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FULL PAPER

# Improved laboratory diagnostics of *Streptococcus pneumoniae* in respiratory tract samples through qPCR

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SUMMARY

The aim of this study was to test the detection performance of the *cpsA*, *lytA* and *ply* genes through qPCR in the identification of *Streptococcus pneumoniae* in respiratory tract samples. Specificity was tested on a panel of 128 streptococci and other bacteria DNA samples. The qPCR assay was tested on a total of 51 respiratory tract samples from patients with community-acquired pneumonia (CAP). The specificity of the *cpsA*, *lytA* and *ply* genes was 100%, 100%, and 86%, respectively. The quantitative assessment, based on *lytA*, determined a cutoff value of ~$2x10^4$, $4x10^2$ and $4x10^2$ DNA copies per 1 mL of valid sputum, tracheal aspirate and bronchial aspirate samples, respectively. The results from the present study suggest that qPCR detection of all three genes would be optimal in the accurate detection of *Streptococcus pneumoniae*.

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## INTRODUCTION

*Streptococcus pneumoniae* is responsible for most cases of community-acquired bacterial pneumonia worldwide; moreover, it is an important etiologic agent of bacterial sinusitis, acute otitis media, bacteraemia, and meningitis (O'Brien *et al*., 2009; Wald, 2011; Garcia-Vidal *et al*., 2012; Strålin *et al*., 2014; Jensen *et al*., 2016). Infections with *S. pneumoniae* are a major threat for public health, especially among children under 5 years of age and in older adults (O'Brien *et al*., 2009; Azzari *et al*., 2011). The accurate identification of *S. pneumoniae* in respiratory samples is clinically important as well as beneficial, since it would enable the correct implementation of targeted antibiotic therapy. Failure to identify *S. pneumoniae* as the etiologic agent of community-acquired pneumonia (CAP) may have serious consequences for the patient, *e.g.* receiving inadequate antibiotics, which also makes treatment expensive (Blaschke, 2011).

The detection of *S. pneumoniae* commonly relies upon microbiological culture; however, its isolation can be negatively influenced by various factors. In contrast, PCR based techniques are able to shorten the time required for its detection and identification besides increasing sensitivity (Park *et al*., 2010; Strålin *et al*., 2014; Lang *et al*., 2015). Despite the advances made in this regard, confirming the etiology of pneumococcal infections from respiratory tract samples remains a complicated affair due to the presence of viridans streptococci, which are genotypically close to pneumococci and thus are difficult to differentiate from each other (Arbique *et al*., 2004; Richter *et al*., 2008). *S. pneumoniae* is classified within the mitis group of streptococci, which currently comprises 20 species including, among others, *S. mitis*, *S. pseudopneumoniae*, *S. oralis* and *S. infantis* (Strålin *et al*., 2014; Jensen *et al*., 2016). Interestingly, these species exhibit more than 99% of homology in the 16S rRNA gene (Arbique *et al*., 2004; Richter *et al*., 2008; Sholz *et al*., 2012; Marín *et al*., 2017; van Prehn *et al*., 2016).

Several PCR based assays have been developed to improve the detection of *S. pneumoniae*. However, these assays are mostly based on the detection of a single gene, *i.e. ply* (Corless *et al*., 2001) or *lytA* (Strålin *et al*., 2005). Unfortunately, these genes can also be found in other species within the mitis group, most notably in *S. mitis* or *S. pseudopneumoniae* (Whatmore *et al*., 2000; Greiner *et al*., 2001; Yang *et al*., 2005; Arbique *et al*., 2004; Strålin *et al*., 2005; Carvalho *et al*., 2007; Azzari *et al*., 2011; Simões *et al*., 2016).

The capsular polysaccharide biosynthesis gene, *cpsA*, is a useful marker in the detection of *S. pneumoniae*, especially because it should be absent in other streptococci species (Park *et al*. 2010). The assumption that all virulent pneumococci are encapsulated enhances the reliability of the *cpsA* gene; however, some *S. pneumoniae* strains, mainly those isolated from conjunctivitis or otitis media samples, may be non-encapsulated (Keller *et al*. 2016).

*Key words:*
*Streptococcus pneumoniae*, qPCR, specificity, quantification, community-acquired pneumonia.

