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Accuracy of Phenotypic Methods for Identification of *Streptococcus pneumoniae* Isolates Included in Surveillance Programs[▽]

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Similarities between *Streptococcus pneumoniae* and viridans group streptococci may result in misidentification of these organisms. In surveillance programs which assess antimicrobial resistance rates among respiratory tract pathogens, such identification errors could lead to overestimates of pneumococcal resistance rates. DNA probe analysis (Gen-Probe, San Diego, CA), the bile solubility test, optochin susceptibility, colony morphology, and the capsular swelling reaction with Omni serum (Staten Serum Institut, Copenhagen, Denmark) were used to characterize 1,733 organisms provisionally identified as *S. pneumoniae* in a 2004 to 2005 antimicrobial resistance surveillance program. These organisms were obtained in 41 U.S. medical centers. Among these, 1,647 (95%) were determined to be *S. pneumoniae* by DNA probe. Elimination of those isolates found not to be *S. pneumoniae* resulted in 1 to 2% decreases in resistance rate estimates with penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole. With AccuProbe as a reference standard, the sensitivities and specificities of each phenotypic method for the identification of *S. pneumoniae* were, respectively, 98.8% and 82.6% for bile solubility, 99.3% and 74.4% for the capsular swelling reaction with Omni serum, and 87.9% and 59.3% for optochin susceptibility. Colony morphology was of limited value, as 391 (23.7%) isolates lacked the typical button or mucoid colony appearance of *S. pneumoniae*.

The accurate distinction of *Streptococcus pneumoniae*, an important cause of pneumonia, meningitis, otitis media, and sinusitis, from relatively avirulent closely related streptococcal species can be difficult (8). Although DNA homology studies provide the most definitive tool for discriminating among the 26 species of viridans group streptococci, this technique is not readily available or practical outside of research settings (5, 22). The utility of 16S rRNA gene analysis is limited for pneumococci due to the >99% 16S rRNA gene sequence similarity between *S. pneumoniae*, *Streptococcus mitis*, and *Streptococcus. oralis* (4, 12). Interspecies recombination allowing transfer of virulence genes (*lytA* and *ply*) to nonpneumococcal species minimizes the usefulness of these genes as a sole method for identification (11, 18, 26). Genomic subtractive hybridization experiments designed to find target genes that could discriminate among *S. pneumoniae*, *S. mitis*, and *S. oralis* succeeded with 2 (spn9802 and spn9828) of 19 designed primer sets (23). A limited study utilizing a four-gene (*lytA*, *ply*, spn9802, and spn9828) PCR for molecular identification of *S. pneumoniae* needs further investigation (24).

Previously published evaluations of identification methods for pneumococci included no genotypic method (14) or relatively small numbers of isolates with results for a genotypic method (2, 11, 17). The AccuProbe DNA probe is the only commercially available molecular method for identification of isolates of pneumococci. Apart from problems due to cross-reactivity with the new species *Streptococcus pseudopneumoniae* reported in 2004 (2), AccuProbe has been considered a reliable identification method (7, 17, 26). Due to reagent and labor cost most clinical labs continue to rely on phenotypic tests such as bile solubility and optochin susceptibility to distinguish *S. pneumoniae* from other alpha-hemolytic streptococci.

It has been suggested that the misidentification of viridans group streptococci as pneumococci in surveillance programs could cause an overestimate of antimicrobial resistance (16, 25). Using AccuProbe as the reference standard, we assessed the accuracy of phenotypic methods (colony morphology, bile solubility, optochin susceptibility, serotyping) to confirm the identity of a large collection of pneumococcal isolates collected in a recent surveillance study and determined the impact of misidentification on estimated resistance rates with *S. pneumoniae*.

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MATERIALS AND METHODS

As part of a longitudinal antimicrobial resistance surveillance program, unique clinical isolates of *S. pneumoniae* were collected from 41 medical centers throughout the United States from 1 November 2004 through 30 April 2005. Fifty consecutive pneumococcal isolates considered by the submitting laboratory to be clinically significant were requested from each center. After receipt of each isolate at the central reference laboratory, subcultures were made and stored on beads at −70°C. Bile solubility, optochin susceptibility, assessment of colony morphology, quellung reactivity, and DNA probe analysis were performed on fresh subcultures of 1,733 isolates provisionally identified as *S. pneumoniae* by participating centers.

