

Comparison of Seeplex PneumoBacter ACE Detection assay and in-house multiplex PCR for the identification of *Streptococcus pneumoniae*

Dane Luznik¹, Mitja Kosnik², Viktorija Tomic¹

¹Laboratory for Respiratory Microbiology, University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia;

²Department of Pneumology, University Clinic of Respiratory and Allergic Diseases, Golnik, Slovenia

SUMMARY

Rapid and accurate identification of *Streptococcus pneumoniae* is important for appropriate and prudent antimicrobial use in the treatment of lower respiratory tract infection. It is difficult to separate *S. pneumoniae* from commensal viridans group streptococci either by classical techniques or molecular methods. Aim of this study was to compare a commercially available multiplex PCR assay Seeplex PneumoBacter ACE Detection assay (Seegene, Seoul, South Korea), and in-house multiplex PCR using primer sets for *lytA* and *cpsA* for ability to differentiate *S. pneumoniae* in a known set of bacteria (*S. pneumoniae* and viridans group streptococci) and clinical samples. Of 20 viridans streptococcal isolates, 8 were misidentified as *S. pneumoniae* by commercial PCR test. Of 209 throat swabs tested with Seeplex PneumoBacter ACE Detection assay, 122 (58,4%) were positive for *S. pneumoniae* while only 11 (5.3%) samples were positive with *lytA* and *cpsA* primers. Therefore, the commercial multiplex PCR test appears to have low specificity in diagnosing *S. pneumoniae*.

KEY WORDS: *Streptococcus pneumoniae*, PCR, Diagnostics, Respiratory samples.

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INTRODUCTION

Despite the introduction of vaccine, *Streptococcus pneumoniae* remains an important cause of morbidity and mortality. *S. pneumoniae* is generally accepted as the most common cause of community-acquired pneumonia (CAP). However, the prevalence of *S. pneumoniae* in CAP varies substantially from 5 to 60% among studies probably reflecting mainly a difference in microbiologic diagnostic procedures (Rozenbaum *et al.*, 2013). In routine clinical practice, *S. pneumoniae* is usually detected by conventional culture which is cheap and facilitates antibiotic susceptibility determination but is time consuming. In addition, the isolation and iden-

tification of *S. pneumoniae* are complicated by antimicrobial suppression of growth in culture and the presence of normal flora viridans group streptococci. When cultured on blood agar, identification of *S. pneumoniae* can be established by 1 or more assays, including optochin susceptibility, bile solubility, carbohydrate utilization, latex agglutination tests and commercially available manual or automated systems. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been described as a rapid, cost-effective, and reliable method for identification of bacteria in the clinical laboratory (Risch *et al.*, 2010; Idelevich *et al.*, 2014). Although very effective in identifying Gram-positive bacteria, including infrequently isolated organisms, its ability to accurately differentiate *S. pneumoniae* from *S. mitis* is limited (Risch *et al.*, 2010; McElvania TeKippe *et al.*, 2013).

Recently, various molecular assays have been developed, including DNA-hybridization test, conventional and real-time PCR tests, and a

Corresponding author

Viktorija Tomic, MD, PhD

Laboratory for Respiratory Microbiology

University Clinic of Respiratory and Allergic Diseases

Golnik, Slovenia

E-mail: viktorija.tomic@klinika-golnik.si

loop-mediated isothermal amplification method (Seki *et al.*, 2005). Molecular assays are promising diagnostic methods as they can be completed quickly, have high sensitivity and specificity, are not disrupted by the presence of other organisms or influenced by previous antimicrobial therapy and can be used to detect pneumococci directly in clinical samples (Cvitkovic Spik *et al.*, 2013; Luo *et al.*, 2012). PCR-based assays for identification of pneumococci have targeted different genes, including autolysin (*lytA*) (McAvin *et al.*, 2001), pneumolysin (*ply*) (Corless *et al.*, 2001), pneumococcal surface antigen A (Morrison *et al.*, 2000), and capsular polysaccharide biosynthesis gene (*cpsA*) (Luo *et al.*, 2012). Ideally, these targets should be specific for *S. pneumoniae* strains only but recent reports suggest that the genes encoding *S. pneumoniae* virulence factors autolysin and pneumolysin are present in oral viridans group streptococci, notably *Streptococcus mitis* and *Streptococcus oralis* (Seki *et al.*, 2005; Verhelst *et al.*, 2003; Whatmore *et al.*, 2000).