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The accurate identification of this etiologic agent is an essential step in avoiding the use of broad-spectrum antimicrobials. Therefore, the aim of the present study was to evaluate the accurate detection of *S. pneumoniae* in respiratory tract samples by qPCR targeting the genes *cpsA*, *lytA*, and *ply*.

## MATERIALS AND METHODS

### *Bacterial strains*

A total of 128 bacterial strains were used in this study (*Table 1*). Reference *Streptococcus* strains (n = 19) were obtained from the Czech National Collection of Type Cultures (CNCTC) in Prague. Clinical *Streptococcus* isolates (n = 97) were obtained from respiratory samples (nasopharyngeal swabs, laryngeal swabs, sputum, tracheal aspirate, bronchoalveolar lavage, and pleural puncture) or from cerebrospinal fluid and blood. Eleven non-streptococcal species (*Table 1*) representing other bacteria associated with respiratory tract infections were added to test the specificity of the assay. The identification of bacterial isolates was performed using the Microflex MALDI-TOF MS platform and the software FlexControl v3.4 (Bruker Daltonics, Bremen, Germany). An identification score of >2.0 was considered reliable to determine genus and species. The identification and differentiation of viridans streptococci from pneumococci was confirmed with bile solubility and optochin susceptibility tests.

### *DNA extraction*

Nucleic acids were isolated directly from the suspension of bacterial strains, respiratory tract samples, or blood and cerebrospinal fluid using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the protocol provided by the manufacturer.

**Table 1** - *qPCR detection of the* cpsA, lytA *and* ply *genes in different streptococci and other bacterial species.*

| *Group* | *Species* | *Genes* | | | |
|---|---|---|---|---|---|
| | | *n* | *cpsA* | *lytA* | *ply* |
| *Streptococcus mitis* group n = 79 | *S. gordonii* | 4 | 0 | 0 | 0 |
| | *S. gordonii* ATCC 10558 | 1 | 0 | 0 | 0 |
| | *S. infantis* | 1 | 0 | 0 | 0 |
| | *S. massiliensis* | 1 | 0 | 0 | 0 |
| | *S. mitis* | 30 | 0 | 0 | 13 |
| | *S. mitis* ATCC 9811 | 1 | 0 | 0 | 0 |
| | *S. oralis* | 7 | 0 | 0 | 0 |
| | *S. oralis* ATCC 10557 | 1 | 0 | 0 | 0 |
| | *S. parasanguinis* | 8 | 0 | 0 | 0 |
| | *S. parasanquinis* ATCC 15912 | 1 | 0 | 0 | 0 |
| | *S. peroris* | 2 | 0 | 0 | 0 |
| | *S. pneumoniae* | 16 | 16 | 16 | 16 |
| | *S. pneumoniae* CNCTC Pn47A 84/74 | 1 | 1 | 1 | 1 |
| | *S. pseudopneumoniae* | 1 | 0 | 0 | 0 |
| | *S. pseudopneumoniae* ATCC BAA-960 | 1 | 0 | 0 | 1 |
| | *S. sanguinis* | 2 | 0 | 0 | 0 |
| | *S. sanguinis* ATCC 10556 | 1 | 0 | 0 | 0 |
| *Streptococcus anginosus* group n = 7 | *S. anginosus* | 2 | 0 | 0 | 0 |
| | *S. anginosus* ATCC 27823 | 1 | 0 | 0 | 0 |
| | *S. constellatus* | 2 | 0 | 0 | 0 |
| | *S. constellatus* ATCC 27823 | 1 | 0 | 0 | 0 |
| | *S. intermedius* | 1 | 0 | 0 | 0 |
| *Streptococcus salivarius* group n = 11 | *S. salivarius* | 7 | 0 | 0 | 0 |
| | *S. salivarius* ATCC 27945 | 1 | 0 | 0 | 0 |
| | *S. vestibularis* | 3 | 0 | 0 | 0 |
| *Streptococcus bovis* group n = 4 | *S. alactolyticus* | 1 | 0 | 0 | 0 |
| | *S. bovis* ATCC 33317 | 1 | 0 | 0 | 0 |
| | *S. gallolyticus* | 1 | 0 | 0 | 0 |
| | *S. lutetiensis* | 1 | 0 | 0 | 0 |

n – number of tested strains.
All species, except the reference strains (marked ATCC or CNCTC), come from clinical isolates.

