A novel quantitative PCR assay for the detection of *Streptococcus pneumoniae* using the competence regulator gene target *comX*

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*Streptococcus pneumoniae* is responsible for an estimated 1.6 million deaths worldwide every year. While rapid detection and timely treatment with appropriate antibiotics is preferred, this is often difficult due to the amount of time that detection with blood cultures takes. In this study, a novel quantitative PCR assay for the detection of *Streptococcus pneumoniae* was developed. To identify novel targets, we analysed the pneumococcal genome for unique, repetitive DNA sequences. This approach identified *comX*, which is conserved and present in duplicate copies in *Streptococcus pneumoniae* but not in other bacterial species. Comparison with *lytA*, the current ‘gold standard’ for detection by quantitative PCR, demonstrated an analytic specificity of 100 % for both assays on a panel of 10 pneumococcal and 18 non-pneumococcal isolates, but a reduction of 3.5 quantitation cycle values (± 0.23 SEM), resulting in an increased analytical detection rate of *comX*. We validated our assay on DNA extracted from the serum of 30 bacteraemic patients who were blood culture positive for *Streptococcus pneumoniae* and 51 serum samples that were culture positive for other bacteria. This resulted in a similar clinical sensitivity between the *comX* and *lytA* assays (47 %) and in a diagnostic specificity of 98.2 and 100 % for the *lytA* and *comX* assays, respectively. In conclusion, we have developed a novel quantitative PCR assay with increased analytical sensitivity for the detection of *Streptococcus pneumoniae*, which may be used to develop a rapid bedside test for the direct detection of *Streptococcus pneumoniae* in clinical specimens.

INTRODUCTION

Despite available vaccines, the bacterial pathogen *Streptococcus pneumoniae* causes an estimated 1.6 million deaths every year (WHO, 2007) and remains a major cause of invasive disease as well as non-invasive infections. As the mortality rate of sepsis increases rapidly with each hour of delay in the initiation of antimicrobial therapy (7.6 % per hour of delayed treatment) (Kumar et al., 2006), broad-spectrum antibiotic treatment is administered as soon as possible. Conversely, targeted antimicrobial treatment instead of broad-spectrum antibiotics increases the survival rate from pneumococcal septic shock by more than twofold, from ~25 % to ~55 % (Kumar et al., 2009), and it has been
suggested that it might also increase the survival rate of patients with pneumococcal bacteraemia (Cremers et al., 2014). In addition, focused treatment is preferred in order to prevent antibiotic resistance. The current ‘gold standard’ for diagnosis of pneumococcal bacteraemia and sepsis is blood culture. Unfortunately, blood cultures generally take between 24 and 48 h to become positive, and antibiotic treatment is normally initiated before the results of blood cultures are known. Additionally, 55–65% of blood cultures remain negative in septic-shock cases (Heffner et al., 2010; Kumar et al., 2009). It is thought that this low sensitivity of blood cultures might be caused by antibiotic use prior to specimen collection, and, in the case of Streptococcus pneumoniae infections, possibly by the release of pneumococcal autolysin, resulting in cell death (Cvitkovic Spik et al., 2013; Resti et al., 2009). Taken together, there is a clear need for more rapid and sensitive molecular detection techniques.

Alternative strategies that could be used in the clinic to diagnose invasive pneumococcal disease are the urine antigen test (e.g. Binax-Now; Alere) or quantitative PCR (qPCR). Although the Binax-Now test is rapid, it has poor positive predictive value in population groups with high pneumococcal colonization rates, such as children, as carriage also leads to a positive test result (Dominguez et al., 2003; Dowell et al., 2001). In contrast, a recent study showed that pneumococcal DNA was not detected in the peripheral blood of individuals colonized by Streptococcus pneumoniae (Rouphael et al., 2011). Moreover, qPCR detection of pneumococcal DNA in blood can give positive results within 2 h, and can theoretically detect as little as one genome copy of DNA. It has been shown that at least one-third of culture-negative clinical samples that were analysed by 16S rRNA gene PCR analysis tested positive for pneumococcal DNA (Harris et al., 2008). As bacterial numbers in the blood of a sepsis patient can be as low as 10 c.f.u. (ml blood) \(^{-1}\) (Boardman et al., 2015; Loonen et al., 2013), it is important that the PCR assay is highly sensitive in order to detect the pathogen without a pre-culture step. Various studies have shown that qPCR has the potential to be a more sensitive detection method than traditional blood culture (Cvitkovic Spik et al., 2013; Resti et al., 2009), although the ability to detect the pathogen is also dependent on the stage of disease, antibiotic treatment (Resti et al., 2009) and the volume of blood that is used for culture or qPCR (Cvitkovic Spik et al., 2013; Isaacman et al., 1996; Loonen et al., 2013).