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TABLE 1. Identification test results for 1,733 isolates provisionally identified as *S. pneumoniae* obtained from 2004 to 2005

Test	Result	No. of isolates (n = 1,733)	No. of isolates (%) with AccuProbe result of:	
			Positive (n = 1,647)	Negative (n = 86)
Bile solubility	Positive	1,642	1,627 (98.8)	15 (17.4)
	Negative	91	20 (1.2)	71 (82.6)
Optochin resistance	Susceptible (>14 mm)	1,482	1,447 (87.9)	35 (40.7)
	Resistant (<14mm)	251	200 (12.1)	51 (59.3)
Omni serum	Positive	1,657	1,635 (99.3)	22 (25.6)
	Negative	76	12 (0.7)	64 (74.4)
Morphology	Button colony	1,150	1,145 (69.5)	5 (5.8)
	Mucoid	192	192 (11.7)	
	Other	391	310 (18.8)	81 (94.2)

Bile solubility plate test. A drop of 3% sodium deoxycholate was placed directly on well-isolated 18- to 24-h colonies on sheep blood agar. The plate was incubated in ambient air at 35 to 37°C for 30 min. Disappearance or flattening of colonies in contact with bile was considered a positive test (15). Bile solubility testing was repeated using 2% sodium deoxycholate for isolates with AccuProbe-positive/bile solubility-negative results.

Optochin susceptibility test. A sheep blood agar plate was inoculated to obtain confluent growth. After placement of a 5-μg optochin disk (Remel Laboratories, Lenexa. KS), the plate was incubated at 35 to 37°C in 5% CO_2 for 18 to 24 h. A zone of inhibition of ≥14 mm was considered indicative of optochin susceptibility (27). A zone of inhibition of <14 mm was considered indicative of optochin resistance. The optochin susceptibility test was repeated with incubation in ambient air for isolates with AccuProbe-positive/bile solubility-negative results.

Colony morphology. Colonies were examined for the presence of a central depression (button) or mucoid appearance typically associated with *S. pneumoniae* (21). Colonies without a button or mucoid appearance were categorized as "other."

Serotyping. Omni serum (Statens Serum Institut, Copenhagen, Denmark) includes antibodies to all recognized *S. pneumoniae* serotypes. A suspension of the test organism was prepared in saline from well-isolated colonies grown on sheep blood agar plates for 18 to 24 h in 5 to 7% CO_2 at 35°C. One drop of this suspension was mixed with 1 drop of antiserum and 1 drop of methylene blue and, after incubation at room temperature for 10 min, examined at 1,000× magnification. Visible evidence of capsular swelling with Omni serum, i.e., a positive quellung reaction, was considered a positive test. Complete serotyping with specific antisera was performed on a subset of isolates with discordant identification test results.

DNA probe analysis. The AccuProbe *Streptococcus pneumoniae* culture identification test (Gen-Probe, San Diego, CA) was performed according to the manufacturer's instructions using well-isolated 18- to 24-h colonies from 5% sheep blood agar. The combining of the DNA probe with target organism rRNA to form a labeled DNA-RNA hybrid was indicated by a luminometer reading greater than or equal to the cutoff value of 50,000 relative light units. Isolates with a luminometer reading of <50,000 relative light units were considered AccuProbe negative.

RESULTS

Among 1,733 isolates submitted by referring laboratories as *S. pneumoniae*, 1,647 (95%) yielded positive results with the DNA probe. The percentages of AccuProbe-positive isolates identified as pneumococci by each phenotypic method were 98.8% for bile solubility, 99.3% for Omni serum, and 87.9% for optochin susceptibility (Table 1). Every mucoid colony and all except 5 of the 1,150 isolates with button morphology were AccuProbe positive. There were 310 isolates without a button or mucoid appearance that were AccuProbe positive.

With AccuProbe as the gold standard, the sensitivity and specificity of bile solubility testing were 98.8% and 82.6%, respectively. Optochin susceptibility, morphology, and Omni serum results for the 15 bile solubility-positive/AccuProbe-negative isolates are shown in Table 2. Thirteen of these 15 isolates were also optochin susceptible.

Optochin testing was repeated with incubation in ambient air for the 20 isolates with negative results by the bile solubility test and positive AccuProbe results (Table 3). Eleven of the 20 isolates were optochin susceptible when the test was performed in ambient air and optochin resistant when it was performed in CO_2, a characteristic consistent with *S. pseudopneumoniae* (2). Bile solubility testing with 2% sodium deoxycholate was repeated on the 20 isolates shown in Table 3, and results reverted to positive for three isolates. Serotyping performed on these isolates confirmed the positive AccuProbe results for the last five isolates in Table 3 (serotypes 19A, 21, 35A [two isolates], and 35F).