Aim of our study was to compare the specificity for detection of *S. pneumoniae* of a commercially available multiplex PCR assay Seeplex PneumoBacter ACE Detection assay (Seegene, Seoul, South Korea), and in-house multiplex PCR using primer sets for *lytA* and *cpsA*, and to evaluate the diagnostic accuracy of both assays for detection of *S. pneumoniae* in clinical samples. Seeplex PneumoBacter ACE Detection assay is a qualitative in vitro test for the detection of 6 common pathogens causing lower respiratory tract infections (LRTI): *Mycoplasma pneumoniae*, *Chlamydomphila pneumoniae*, *Legionella pneumophila*, *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Bordetella pertussis*. By applying a dual priming oligonucleotide technology (DUO™) it should be accurate in detecting 6 bacteria causing pneumonia in respiratory specimens such as sputum, nasopharyngeal swabs and aspirates, as well as bronchoalveolar lavages.

METHODS

Bacterial strains and throat swab culture

The bacterial strains used in this study were (N): *Streptococcus constellatus* (1), *S. mitis*

(3), *S. oralis* (4), *Streptococcus salivarius* (2), *Streptococcus intermedius* (4), *Streptococcus sanguinis* (3), *Streptococcus vestibularis* (1) and *Streptococcus parasanguis* (2). These alpha-haemolytic streptococcal strains were isolated from clinical respiratory samples. They were non-susceptible to optochin, had negative Slidex® pneumo-kit agglutination test (bioMerieux, Marcy-l'Etoile, France) and were identified to species level by commercially available identification assay BBL™ Crystal™ Gram Positive ID kit (Becton, Dickinson and Company, Sparks, MD, USA). We also used 20 *S. pneumoniae* isolates detected previously in blood cultures and stored as a part of the blood culture isolates archive maintained in the Laboratory of Respiratory Microbiology at the University Clinic of Respiratory and Allergic Diseases Golnik, Slovenia (UCRAD). Isolates were stored in Microbank vials with beads (Pro-Lab Diagnostics, Richmond Hill, Canada) at -70°C. All *S. pneumoniae* isolates were identified by routine laboratory protocol (optochin susceptibility, positive Slidex® pneumo-kit agglutination test, positive DNA-hybridization AccuProbe test (GeneProbe, San Diego, CA, USA)). Throat swabs were cultivated on Trypticase soy agar supplemented with 5% defibrinated sheep blood.

Study population

To estimate the detection accuracy of *S. pneumoniae* in clinical samples we collected 209 throat swabs from patients admitted to UCRAD for suspected LRTI between November 2010 and June 2011. All patients were adults (mean age 54.8 years, range 18-88 years). Samples were collected with Sigma VCM swabs, consisting of a large vial with 3.0 ml medium and a standard Sigma swab made of cellular foam (Medical Wire & Equipment, Corsham, UK). All patients understood the nature of the research and gave informed consent.

DNA preparation

DNA extraction from the throat swabs was achieved using QIAamp® DNA Blood Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Extracted bacterial DNA was kept frozen at -30°C until further use.

PCR assays

Amplification reactions were conducted using Thermocycler T3000 (Biometra GmbH, Goettingen, Germany). Seplex Pneumobacter ACE Detection assay (Seegene) was performed according to the manufacturer's instructions. Results for *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *H. influenzae* and *B. pertussis* were not analyzed for this study. PCR products were visualized after agarose gel electrophoresis on pre-cast 2% E-gel agarose gels (Invitrogen/Life Technologies, Carlsbad, CA, USA). The estimated size of the PCR product of *S. pneumoniae* on agarose gel was 349 bp. Each amplification reaction contained DNA as an internal control and each run was accompanied by a positive control containing a mixture of clones for 6 pathogens and sterilized distilled water as a negative control.