To date, several targets for qPCR-based detection of Streptococcus pneumoniae have been described, amplifying genes such as the autolysin gene (lytA; Carvalho et al., 2007), pneumolysin gene (ply; Selva et al., 2010), pneumococcal surface adhesion A gene (psaA; Carvalho et al., 2007), topoisomerase gene (parE; Tissari et al., 2010), spn 9802 gene fragment (Abdelaim et al., 2010) and 16S rRNA gene (Hansen et al., 2010). Of these targets, the lytA primer/probe set designed by Carvalho et al. (2007) has been tested most extensively in clinical studies (Cvitkovic Spik et al., 2013; Wu et al., 2013). This target has been shown not to cross-react with the recently described species Streptococcus pseudopneumoniae (Arbique et al., 2004), which is genetically very similar to Streptococcus pneumoniae and can be misidentified as atypical pneumococci with traditional identification methods (Leung et al., 2012; Rolo et al., 2013). Many of the other qPCR assays also give a positive result for Streptococcus pseudopneumoniae (Carvalho et al., 2007) and are therefore not specific for Streptococcus pneumoniae. Although it remains unclear what the exact clinical relevance of Streptococcus pseudopneumoniae is as a cause of sepsis, pneumococcal isolates do have a different antimicrobial resistance profile compared with Streptococcus pneumoniae (Keith et al., 2006; Laurens et al., 2012; Rolo et al., 2013). Therefore, correct identification of these two species is important for the appropriate administration of antibiotics.

All the targets described to date, with the exception of the 16S rRNA gene, are present in the genome as a single copy. A multicopy target is likely to increase the sensitivity of detection. Although the 16S rRNA gene is also present in multiple copies, due to the high similarity of the 16S rRNA gene between Streptococcus pneumoniae and other closely related streptococcal species (Kawamura et al., 1995), it is difficult to design probes and primers that specifically detect Streptococcus pneumoniae. In this study, we searched for a multicopy, conserved and species-specific DNA region in Streptococcus pneumoniae. Subsequently, the specificity and sensitivity of this assay were evaluated and compared with the lytA assay described by Carvalho et al. (2007). Finally, we evaluated our assay using clinical samples obtained from patients with bacteremic pneumococcal or non-pneumococcal infections.

**METHODS**

**Bacterial isolates.** Streptococcus pneumoniae TIGR4 was obtained from Professor H. Tettelin (Tettelin et al., 2001). The panel of bacterial isolates used to determine analytic intraspecies specificity consisted of 10 different serotypes (serotypes 1, 3, 4, 5, 6A, 7F, 8, 9V, 14 and 19A) of Streptococcus pneumoniae that were obtained from patients with pneumococcal bacteraemia. The panel to determine interspecies specificity consisted of Enterococcus faecalis (CDC NY 5), Enterococcus faecium (clinical isolate), Staphylococcus aureus (methicillin-resistant Staphylococcus aureus m2 and ATCC 29213), Staphylococcus epidermidis (ATCC 14990), Staphylococcus hominis (clinical isolate), Klebsiella oxytoca (ATCC 13182/DSM5175), Klebsiella pneumoniae (ATCC 13883), Pseudomonas aeruginosa (ATCC 10145), Escherichia coli (ATCC 11775), Acinetobacter baumannii (ATCC 19606/DSM 6974), Acinetobacter calcoaceticus (ATCC 23055), Acinetobacter genomospecies 13TU (ATCC 17903), Acinetobacter genomospecies 3 (ATCC 19004), Veillonella parvula (DSM 2008/ATCC 10790), Strepptococcus oralis DSM 20627 (ATCC 35037), Strepptococcus mitis DSM 12643 (ATCC 49456) and Strepptococcus pseudopneumoniae (ATCC BAA-960).

