Streptococcus pseudopneumoniae: prospective detection among community lower respiratory isolates in Eastern Saudi Arabia

A. M. ALNIMR
King Fahad Hospital of the University College of Medicine, University of Dammam, Saudi Arabia

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ABSTRACT

Streptococcus pseudopneumoniae is a distinct pathogen and the available evidence suggests its significance in lower respiratory tract infections. The purpose of this study is to investigate the occurrence of this new species in patients presenting with community-acquired pneumonia in a university hospital setting using routine laboratory tests. Sputum samples (n=213) submitted to the microbiology laboratory for culture from cases clinically diagnosed as community-acquired pneumonia are studied. Optochin variability in ambient air and 5% CO_2 was the criterion used to identify *S. pseudopneumoniae*. Disc-diffusion and E-test on Muller Hinton blood-based agar were used for antibiotic susceptibility testing following Clinical Laboratory Standard Institute breakpoints. Out of the screened isolates, only six (3%) were identified as *S. pseudopneumoniae*. None of the commercial tests differentiated the pathogens from viridans streptococci so diagnostic laboratories need to provide an alternative identification algorithm. The six isolates showed susceptibility to penicillin, and five isolates were susceptible to vancomycin, but one exhibited low-level resistance (minimum inhibitory concentration [MIC]: 1 μg/mL). All isolates showed high-level resistance to macrolides (MIC: 24–>256 μg/mL). Although *S. pseudopneumoniae* strains were not sufficiently frequent to justify routine additional incubation of sputum samples in ambient air, the high resistance pattern is alarming. Further surveillance is needed in diagnostic laboratories to understand the epidemiology and clinical significance of the newly described pathogen.

KEY WORDS: Respiratory tract infection.
Streptococcus.
Streptococcus mitis.

Introduction

Rapid and accurate diagnosis of infectious diseases is an essential role for microbiology laboratories that is occasionally challenging. The pseudopneumococcus *Streptococcus pseudopneumoniae* was recently classified as a species phenotypically and genotypically distinct from *S. pneumoniae*.[1] Both organisms belong to the same phylogenetic lineage, the closely related pneumoniae-mitis-pseudopneumoniae cluster, originating from a common ancestor, as suggested from the work of Kilian *et al* that sequenced four housekeeping genes among these strains.[2] It has been implicated as a potential aetiological agent of lower respiratory tract infections, especially in patients known to have chronic obstructive pulmonary diseases (COPD).[1,3–5] Despite the fact that most viridans streptococci belonging to the mitis group remain susceptible to antimicrobial agents, the available evidence suggests the occurrence of tolerance and resistance genes in *S. pseudopneumoniae*.[5,6] This is also supported by *in vitro* phenotypic resistance of the organism to a number of agents.[4,7]

There is no gold-standard method to identify *S. pseudopneumoniae* in routine clinical settings. Neither the colonial morphology nor microscopy can differentiate the pneumococcus and pseudopneumococcus.[4] Furthermore, *S. pseudopneumoniae* is difficult to differentiate from other streptococcal strains belonging to the *S. mitis* and *S. oralis* groups. Accurate identification of the organism in respiratory samples is important in diagnostic practice to highlight its pathogenic potential compared to other viridans streptocci. Phenotypically, *S. pseudopneumoniae* is α-haemolytic, bile-insoluble and non-capsulated. Characteristically, it tends to be optochin-resistant in 5% CO_2, the atmosphere used routinely for streptococci, but susceptible in ambient O_2.[1]

Since the first recognition of *S. pseudopneumoniae* as a human pathogen in 2004, the organism has not investigated or reported in the Middle East. The present study prospectively examines the frequency of isolation and susceptibility patterns of *S. pseudopneumoniae* from sputum samples of patients presenting with lower respiratory tract infections. The performance of various commercial tests to identify this species in routine laboratory settings, along with susceptibility pattern, is also investigated.

Correspondence to: Dr. A. M. Alnimr
Email: amalnimr@uod.edu.sa

Materials and methods

This was a hospital-based study in which all viridans streptococci isolated as pure or predominating growth from sputum samples between January 2013 and February 2014

Table 1. Identification of the six *S. pseudopneumoniae* strains by commercial phenotypic systems.