**Table 1** - *continued: qPCR detection of the* cpsA, lytA *and* ply *genes in different streptococci and other bacterial species.*

| *Group* | *Species* | *Genes* | | | |
|---|---|---|---|---|---|
| | | *n* | *cpsA* | *lytA* | *ply* |
| *Streptococcus mutans* group n = 2 | *S. mutans* ATCC 25175 | 1 | 0 | 0 | 0 |
| | *S. sobrinus* ATCC 33478 | 1 | 0 | 0 | 0 |
| Other viridans streptococci n = 4 | *S. minor* | 1 | 0 | 0 | 0 |
| | *S. uberis* | 1 | 0 | 0 | 0 |
| | *S. urinalis* | 1 | 0 | 0 | 0 |
| | *S. suis* ATCC 43765 | 1 | 0 | 0 | 0 |
| Beta-hemolytic streptococci n = 10 | *S. agalactiae* | 2 | 0 | 0 | 0 |
| | *S. agalactiae* ATCC 12386 | 1 | 0 | 0 | 0 |
| | *S. canis* ATCC 43496 | 1 | 0 | 0 | 0 |
| | *S. dysgalactiae* | 2 | 0 | 0 | 0 |
| | *S. dysgalactiae* ATCC 35666 | 1 | 0 | 0 | 0 |
| | *S. pyogenes* | 1 | 0 | 0 | 0 |
| | *S. pyogenes* CNCTC Str 6/58 | 1 | 0 | 0 | 0 |
| | *S. iniae* ATCC 29178 | 1 | 0 | 0 | 0 |
| Other bacteria n = 11 | *Moraxella catarrhalis* | 1 | 0 | 0 | 0 |
| | *Klebsiella pneumoniae* | 1 | 0 | 0 | 0 |
| | *Staphylococcus epidermidis* | 1 | 0 | 0 | 0 |
| | *Haemophilus parainfluenzae* | 1 | 0 | 0 | 0 |
| | *Staphylococcus aureus* | 1 | 0 | 0 | 0 |
| | *Haemophilus influenzae* | 1 | 0 | 0 | 0 |
| | *Pseudomonas aeruginosa* | 1 | 0 | 0 | 0 |
| | *Mycoplasma pneumoniae*[1] | 1 | 0 | 0 | 0 |
| | *Chlamydia pneumoniae*[1] | 1 | 0 | 0 | 0 |
| | *Legionella pneumophila* | 1 | 0 | 0 | 0 |
| | *Escherichia coli* | 1 | 0 | 0 | 0 |

n – number of tested strains.
All species, except the reference strains (marked ATCC or CNCTC), come from clinical isolates.
[1]DNA obtained from an independent International External Quality Assessment QCMD.

### *Primers and probes*

qPCR assays were performed in a volume of 25 µL containing the universal 2x gb IPC PCR Master Mix (Generi Biotech, Czech Republic), 400 nM primers, and dual labeled hydrolysis TagMan® probes (FAM-BHQ1) at a concentration of 200 nM; these assays included an internal positive control. Primers and probes (Generi Biotech; Czech Republic) targeting *cpsA*, *lytA* and *ply* are listed in *Table 2*. The amplification parameters were as follows: 95°C/5 min; followed by 95°C/15 s, and 60°C/ 30 s (45 cycles). The assays were performed in a Rotor-Gene Q thermal cycler (Qiagen, Hilden, Germany). The obtained data was analyzed using the Rotor-Gene Q Series Software v1.7.

### *Quantification and calibration curve*

Cloned sequences of the *cpsA*, *lytA* and *ply* genes were used in the calibration curve to determine the absolute number of DNA copies. A total of $10^7$ copies/µL from each *cpsA*, *lytA* and *ply* plasmid clones were obtained from Generi Biotech (Czech Republic). Standard calibration curves were obtained for each of the targeted genes in serial 10-fold dilutions. The cycle threshold values (Ct) from the standard calibration curves are shown in *Table 4*.

### *qPCR performance in clinical samples*

The detection capacity of *S. pneumoniae* through qPCR analysis of *cpsA*, *lytA* and *ply* was tested on respiratory samples from patients with community-acquired pneumonia (n = 51), whose criteria included a clinical diagnosis based on chest radiographs, the presence of significant bacterial pathogens in blood and/or bronchoalveolar lavage fluid, bronchial aspirate, tracheal aspirate or valid sputum and/or pneumococcal or legionella urinary antigen and inflammation markers (CRP >40 mg/L, >25 leukocytes per low magnification field x100).