In-house multiplex PCR specific for *S. pneumoniae* was created using oligonucleotide primer sets published previously and listed in Table 1 (McAvin *et al.*, 2001; Park *et al.*, 2010; Prere and Fayet, 2011). The *cpsA* primer set was designed on the basis of 492 bp sequence of the *cpsA* gene from suppression subtractive hybridiza-

tion clone. Length of PCR amplicon was 350 bp. Within the 901 bp highly conserved sequence of *lytA* gene the 101 bp sequence was selected to develop primers. As an internal control pair of primers to amplify the 478 bp fragment from gene coding a small ribosomal subunit RNA (16S-rRNA) was included. The reaction mix and PCR conditions were set according to the manufacturer's instructions. (Fermentas/Thermo scientific, Waltham, MA, USA). Reaction conditions for the test were: initial denaturation at 95°C for 3 minutes, 40 cycles of 95°C for 30 seconds, 54°C for 30 seconds, 72°C for 30 seconds and final extension at 72°C for 10 minutes.

RESULTS

First, in-house multiplex PCR was performed on known *S. pneumoniae* strains and we considered the PCR test to be positive for *S. pneumoniae* only when both primers were positive. It produced specific band sizes for *lytA* and *cpsA* in all 20 isolates. Table 2 and Figures 1-4 show the results of detection of specific pneumococ-

TABLE 1 - Sequence of oligonucleotide primers used for in-house PCR.

Primer	Sequence (5' ->3')	Amplicon size (bp)	Reference
<i>cpsA</i> -382F	ACGCAACTGACGAGTGTGAC	350	Park <i>et al.</i> (2010)
<i>cpsA</i> -735R	GATCGCGACACCGAACTAAT	350	Park <i>et al.</i> (2010)
<i>lytA</i> -F	ACGCAATCTAGCAGATGAAGC	101	McAvin <i>et al.</i> (2001)
<i>lytA</i> -R	TGTTTGGTTGGTTATTCGTGC	101	McAvin <i>et al.</i> (2001)
MFP889-F	TCAAATGAATTGACGGGGGC	478	Prere <i>et al.</i> (2011)
MFP890-R	GGCCCGGGAACGTATTCAC	478	Prere <i>et al.</i> (2011)

TABLE 2 - Detection of pneumococcal genes in *S. viridans* group and *S. pneumoniae* isolates by Seplex PneumoBacter ACE Detection assay and in-house multiplex PCR.

Species (No. of isolates)	<i>cpsA</i> (No. of isolates)	<i>lytA</i> (No. of isolates)	Seplex PB ACE Detection assay (No. of isolates)
<i>S. constellatus</i> (1)	-	-	+ (1)
<i>S. intermedius</i> (4)	-	+ (1)	+ (2)
<i>S. mitis</i> (3)	-	+ (3)	+ (2)
<i>S. oralis</i> (4)	-	+ (1)	+ (2)
<i>S. salivarius</i> (2)	-	-	-
<i>S. sanguinis</i> (3)	-	-	-
<i>S. vestibularis</i> (1)	-	-	+ (1)
<i>S. parasanguis</i> (2)	-	-	-
<i>S. pneumoniae</i> (20)	+ (20)	+ (20)	+ (20)

