratory viruses (5, 32). Real-time PCR assays for *S. pneumoniae* have been reported in the literature (8, 15, 16, 20, 26, 29). These assays, mostly based on amplification of the *lytA* and *ply* genes, are not in routine use but have been used in research studies for the detection of pneumococcal DNA and for evaluation of their suitability when they are used with different clinical specimen types (2, 8, 20, 23, 26–29, 33). In this study, we have developed three new real-time PCR assays for the detection of specific sequence regions of the pneumococcal *psaA*, *lytA*, and *ply* genes. These newly developed *lytA* and *ply* assays use primer/probe sequences different from those used in assays described previously. We have taken advantage of the extensive strain collection of the CDC *Streptococcus* Laboratory to thoroughly evaluate these new PCRs with a stringent specificity panel.

An adequate, extensive, and simultaneous evaluation of the published real-time PCRs performed with the same samples to ascertain the comparative sensitivities and specificities of the assays has not been done to date. Therefore, it is unclear if the use of one assay is more advantageous than the use of another or if they have equal sensitivities and specificities. In this study, the three newly developed assays were compared to two previously published assays, the *ply* assay developed by Corless et al. (8) and the *lytA* assay developed by McAvin et al. (20), to determine relative sensitivities and specificities. These assays were chosen because they were performed in-house in our laboratories. One goal of this study was to thoroughly evaluate the performance of these assays as well as those of the new assays by using the same set of isolates and clinical specimens to assess their relative usefulness. We also wanted to demonstrate that it is possible, with the use of and access to an exacting strain collection, to design a highly specific real-time assay for *S. pneumoniae* which would not require additional postprocessing steps.

MATERIALS AND METHODS

Bacterial isolates. The panel consisted of 67 strains representing 44 different serotypes of *S. pneumoniae* (serotypes 1, 2, 4, 5, 6A, 6B, 7B, 7C, 7F, 8, 9N, 9V, 10A, 11A, 12F, 13, 14, 15B, 15A, 15C, 16F, 17A, 17F, 18B, 18C, 18F, 19A, 19C, 19F, 20, 21, 22A, 22F, 23B, 23F, 24A, 24B, 28A, 28F, 32F, 33A, 33F, 35A, 35F, and 40 and 3 nonencapsulated strains from conjunctivitis outbreaks) and 104 nonpneumococcal isolates, including *S. pseudopneumoniae*, *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus sanguinis*, *Streptococcus parasanguinis*, *Streptococcus peroris*, *Streptococcus infantis*, *Streptococcus gordonii*, *Streptococcus cristatus*, *Streptococcus salivarius*, *Streptococcus vestibularis*, *Streptococcus australis*, *Streptococcus sinensis*, *Streptococcus oligofermentans*, *Streptococcus intestinalis*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus canis*, *Streptococcus anginosus*, *Streptococcus equi* subsp. *equi*, *Streptococcus equi* subsp. *zooepidemicus*, *Streptococcus porcinus*, *Streptococcus dysgalactiae*, *Streptococcus constellatus*, *Streptococcus iniae*, *Streptococcus intermedius*, *Staphylococcus aureus*, *Staphylococcus warneri*, 13 viridans group streptococci not identified to the species level, *Dolosigranulum pigrum*, *Enterococcus faecalis*, *Escherichia coli*, *Chlamydia pneumoniae*, *Chlamydia psittaci*, *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Haemophilus influenzae* types a to f and nontypeable, *Haemophilus parainfluenzae*, *Corynebacterium diphtheriae*, *Corynebacterium pseudotuberculosis*, *Nocardia farcinica*, *Nocardia asteroides*, *Klebsiella pneumoniae*, *Mycobacterium fortuitum*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, and *Bordetella bronchiseptica*. *S. pneumoniae* ATCC strain 33400 was used as a positive control in all assays. All bacterial isolates were obtained from the culture collections of CDC laboratories (*Streptococcus* Laboratory, Respiratory Diseases Branch and Meningitis Laboratory, Meningitis and Vaccine Preventable Diseases Branch).

OPT. The optochin susceptibility test (OPT) was performed on 5% sheep blood agar plates in 5% CO_2 environments, as described by Arbique et al. (3).

BS test. The tube bile solubility (BS) test was performed as described previously (3, 25).

DNA probe hybridization test. The AccuProbe *Streptococcus pneumoniae* culture identification test, based on the rRNA gene sequence, was performed according to the manufacturer's instructions (Gen-Probe, San Diego, CA).

DNA-DNA reassociation. Growth, harvesting, and lysis of the bacterial cells were performed as described previously (3, 7). Extraction and purification of DNA and DNA-DNA reassociation studies, including determination of DNA relatedness by the hydroxyapatite hybridization method, were performed as described by Brenner and colleagues (7). DNA hybridization experiments were performed at 55°C for optimal DNA reassociation and at the stringent DNA reassociation temperature of 70°C. The levels of divergence within related sequences were determined by assuming that each degree of heteroduplex instability was caused by 1% unpaired bases. Divergence, expressed by the change in melting temperature, is the decrease in the thermal stability (in degrees Celsius) of the heterologous DNA duplex relative to that of the homologous duplexes. Divergence was calculated to the nearest 0.5%.