### *Data analysis*

The qPCR analyses of *cpsA*, *lytA* and *ply* from a panel of 128 bacterial strains were compared by determining the parameters of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) (Jennings *et al.*, 2009). $Log^{10}$ copies/mL of *lytA* in the clinical samples were used in the statistical calculations. The software Graphpad (v8.1.2) was used to analyze and compare the receiver operating characteristic (ROC) curves and determine the cutoff values.

## RESULTS

### *Specificity test*

The specificity test included the detection of *cpsA*, *lytA* and *ply* in 16 *S. pneumoniae* clinical isolates and from one reference strain (CNCTC Pn47A 84/74), resulting positive for all genes. We used strains isolated from different respiratory tract samples or from cerebrospinal fluid and blood to encompass the variability of *S. pneumoniae*. The negative specificity of the targeted genes was also tested on 111 non-pneumoniae isolates (*Table 1*). The observed non-pneumoniae viridans streptococci were mainly of the mitis group, followed by anginosus, salivarius or bovis groups (*Table 1*). The qPCR was negative for *cpsA* and *lytA* genes in 111 non-pneumoniae isolates; however, *ply* was positive in 13 (43%) and in 2 (100%) of the tested *S. mitis* and *S. pseudopneumoniae* strains, respectively, thus showing its low specificity in the detection of *S. pneumoniae*.

The specificity, positive predictive value (PPV) and negative predictive value (NPV) of the qPCR tests are shown in *Table 3*. The results from both negative specificity and NPV indicated clear differences among the three tested genes. However, the low specificity (86%) and low NPV (50%) were determined only for *ply*.

### *Detection of Streptococcus pneumoniae*

The algorithm used in the detection of *S. pneumoniae* was based on qPCR analysis of *cpsA*, *lytA* and *ply* (*Table 5*). The detection of *cpsA*, *lytA* and *ply* was considered as clear positive presence of *S. pneumoniae*, thus indicating that these genes should not be amplified at all when *S. pneumoniae* is absent. Some samples may show an inconclusive result, thus requiring a repeat of the test and/or careful cross-examination with other laboratory tests and the clinical status of the patient.

### *Quantitation and calibration curve*

The detection limit for *S. pneumoniae* DNA through qPCR was calculated with the serial dilution of plasmids carrying the target sequence of the *cpsA*, *lytA* and *ply* genes; the

**Table 2** - *Primers and probes.*

| *Name* | *Sequence (5' to 3')* | *Amplicon size* | *Reference* |
|---|---|---|---|
| *cpsA* - F | ATG CGA CTT CTA ATT ACT CAG | | |
| *cpsA* - R | CAT TAT CAG TCC CAG TCG GT | 114 bp | Lang *et al.*, 2015 |
| *cpsA* - probe | FAM-ACG CAA CTG ACG AGT GTG AC-BHQ1 | | |
| *lytA* - F | ACG CAA TCT AGC AGA TGA AGC A | | |
| *lytA* - R | TCG TGC GTT TTA ATT CCA GCT | 75 bp | Morais *et al.*, 2007 |
| *lytA* - probe | FAM-TGC CGA AAA CGC TTG ATA CAG GGA G-BHQ1 | | |
| *ply* - F | TGCAGAGCGTCCTTTGGTCTAT | | |
| *ply* - R | CTCTTACTCGTGGTTTCCAACTTGA | 81 bp | Corless *et al.*, 2001 |
| *ply* - probe | TGGCGCCCATAAGCAACACTCGAA | | |

Abbreviations: 6-carboxyfluorescein (FAM), Black hole quencher1 (BHQ1).