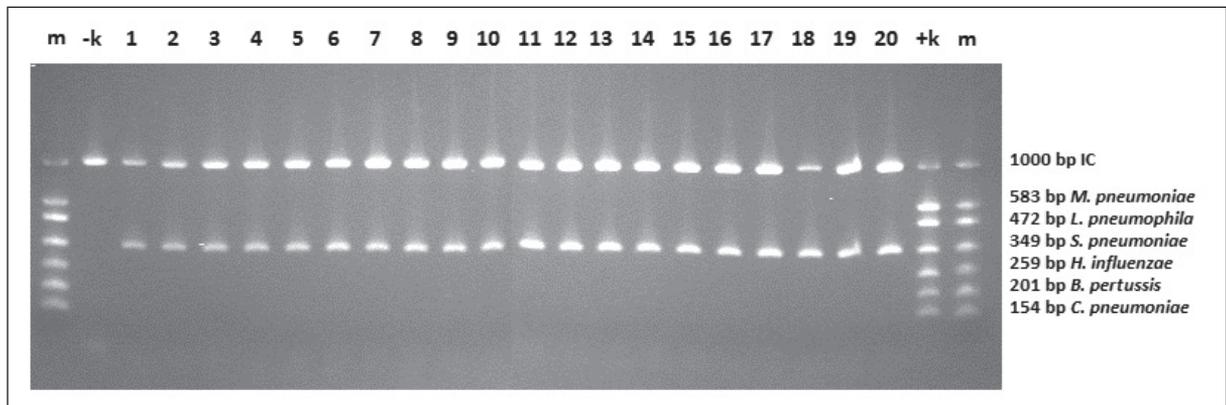


FIGURE 1 - *Seeplex PneumoBacter ACE Detection* assay for detection of *S. pneumoniae* performed on known *S. pneumoniae* isolates. Lane *m*: amplicon size marker; lane *-k*: negative control; lanes 1- 20: *S. pneumoniae* isolates; lane *+k*: positive control.

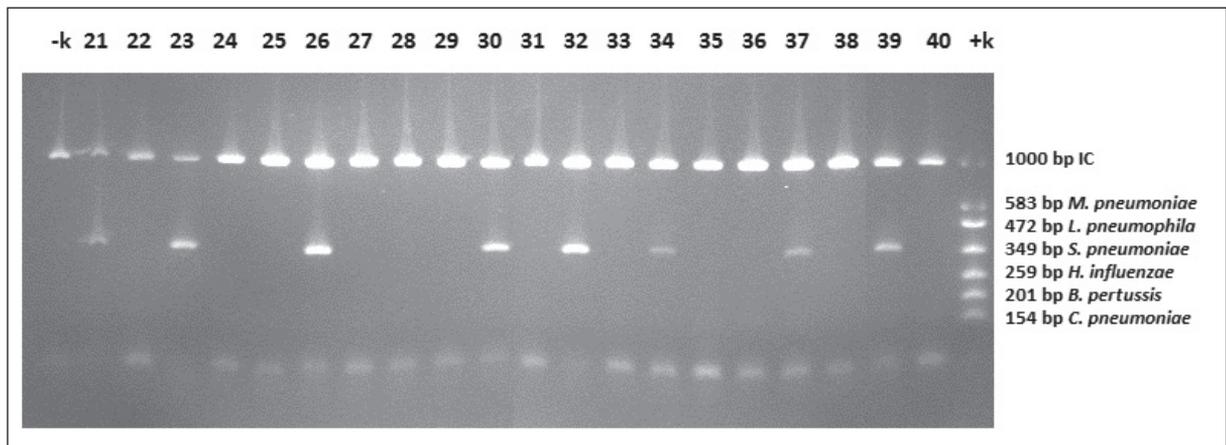


FIGURE 2 - *Seeplex PneumoBacter ACE Detection* assay for detection of *S. pneumoniae* performed on different isolates belonging to *S. viridans* group. Lane *-k*: negative control; lanes 21- 40: *S. viridans* group isolates; lane *+k*: positive control.