Clinical specimens. Clinical specimens consisted of serum, middle ear fluids (MEFs), and cerebrospinal fluids (CSFs) and were obtained in accordance with CDC Institutional Review Board stipulations. The sera and MEFs were obtained from the Soroka University Hospital in Beer-Sheva, Israel. Serum specimens were collected from 15 patients with pneumococcal bacteremia and 15 age-matched, ethnic group-matched, healthy control children for whom nasopharyngeal swab cultures were negative for *S. pneumoniae*. MEF specimens consisted of 10 *S. pneumoniae* culture-positive MEF specimens and 10 *S. pneumoniae* culture-negative but *H. influenzae*-positive MEF specimens. Twenty-five CSF specimens were obtained from the Laboratorio Central do Estado do Rio Grande do Sul, Porto Alegre, Brazil, and consisted of 15 specimens from meningitis patients who were pneumococcal culture positive and 10 CSF specimens from pneumococcus-negative, *Neisseria meningitidis*-positive patients. The specimens were shipped on dry ice and were frozen at −70°C upon arrival.

DNA extraction for real-time PCR analysis. DNA was extracted from the isolates by a modification of the QIAGEN DNA Mini kit (QIAGEN Inc., Valencia, CA) method. Briefly, a loopful of the overnight growth from a blood agar plate was resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0, 2.0 mM EDTA, 1.2% Triton X-100) containing 0.04 g/ml lysozyme and 75 U/ml of mutanolysin (Sigma Chemical Co., St. Louis, MO) and incubated for 1 h at 37°C in a water bath. The remaining procedures followed the manufacturer's instructions.

For clinical specimens, 200 μl of clinical material was added to 100 μl of TE (Tris-EDTA) buffer containing 0.04 g/ml lysozyme and 75 U/ml of mutanolysin (Sigma Chemical Co.), and the mixture was incubated for 1 h in a 37°C water bath. All subsequent steps were as outlined in the QIAGEN DNA Mini protocol

TABLE 1. Real-time PCR primers and probes[d]

Oligonucleotide	Sequence	Nucleotide position	GenBank accession no.
lytA-CDC forward	5′-ACGCAATCTAGCAGATGAAGCA-3′	1841014	AE005672
lytA-CDC reverse	5′-TCGTGCGTTTTAATTCCAGCT-3′	1840961	
lytA-CDC probe	5′-FAM-GCCGAAAACGCTTGATACAGGGAG-3′-BHQ1	1840985	
psaA forward	5′-GCCCTAATAAATTGGAGGATCTAATGA-3′	166	U53509
psaA reverse	5′-GACCAGAAGTTGTATCTTTTTTTCCG-3′	279	
psaA probe[a,b]	5′-HEX-CTAGCACATGCTACAAGAATGATTGCAGAAA GAAA-3′-phosphate	219	
ply-CDC forward	5′-GCTTATGGGCGCCAAGTCTA-3′	721	AE008539
ply-CDC reverse	5′-CAAAGCTTCAAAAGCAGCCTC TA-3′	798	
ply-CDC probe[b,c]	5′-FAM-CTCAAGTTGGAAACCACGAGTAAGAGTGAT GAA-3′-phosphate	742	

[a] The *psaA* probe is designed to bind to the reverse strand of the amplicon.
[b] T, the thymidine on which the internal BHQ quencher was attached.
[c] For multiplex detection the 5′ end label was changed to CAL Flour 610.
[d] After this study was completed a modification to the probe for the *lytA*-CDC assay by the addition of a T residue (in boldface) to the 5′ end, which yielded the probe sequence 5′-FAM-**T**GCCGAAAACGCTTGATACAGGGAG-3′-BHQ1, was shown to improve the intensity of the signal but not change the LLD with purified DNA or the sensitivity or specificity with DNA from isolates. Unfortunately, the supply of the clinical specimens used in the study and the DNA from them was depleted, so we were unable to completely evaluate the new probe. All our preliminary experiments with this new probe suggest that it performs as well as the original probe. We recommend its use and have begun a complete evaluation and validation study.

booklet. DNA was eluted in 100 μl of QIAGEN elution buffer and stored at −20°C. The concentrations of the DNA extracted from the bacterial cultures were determined by the Nanodrop method (Nanodrop Technologies, Wilmington, DE).