Optochin disk testing did not perform as well as bile solubility testing, with lower sensitivity (87.9%) and specificity (59.3%), using AccuProbe as the reference method. Only 18 of the 200 isolates with presumed false-negative optochin test results (i.e., negative optochin result, positive AccuProbe result) were bile solubility negative, and only 10 of these 200 isolates were Omni serum negative. Thirteen of the 35 op-

TABLE 2. Test results obtained with 15 isolates that were bile solubility positive but negative with the AccuProbe test

Isolate	Result by:		
	Optochin susceptibility	Omni serum	Morphology[a]
172	Resistant	Negative	Other
235	Susceptible	Negative	Button
356	Susceptible	Negative	Other
397	Susceptible	Negative	Other
419	Susceptible	Positive	Other
447	Susceptible	Negative	Other
725	Susceptible	Positive	Other
751	Susceptible	Negative	Other
1198	Susceptible	Negative	Other
1220	Susceptible	Negative	Button
1255	Susceptible	Positive	Other
1455	Susceptible	Positive	Other
1553	Susceptible	Negative	Other
1561	Resistant	Negative	Other
1596	Susceptible	Positive	Other

[a] Button morphology refers to central depression of colonies; "other" denotes alpha-hemolytic colonies without button or mucoid morphology.

TABLE 3. Test results obtained with 20 isolates that were bile solubility negative but had positive AccuProbe results

Isolate	Result by:				
	Optochin susceptibility		Omni serum	Serotyping	Morphology[a]
	CO_2	Air			
12[b]	Susceptible	No growth	Positive		Button
158[b]	Susceptible	Susceptible	Positive		Other
423	Resistant	Susceptible	Negative		Other
504	Resistant	Susceptible	Positive		Other
779	Resistant	Susceptible	Positive		Other
1004	Resistant	Susceptible	Positive		Other
1019	Resistant	Susceptible	Positive		Other
1065	Resistant	Susceptible	Positive		Other
1070	Resistant	Susceptible	Negative		Other
1088	Resistant	Susceptible	Negative		Other
1103	Resistant	Susceptible	Negative		Other
1134	Resistant	Susceptible	Positive		Other
1324	Resistant	Susceptible	Negative		Other
407	Resistant	Resistant	Positive		Button
1124	Resistant	Resistant	Negative		Other
1329	Resistant	Resistant	Positive	35F	Other
1584	Resistant	Resistant	Positive	21	Other
1652	Resistant	Resistant	Negative	19A	Button
1676	Resistant	Resistant	Negative	35A	Other
1704[b]	Resistant	No growth	Negative	35A	Other

[a] Button morphology refers to central depression of colonies; "other" denotes alpha-hemolytic colonies without button or mucoid morphology.
[b] Repeat bile solubility testing of this isolate with 2% sodium deoxycholate was positive.

tochin-susceptible, AccuProbe-negative isolates had positive bile solubility results. The optochin result concurred with the bile solubility result for most isolates (87 to 90%), with discordant results between the bile solubility and probe tests (Tables 2 and 3).

Compared to AccuProbe, Omni serum testing had the highest sensitivity (99.3%) but lower specificity (74.4%) than bile solubility testing. Three of the 12 Omni serum-negative, AccuProbe-positive isolates were typeable when tested against specific antisera (serotypes 19A and 35A [two isolates]). The remaining nine isolates may represent strains of *S. pneumoniae* that lack a capsular antigen. None of the 22 Omni serum-positive, AccuProbe-negative isolates were typeable with specific antisera.

The impact on estimates of resistance of false identification of *S. pneumoniae* in our surveillance program was determined by comparing the percentages of strains determined to be susceptible to penicillin, erythromycin, tetracycline, and trimethoprim-sulfamethoxazole (TMP-SMX) both before (n = 1,733) and after (n = 1,647) arbitration of submitted isolates with the AccuProbe test. The percentages of susceptible isolates changed from 65.3 to 67.5% with penicillin, 68.6 to 70.9% with erythromycin, 82.5 to 84.1% with tetracycline, and 72.2 to 73.2% with TMP-SMX.

DISCUSSION

This study provides insight into the performance characteristics of various phenotypic methods for identifying *S. pneumoniae.* The isolates examined in this study had previously been identified as pneumococci by laboratories participating in a large, multicenter, longitudinal surveillance program of antimicrobial resistance among respiratory tract pathogens.

The reported accuracy of AccuProbe in comparison to bile solubility, optochin susceptibility, and the quellung reaction for discriminating between *S. pneumoniae* and nonpneumococcal isolates has been excellent (7, 17). The manufacturer's package insert states that AccuProbe detected "specific rRNA sequences that are unique to *S. pneumoniae*" with a sensitivity and specificity of 100% in a two-center evaluation of 662 isolates (305 pneumococci, 185 other *Streptococcus* spp., and 172 nonstreptococcal isolates from 25 genera). The sequences targeted by the AccuProbe assay have not been published. A 1992 study evaluating 172 pneumococci and 204 other isolates also reported 100% sensitivity and specificity for AccuProbe (7). More recently, Mundy and colleagues reported AccuProbe's detection of 74 pneumococcal and 56 nonpneumococcal isolates with 100% accuracy (17). Finally, Carvalho et al. confirmed the identity of 11 AccuProbe-positive nontypeable isolates causing conjunctivitis as *S. pneumoniae* with DNA-DNA reassociation studies (5).