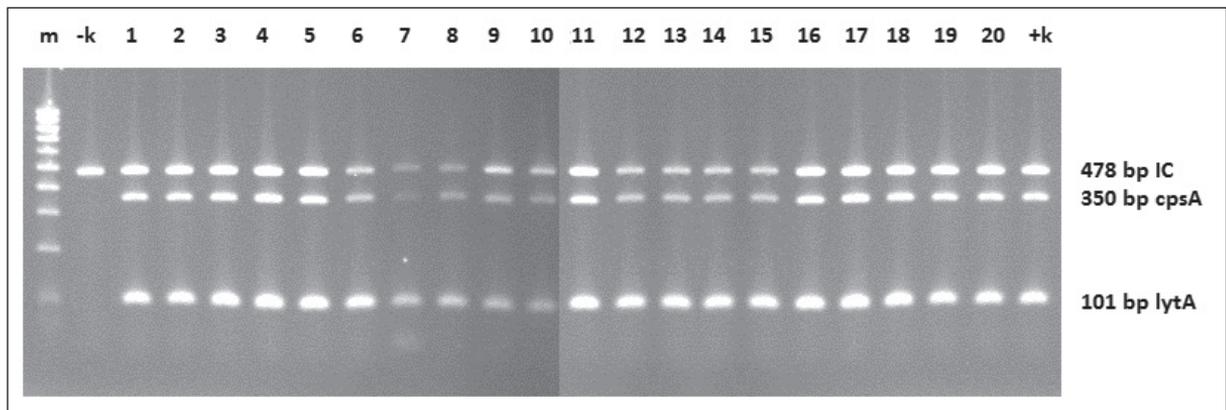


FIGURE 3 - *In-house PCR* assay for detection of *S. pneumoniae* performed on known *S. pneumoniae* isolates. Lane *m*: 100 bp DNA ladder; lane *-k*: negative control; lanes 1- 20: *S. pneumoniae* isolates; lane *+k*: positive control.

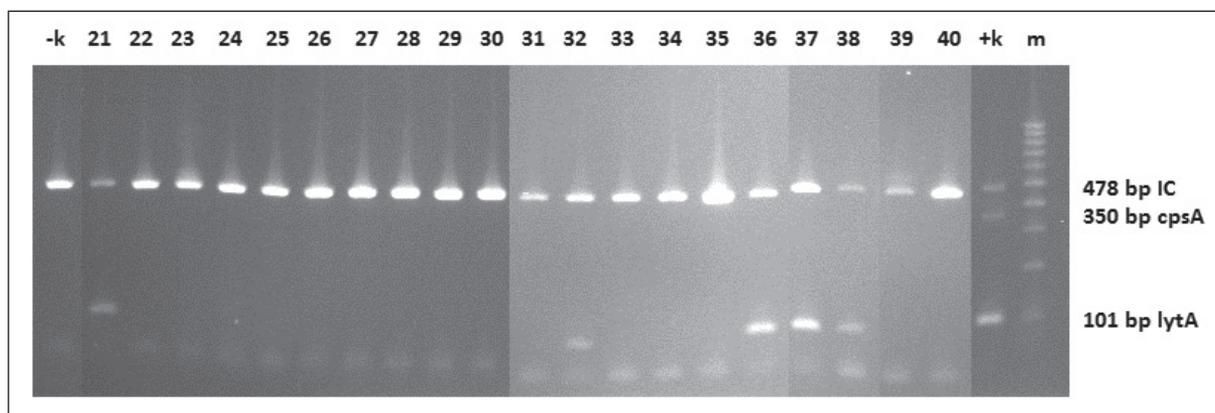


FIGURE 4 - In-house PCR assay for detection of *S. pneumoniae* performed on known isolates belonging to *S. viridans* group. Lane -k: negative control; lanes 21-40: *S. viridans* group isolates; lane +k: positive control; lane m: 100 bp DNA ladder.

