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Comparison of Five Genotypic Techniques for Identification of Optochin-Resistant Pneumococcus-Like Isolates

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Three PCR techniques (amplification of the *psaA*, *ply*, and *lytA* genes) and a commercial kit (AccuProbe [GenProbe, San Diego, Calif.], based on hybridization with the 16S rRNA gene), all four of which claimed to be specific for *Streptococcus pneumoniae*, were used to identify 49 alpha-hemolytic streptococcal isolates suspected of being pneumococci. The definite phenotypic identification of these organisms as *S. pneumoniae* was difficult when optochin susceptibility and the presence of a capsule were taken as markers. Furthermore, *Rsa*I digestion of the amplified 16S rRNA gene was applied. All 49 strains were optochin resistant. Eleven of these were encapsulated and were identified as pneumococci by all tests. Twenty of the 38 unencapsulated strains were unambiguously identified as nonpneumococci by all tests. The identities of another 18 unencapsulated strains remained inconclusive due to highly variable reactions for all phenotypic and genotypic techniques applied. The AccuProbe test was positive for seven strains for which the results of the other tests were inconclusive. *Rsa*I restriction of the amplified 16S rRNA gene confirmed the AccuProbe result for all strains, while the result of the *psaA*-specific PCR was in concordance with encapsulation for all strains. The results presented here indicate that identification problems continue to exist for some strains, despite the application of genotypic and phenotypic tests in combination. We found the *psaA*-specific PCR to be the genotypic technique best suited for the identification of genuine pneumococci and optochin-resistant pneumococci.

Optochin susceptibility and encapsulation are the phenotypic characteristics that are the most frequently used to differentiate between *Streptococcus pneumoniae* and other streptococci (22). However, optochin-resistant *S. pneumoniae* strains are being isolated more frequently (2, 29) and are probably largely overlooked, since in many laboratories primary isolation of pneumococci on culture medium relies on optochin susceptibility itself. The occasional occurrence of encapsulated *S. mitis* and *S. oralis* strains and the fact that nontypeable, unencapsulated pneumococci have been reported to comprise 2% of the isolates from normally sterile sites (3) and up to 20% of the conjunctival isolates (12) further complicate the identification of pneumococci. Commercial systems like the API 20S and Vitek2 systems occasionally fail to identify pneumococcal isolates or identify other streptococci as pneumococci (4). Thus, even though the phenotypic identification of typical pneumococci is unambiguous, the existence of optochin-resistant isolates may increasingly cause problems in clinical bacteriological laboratories.

In the last decade, new gene amplification methods, based on the detection of pneumococcal virulence factors, have been developed to identify pneumococcal strains (18, 23, 24, 34) and to detect pneumococci directly from clinical samples (8, 13, 14, 19, 31–33, 35, 36). Hybridization methods (10, 30) have also been used for the identification of pneumococci. A commercial hybridization method (AccuProbe; GenProbe, San Diego, Calif.) that is based on the complementarity of a DNA probe with the 16S rRNA of *S. pneumoniae* is increasingly being used (6) in clinical laboratories.

Our aim was to evaluate recently described genotypic methods for their abilities to identify pneumococci. We tested the applicabilities of the commercial AccuProbe test and of enzymatic amplification of three different genes, those encoding pneumolysin (*ply*) (31), pneumococcal surface antigen A (*psaA*) (24), and autolysin (*lytA*) (23), for the identification of 49 optochin-resistant pneumococcus-like strains that were sent to the Reference Laboratory for Pneumococcus (KTL), National Public Health Institute, Oulu, Finland, during the last few years. In addition, *Rsa*I restriction digestion of the amplified 16S rRNA gene (rDNA) (amplified rDNA restriction analysis [ARDRA]) was carried out (5, 16).