Real-time PCRs for *lytA*, *ply*, and *psaA*. The two previously published real-time PCR assays were performed as described previously (8, 20). For development of the new assays, oligonucleotide primers and fluorescent dye-labeled probes were designed on the basis of the previously published *lytA*, *ply*, and *psaA* gene sequences and the sequences available in GenBank by using Primer Express software (Applied Biosystems, Foster City, CA). The probes were labeled at the 5′ end with either 6-carboxyfluorescein (FAM) or, in the case of the *psaA* probe, hexachloro-6-carboxyfluorescein (HEX). Black hole quencher 1 (BHQ1; Biosearch Technologies, Novato, CA) was placed either at the 3′ end of the probe or internally on a thymidine (Table 1). If the sequence was internally quenched, the 3′ end was capped with a phosphate group to prevent extension of the probe. The primer and probe sequences are listed in Table 1. The assays were carried out in a final 25-μl reaction volume and were performed by use of the TaqMan Universal Master Mix kit (Applied Biosystems), according to the instructions of the manufacturer, with 2.5 μl of sample DNA. The primer and probe concentrations for each of the three assays were optimized; and in accordance with the experimentally optimized concentrations, 500, 200, and 200 nM *psaA*-, *lytA*-, and *ply*-specific primers, respectively, and 100, 200, and 200 nM *psaA*-, *lytA*-, and *ply*-specific probes, respectively, were used for all subsequent experiments. A no-template control and an *S. pneumoniae*-positive DNA control (*S. pneumoniae* ATCC 33400) were included in every run. DNA was amplified with the Mx3000P system (Stratagene, La Jolla, CA) or the 7500 Real Time PCR system (Applied Biosystems) by using the following cycling parameters: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Amplification data were analyzed by instrument software (Stratagene or Applied Biosystems). Negative samples were defined as those with cycle threshold (C_T) values greater than >40. The new assays are designated *lytA*-CDC, *ply*-CDC, and *psaA*.

Analytical sensitivity and specificity determinations of real-time PCRs. For assessments of the lower limits of detection (LLDs), serial 10-fold dilutions (equivalent to from 6,666 to 6.6 copies) of purified DNA from pneumococcal reference strain ATCC 33400 were prepared, and aliquots were tested by all five real-time PCR protocols. Specificity determinations were made by testing at 5 ng/μl the DNAs extracted from 67 *S. pneumoniae* isolates and 104 nonpneumococcal isolates (listed above) by all five assays.

Real-time PCR of clinical samples. The detection of *S. pneumoniae* DNA in the serum, MEF, and CSF specimens was performed in parallel with aliquots of the same specimen for all assays. DNA extracted (2.5 μl of undiluted DNA or 2.5 μl of a 1:3 dilution) from serum, MEF, or CSF specimens was used in the amplification reactions. All assays with each clinical sample were performed in triplicate. A specimen was considered positive if two of the three triplicates gave a positive result within the <40-cycle cutoff. The assay protocols were as described above. A control reaction with RNase P human gene was performed independently with each sample to check for the presence of inhibitors (13). A failure to get amplification in this reaction was considered indicative of the presence of inhibitors.

Mutiplex *psaA*, *lytA*-CDC, and *ply*-CDC real-time PCRs. The three sets of primers and probes were combined into a single reaction mixture for multiplex detection. Modifications to the single-gene detection assays included the use of QIAGEN′s QuantiTect Multiplex PCR NoROX master mixture, changing of the *ply* gene probe fluorescent label from FAM to CAL Flour Red 610 at the 5′ end and BHQ2 (both from Biosearch Technologies) at the 3′ end, and reduction of the concentration of the *lytA* FAM probe to 100 nM from the original 200 nM. The temperatures and the numbers of cycles remained the same as described above for the original single-PCR protocols.

RESULTS

LLDs of assays for *S. pneumoniae* detection. The analytical LLDs for all five assays were measured by amplifying serial dilutions of purified extracted genomic DNA from positive control strain *S. pneumoniae* ATCC 33400. All five assays showed high sensitivities with their respective primer pairs and probes, with a limit of detection equivalent to <10 copies for all PCRs except the *psaA* PCR, which was approximately twofold less sensitive. All standard curves generated had slopes of −3.4 to −3.2, with the R^2 value being >0.96. The efficiencies of the assays were very similar and ranged from 96% to 100%. Evaluation of the five assays for their abilities to amplify DNA from a panel of 67 *S. pneumoniae* strains representing 45 serotypes and nontypeable *S. pneumoniae* strains resulted in 100% amplification or the detection of DNA from all *S. pneumoniae* strains tested.

Specificities of assays for *S. pneumoniae* detection. The analytical specificity of each of the five assays was evaluated and the specificities were compared by amplifying DNA extracted from 104 strains of nonpneumococcal bacteria. These strains represented several genera of gram-positive and gram-negative bacteria, some of which inhabit the oral cavity. No amplification occurred with any of the nonstreptococci in the specificity panel. There was, however, amplification with some strains of

TABLE 2. DNA-DNA hybridization and real-time PCR for unidentified viridans streptococci and *Streptococcus pseudopneumoniae*[a]