cal genes with in-house PCR and the commercial PCR kit. All 20 viridans group streptococcal isolates had a negative result with the *cpsA* primer. Specific bands for *lytA* were present in 5 non-pneumococcal viridans streptococcal strains: *S. mitis* (3/3), *S. oralis* (1/4) and *S. intermedius* (1/4). The calculated sensitivity of both primers was 100%, the specificity of primers *cpsA* and *lytA* was 100% and 75%, respectively. *S. pneumoniae* strains and viridans streptococcal strains were also tested with Seplex Pneumobacter ACE Detection assay. All 20 *S. pneumoniae* strains were positive with Seplex Pneumobacter ACE Detection assay. Of 20 viridans streptococcal isolates, 8 were positive and misidentified as *S. pneumoniae*. Misidentified isolates were as follows: *S. mitis* (2), *S. intermedius* (2), *S. oralis* (2), *S. constellatus* (1), and *S. vestibularis* (1). Two positive *S. mitis* strains were also positive for *lytA* in our in-house PCR. Calculated specificity of Seplex Pneumobacter ACE Detection assay for known strains was 60% and calculated sensitivity was 100%.

Afterwards, 209 throat swabs of patients with LRTI and a low clinical probability of pneumococcal pneumonia were tested with both See-

plex Pneumobacter ACE Detection assay and in-house multiplex PCR. All samples had valid internal control results. Of 209 samples tested with Seplex Pneumobacter ACE Detection assay, 122 (58,4%) were positive for *S. pneumoniae* (Table 3). Using in-house multiplex PCR specific for *S. pneumoniae* only 11 (5,3%) samples were positive with *lytA* and *cpsA* primers. All throat swabs grew alpha-haemolytic streptococci. In 11 out of 209 throat swabs alpha-haemolytic streptococci were positive with optochin disc and latex agglutination test for *S. pneumoniae*. Non-pneumococcal alpha-haemolytic streptococci were not identified further to species level.

The overall concordance rate for the two methods was 46.9% (98/209). Agreement of positive results between both methods was 9.0% (11/122). 111 samples had discrepant results; all of them were Seplex Pneumobacter ACE Detection Assay positive and in-house PCR negative. All negative results with Seplex Pneumobacter ACE Detection assay were also negative with in-house multiplex PCR specific to *S. pneumoniae*. All positive results with in-house multiplex PCR specific to *S. pneumoniae* were

TABLE 3 - Detection of *S. pneumoniae* in throat swabs of patients by Seplex PneumoBacter ACE Detection assay and in-house multiplex PCR.

	No. of samples (%)	In-house PCR for <i>S. pneumoniae</i>	
		Positive	Negative
Seplex PneumoBacter ACE Detection assay	Positive	11 (5,3)	111 (53,1)
	Negative	0 (0)	87 (41,6)

also positive with Seeplex Pneumobacter ACE Detection assay (Table 3).

Results of in-house multiplex PCR specific to *S. pneumoniae* were used to calculate the specificity and sensitivity of Seeplex Pneumobacter ACE Detection assay for *S. pneumoniae*. Calculated specificity was 64% and sensitivity was 100%.

DISCUSSION

Primarily to improve the detection of causative agents of atypical community-acquired pneumonia, namely *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* we evaluated the commercially available PCR test Seeplex PneumoBacter ACE Detection assay. The assay is a qualitative in vitro test for the detection of above mentioned pathogens as well as *S. pneumoniae*, *H. influenzae* and *B. pertussis*. During initial evaluation of Seeplex PneumoBacter ACE Detection assay on various respiratory samples before intended introduction into routine laboratory use we unexpectedly found a high rate of positive results for *S. pneumoniae*. To evaluate the performance and accuracy of the Seeplex PneumoBacter ACE Detection assay we designed a study including known bacterial strains as well as throat swabs from adult patients with LRTI and a low probability of pneumococcal infection. For the purpose of comparison we used an in-house multiplex PCR test targeting *lytA* and *cpsA* genes.