**Clinical specimens.** Two collections of serum samples were used for this study. The first consisted of sera from 30 patients, hospitalized at a Dutch hospital with a blood culture-proven pneumococcal bacteraemia, collected prior to antibiotic treatment. The second consisted of sera from 51 patients, hospitalized at a Dutch hospital, with bacteremia caused by a non-pneumococcal pathogen (see Table 2). Culture of the blood was initiated on the same day as collection of the serum. This study was approved by the Local Medical Ethics Committee of the Canisius-Wilhelmina Hospital.
Novel qPCR assay for the detection of *S. pneumoniae*

DNA extraction for qPCR analysis. DNA from *Streptococcus pneumoniae*, *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus pseudopneumoniae* was extracted using a modified protocol from the QiaGen DNA Mini kit. Bacterial pellets were incubated for 30 min at 37 °C in a chemical lysis buffer (20 mM Tris/HCl, pH 8.0, 2 mM sodium EDTA, 1.2 % Triton X-100) supplemented with 10 mg lysozyme ml⁻¹ (Merck), followed by a 30 min incubation at 56 °C with 1.5 mg proteinase K (Qiagen) ml⁻¹. The bacterial lysate was then processed according to the manufacturer’s instructions, eluted in 50 μl DNase-free water (Sigma) and stored at −20 °C until further use. DNA concentrations were determined by measuring the absorbance at 260 nm using a Nanodrop instrument (Thermo Scientific). DNA from other species was isolated and quantified as described previously (van den Brand et al., 2014).

Total DNA from the clinical samples was extracted from 200 μl thawed patient serum using a MagNA Pure 96 Instrument and a MagNA Pure 96 DNA and Viral NA Small Volume kit (Roche Diagnostics). Extracted DNA was eluted in 100 μl elution buffer, which was stored at −20 °C until further use. A positive control for the extraction consisted of phocine herpes virus (van Doornum et al., 2003), which was spiked into the samples prior to extraction and was assayed separately.

Design of comX primers and probe. For the identification of species-specific pneumococcal repeat regions, the online analysis tool MultiMPrimer was used (http://bioinfo.ut.ee/multimprimer3) (Korressaar et al., 2009). Seventeen pneumococcal strains (GenBank accession nos FQ312030, FQ312029, FQ312027, FM211187, CP002176, CP002121, CP001993, CP001033, CP001015, CP000936, CP000921, CP000920, CP000919, CP000918, CP000410, AE007317 and AE005672) of which the closed genome sequences were known were screened for repetitive regions and compared with a non-target database. This strategy identified a repeat region, designated comX (GenBank accession no. AE005672; SP_0014 and SP_2006), which was then checked against the NCBI nucleotide database using BLAST search (http://blast.ncbi.nlm.nih.gov) (Altschul et al., 1990). For the pneumococcus-specific oligonucleotide primers and a fluorescent dye-labelled probe design, comX gene sequences of 25 *Streptococcus pneumoniae* strains, two *Streptococcus pseudopneumoniae* strains and two *Streptococcus mitis* strains were aligned with CLUSTAL W (http://www.ebi.ac.uk). Primers and probe were designed according to TaqMan guidelines. A whole-genome shotgun BLAST search was performed on the NCBI website (http://blast.ncbi.nlm.nih.gov). All Blast searches were performed on 27 January 2015. Primer efficiencies were calculated using the formula: primer efficiency (percentage) = 10(1−(slope)−1) × 100.

qPCR for comX and lytA. The oligonucleotide primers and fluorescently labelled probes for the qPCR of lytA and comX are listed in Table 1.

The primer and probe sequences for lytA have been published previously (Carvalho et al., 2007). Amplicon lengths were 76 bp for lytA-CDC and 170 bp for comX. The PCR was carried out in a final 25 μl reaction volume and was performed in PCR buffer [50 mM KCl (Sigma BioUltra), 10 mM Trizma base (Sigma BioUltra), 200 nM dNTPs (Roche), 5 mM MgCl₂ (Sigma BioUltra), 0.5 mg BSA (Sigma) ml⁻¹, 0.39 % trehalose (Sigma) and 1 U FastStart Tag DNA polymerase (Roche)] with 10 μl template DNA. Primer and probe concentrations were 200 nM for both assays. In every run, a no-template control and a positive control (*Streptococcus pneumoniae* TIGR4), were included. PCR was performed on the 7500 Real-time PCR System (Applied Biosystems) with the following cycling parameters: 10 min at 95 °C, followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min. The data were analysed using 7500 Fast Systems Software (Applied Biosystems), with a threshold of 220 436 relative fluorescence units (RFU) for comX and 86 559 RFU for lytA.