**Table 3** - *Sensitivity, specificity, PPV and NPV of the cpsA, lytA and ply genes.*

| *Gene* | *Sensitivity (%)* | *Specificity (%)* | *NPV (%)* | *PPV (%)* |
|---|---|---|---|---|
| *ply* | 100 | 86 | 50 | 100 |
| *lytA* | 100 | 100 | 100 | 100 |
| *cpsA* | 100 | 100 | 100 | 100 |

**Table 4** - *Ct values from a standard calibration curve.*

| *Plasmid DNA (copies per 1 µL)* | *DNA concentration (ng/µL)* | *cpsA Ct (mean ± SE)* | *lytA Ct (mean ± SE)* | *ply Ct (mean ± SE)* |
|---|---|---|---|---|
| $10^7$ | $2.2 \times 10^1$ | 15.28 ± 0.04 | 14.68 ± 0.16 | 16.12 ± 0.23 |
| $10^6$ | $2.2 \times 10^0$ | 18.51 ± 0.08 | 18.33 ± 0.11 | 19.60 ± 0.26 |
| $10^5$ | $2.2 \times 10^{-1}$ | 21.58 ± 0.05 | 21.31 ± 0.05 | 22.66 ± 0.31 |
| $10^4$ | $2.2 \times 10^{-2}$ | 25.26 ± 0.15 | 24.76 ± 0.05 | 26.51 ± 0.37 |
| $10^3$ | $2.2 \times 10^{-3}$ | 28.41 ± 0.14 | 28.29 ± 0.22 | 29.83 ± 0.13 |
| $10^2$ | $2.2 \times 10^{-4}$ | 32.85 ± 0.05 | 31.66 ± 0.36 | 32.94 ± 0.16 |
| $10^1$ | $2.2 \times 10^{-5}$ | 35.16 ± 0.47 | 35.41 ± 0.30 | 37.02 ± 0.05 |
| $10^0$ | $2.2 \times 10^{-6}$ | 41.28 ± 0.46 | 40.07 ± 0.68 | 39.59 ± 0.83 |
| negative control | – | 0 | 0 | 0 |

SE – standard error.

**Table 5** - *Algorithm used for the detection of Streptococcus pneumoniae based on qPCR assay (*cpsA, lytA *and* ply*).*

| *cpsA* | *lytA* | *ply* | *Evaluation of S. pneumoniae detection* |
|---|---|---|---|
| + | + | + | Positive |
| – | – | – | Negative |
| – | – | + | Negative |
| – | + | – | Negative |
| – | + | + | Inconclusive or non-encapsulated *S. pneumoniae* |
| + | – | + | Inconclusive or *lytA* negative *S. pneumoniae* |
| + | – | – | Negative or repeat the test |

Ct values of these serial dilutions are shown in *Table 4*. The minimal detection limit, calculated based in the number of copies per 1 µL, was of ~100 copies per mL. After evaluating the calibration curves, we selected the *lytA* gene for the quantification of *S. pneumoniae* DNA in clinical samples. Although *cpsA* would appear as more reliable, 1 in 17 confirmed *S. pneumoniae* strains displayed decreased positivity for this gene (>100x) in comparison with the other tested genes.

*qPCR detection of cpsA, lytA and ply in clinical samples*

In this study, we evaluated the accuracy of qPCR in the detection of *S. pneumoniae* by targeting the *cpsA*, *lytA* and *ply* genes in non-sterile clinical samples (*e.g.* sputum, tracheal aspirate, bronchial aspirate or bronchoalveolar lavage) from patients with CAP (n = 51), finding the presence of *S. pneumoniae* in 32 (63%) samples. Further, *S. pneumoniae* was identified by qPCR in 15 (29%) clinical culture samples (*i.e.,* respiratory samples and blood) and/or by urinary antigen. Pneumococcal pneumonia was confirmed in 26 of 32 *S. pneumoniae* DNA positive samples, 6 of which were evaluated as carriage due to the detection of another significant pathogen (*Table 6*). The ROC analysis predicting pneumococcal pneumonia using $\log^{10}$ DNA copies/mL of *lytA* in sputum, tracheal aspirate and bronchial aspirate samples revealed a cutoff value of ~$2x10^4$, $4x10^2$ and $4x10^2$ DNA copies per 1 mL of sample, respectively. Bronchoalveolar lavage samples were not included in the calculation of the cutoff value because of the low number of available samples.

## DISCUSSION

In the present study, the detection of *cpsA, lytA and ply* by qPCR showed the potential of this assay in the identification of *S. pneumoniae* through specificity, sensitivity, PPV, and NPV testing; further, it allowed us to determine a cutoff value for clinical samples such as sputum, and tracheal and bronchial aspirate samples. Previously, the identification of *S. pneumoniae* relied on the detection of single genes encoding capsule biosynthesis proteins (*e.g., cpsA*) (Park *et al*., 2010; Lang *et al*., 2015) or other virulence factors, such as autolysin (*lytA*) (Strålin *et al*., 2005; Greve and Møller, 2012; Simões *et al*., 2016) or pneumolysin (*ply*) (Corless *et al*., 2001; Kais *et al*., 2006; Abdeldaim *et al*., 2009); however, this approach may be problematic due to potential misidentification (Scholz *et al*., 2012).