Strain	Geographic origin	Specimen	Identification test result			No. of *S. pneumoniae* ATCC 33400^T/no. of *S. pseudopneumoniae* ATCC BAA 960^T reference strains whose DNA was labeled			Real-time PCR result				
			OPT	BS test	GP	RBR at 55°C	RBR at 70°C	*D*	*psaA*	*lytA*	*lytA*-CDC	*ply*	*ply*-CDC
S. pneumoniae ATCC 33400^T			S	+	+	100/55	100/28	0.0/3.5	+	+	+	+	+
Unidentified viridans streptococci													
868-84	MD	Blood	R	−	−	66/ND	59/ND	4.5/ND	−	−	−	+	+
2901-90	AL	Throat	R	+	−	65/ND	51/ND	6.0/ND	−	−	−	+	+
2904-90	AL	Throat	S	+	−	61/ND	49/ND	5.5/ND	−	−	−	+	−
2909-90	AL	Throat	R	−	−	61/ND	46/ND	6.0/ND	−	−	−	+	+
2913-90	AL	Throat	S	−	−	65/52	58/44	4.5/4.5	−	−	−	+	+
2916-90	AL	Throat	R	−	−	58/ND	42/ND	6.0/ND	−	−	−	+	+
2918-90	AL	Throat	S	+	−	63/ND	54/ND	6.0/ND	−	−	−	+	+
2919-90	AL	Throat	R	−	−	63/ND	54/ND	4.5/ND	−	−	−	+	−
2920-90	AL	Throat	S	+	−	62/ND	53/ND	6.5/ND	−	−	−	+	+
2921-90	AL	Throat	R	−	−	62/ND	58/ND	4.5/ND	−	−	−	+	−
2939-90	AL	Throat	R	−	−	66/ND	57/ND	4.0/ND	−	−	−	+	+
Streptococcus pseudopneumoniae													
ATCC BAA-960^T	NS-CA	Sputum	R	−	+	62/100	56/100	4.0/0.0	−	−	−	+	+
253-03	NS-CA	Sputum	R	−	+	70/84	60/74	4.0/2.0	−	−	−	+	+
276-03	NS-CA	Sputum	R	−	+	68/83	53/77	4.0/1.0	−	−	−	+	+
288-03	NS-CA	Sputum	R	−	+	70/82	57/50	4.0/0.5	+	+	−	+	+
290-03	NS-CA	Sputum	R	−	+	70/81	64/75	4.0/1.5	−	−	−	+	+
2482-91	AL	Throat	R	−	+	58/72	46/71	3.0/1.0	−	+	−	+	+
2483-91	AL	Throat	R	−	+	58/76	46/71	3.5/1.5	−	−	−	+	+
2497-91	AL	Throat	R	−	+	37/82	49/80	3.5/2.0	+	−	−	+	+
2946-98	AL	Throat	R	−	+	61/76	54/76	3.0/1.5	−	+	−	+	+
2987-98	AZ	NP swab	R	−	+	61/98	50/73	3.0/1.0	−	+	−	+	+

[a] T, type strain; GP, GenProbe Accuprobe Pneumococcus culture identification test; RBR, relative binding relation at 55°C (optimal temperature) and 70°C (stringent temperature); *D*, divergence calculated to the nearest 0.5%; ND, not done; AL, Alaska; AZ, Arizona; MD, Maryland; NS-CA, Nova Scotia, Canada; R, resistant; S, susceptible; NP, nasopharyngeal.

P-LVS and *S. pseudopneumoniae*. The strains of P-LVS were specifically selected from among strains that were submitted to the CDC *Streptococcus* Laboratory which had been difficult to identify or classify by using the standard methodology criteria. DNA-DNA reassociation analysis had been performed with these isolates, in addition to the BS test, OPT, and the AccuProbe assay. The DNA-DNA reassociation values revealed that these P-LVS and the *S. pseudopneumoniae* strains (Table 2) were not *S. pneumoniae*. Analysis of these strains by real-time PCR demonstrated that the new *lytA*-CDC real-time PCR assay was the most specific (100%), showing no detectable fluorescent signal with DNA from the non-*S. pneumoniae* organisms in the specificity panel (Table 2). This was followed by the *psaA* real-time PCR (98%), which gave positive results with two of the *S. pseudopneumoniae* isolates, and the *lytA* real-time PCR (96%) published by McAvin et al. (20), which was positive for four *S. pseudopneumoniae* isolates. No amplification occurred with DNAs from P-LVS with the *lytA*, *lytA*-CDC, or *psaA* primer/probe sets (Table 2). The two *ply* assays gave positive reactions with all *S. pseudopneumoniae* isolates; of the other isolates of P-LVS, positive reactions occurred with both the *ply* (13 of 13) and the *ply*-CDC (10 of 13) assays, making final specificities of 78% and 81%, respectively.

Detection of *S. pneumoniae* in clinical samples. The five assays were used with three types of clinical specimens (described above) to evaluate and compare the results of the assays (Table 3). The results of all five assays showed excellent correlations. When specimens were positive or negative by one assay, the identical specimens were generally positive or negative, respectively, by the other assays. Only differences in the total numbers of positive and negative specimens for each were detected. Sensitivities were calculated on the basis of the results for the 15 culture-positive serum specimens, even though one of these was RNase P negative, indicating inhibition. The sensitivities with serum samples were 53% (8/15) for the *lytA*-CDC and *ply*-CDC assays (1 additional positive specimen for each assay, but the specimens were different), 47% (7/15) for the *lytA* and *psaA* assays, and 40% (6/15) for the *ply* assay. Analysis of the results for the *S. pneumoniae* culture-negative sera showed that there was a good correlation between the results of the assays and that the specificities were good. No positive results occurred by the *psaA*, *lytA*, and *ply* assays, resulting in 100% specificities with serum.