Strain	Age (years)/gender	Optochin (mm)		Identification by commercial system			Typing
		CO_2	Air	VITEK 2	API Strep	API Rapid Strep	Pneumolatex kit
1	5/male	6	20	*S. oralis*	*S. oralis*	*S. oralis*	Non-typable
2	10/female	10	21	*S. oralis*	*S. mitis*	*S. mitis*	Non-typable
3	11/male	6	22	*S. oralis*	*S. oralis*	No ID for the code	Non-typable
4	27/male	6	18	*S. anginosus*	*S. oralis*	*S. oralis*	Non-typable
5	42/female	9	20	*S. mitis*	*S. oralis*	*S. oralis*	Non-typable
6	16/female	6	19	50% *S. mitis* 50% *Gemella morbillorum*	*S. mitis*	*Gemella* spp.	Non-typable
Concordance				42% with API Strep 25% with API Rapid Strep	67% with API Rapid Strep 42% with VITEK 2	67% with API Strep 25% with VITEK 2	–

in the microbiology diagnostic laboratory at King Fahad Hospital (KFHU) were collected. Identification was based on colonial morphology with α-haemolysis on Columbia blood agar (CBA; SPML, Saudi Arabia) in 5% CO_2, negative catalase test and resistance to a 6-mm optochin disc (5 g).

The purulent portions of sputa were selected from good-quality specimens that showed more than 25 leucocytes/low power field and less than 10 epithelial cells per low power field on screening microscopy. Samples from patients admitted for a period longer than five days before submitting sputum samples were excluded, as these were likely to represent hospital-acquired pneumonia.[8,9]

Optochin susceptibility

In order to identify *S. pseudopneumoniae* strains, the isolates were retested for optochin sensitivity in ambient air by inoculating a single colony of each isolate onto CBA and placing a 6-mm optochin disc (5 μg). The disc was placed at the junction of the primary inoculum and second streak and incubated without CO_2 for 18 h. Zone sizes were measured and interpreted in accordance with Clinical Laboratory Standard Institute (CLSI) criteria (resistant: <14 mm; susceptible: ≥14 mm).

VITEK 2

VITEK 2 is an automated system for bacterial identification and antimicrobial susceptibility testing based on kinetic analysis of the fluorescence and colorimetric tests for enzymes, carbon utilisation and antibiotic resistance profile. The manufacturer's instructions were followed for inoculation, reading and interpretation of VITEK 2 cards. Briefly, isolates were grown on CBA for 18 h at 37°C and the inoculum prepared for card inoculation was a bacterial suspension adjusted to a McFarland standard of 0.5 in sterile saline. The 64-well disposable cards were automatically filled by a vacuum device, sealed and then loaded in the VITEK 2 instrument. The card was subjected to a kinetic fluorescence measurement every 15 minutes by the VITEK 2 reader-incubator module, and the results were interpreted by the ID-GPC computer software (Version 5.03) through comparison of the biochemical profile of the strain tested against the strains present in the software database (includes 33 streptococcal species).[10]

API 20 Strep and API 32 Rapid Strep

The isolates were also identified by the API 20 Strep and API 32 Rapid Strep according to the manufacturer's instructions (bioMérieux, France). The API 20 Strep kit consists of plastic strips each with 20 microtubes containing substrates for detection of biochemical reactions. As leucine arylamidase (LAP) is a marker for the genus *Streptococcus*, a negative result was interpreted as poor inoculum and the test was repeated. The rapid ID 32 Strep system includes 32 wells containing various biochemical tests in dehydrated form. After growth on CBA, a bacterial suspension was prepared in 2 mL sterile distilled water to prepare a dense inoculum equivalent to 4.0 McFarland. Suspension (55 μL) was placed into each of the strip cupules. The inoculated strips were incubated at 37°C under aerobic conditions.

Reading the API 20 Strep and API 32 Rapid Strep was performed at 24 h and 4 h following incubation, respectively. The reactions were compared with the reading chart provided and recorded as positive or negative. A seven or 11-digit profile number was obtained by assigning each group of three tests a numerical value of 1, 2, and 4 sequentially and scoring positive tests within each group. Interpretation of the numerical code profile for API 20 Strep and API 32 Rapid Strep was performed with the API database versions 5.2 and 2.2, respectively. Identification at species level was accepted as correct if the corresponding probability was at least 0.9. Isolates with an unknown profile were declared non-identifiable.

Serotyping

The Pneumotest-Latex kit (Statens Serum Institute [SSI], Copenhagen, Denmark), which is specific for 87 serotypes/groups, was utilised for capsular typing of the isolates, as previously described.[11] The kit represents a chessboard modification of the quellung method in which two sets of pneumococcal combined antiserum pools (A to I, P to T) and omniserum (SSI) contains batches of pneumococcal antisera that are covalently linked to latex particles. Briefly, an aliquot (10 μL) of an overnight pneumococcal fresh culture (brain heart infusion broth) was mixed with 10 μL latex reagent on a reagent card (SSI), and thereafter the card was manually rocked for 5–10 sec. A positive reaction is read with the naked eye within 5–10 sec if the latex reagent contained antiserum homologous to the

Table 2. *In vitro* susceptibility of six *S. pseudopneumoniae* isolates to antimicrobial agents as determined by E-test based on breakpoints established by the Clinical and Laboratory Standards Institute for α-haemolytic streptococci.