One concern in the current study, given that we utilized the AccuProbe assay as our reference standard method, was the potential for at least small numbers of erroneous results with AccuProbe, given the high degree of similarity of *S. pneumoniae* 16S rRNA (>99%) to that of other viridans group streptococci (*S. mitis* and *S. oralis*) (4, 12). In 2004, Arbique et al. described a new species of viridans streptococci, *S. pseudopneumoniae*, that AccuProbe could not discriminate from *S. pneumoniae* (2). A subsequent investigation demonstrated the presence of the *ply* gene in clinical isolates of *S. pseudopneumoniae* and pathogenic potential using a mouse peritonitis/sepsis model (9). Keith et al. reported the prevalence of *S. pseudopneumoniae* among alpha-hemolytic streptococci from sputum as 4% and noted that all isolates (n = 35) were from patients with respiratory symptoms (79% had chronic obstructive pulmonary disease, 33% had pulmonary infiltrates) (13).

The phenotypic reactions of 11 AccuProbe-positive isolates in our study that were bile solubility negative, nonreactive with capsular antisera, and optochin susceptible only when incubated in ambient air are consistent with an identification of *S. pseudopneumoniae* (Table 3) (2, 13). Susceptibility testing with the CLSI broth microdilution method (6) was performed on these 11 isolates and revealed 5 isolates (45%) with intermediate susceptibility to penicillin (MIC, 0.25 to 1 μg/ml), 7 isolates (64%) with resistance to erythromycin (MIC, 2 to >128 μg/ml), and 8 isolates (73%) with resistance to tetracycline (MIC, 16 to 32 μg/ml). These results are similar to the susceptibility profiles previously reported for 35 isolates of *S. pseudopneumoniae* collected from 2001 to 2004 (13).

Of the methods used, bile solubility had the highest agreement with AccuProbe (98.8% sensitivity, 82.5% specificity). If the 11 bile solubility-negative isolates that were thought to be *S. pseudopneumoniae* are excluded, the sensitivity of bile solubility increases from 98.8% to 99.4% (1,627/1,636) (Table 3). Three of the other false-negative bile solubility results turned positive with repeat testing, but four were confirmed as false negative by positive serotyping. That leaves two nontypeable bile-negative, AccuProbe-positive isolates that could represent failure of the bile solubility test to detect pneumococci or another viridans group streptococcal species that yields false-positive AccuProbe results similar to those obtained with *S. pseudopneumoniae*.

The high percentage of optochin-resistant isolates (79.7%) that were probe positive supports the recommendation to perform additional testing (i.e., bile solubility or AccuProbe) on these strains (1, 20). Pikis et al. reported four optochin-resistant pneumococcal isolates and raised the possibility that quinine exposure could be a factor in the emergence of optochin resistance (20). Studies from Portugal found that 3.2% of pneumococcal isolates causing disease collected in 2005 (1) and 2% of *S. pneumoniae* isolates colonizing children obtained from 2001 to 2006 were optochin resistant (19). Although our prevalence of optochin resistance among AccuProbe-positive isolates would decrease from 12.1% to 11.6% if the *S. pseudopneumoniae* isolates were excluded, both values are higher than those in prior reports.

Optochin-susceptible nonpneumococcal isolates with negative AccuProbe results have also been reported previously (3). We noted 35 false-positive optochin results.

Some of the errors observed with Omni serum can be explained by the inherent subjectivity of interpreting a quellung reaction as positive or negative. Sensitivity issues arise from Omni serum-negative results for acapsular strains. Omni serum has been shown to react with nonpneumococcal streptococci (10). The Omni serum-positive results for 6 of the 11 isolates that we considered *S. pseudopneumoniae* should be considered false-positive results despite agreement with the AccuProbe result.

In conclusion, in comparison to the DNA probe, bile solubility and Omni serum reactivity were found to be reliable single methods for the identification of *S. pneumoniae*. Optochin testing and assessment of colony morphology, used alone, resulted in unacceptably high rates of misidentification. Confirmation of organisms identified as *S. pneumoniae* in surveillance programs is important. Approximately 5% of the isolates characterized in our study were found not to be pneumococci, and these would have led to overestimates of resistance rates with penicillin, erythromycin, tetracycline, and TMP-SMX of 1 to 2%.

Finally, the results of this study may be relevant to routine clinical laboratory practice, as many laboratories rely only on optochin testing for identifying clinical isolates of *S. pneumoniae*. Misidentification of this pathogen can have important clinical ramifications.

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