Analysis of the MEF and CSF specimens revealed that all five assays gave positive results for all 10 of the *S. pneumoniae*-positive MEF specimens and all 15 CSF specimens, yielding

TABLE 3. Assay results for clinical specimens for all five real-time PCRs

Specimen	*S. pneumoniae* culture result	No. of specimens	Real time-PCR result									
			psaA		*lytA*		*lytA*-CDC		*ply*-CDC		*ply*	
			Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Serum	+	15[a]	7	8	7	8	8[b]	7	8[b]	7	6	9
	−	15	0	15	0	15	1[b]	14	1[b]	14	0	15
MEF	+	10	10	0	10	0	10	0	10	0	10	0
	−	10	6	4	6	4	6	4	7[b]	3	6	4
CSF	+	15	15	0	15	0	15	0	15	0	15	0
	−	10	1[c]	9	2[b,c]	8	3[b,c]	7	2[b,c]	8	2[c]	8

[a] One serum specimen was RNase P gene negative.
[b] The averages of the C_T values was ≤38 for the *lytA*, *lytA*-CDC, and *ply*-CDC assays.
[c] C_T values were ≤37 for all five PCRs with the one specimen.

sensitivities of 100%. Evaluations of the specificities of the assays with the 10 culture-negative MEF specimens resulted in positive results with the same six MEF specimens by the *psaA*, *lytA*, *lytA*-CDC, and *ply* real-time PCR assays, yielding specificities of 40%. An additional specimen was positive by the *ply*-CDC assay, for a 30% specificity. Examination of the *S. pneumoniae*-negative CSF specimens showed good specificity. The numbers of positive results were 1 of 10 (90% specificity) for the *psaA* assay; 2 of 10 (80% specificity) for the *lytA*, *ply*-CDC, and *ply* assays; and 3 of 10 (70% specificity) for the *lytA*-CDC assay.

Mutiplex CDC real-time PCRs for *psaA*, *lytA*, and *ply*. To ascertain if use of a combination of the primer/probe sets for the detection all three genes (*psaA*, *lytA*, and *ply*) at once would be advantageous in improving sensitivity, we constructed a multiplex of the three newly developed assays. Evaluations of the LLDs with serial dilutions of known concentrations of the pneumococcal positive control strain ATCC 33400 were done, and the results were compared to those of the singleplex assay for each gene. These studies showed that the variation was less than ±1 C_T value (the cycle number at which the fluorescence value crosses the threshold) compared to the C_T values for all the singleplex PCRs. Evaluation of the *S. pseudopneumoniae* and other P-LVS bacteria yielded results similar to those of the individual assays. There were no additional positive or negative reactions.

DISCUSSION

Newer nucleic acid techniques, particularly real-time PCR, offer an opportunity to readdress the problem of the diagnosis of *S. pneumoniae* disease. In the present study we developed three new *S. pneumoniae*-specific real-time PCRs and demonstrated their abilities to detect *S. pneumoniae* DNA from cultures and three different types of clinical samples. These new assays were compared with each other and with two previously described real-time PCRs, with promising results.

The LLDs for all five assays were excellent. All demonstrated a linear detection range of 6 orders of magnitude and gave positive amplifications signals with all *S. pneumoniae* serotypes and nontypeable *S. pneumoniae* strains tested. Specificity evaluations, however, indicated differences among the assays. None of the five assays amplified any of the nonstreptococcal species tested, but in contrast, the specificity was problematic for both *ply* real-time PCRs when the amplification of *S. pseudopneumoniae* and other isolates of P-LVS was evaluated; these assays lacked sufficient specificity and amplified these *S. pneumoniae*-related strains. Several researchers have investigated and reported on atypical alpha-hemolytic streptococci and their likenesses in their phenotypic and genotypic properties to *S. pneumoniae* strains (3, 17). Arbique et al. (3) described *S. pseudopneumoniae* species and thoroughly discussed the problems posed for *S. pneumoniae* disease diagnostics due to both the detection of the *ply* and the *lytA* genes in these organisms and also the high degree of sequence similarity found in the rRNA gene which is used in the GeneProbe AccuProbe Pneumococcus identification test. The present study added five more isolates of *S. pseudopneumoniae* whose identities were confirmed by DNA-DNA reassociation to the original ones described by Arbique et al. (3), and again the rRNA gene sequence region chosen by the GenProbe investigators for their test failed to discriminate between *S. pneumoniae* and *S. pseudopneumoniae*. The detection of *ply* in several P-LVS isolates and the detection of *psaA* and *lytA* in *S. pseudopneumoniae* isolates in this study reinforce the call for caution in choosing sequence regions when these genes are used for the development of diagnostic assays. In nature, a taxonomic situation exists in which many mosaic organisms that do not clearly fit any particular species is evolving over time, and this could explain the detection of these genes in pneumococcus-like organisms (3, 12, 17). The role of P-LVS, if any, in disease has yet to be elucidated. However, a recent publication by Keith et al. (17) describing a study of patients with chronic obstructive pulmonary disease (COPD) suggests that the isolation of *S. pseudopneumoniae* from sputum specimens was associated with both a history of COPD and the exacerbation of COPD (17). The increasing ability of laboratories to identify this species will help to elucidate its prevalence and clinical relevance. It is difficult to say if the lack of the ability to discriminate between them may have affected previous clinical studies on the detection and identification of *S. pneumoniae*, but this cannot be ruled out.