Strain	Age (years)/gender	Minimum inhibitory concentration (μg/mL)							D-test
		Penicillin	Vancomycin	Linezolid	Levofloxacin	Doxycycline	Erythromycin	Clindamycin	
1	5/male	0.016	0.125	0.016	2	**>256**	**>256**	**16**	Positive
2	10/female	0.047	0.5	0.125	**12**	**8**	**32**	**2**	Non-applicable*
3	11/male	0.25	**1**	0.125	**8**	**24**	**128**	**1.5**	Positive
4	27/male	0.125	0.38	0.125	**8**	**16**	**48**	**3**	Non-applicable*
5	42/female	0.016	0.094	0.094	**16**	2	**24**	0.25	Negative
6	16/female	0.094	0.064	0.064	1	**>256**	**>256**	**4**	Positive
Susceptible (%)		6 (100)	5 (83)	6 (100)	**2 (33)**	**1 (17)**	**0 (0)**	**1 (17)**	–

*Both macrolide and clindamycin tests showed resistance without D-test to induce resistance.
Bold figures signify resistance.

capsule of the organism. Late agglutinations were ignored. The isolates were subjected to a second test to verify the presence of a capsule by mixing with an omniserum (SSI), which is specific for 91 serotypes to be examined by light microscopy (x1000 oil immersion lens) for agglutination. The serotype, serogroup or pool of serogroups is assigned according to the chessboard system provided with the kit after testing with the 14 latex reagents. Isolates that react with only the A to I pool, and not the P to T pool, is identified only to the pool level. Isolates that do not react with any of the pools but react with omniserum are classified as non-typeable, and those with no reactivity to antisera as non-capsulated.

Drug susceptibility testing

Susceptibility testing was performed following CLSI 2013 guidelines on MHA with 5% sheep blood (SPML, Saudi Arabia) using 0.5 McFarland inoculum. Results were interpreted using the zone diameters and MIC Interpretive criteria for viridans streptococci. The following discs were included: vancomycin (30 μg), linezolid (30 μg), levofloxacin (5 μg), doxycline (30 μg), erythromycin (15 μg) and clindamycin (2 μg). In addition, Etest strips (AB Biodisk, Sweden) of the following agents were used: penicillin, vancomycin, levofloxacin, doxycline, erythromycin and clindamycin. Where no zone diameter criteria were available (penicillin) in the CLSI, only E-test-based MIC was carried out. D-test for detection of the MLS_B phenotype was carried out when the erythromycin disc showed resistance, while a clindamycin zone indicated apparent susceptibility (CLSI 2013). The two discs of erythromycin (15 μg) and clindamycin (2 μg) were placed 20 mm apart and incubated overnight. Flattening of the clindamycin zone indicated the presence of the MLS_B phenotype.

Table 3. Common test results noted for the six *S. pseudopneumoniae* using API Strep/Rapid Strep.

Test	No (%) positive
Trebalose fermentation test (TRE)	0 (0)
Acid from raffinose (RAF)	0 (0)
α-galactosidase (GAL)	0 (0)
Alkaline phosphatase (PAL)	6 (100)

Quality control and storage of the isolates

The isolates and supermaster stock of control ATCC strains were stored in cryovials containing brain heart infusion (BHI) broth with 12% glycerol (SPML, Saudi Arabia) at –70°C. As recommended by CLSI, *S. pneumoniae* ATCC 49619 was used as the quality control (QC) strain for all tests except serotyping, for which positive and negative controls were prepared from wild-type clinical strains of *S. pneumoniae* and viridans streptococci, respectively; the ATCC strains of *S. pneumoniae* were not used because they lose their capsule during serial subcultures, leading to false-negative results. For retrieval prior to testing, the strains were taken out of the cryovial and allowed to thaw slightly at room temperature before subculture by aseptic techniques on CBA incubated in 5% CO_2 at 37°C. After 18 hours' incubation, the culture plates were examined to check that the morphological characteristics are comparable to the desired organism and for the presence of pure growth. Whenever susceptibility testing is performed, the results were accepted only if the control strain fitted the expected MIC or zone range. Purity plates were included from each streptococcal suspension for bacterial identification by VITEK 2, API Strep, and API 32 Rapid Strep.