MATERIALS AND METHODS

Bacterial strains. The following reference strains were used: *S. pneumoniae* LMG 14545^T, LMG 15155, LMG 16738, GUH S91 01273, GUH H91 04493, GUH 93 08 1310, GUH 93 0 3230, GUH 93 09 1111, GUH 98 10 1630, GUH 98 10 3326, and GUH 98 10 3367; *S. mitis* LMG 14557^T, LMG 14553, LMG 14552, GUH 94 03 0728, GUH 94 04 0401, GUH 97 03 2943, GUH 98 05 5898, GUH 98 07 1207, GUH 98 09 0066, KTL 101, KTL 102, and KTL 103; *S. oralis* LMG 14532^T, LMG 14533, LMG 14534, GUH 94 08 5574, GUH 98 05 5050, and GUH 98 10 1512; and *S. sanguinis* LMG 14656 and LMG 14657. Table 1 lists the 49 optochin-resistant alpha-hemolytic streptococcal isolates included in this study. These strains were originally identified as pneumococci or suspected of being pneumococci on the basis of the results of the optochin susceptibility test and evaluation of their colony morphologies, performed in routine clinical laboratories. They were sent to the reference laboratory (KTL) for identification and serotyping because they were isolated from blood or cerebrospinal fluid or because they were not fully susceptible to penicillin. They were included in this study because they were found to be optochin resistant in the reference laboratory.

Phenotypic assays. Optochin resistance testing, the tube bile solubility test, and the capsular swelling test (the Quellung reaction) were performed as described by Kaijalainen et al. (17). Briefly, after four commercially available tests

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TABLE 1. Phenotypic and genotypic identification results for 49 optochin-resistant alpha-hemolytic pneumococcus-like *Streptococcus* spp.

Group and study isolate no.	Original isolate no.	AccuProbe result	Capsule formation	PCR result			ARDRA pattern[a]	Bile solubility
				psaA	*lytA*	*ply*		
Group I								
STR119	KTL 004	+	+	+	+	+	p	+
STR120	KTL 005	+	+	+	+	+	ND	+
STR125	KTL 013	+	+	+	+	+	p	+
STR127	KTL 017	+	+	+	+	+	ND	+
STR141	KTL 043	+	+	+	+	+	p	+
STR144	KTL 051	+	+	+	+	+	ND	+
STR147	KTL 056	+	+	+	+	+	ND	+
STR149	KTL 063	+	+	+	+	+	p	+
STR155a	KTL 076	+	+	+	+	+	ND	+
STR163	KTL 091	+	+	+	+	+	ND	+
STR164	KTL 093	+	+	+	+	+	ND	+
Group IIa								
STR157	KTL 079	+	−	−	w[b]	+	p	−
STR148b	KTL 062b	+	−	−	w	+	p	−
STR150	KTL 065	+	−	−	w	+	p	−
STR162	KTL 089	+	−	−	−	+	p	−
STR165	KTL 096	+	−	−	w	+	p	+
STR142b	KTL 046b	+	−	−	−	+	p	+
STR146b	KTL 055b	+	w[c]	−	−	−	p	−
Group IIb								
STR122	KTL 007	−	−	−	−	+	m	−
STR133	KTL 028	−	−	−	−	+	ND	−
STR126b	KTL 016b	−	−	−	w	+	m	−
STR129	KTL 020	−	−	−	w	+	m	−
STR130	KTL 021	−	−	−	−	+	ND	+
STR137	KTL 035	−	−	−	w	+	m	+
STR152	KTL 069	−	−	−	w	+	m	+
STR153	KTL 072	−	−	−	−	+	m	+
STR166b	KTL 099b	−	−	−	−	+	m	+
STR131	KTL 022	−	−	−	w	−	m	+
STR151	KTL 068	−	−	−	−	−	ND	+
Group IIc								
STR118	KTL 003	−	−	−	−	−	ND	−
STR121	KTL 006	−	−	−	−	−	ND	−
STR136	KTL 034	−	−	−	−	−	ND	−
STR138	KTL 038	−	−	−	−	−	ND	−
STR145	KTL 054	−	−	−	−	−	ND	−
STR154	KTL 073	−	−	−	−	−	ND	−
STR123	KTL 008	−	−	−	−	−	ND	−
STR128	KTL 019	−	−	−	−	−	m	−
STR132	KTL 023	−	−	−	−	−	ND	−
STR134	KTL 029	−	−	−	−	−	ND	−
STR139	KTL 039	−	−	−	−	−	m	−
STR158	KTL 081	−	−	−	−	−	ND	−
STR159	KTL 083	−	−	−	−	−	ND	−
STR156	KTL 077	−	−	−	−	−	m	−
STR117	KTL 002	−	−	−	−	−	ND	−
STR140	KTL 041	−	−	−	−	−	ND	−
STR160	KTL 085	−	−	−	−	−	ND	−
STR161	KTL 087	−	−	−	−	−	ND	−
STR135	KTL 030	−	w	−	−	−	m	−
STR143	KTL 050	−	w	−	−	−	m	−