On the other hand, the qPCR revealed the absence of the *cpsA* gene in all of the 111 non-pneumoniae isolates. As previously demonstrated, *cpsA* was detected only in *S. pneumoniae* isolates, including the reference strain (Park *et al*., 2010; Lang *et al*., 2015). The suitability of qPCR in the detection of *cpsA* gene as a discriminatory tool is strongly supported by its specificity, NPV, and PPV (*Table 3*), particularly because this capsular polysaccharide, essential for pneumococcal virulence, is produced only by *S. pneumoniae* (Park *et al*., 2010). Further, targeting *cpsA* enables the accurate discrimination of *S. pneumoniae* from the other problematic and more abundant species within the mitis group, *e.g. S. mitis* (Park *et al*., 2010; Lang *et al*., 2015; Gillis *et al*., 2017). It must be mentioned that a possible pitfall in the detection of *S. pneumoniae* through *cpsA* could be the presence of non-encapsulated strains in the respiratory tract. However, such pneumococci strains are considered to be less virulent than their encapsulated counterparts (Keller *et al*., 2016); furthermore, these strains are relatively uncommon. A previous study by Park *et al*. (2014) reported only 0.6% non-encapsulated pneumococci from 14,328 strains causing invasive pneumococ-

**Table 6** - *Performance of the qPCR assay (*cpsA, lytA *and* ply*) on clinical respiratory samples (n = 51) for the detection of Streptococcus pneumoniae etiology in patients with community-acquired pneumonia in comparison with routine culture and urinary antigen testing.*

| | *S. pneumoniae qPCR positive CAP samples (n = 26)* | | | | *S. pneumoniae qPCR positive non-pneumococcal CAP samples (n = 6)* | | *S. pneumoniae qPCR negative non-pneumococcal CAP samples (n = 17)* | | *qPCR lytA based cutoff (copies/mL)* |
|---|---|---|---|---|---|---|---|---|---|
| *Specimen type* | *n* | *Quantity range (copies/µl)* | *Median (copies/µl)* | *Culture and urinary antigen positivity* | *n* | *Culture and urinary antigen positivity* | *n* | *Culture and urinary antigen positivity* | |
| Sputum | 7 | $5x10^3$ – $1x10^8$ | $10^7$ | 2 | 2 | 0 | 11 | 0 | $2x10^4$ |
| Tracheal aspirate | 8 | $1x10^4$ – $3x10^9$ | $10^8$ | 6 | 1 | 0 | 1 | 0 | $4x10^2$ |
| Bronchial aspirate | 6 | $1x10^4$ – $3x10^8$ | $10^6$ | 5 | 1 | 0 | 2 | 0 | $4x10^2$ |
| Bronchoalveolar lavage | 5 | $3x10^2$ – $3x10^4$ | $10^3$ | 2 | 2 | 0 | 3 | 0 | ND |

Detection of *S. pneumoniae* DNA based on positivity for cpsA, lytA and ply genes.
Quantification of *S. pneumoniae* DNA based on a plasmid calibration curve for the lytA gene.
Results of two discrepant samples are discussed in Discussion section and are not mentioned in the Table 6.
ND – not determined.

cal disease. Regardless, the percentage of non-encapsulated strains from non-invasive infections is higher. Pneumococci isolated from conjunctivitis samples were reported as non-encapsulated in up to 90% and from otitis media in up to 8% (Keller *et al*., 2016). Interestingly, two groups of non-encapsulated pneumococci have been reported, the isolates from group I containing a conventional, although defective, *cps* locus; on the other hand, group II lacks the *cpsA* gene entirely, thus showing as a false negative during qPCR detection (Hathaway *et al*., 2004; Keller *et al*., 2016; Langereis and Jonge, 2017).