Results

Of the 213 sputum samples tested, six were identified as *S. pseudopneumoniae* based on optochin variability; these isolates were resistant to optochin in CO_2 but were susceptible to optochin in ambient air. The colonial morphology of the six isolates showed small pinpoint colonies on CBA after overnight incubation in 5% CO_2. The biochemical profiles given by the three identification systems (VITEK 2, API Strep, API Rapid Strep) in the majority of reactions (14.5/18) were identified as *S. mitis/oralis* (81%) (Table 1). One isolate was non-identifiable by the API Rapid Strep; one was assigned as *Gemella* sp. by the same system as well as VITEK 2 (50%). VITEK 2 identified

one strain as *S.anginosus.* Omniserum was negative so no further subtyping was carried out. The six isolates did not possess a pneumococcal capsule, confirming that these were non-pneumococcal strains (Tables 1–3).

Discussion

The α-haemolytic streptococci include four phylogenetic clusters: the mitis, mutans, salivarius and anginosus groups.[12] Both *S. pneumoniae* and *S. pseudopneumoniae* belong to the mitis group of streptococci and they share 100% sequence homology with *S. mitis* and *S. oralis*, but *S. pseudopneumoniae* can be differentiated from the group by variable optochin susceptibility and lack of capsule.[1,13] In this study, the discrepancy in optochin testing results in 5% CO_2 (average zone diameter of 7.2 mm) and ambient air (average zone diameter of 20 mm) was used to identify *S. pseudopneumoniae* and compared with the routine commercial identification systems for streptococci (i.e., VITEK 2, API Strep and API Rapid Strep). The data suggest a low prevalence (2.8%) of the pathogen among α-haemolytic streptococci grown in pure or dominating growth from good-quality sputum specimens that could not be classified as *S. pneumoniae* on the basis of available phenotypic tests, which was similar to other studies performed in Asia and Europe.[14,15]

At the moment, the CLSI recommends testing optochin sensitivity in 5% CO_2 only for presumptive identification of *S. pneumoniae* so *S. pseudopneumoniae* isolates are misidentified as viridans streptococci and thus not reported in most diagnostic laboratories. Incubation in ambient air discriminates the organisms but this should be done as an additional test to be distinguished from *S. pneumoniae*. Also, 8% of *S. pneumoniae* clinical strains do not grow without supplemental CO_2 so they will be missed if only ambient air is used for incubation.[16] The results of optochin susceptibility need to be supplemented by another test such as capsular detection or bile solubility before ruling out pneumococci, as some pneumococcal isolates are optochin-resistant.[17,18]

The six strains identified as pseudopneumococci were shown to be non-capsulated as they were non-reactive to any of the omnisera used. With the emergence of new streptococcal species such as *S. pseudopneumoniae* and optochin-resistant pneumococci, the laboratory needs to adopt a complementary test to discriminate closely related respiratory streptococcal species. This is particularly important because the results of the optochin test are subject to the inoculum effect as shown by Gardam *et al*.[19]

The API Strep and API Rapid Strep remain among the most frequently used kits in a routine laboratory for streptococci identification. However, the results shown in Table 1 illustrate that the commercially available versions can only provide reliable identification to the group level. Previous work showed inaccuracy of the technique for speciation of other members of the mitis group of streptococci, which is in agreement with the data shown.[20–22] Arbique *et al*. have suggested the alkaline phosphatase test to be a useful indicator for the identification of *S. pseudopneumoniae*, along with optochin variability.[1] In the presented data, all six isolates were positive for the test (Table 3).

Another frequently used identification system in clinical laboratories is the VITEK 2, along with other automated platforms that speciate streptococci on the basis of their biochemical properties. Difficulties in discriminating α-haemolytic streptococci by VITEK 2 have been reported as the system could achieve only 55% accurate speciation of these organisms compared to 79% by API Rapid Strep.[23] The newly introduced matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) platform suffers from some resolution problems in differentiating the closely related pneumoniae-mitis-pseudopneumoniae cluster, but newer targets in the technology are promising and are being investigated.[24]

The clinical significance of *S. pseudopneumoniae* has not been clearly established, but its pathogenicity has been shown in a murine model of peritonitis and sepsis, where all mice injected peritoneally with the organism died within 36 hours.[15] Currently available data propose an association with COPD.[3] This was not the case in the present study as none of the six patients was diagnosed as a case of COPD prior to or following this episode of infection. No definitive evidence exists about the role of *S. pseudopneumoniae* in COPD patients and more data are required to support the pathogenic potential of the organism in this group in whom potential pathogens may occur as colonisers during times of clinical stability.[25]