[a] ARDRA, PCR-based amplification of the 16S rRNA-gene followed by *Rsa*I digestion, agarose electrophoresis, and ethidium bromide staining. m, *S. mitis*-specific pattern, p, *S. pneumoniae*-specific pattern, ND, not determined.
[b] Weak band after agarose gel electrophoresis and ethidium bromide staining.
[c] Weak capsule reaction, with the strain considered unencapsulated.

for optochin susceptibility were compared, it was decided to use the result obtained with the Biodisk (Biodisk PDM Diagnostic Disks, Solna, Sweden) as the result of record. Bile solubility was tested by the method described by Facklam and Washington (9). The Quellung reaction test was performed as described by Austrian (1).

AccuProbe test. DNA hybridization with *S. pneumoniae*-specific 16S rRNA sequences was performed by using the AccuProbe *S. pneumoniae* culture identification test (GenProbe) according to the instructions of the manufacturer (17).

PCR assays. Amplification of the pneumolysin (*ply*) gene fragment was performed as described by Rintamäki et al. (31). Briefly, one pneumococcal colony

was suspended in 100 μl of the PCR mixture, and primers WO506 (5′-biotin-C CCACTCTTCTTGCGGTTGA) and WO507 (5′-TGAGCCGTTATTTTTTCA TACTG) amplified a 209-bp region of the pneumolysin gene.

Amplification was done with the following PCR program: predenaturation for 10 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C; and postextension for 6 min at 72°C. The amplification products were identified by agarose gel electrophoresis with ethidium bromide staining and were confirmed by solution hybridization with a europium-labeled probe.

For amplification of the pneumococcal surface antigen A (*psaA*) gene and the autolysin (*lytA*) gene, DNA was extracted by alkaline lysis, which was carried out by suspending one colony in 20 μl of 0.25% sodium dodecyl sulfate–0.05 N NaOH and heating of the mixture at 95°C for 15 min, followed by a final dilution with 180 μl of distilled water.

Part of the *psaA* gene (838 bp) was amplified with 1.6 μM each primers psaA1 (5′-CTT TCT GCA ATC ATT CTT G) and psaA2 (5′-GCC TTC TTT ACC TTG TTC TGC), designed by Morrison et al. (24). Amplification was performed in a reaction mixture of 10 μl containing 5 μl of PCR Master Mix (Promega Benelux, Leiden, The Netherlands) and 1 μl of the DNA extract in a GeneAmp PCR System 9600 instrument (Applied Biosystems, Foster City, Calif.) by using the following cycling parameters: 95°C for 5 min, followed by 30 cycles of 30 s at 94°C, 30 s at 52°C, and 2 min at 72°C, with a final extension for 8 min at 72°C.

One micromolar each of primers lytAF (5′-ACG CAA TCT AGC AGA TGA AGC) and lytAR (5′-TGT TTG GTT GGT TAT TCG TGC), designed by McAvin et al. (23), were used to amplify a 101-bp fragment of the *lytA* gene. The cycling parameters for the *lytA* gene were 95°C for 5 min, followed by 35 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C, with a final extension at 72°C for 8 min.

The presence of *psaA* and *lytA* amplification products was checked by electrophoresis on 2% agarose gels stained with ethidium bromide.