In contrast, we observed a low specificity for *ply* (NPV - 50%), as revealed by the detection of this gene in a surprisingly high number - 13 (43%) out of 30 tested *S. mitis* strains (*Table 1*), a similar result that has been previously reported as well in *S. pseudopneumoniae* (Carvalho *et al*. 2007). Therefore, this gene has low specificity and poor NPV in the detection of *S. pneumoniae*. The detection of *ply* was, however, initially designed to detect *S. pneumoniae* in primary sterile samples, mainly cerebrospinal fluid (Corless *at al*., 2001). Taken together, the detection of *ply* alone is inappropriate when determining the presence of pneumococci in the respiratory tract. The results of the present study are consistent with previous documentation regarding the non-specificity of *ply*, indicating that both *S. mitis* and *S. pseudopneumoniae* may carry this gene, thus complicating the identification of *S. pneumoniae* (Whatmore *et al*., 2000; Messmer *et al*., 2004; Carvalho *et al*., 2007; Abdeldaim *et al*., 2009; Greve and Møller, 2012).

The detection of *lytA* in the present study showed excellent results regarding specificity, sensitivity, NPV and PPV (*Table 3*), thus having the potential to accurately identify *S. pneumoniae*, an observation that is consistent with other studies (Strålin *et al*., 2005; Greve and Møller, 2012; Hajia *et al*., 2014; Strålin *et al*., 2014). However, the presence of *lytA* in the relatively novel species *S. pseudopneumoniae*, and even in *S. mitis*, has been repeatedly reported, suggesting that the detection of this gene alone should be avoided in the identification of *S. pneumoniae* (Arbique *et al*., 2004; Strålin *et al*., 2005; Carvalho *et al*., 2007; Azzari *et al*., 2010; Simões *et al*., 2016). Moreover, Greve and Møller (2012) and Simões *et al*. (2016) reported *lytA* negative strains of *S. pneumoniae*. In our study, the positivity of *lytA* in *S. pseudopneumoniae* was not confirmed; however, it must be mentioned that the number of available strains in this study was limited to only two (*Table 1*).

The specificity analysis of *cpsA*, *lytA* and *ply* in other bacterial strains revealed a high positivity incidence of *ply* in *S. mitis* isolates (*Table 1*) as well as in non-sterile respiratory tract samples, being detected in 9 (53%) out of 17 *S. mitis* culture positive and pneumococci negative respiratory tract samples (unpublished results). This data confirms that the presence of *S. mitis* and *S. pseudopneumoniae* could be a potential issue in the diagnostics of *S. pneumoniae* since these species also can carry the *ply* gene.

Based on these results, the identification of *S. pneumoniae* should rely on the detection of *cpsA*, *lytA* and *ply* as a whole to reduce the possibility of false positive/negative results, especially because the specificity of the targeted genes allows the accurate detection of pneumococcal DNA. The results from the detection algorithm based on the amplification of the *cpsA*, *lytA* and *ply* genes of *S. pneumoniae* are summarized in *Table 5*. The combined detection of *cpsA*, *lytA* and *ply* through qPCR is assumed to be beneficial, and while it is true that *ply* has a low negative predictive value, this is overcome by its positive predictive value in *S. pneumoniae* (100%). See *Table 3*.

The quantification of pneumococcal DNA should be of assistance when assessing the colonization status of the pathogen, mostly in the case of sputum samples and contaminated tracheal or bronchial aspirate samples. For comparison, older studies (Yang *et al*., 2005; Kais *al*., 2006) reported a cutoff value of $4x10^4$ in sputum samples and $10^5$ DNA genomic equivalents in 1 mL, respectively; however, this was based on *ply*, which has proven to be unreliable. In this regard, Nomanpour *et al*. (2011) showed a cutoff value of ~$10^3$ DNA genomic equivalents in 1 mL for various respiratory samples based on a conserved sequence in the choline binding protein A gene (*cbpA*). On the other hand, Saukkoriipi *et al*. (2017) used a predefined cutoff value of $10^4$ genomic equivalents in 1 mL, although it relied only on sputum samples.

We found two more discrepancies among these clinical samples (*Table 6*). The first was the positivity of *cpsA* and *ply* in the clinical samples and *S. pneumoniae* negativity in the clinical culture from a patient with secondary pneumonia after infection with influenza virus, suggesting the existence of an indeterminate result or *lytA* negative strain of *S. pneumoniae*. The second was the positivity of *lytA* and *ply* and negativity of *cpsA* in the sputum samples from an oncologic patient, although the presence of *S. pneumoniae* was strongly supported by a positive urinary antigen test. The culture from sputum samples allowed the identification of *S. pseudopneumoniae* (not included in the specificity test), which has been reported as a possible cause of false positive hits during a pneumococcal urinary antigen analysis (Keith *et al*., 2006; Eletu *et al*., 2017).