Determination of analytical sensitivity and specificity. The MIQE guidelines for validation were followed (Bustin et al., 2009). For determination of the analytical sensitivity and the lower limit of detection (LLOD) of the comX and lytA PCRs, 10-fold serial dilutions of genomic DNA of *Streptococcus pneumoniae* (TIGR4) in the range of 1 fg to 100 pg per reaction were used, and experiments were performed in triplicate. In order to determine if the lower quantitation cycle (Cₚ) values of comX resulted in a higher analytical detection rate, the PCR containing 1 fg template DNA was repeated 39 times (in four independent runs). Determination of the analytical specificity was performed with DNA equivalent to 1000 c.f.u. and with 10 pg per reaction for *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus pseudopneumoniae*, to maximize the chances of detecting any false-positive results with the most closely related bacterial species. To determine the intraspecies analytical specificity for different pneumococcal serotypes, 10 pg DNA per reaction was used. qPCR on the clinical specimens was performed using a single run of 5 μl eluate from the DNA extraction.

Statistical analysis was performed using Graphpad Prism Software v.5.03. Paired t-tests were performed to determine the statistical significance between two groups of samples with detectable Cₚ values. The statistical significance threshold was set at P<0.05.

RESULTS

comX is a unique multicopy gene in *Streptococcus pneumoniae*

In order to develop a qPCR with increased sensitivity, we analysed the pneumococcal genome for unique repetitive DNA sequences. Using the online web tool MultiMPrimer, we...
identified competence regulator comX as a unique sequence for *Streptococcus pneumoniae*, of which two copies are present per pneumococcal genome. Oligonucleotide primers and a fluorescent dye probe were designed, and a BLAST search of the amplicon (170 bp) against all 27 published complete whole-genome sequences on the NCBI database showed that all genomes contained the amplicon in duplicate with a similarity of 90–93 %, indicating that this potential target is highly conserved among *Streptococcus pneumoniae*. For comparison, we also performed the same BLAST search with the amplicon of the lytA assay as described by Carvalho et al. (2007), hereafter referred to as lytA-CDC, for which we also found a coverage of 100 %.

To predict the specificity of our assay, we performed a BLAST search of the *comX* amplicon against the complete NCBI nucleotide database minus *Streptococcus pneumoniae*. This yielded hits only on *Streptococcus pseudopneumoniae* (93 % coverage) and *Streptococcus mitis* (92 % coverage). Since only one complete whole-genome sequence was present in the NCBI database, we also performed a BLAST search on the six whole-genome shotgun sequences of *Streptococcus pseudopneumoniae* that were present in the database. All six genomes contained a single-copy sequence similar to the *comX* amplicon with a similarity of 90–93 %. Further analysis showed that these sequences had at least two nucleotide differences within the *comX* forward primer binding site, three nucleotide differences with the *comX* probe and two nucleotide differences with the reverse primer. Similarly, for *Streptococcus mitis*, the 38 whole-genome shotgun sequences showed 87–95 % sequence identity to the *comX* amplicon. All these strains differed in at least one nucleotide from the *comX* forward primer and in two nucleotides from the *comX* probe.

In summary, in silico analysis showed that the *comX* target was present with two copies in all *Streptococcus pneumoniae* genome sequences present in the public domain. Although *Streptococcus pseudopneumoniae* and *Streptococcus mitis* also contained a single *comX*-like copy, nucleotide differences in the primer–probe binding regions for these two species suggested that the PCR would be specific.