ARDRA. ARDRA, i.e., amplification of the 16S rRNA gene and subsequent restriction digestion with *Rsa*I followed by agarose electrophoresis, was carried out as described previously (5, 16).

RESULTS

A set of three phenotypic and five genotypic identification techniques was carried out with a total of 78 *Streptococcus mitis* group strains, comprising 12 *S. mitis*, 6 *S. oralis*, and 11 encapsulated optochin-sensitive *S. pneumoniae* strains, and with 49 optochin-resistant streptococci of uncertain identities. A complete list of the optochin-resistant strains and their reactions are given in Table 1.

The phenotypic identification techniques used were optochin susceptibility, bile solubility, and encapsulation. The genotypic identification methods used were amplification of the pneumolysin gene (*ply*), the autolysin gene (*lytA*), and the pneumococcal surface antigen A gene (*psaA*); hybridization with 16S rRNA (the AccuProbe test); and *Rsa*I restriction of the amplified 16S rRNA gene (ARDRA).

For the group of phenotypically well-identified *S. mitis*, *S. oralis*, and *S. pneumoniae* strains, the results of all phenotypic and genotypic techniques were in agreement with published data. *S. mitis* and *S. oralis* were optochin resistant, unencapsulated, bile insoluble, and negative by the AccuProbe test; gave no amplification with *ply*-, *lytA*-, and *psaA*-specific primers; and revealed the mitis group-specific *Rsa*I ARDRA pattern (restriction fragments of 114, 146, 262, 355, and 625 bp). The *S. pneumoniae* strains, however, had opposite characteristics and a different *Rsa*I restriction pattern, composed of fragments of 114, 146, 335, and 887 bp. The latter pattern was also observed for the two *S. sanguinis* isolates tested.

For a better understanding, the 49 optochin-resistant pneumococcus-like strains were split into a group consisting of the 11 encapsulated strains (group I) and a group consisting of the remaining 38 strains, of which 35 were nonencapsulated and 3 had a weak Quellung reaction (group II). The group II strains were further subdivided into groups IIa, IIb, and IIc, according to their reactions by the different genotypic tests.

For the group I isolates (optochin resistant and encapsulated), the results obtained by all other techniques were positive, pointing to an unequivocal identification as *S. pneumoniae*.

The seven optochin-resistant, nonencapsulated isolates of group IIa were positive by the AccuProbe test and had an ARDRA pattern typical for *S. pneumoniae* (ARDRA positive), although five isolates were negative by all three phenotypic assays and two were only bile soluble. Six of the seven strains were also positive for *ply*, and four of seven of the isolates had a weakly positive *lytA* amplification signal.

The 11 optochin-resistant, nonencapsulated isolates in group IIb were positive only by the *ply*-specific PCR and/or bile positive. Four isolates also showed a weak positive reaction by the *lytA*-specific PCR.

For the 20 optochin-resistant, nonencapsulated isolates of group IIc, all techniques pointed to an identification as nonpneumococci.

In conclusion, all optochin-resistant and encapsulated isolates (group I) were identified as pneumococci by all other phenotypic and genotypic techniques. Twenty of the 38 optochin-resistant, unencapsulated isolates (group IIc) were identified as nonpneumococci by all techniques, but 18 of the 38 optochin-resistant, nonencapsulated isolates (groups IIa and IIb) could not be unequivocally identified, due to the contradicting results of the phenotypic and genotypic tests.

The results of *Rsa*I restriction of the amplified 16S rDNA were always in agreement with the results obtained with the AccuProbe system, and the *psaA*-specific PCR result was always in agreement with encapsulation.