S. pseudopneumoniae have been identified as colonisers in respiratory samples from children.[7] In the presented study, half of the isolates (3/6) originated from paediatric cases who presented with clinical and radiological features suggestive of bacterial pneumonia. No other pathogen was isolated in these patients, suggesting a potential role of *S. pseudopneumoniae* isolated. The organism was isolated from good-quality purulent sputum samples obtained as pure growth in two and as a predominant organism in the third case. The pathogenic role of the organism in paediatrics and COPD patients is still to be elucidated.

The other 207 samples grew viridans streptococci which, despite being a part of the oral commensal flora, have been implicated as respiratory pathogens in several studies of pneumonia complicated by bacteremia.[26–28] Despite the debatable potential of viridans streptococci to be respiratory pathogens, it is crucial for the laboratory to differentiate them accurately from *S. pneumoniae* as the latter is highly virulent and has different antimicrobial interpretive criteria. The inaccuracy in laboratory identification is thought to contribute to resistance over-estimation in *S. pneumoniae* when the wrong breakpoints are applied.[29]

The findings of this study showed universal susceptibility to penicillin among the six pseudopneumococcal isolates. All strains showed decreased susceptibility to macrolide antibiotics, with 5/6 resistant to clindamycin and doxycycline. Inducible MLSβ resistance was noted in three of the six erythromycin-resistant strains and those isolates had high MIC to both macrolides and clindamycin compared to the two isolates with constitutive resistance (Table 2). Two-thirds of the isolates exhibited resistance to the fluoroquinolone tested and, of note, one isolate showed resistance to vancomycin, although the resistance isolate was susceptible to linezolid and penicillin. Susceptibilities of viridans streptococci are rarely reported and thus it is difficult to infer the comparability of the findings, but reduced susceptibility to β-lactams is increasing among these organisms,[30] along with increased resistance

to the macrolides and fluoroquinolones worldwide.[31–34]

Members of the *S. mitis* group have been noted to be most resistant among the streptococci, due to their natural competence.[35] *S. pseudopneumoniae* is thought to be a hybrid species between *S. pneumoniae* and *S. mitis* and thus it may facilitate gene transfer between streptococcal species through transformation.[5] This horizontal gene transfer is thought to contribute to the evolving resistance to β-lactam antimicrobial agents among *S. mitis*,[36] although this was not the case here. Growing evidence suggests that recombination occurs at a high rate within the housekeeping alleles of the *S. pseudopneumoniae* population, which could be related to absence of capsule as in the case of non-typeable pneumococci.[37] Thus, resistance in *S. pseudopneumoniae* may complicate the problem of increasing resistance in other respiratory pathogens.

The study presented has several limitations. The small number of isolates found imposes the need for larger active surveillance studies on different patient populations and from other sample types in order to define the pathogenic role of the *S. pseudopneumoniae*. The patient information was retrieved retrospectively from clinical records. Genomic-based methods were not used to confirm the identity as the intention was to rely on routine phenotypic testing that is available for diagnostic purposes in low-resource settings. A major drawback was the inadequacy of the diagnostic commercial streptococcal databases. However, the possibility of *S. pneumoniae* was ruled out by serotyping and optochin testing in CO_2. It is unlikely for a pneumococcus strain to be non-capsulated and optochin-susceptible simultaneously.[20] Other viridans streptococci were eliminated by optochin susceptibility in ambient air. The results presented in this study reflect the complex taxonomy of the streptococci and the inherent difficulties in defining the gold-standard method that accurately identifies streptococci to a species level. Further research is needed to simplify the approach to differentiate the pneumoniae-mitis-pseudopneumoniae members.

Conclusions

The author believes this study reports the first subset of *S. pseudopneumoniae* isolates in the Middle East using multiple, parallel phenotypic methods. The identification of the organism is challenging in low-resource clinical laboratories and it requires attention when pure non-pneumococcal streptococci are isolated from lower respiratory samples. Currently, none of the commercial kits tested (i.e., VITEK 2, API Strep and API Rapid Strep) can identify this species from other viridans streptococci. Commercial databases used in diagnostic settings need to be updated to include this overlooked pathogen. The inadequacy of identification systems can lead to major effects on the accuracy of organism reporting and antimicrobial susceptibility breakpoints. The pathogenic role of *S. pseudopneumoniae* is not yet certain but its antimicrobial susceptibility trend is a cause for concern. □

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