This study described rapid, reliable assays that clearly discriminate *S. pseudopneumoniae* and other P-LVS from *S. pneumoniae*. The P-LVS isolates in our specificity panel were intentionally selected because of the difficulty in classifying them,

and thus, their use provides a strict criterion for assay specificity determinations. Both the *lytA*-CDC and the *psaA* real-time PCRs were highly specific, showing no amplification with P-LVS isolates. The *psaA* real-time PCR was slightly less specific, amplifying two of the *S. pseudopneumoniae* isolates. These results correlate with those of an earlier study that used conventional PCR, showing the utility of these genes in discriminating *S. pneumoniae* strains (21). Undoubtedly, the increasing incidence of isolation of P-LVS is certain to complicate diagnosis and create additional obstacles for pneumococcal assay design; therefore, their inclusion may be required in future specificity evaluations for pneumococcal assay development.

The clinical performance of these pneumococcal real-time PCR assays was assessed with a limited number of samples of three different clinical specimen types. Our studies and those of others indicate that the choice of specimen comes with its own set of problems and that many different factors affect the sensitivities of the assays. Overall, PCR of blood and blood fractions has been reported to be very unpredictable and challenging due to the presence of inhibitors in blood and the low numbers of pneumococci (10, 28, 33). In previous studies, others have shown that the rate of positivity is greater by PCR than by culture and have suggested that PCR is more sensitive (10, 28, 33). In our analysis of pneumococcal culture-positive serum specimens, the rates of positivity by the five real-time PCRs correlated well among the various assay but were lower than expected compared with the culture results. There are several possible explanations for this. These include delays in processing and storage of specimens, the drawing of blood at times different from those at which blood for culture was drawn, and the presence of low levels of inhibitors. Additionally, while viable organisms are not required for PCR amplification, the length of time after the initiation of antibiotic treatment and specimen collection may affect the reactivity of the PCR, as reported by Dagan et al. (10). Other PCR studies that have evaluated blood have cited one or more of these reasons for discordant results (28, 29). For the specimens evaluated in this study, it is possible that both the storage conditions and the inability to perform PCR at the same time that the blood culture was performed may be factors. Thus, a prospective study with serum specimens collected and stored specifically for PCR would be a better indicator of the true value of these real-time PCRs with serum specimens. Use of MEF and CSF specimens, on the other hand, proved to be 100% sensitive, possibly reflecting the lack of or the presence of lower levels of inhibitors or the ease of removal of inhibitors during extraction and the higher bacterial counts generally seen in MEF specimens than in blood.

Among the culture-negative samples of all three specimen types (serum, MEF, and CSF), some real-time PCR amplification of the samples occurred. Again, this occurred in most cases with identical samples, and there was close to 100% agreement between all five assays, with one or two specimen outliers. In cases in which this occurred, the average of the C_T value was >38, which is very close to the assay C_T limit of 40. The amplification of samples that are culture negative has been reported in previous publications and has been attributed to one or more of the following: (i) the superior sensitivity of PCR methods over that of culture, (ii) amplification of viridans group streptococcus-related sequences, (iii) detection of bacterial DNA from dead organisms, or (iv) contamination (15, 16, 27). The fact that we detected *S. pneumoniae* in MEF specimens that were culture positive only for *H. influenzae* is not surprising; other investigators have reported that up to approximately 24% of MEF samples from patients result in mixed *S. pneumoniae-H. influenzae* culture isolations (4, 9, 31). Thus, another plausible explanation for these *S. pneumoniae* culture-negative, PCR-positive MEF specimens is dual infection not revealed by culture, for even though *S. pneumoniae* was undetectable by culture, its presence and/or the presence of its DNA cannot be ruled out. Additionally, *S. pneumoniae* has previously been detected in healthy children by PCR (for the *ply* gene) of serum samples and was attributed to the carriage of *S. pneumoniae* (10), which might be doubtful, since *ply* gene PCRs have been found to be nonspecific (21). In our study, the high specificities of the *lytA* and *psaA* assays make it highly unlikely that atypical alpha-hemolytic streptococci are responsible for the amplification of these culture-negative clinical samples. In addition, our strict laboratory methods regarding PCR procedures and the fact that the same samples that were positive by one assay were positive by the other assays lend support to the true-positive results for these samples and not positive results due to contamination by PCR amplicons. Moreover, with samples from nonsterile sites such as MEF and the difficulty of pneumococcal culture, the real-time PCR positivity of culture negative specimens is not unexpected. Therefore, we suspect that amplification for these culture-negative specimens is most likely indicative of the greater sensitivity of real-time PCR or the amplification of DNA still present in specimens from patients treated with antibiotics.

Despite the limited numbers of specimens tested, there was very good agreement among the results of the assays. The results with clinical samples were good overall and very promising. LLD estimations indicate that the real-time assays are highly sensitive, detecting at least 10 copies for *ply* and *lytA*. However, a true clinical assessment must await the execution of much larger, well-defined clinical studies with different types of specimens. Our results clearly demonstrate the enhanced specificities of the *lytA*-CDC and *psaA* assays for the detection of true *S. pneumoniae* strains. Although the two *ply* assays were shown to be very sensitive, they were much less specific and their use could be problematic, especially if they were applied to the analysis of specimens from nonsterile sites. The true value of real-time PCR for the diagnosis of pneumococcal disease is not yet firmly established, and thus, no one assay is routinely used. The newly developed assays described here present the opportunity to use not only assays with high sensitivities but also assays that have improved specificities compared with those of the assays currently in use. The improvement in specificity allows their use with specimens from nonsterile sites as well as sterile sites, making them suitable for use for diagnosis and in carriage studies.