DISCUSSION

Susceptibility to optochin is a cornerstone in the phenotypic identification of pneumococci and is often used in laboratories as the primary and sometimes the only identification method (21). The occurrence of optochin-resistant *S. pneumoniae* strains, which can result from mutations in the H^+-ATPase (11), is not uncommon (2, 10, 21, 25, 26, 28), jeopardizing the detection and correct identification of pneumococci. Other problems with optochin susceptibility testing may be caused by alpha-hemolytic streptococci and aerococci, which show small zones of inhibition around optochin disks (23). Besides optochin susceptibility testing, several problems exist with other phenotypic tests for the identification of pneumococci (1, 7, 10, 23, 25, 27). An extensive comparison of the available biochemical and serological methods was published recently, and that analysis pointed to the high degrees of sensitivity but limited specificities of most of the phenotypic tests (20).

The capsular reaction is sometimes difficult to interpret, as was the case for two AccuProbe test-negative strains and one AccuProbe test-positive strain included in the present study. To simplify the discussion, these three strains were considered unencapsulated, and therefore, in this study only strong capsular swelling was considered indicative of an identification as *S. pneumoniae*.

This study was set up to evaluate which of some of the recently described genotypic techniques for the identification of pneumococci are best suited for the unambiguous identifi-

cation of a series of 49 strains that were sent to KTL in recent years for serotyping or because they were "possibly pneumococcal."

The results of this study can be summarized as follows. The group I and group IIc isolates posed no identification problems. Although the 11 group I isolates are optochin resistant, they were readily recognized as pneumococci by the presence of a capsule, by AccuProbe assay positivity, and by a positive *psaA*-specific PCR result. Group IIc isolates were readily identified as nonpneumococcal alpha-hemolytic streptococci since they were negative by all phenotypic and genotypic methods applied in this study.

Taking into account all of the tests carried out, one is inclined to consider the group IIa and IIb isolates to be nonpneumococcal alpha-hemolytic streptococci which, however, show one or more characteristics of pneumococci. Most problematic were the positive results by the AccuProbe assay (confirmed by ARDRA) for the seven group IIa isolates. Group IIa isolates showed several other reactions characteristic of those of pneumococci, like positivity by the *ply*- and *lytA*-specific PCRs and bile solubility, but all were unencapsulated and *psaA*-specific PCR negative. The isolates in group IIb were only *ply*-specific PCR positive and/or bile soluble, and four group IIb isolates also showed a weak positive reaction by the *lytA*-specific PCR.

This prompted us to consider the specificity of the AccuProbe test, which has been used as a "gold standard" for the identification of pneumococci in several studies (6, 37).

Comparison of all 16S rRNA sequences of the *S. mitis* group species showed that *S. pneumoniae* and *S. sanguinis* lack a *Rsa*I restriction site (GTAC) at *Escherichia coli* position 635, which is present in *S. mitis*, *S. oralis*, and *S. parasanguinis. S. sanguinis* is a species for which *Rsa*I digestion of the 16S rDNA results in a pattern that is similar to that for *S. pneumoniae* but that is due to another point mutation. At *E. coli* position 635, the sequences are GTAG in *S. pneumoniae* and GTAT in *S. sanguinis*. We found complete concordance between the results of the AccuProbe test and ARDRA, probably because the AccuProbe test targets the same region as ARDRA with *Rsa*I digestion. This points to the possibility that digestion of amplified 16S rDNA with *Rsa*I could be a valuable and cheaper alternative for differentiation between pneumococci and nonpneumococcal streptococci, although the false-positive results for *S. sanguinis* isolates must be taken into account.

Although the AccuProbe test and *Rsa*I digestion of 16S rDNA appear to enable identification of all genuine *S. pneumoniae* isolates, the possibility that closely related nonpneumococcal *S. mitis* strains have (nearly) identical 16S rDNAs, resulting in false-positive AccuProbe test and ARDRA results, cannot be excluded. On the basis of all data gathered in the present study and in the study of Whatmore et al. (37), we believe that not only the AccuProbe test-negative group IIb strains but also the AccuProbe test-positive group IIa strains are indeed not pneumococci. Mundy et al. (25) also reported that of 115 atypical pneumococci with inconsistent combinations of optochin susceptibilities, tube bile solubilities, and Quellung reactions, 33 reacted by the AccuProbe assay. Of these 33 isolates, only 3 showed a capsule that reacted in the Quellung test. All group IIa strains were isolated from the upper respiratory tract and not from patients with invasive disease. Whatmore et al. (37) conclude, "Our results indicated that many of the isolates identified as pneumococci by AccuProbe could actually represent organisms which are genetically rather divergent from typical pneumococci."