The results from the present study suggest that the detection of *cpsA*, *lytA* and *ply* enable the accurate identification of *S. pneumoniae* and its differentiation from *S. pseudopneumoniae* and *S. mitis*, which have a highly homogenous DNA sequence. In this regard, a pairwise comparison revealed 99.7% sequence homogeneity in the 16S rRNA gene, *i.e.,* a difference of only 5 bp between them (Arbique *et al*., 2004). Considering the high occurrence of *S. mitis* in respiratory tract samples and the relatively low pathogenic potential of *S. pseudopneumoniae*, although with a higher rate of antibiotic resistance (*e.g.,* penicillin, macrolides, trimethoprim/sulfamethoxazole, and tetracycline), the accurate identification of *S. pneumoniae* is, therefore, of utmost importance (Keith *et al*., 2006; Garriss *et al*., 2019).

The detection of *cpsA*, *lytA* and *ply* through qPCR has been introduced and rapidly implemented in our laboratory due to the increasing requirements of clinicians to identify *S. pneumoniae* in respiratory tract samples. It is not surprising, then, that a population of patients with high suspicion of pneumonia and/or in serious condition have received broad-spectrum antibiotics, or their combination, before the collection of samples. Moreover, the laboratory diagnostics of pneumococci is commonly based on culture methods, which have lower sensitivity and are made difficult when colonies are hidden among other bacteria with a tendency towards autolysis during the stationary phase, are not typically mucoid, or are not susceptible to optochin or are insoluble in bile (Blaschke, 2011). Therefore, the detection of pneumococcal DNA by targeting and amplifying the *cpsA*, *lytA* and *ply* genes remains a method of choice when the correct identification of *S. pneumoniae* is necessary for targeted penicillin therapy in CAP patients, especially since pneumococci resistance to penicillin is

very low in the Czech Republic (<5%) (Strålin *et al*., 2014; Torumkuney *et al*., 2018).

*S. pneumoniae* DNA detection is possible from valid sputum, bronchial and tracheal aspirate, or bronchoalveolar lavage samples. The results from *Table 6* suggest that the DNA detection of *S. pneumoniae* is beneficial mainly in sputum when compared to culture and antigen detection. Strålin *et al*. (2014) tested *lytA* and *Spn9802* fragment-based qPCR methods, reporting a high positivity level in the identification of pneumococcal DNA in sputum samples but only a moderate positivity level in nasopharyngeal aspirate samples. Nasopharyngeal samples may, therefore, be a relatively reliable alternative for patients who are unable to produce valid sputum (Strålin *et al*., 2014).

Our results notwithstanding, this study was not without limitations: not all pneumococcal strains are encapsulated; therefore, they may be *cpsA* negative (Greve and Møller, 2012; Park *et al*., 2014; Keller *et al*., 2016). Some pneumococci may be *lytA* negative (Simões *et al*., 2016); in such cases, it is necessary to make the diagnosis according the other findings in the patient with suspected CAP. The cutoff value could be another limitation, since it would be more appropriate to calculate it from a higher number of clinical samples.

## CONCLUSION

The identification of *S. pneumoniae* is frequently based on culture methods which are known to lack sensitivity, especially in patients under antibiotic treatment. On the other hand, the detection of *S. pneumoniae* through qPCR does not require viable bacteria. Regardless, the detection of *lytA* and *ply* could be problematic due to low specificity, especially when tested alone. The present study determined the specificity and sensitivity of qPCR tests targeting the *cpsA*, *lytA* and *ply* genes in the accurate detection of *S. pneumoniae*. We propose that the combination of these genes is optimal for its accurate detection and differentiation from other closely related species. Therefore, the combined detection of *cpsA*, *lytA* and *ply* through qPCR is now routinely used in our laboratory for the molecular identification of *S. pneumoniae* in different respiratory tract samples. Correct identification and determination of copy number would be a strong support when deciding to administer targeted antibiotic therapy in the treatment of community-acquired pneumococcal infections.

### Conflicts of interest

The authors declare no conflicts of interest.

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