Currently, the trend is to use multiplex assays for the simultaneous detection of various pathogens, providing a savings in time and money. We have done this for a single pathogen but propose that our *lytA*-CDC or *psaA* primer/probe sets would provide high specificity in an array for respiratory pathogen detection multiplexed with primers and probes for the detec-

tion of other respiratory pathogens. This potential for multiplexing and the speed of performance make these assays promising tools for molecular detection and epidemiologic carriage studies. This technology should offer an added advantage when it is used in conjunction with other assays for pneumococcal disease diagnosis.

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REFERENCES

1. **Advisory Committee on Immunization Practices.** 2000. Preventing pneumococcal disease among infants and young children: recommendations of the Advisory Committee on Immunization Practices (ACIP). Morb. Mortal. Wkly. Rep. Recomm. Rep. **49:**1–35.
2. **Apfalter, P., B. Stoiser, W. Barousch, M. Nehr, L. Kramer, and H. Burgmann.** 2005. Community-acquired bacteria frequently detected by means of quantitative polymerase chain reaction in nosocomial early-onset ventilator-associated pneumonia. Crit. Care Med. **33:**1492–1498.
3. **Arbique, J. C., C. Poyart, P. Trieu-Cuot, G. Quesne, M. G. S. Carvalho, A. G. Steigerwalt, R. E. Morey, D. Jackson, R. J. Davidson, and R. R. Facklam.** 2004. Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov. J. Clin. Microbiol. **42:**4686–4696.
4. **Arguedas, A., R. Dagan, E. Leibovitz, A. Hoberman, M. Pichichero, and M. Paris.** 2006. A multicenter, open label, double tympanocentesis study of high dose cefdinir in children with acute otitis media at high risk of persistent or recurrent infection. Pediatr. Infect. Dis. J. **25:**211–218.
5. **Barenfanger, J., C. Drake, N. Leon, T. Mueller, and T. Trout.** 2000. Clinical and financial benefits of rapid detection of respiratory viruses: an outcomes study. J. Clin. Microbiol. **38:**2824–2828.
6. **Barzilay, E. J., K. L. O'Brien, Y. S. Kwok, R. M. Hoekstra, E. R. Zell, R. Reid, M. Santosham, C. G. Whitney, and D. R. Feikin.** 2006. Could a single dose of pneumococcal conjugate vaccine in children be effective? Modeling the optimal age of vaccination. Vaccine **24:**904–913.
7. **Brenner, D. J., A. C. McWhorter, J. K. Leete Knutson, and A. G. Steigerwalt.** 1982. *Escherichia vulneris*: a new species of *Enterobacteriaceae* associated with human wounds. J. Clin. Microbiol. **15:**1133–1140.
8. **Corless, C. E., M. Guiver, R. Borrow, V. Edwards-Jones, A. J. Fox, and E. B. Kaczmarski.** 2001. Simultaneous detection of *Neisseria meningitis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J. Clin. Microbiol. **39:**1553–1558.
9. **Dagan, R., C. E. Johnson, S. McLinn, N. Abughali, J. Feris, E. Leibovitz, D. J. Burch, and M. R. Jacobs.** 2000. Bacteriologic and clinical efficacy of amoxicillin/clavulanate vs. azithromycin in acute otitis media. Pediatr. Infect. Dis. J. **19:**95–104.
10. **Dagan, R., O. Shriker, I. Hazan, E. Leibovitz, D. Greenberg, F. Schlaeffer, and R. Levy.** 1998. Prospective study to determine clinical relevance of detection of pneumococcal DNA in sera of children by PCR. J. Clin. Microbiol. **36:**669–673.
11. **Dowell, S. F., R. L. Garman, G. Liu, O. S. Levine, and Y. H. Yang.** 2001. Evaluation of Binax NOW, an assay for detection of pneumococcal antigen in urine samples performed among pediatric patients. Clin. Infect. Dis. **32:**824–825.
12. **Dowson, C. G.** 2004. What is a pneumococcus? p. 3–14. *In* E. I. Tuomanen, T. J. Mitchell, D. A. Morrison, and B. G. Spratt (ed.), The pneumococcus. American Society for Microbiology, Washington, DC.
13. **Emery, S. L., D. D. Erdman, M. D. Bowen, B. R. Newton, J. M. Winchell, R. F. Meyer, S. Tong, B. T. Cook, B. P. Holloway, K. A. McCaustland, P. A. Rota, B. Bankamp, L. E. Lowe, T. G. Ksiazek, W. J. Bellini, and L. J. Anderson.** 2004. Real-time reverse transcription-polymerase chain reaction assay for SARS-associated coronavirus. Emerg. Infect. Dis. **10:**311–316.
14. **Facklam, R. R., and N. Pigott.** 1994. Description of phenotypic characteristics to aid in the identification of *Streptococcus pneumoniae. In* A. Totollan (ed.), Pathogenic streptococci: present and future. Lancer Publications, St. Petersburg, Russia.