The probable close evolutionary relationship between genuine and atypical pneumococci and the sporadic horizontal transfer of some pneumococcal genes to their closest relatives may explain the existence of nonpneumococcal *S. mitis* cluster strains with a pneumococcal 16S rDNA sequence and with sporadic pneumococcal genes, and this may be an explanation for the identification problems posed by such atypical pneumococci.

Although we tend to consider most of the atypical pneumococcus isolates of group IIa to be nonpneumococcal isolates, despite a positive AccuProbe test result, it may be prudent to treat these isolates as pneumococcal isolates anyway, since they can harbor some pneumococcal virulence genes. In this study, the presence of several pneumococcal virulence genes in the AccuProbe test-positive nonpneumococcal *S. mitis* cluster strains was obvious, and Whatmore et al. (37) also described the existence of pathogenic *S. mitis* strains harboring the virulence determinants pneumolysin and autolysin, which are classically associated with *S. pneumoniae*. Fortunately, the presence of one or another pneumococcal virulence gene seems to coincide in many cases with a positive AccuProbe test result; therefore, the AccuProbe assay could be continued to be used as the gold standard for the detection of pneumococcal virulence genes.

Although Jado et al. (15) reported that the *psaA* gene could be amplified from isolates of *S. mitis*, *S. oralis*, and *S. anginosus* with their primers, the primers described by Morrison et al. (24), which enabled amplification of the *psaA* gene from all 90 serotypes of *S. pneumoniae* and which were used in this study, appear to be very specific for *S. pneumoniae*. The *psaA* gene was amplified with the primers of Morrison et al. (24) only from genuine pneumococci and the optochin-resistant but encapsulated pneumococci of group I. This positivity by the *psaA*-specific PCR always corresponded to encapsulation.

In conclusion, the results presented here indicate that identification problems continue to exist for some strains, despite the application of combined genotypic and phenotypic tests. On the basis of our observations and those of other researchers, we propose that the following recommendations be put into practice in our laboratories and be considered by other researchers. The optochin sensitivity test, when performed carefully, is the standard method for the identification of pneumococci. An optochin-sensitive alpha-hemolytic *Streptococcus* is almost always a pneumococcus. For optochin-resistant isolates, the presence or absence of a capsule should be verified by the Quellung reaction or the counterimmunoelectrophoresis method, if, on the basis of the origin (e.g., blood or cerebrospinal fluid) or the colony morphology (e.g., a slimy colony) of the isolate, there is a good reason to suspect the presence of pneumococci. An optochin-resistant, encapsulated bacterium is then identified as a pneumococcus. Since we found in this study that a positive result by the *psaA*-specific PCR with the primers described by Morrison et al. (24) is in perfect correspondence with encapsulation, this genotypic technique is best suited for the identification of genuine pneumococci and can possibly replace both the optochin sensitivity test and the

Quellung reaction. The AccuProbe test was considered to be somewhat less specific than the *psaA*-specific PCR, but the AccuProbe test result largely coincided with the presence of pneumococcal virulence genes in nonpneumococci, and it therefore retains its value. Digestion of amplified 16S rDNA with *Rsa*I (ARDRA) is a cheap and valuable alternative to the AccuProbe test. Amplification of the pneumolysin gene (*ply*-specific PCR) and the autolysin gene (*lytA*-specific PCR) is somewhat less specific than the AccuProbe test and probably leads to overestimation of the number of pneumococcal infections. Even though genotypic methods provide new possibilities for the identification of pneumococci, it is obvious that due to the presence of pneumococcal virulence genes in nonpneumococcal *S. mitis* isolates, it is not always easy to draw a clear line between pneumococcal and nonpneumococcal isolates.

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