When evaluating the performance and accuracy of a PCR test, cultivation would normally be used as a "gold standard". With known limitations to pneumococcal cultivation and differentiation from closely related alpha-haemolytic streptococci which are found in abundance in respiratory samples, we opted for an in-house PCR test using a reliable set of primers as a comparison. Over the years various primers have been used for *S. pneumoniae* detection showing various levels of success (Corless *et al.*, 2001; Verhelst *et al.*, 2003; Prere and Fayet, 2011). It has been shown that commonly used target genes *ply* and *lytA* are also present in closely related species of the viridans streptococcal group such as *S. mitis*. Judging from previous studies *ply* appeared to be an unspecific PCR

target gene for *S. pneumoniae* detection while *lytA*, Spn9802, Spn9828, MFP1029, MFP1048, *gyrB* and *cpsA* showed high specificity (Cvitkovic Spik *et al.*, 2013; Luo *et al.*, 2012; Verhelst *et al.*, 2003; Teles *et al.*, 2011; Kim *et al.*, 2013; Abdeldaim *et al.*, 2008; Messmer *et al.*, 2004; Mackenzie *et al.*, 2010). According to published studies, both *cpsA* and *lytA* primer sets should have excellent sensitivity and specificity. Park *et al.* showed that *cpsA*-specific primer set differentiated *S. pneumoniae* from *S. pseudopneumoniae* (2010). These two species are very closely related and their 16S rRNA gene sequences are almost identical, with a difference of only 5 bp, corresponding to 99.7 % identity (Park *et al.*, 2010). Messmer *et al.* showed the *lytA* PCR to be 100% specific, amplifying all encapsulated and nontypable pneumococci with no cross-reaction to any other strain, including reference and clinical viridans strains, optochin-positive and bile soluble viridans strains (2004). McAvin *et al.* (2001) also reported a 100% specificity for *lytA* primer set using real-time fluorescence PCR (2001). We chose to use *lytA* and *cpsA* primer sets and postulated that both primers should give a positive result for *S. pneumoniae* identification. Although we had 5 false positive amplifications with *lytA* primer sets when combined amplification results for both primer sets were analyzed the specificity of our in-house PCR test was 100%.

Besides cross-reactivity with closely related viridans streptococci from the oropharyngeal flora, there is also a problem of colonization of *S. pneumoniae* in the respiratory tract when using PCR as a diagnostic tool for timely and accurate diagnosis of LRTI. On initial assessment of the Seeplex PneumoBacter ACE Detection assay before putting it into regular diagnostic use we detected a high rate of positive results for *S. pneumoniae* (>60%, data not shown). Since the initial assessment of the assay performance was performed in a group of patients with a low clinical probability of *S. pneumoniae* infection the results implied a high rate of colonization of *S. pneumoniae* in this adult group of patients. Mackenzie *et al.* conducted a survey of nasopharyngeal carriage of *S. pneumoniae* among adults and children in Aboriginal rural communities (2010). Pneumococcal carriage prevalence was 26% among adults and

67% among children. Le Polain de Waroux *et al.* conducted a systematic review of studies providing carriage estimates across age groups in healthy populations not previously exposed to pneumococcal conjugate vaccine (2014). The *S. pneumoniae* carriage rate in the age group >18 years was between 0 and 27% with only one study showing a high carriage rate of 55%. We suspected that the high rate of positive results for *S. pneumoniae* found in our group of patients could be at least partially due to false positive results.

Our suspicion was confirmed when in-house multiplex PCR specific to *S. pneumoniae* and Seeplex PneumoBacter ACE Detection assay were used on throat swabs. Ninety percent of throat swabs positive with the commercial PCR test were negative with our in-house multiplex PCR specific to *S. pneumoniae*. According to these results, the calculated specificity of Seeplex PneumoBacter ACE Detection assay was 64%.

Although Seeplex PneumoBacter ACE Detection assay was developed for timely detection of most common pathogens causing LRTI, the low specificity for *S. pneumoniae* makes the results for this specific pathogen irrelevant for clinicians although performance for *M. pneumoniae*, *C. pneumoniae* and *L. pneumophila* are of high reliability according to our experience (data not shown). Our findings suggest that the combination of *cpsA* and *lytA* specific primers can discriminate *S. pneumoniae* from closely related viridans group streptococci and can be used for the diagnosis and identification of presumptive *S. pneumoniae* isolates. The clinical benefit of in-house PCR for timely diagnosis of community-acquired pneumonia is being evaluated in an ongoing study.

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