15. **Greiner, O., P. J. R. Day, P. P. Bosshard, F. Imeri, M. Altwegg, and D. Nadal.** 2001. Quantitative detection of *Streptococcus pneumoniae* in nasopharyngeal secretions by real-time PCR. J. Clin. Microbiol. **39:**3129–3134.
16. **Kaijalainen, T., S. Rintamaki, E. Herva, and M. Leinonen.** 2002. Evaluation of gene-technological and conventional methods in the identification of *Streptococcus pneumoniae*. J. Microbiol. Methods **51:**111–118.
17. **Keith, E. R., R. G. Podmore, T. P. Anderson, and D. R. Murdoch.** 2006. Characteristics of *Streptococcus pseudopneumoniae* isolated from purulent sputum samples. J. Clin. Microbiol. **44:**923–927.
18. **Korppi, M., M. Koskela, E. Jalonen, and M. Leinonen.** 1992. Serologically indicated pneumococcal respiratory infection in children. Scand. J. Infect. Dis. **24:**437.
19. **Llull, D., R. Lopez, and E. Garcia.** 2006. Characteristic signatures of the *lytA* gene provide a basis for rapid and reliable diagnosis of *Streptococcus pneumoniae* infections. J. Clin. Microbiol. **44:**1250–1256.
20. **McAvin, J. C., P. A. Reilly, R. M. Roudabush, W. J. Barnes, A. Salmen, G. W. Jackson, K. K. Beninga, A. Astorga, F. K. McCleskey, W. B. Huff, D. Niemeyer, and K. L. Lohman.** 2001. Sensitive and specific method for rapid identification of *Streptococcus pneumoniae* using real-time fluorescence PCR. J. Clin. Microbiol. **39:**3446–3451.
21. **Messmer, T. O., J. S. Sampson, A. Stinson, B. Wong, G. M. Carlone, and R. R. Facklam.** 2004. Comparison of four polymerase chain reaction assays for specificity in the identification of *Streptococcus pneumoniae*. Diagn. Microbiol. Infect. Dis. **49:**249–254.
22. **Murdoch, D. R.** 2005. Impact of rapid microbiological testing on the management of lower respiratory tract infection. Clin. Infect. Dis. **41:**1445–1447.
23. **Murdoch, D. R., R. T. R. Laing, G. D. Mills, N. C. Karalus, G. I. Town, S. Mirrett, and L. B. Reller.** 2001. Evaluation of a rapid immunochromatographic test for detection of *Streptococcus pneumoniae* antigen in urine samples from adults with community-acquired pneumonia. J. Clin. Microbiol. **39:**3495–3498.
24. **Norrby, S. R., and K. A. Pope.** 1979. Pneumococcal pneumonia. J. Infect. **1:**109–120.
25. **Ruoff, K., L. R. A. Whiley, and D. Beighton.** 2003. *Streptococcus*, p. 405–421. *In* P. R. Murray, E. J. Barron, J. H. Jorgensen, M. A. Pfaller, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, DC.
26. **Saukkoriipi, A., K. Leskela, E. Herva, and M. Leinonen.** 2004. *Streptococcus pneumoniae* in nasopharyngeal secretions of healthy children: comparison of real-time PCR and culture from STGG-transport medium. Mol. Cell. Probes **18:**147–153.
27. **Saukkoriipi, A., A. Palmu, T. Kilpi, and M. Leinonen.** 2002. Real-time quantitative PCR for the detection of *Streptococcus pneumoniae* in the middle ear fluid of children with acute otitis media. Mol. Cell. Probes **16:**385–390.
28. **Sheppard, C. L., T. G. Harrison, R. Morris, A. Hogan, and R. C. George.** 2004. Autolysin-targeted LightCycler assay including internal process control for detection of *Streptococcus pneumoniae* DNA in clinical samples. J. Med. Microbiol. **53:**189–195.
29. **Skerrett, S. J.** 1999. Diagnostic testing for community-acquired pneumonia. Clin. Chest Med. **20:**531–548.
30. **Sorensen, U. B. S., and J. Heinrichsen.** 1987. Cross-reactions between pneumococci and other streptococci due to C polysaccharide and F antigen. J. Clin. Microbiol. **25:**1854–1859.
31. **Soriano, F., A. Parra, C. Cenjor, E. Nieto, G. Garcia-Calvo, M. J. Gimenez, L. Aguilar, and C. Ponte.** 2000. Role of *Streptococcus pneumoniae* and *Haemophilus influenzae* in the development of acute otitis media and otitis media with effusion in a gerbil model. J. Infect. Dis. **181:**646–652.
32. **van der Hoek, L., K. Pyrc, M. Jebbink, W. Vermeulen-Oost, R. Berkout, K. Wolthers, P. Wertheim-van Dillen, J. Kaandorp, J. Spaargaren, and B. Berkhout.** 2004. Identification of a new human coronavirus. Nat. Med. **10:**368–373.
33. **van Haeften, R., S. Palladino, I. Kay, T. Keil, C. Health, and G. W. Waterer.** 2003. A quantitative LightCycler PCR to detect *Streptococcus pneumoniae* in blood and CSF. Diagn. Microbiol. Infect. Dis. **47:**407–414.