### The comX qPCR assay detects *Streptococcus pneumoniae* with high analytical sensitivity

To determine the LLOD of the novel *comX* assay and the reference lytA-CDC assay, we performed qPCR on serial dilutions of genomic DNA from *Streptococcus pneumoniae* TIGR4. Both lytA-CDC and *comX* showed high sensitivity with an LLOD of 10 fg template DNA per reaction, equivalent to four genomic copies (calculated using the genomic weight of *Streptococcus pneumoniae* TIGR4). The *comX* PCR was more efficient, detecting its target at a significantly lower C<sub>q</sub> value (3.46 ± 0.23 SEM) than the lytA-CDC PCR (Fig. 1a), while primer efficiencies were comparable (97 and 103 %, respectively). Both qPCR assays were performed using different commercial PCR buffers and different magnesium concentrations, but the *comX* qPCR kept a lower C<sub>q</sub> value compared with the lytA assay, indicating that buffer conditions were not playing a role (results not shown).

**Fig. 1.** The novel target *comX* has a higher analytical sensitivity than lytA-CDC and has a similar primer efficiency. (a) Standard curve of the primer efficiency on *Streptococcus pneumoniae* genomic DNA (*n*=3). The primer efficiency for lytA-CDC (●) was 103 % and was 97 % for *comX* (▲). *comX* detected pneumococcal DNA at mean ± SEM C<sub>q</sub> values of 3.46 ± 0.23 lower than lytA-CDC. (b) Analytical detection rate at 1 fg *Streptococcus pneumoniae* TIGR4 genomic DNA per PCR compared with lytA-CDC (*n*=39). The dotted line indicates the detection limit (number of amplification cycles of the PCR). Statistics was performed with a paired t-test of samples that had detectable C<sub>q</sub> values. ***P<0.001.
To determine if the lower \( C_q \) values of the \( \text{comX} \) assay translated into an increased detection rate, we repeated the qPCR 39 times at a concentration 10 times lower than the LLOD (1 fg per PCR). PCR analysis showed that in 54% of cases (21/39) \( \text{comX} \) was detected, while \( \text{lytA-CDC} \) was detected in 36% of the repeat experiments (14/39). The mean of the difference in \( C_q \) value between the two assays at this concentration was 4.2 (\( P<0.0001 \)) (Fig. 1b). These results indicated that the lower \( C_q \) value at which the \( \text{comX} \) PCR detected its target indeed resulted in an increased analytical detection rate in comparison with the \( \text{lytA} \) qPCR.

**The \( \text{comX} \) qPCR assay is specific for *Streptococcus pneumoniae***

To evaluate the analytical specificity of the \( \text{comX} \) assay, qPCR was performed on genomic DNA of 18 different non-pneumococcal species, including the most common causative agents of sepsis. The most closely related species *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus pseudopneumoniae* were also included. No amplification was observed with any of the species of this panel, as was predicted based on our *in silico* analysis (see above), resulting in an analytic specificity of 100%. We also tested the performance of the \( \text{comX} \) assay on a panel of 10 different pneumococcal serotype strains. Amplification of genomic DNA from these strains showed that all serotypes gave similar \( C_q \) values (Fig. 2).

**The \( \text{comX} \) qPCR assay detects *Streptococcus pneumoniae* with equivalent clinical sensitivity as the \( \text{lytA} \) assay**

To evaluate the results of our new assay in a clinical setting, DNA was extracted from 200 \( \mu l \) serum of 30 blood-culture-positive pneumococcal bacteraemia patients who had not received antibiotics prior to sampling. qPCR was performed on the equivalent of 10 \( \mu l \) serum per PCR. Similar to our previous observations, the \( C_q \) values of the \( \text{comX} \) assay were significantly lower than those of the \( \text{lytA-CDC} \) assay (\( P=0.0006 \)) (Fig. 3). With both the \( \text{comX} \) and the \( \text{lytA-CDC} \) assays, 14 out of 30 culture-positive patients had a positive qPCR result, indicating that the clinical sensitivity of both assays was the same. The \( \text{comX} \) assay detected three patients that tested negative with the \( \text{lytA-CDC} \) PCR. Vice versa, the \( \text{lytA-CDC} \) PCR also detected three patients that tested negative with the \( \text{comX} \) PCR. It is important to note that these patients all had \( C_q \) values of >40 for the \( \text{comX} \) PCR and >43 for the \( \text{lytA-CDC} \) PCR, indicating that this was most likely caused by the presence of very low copy numbers of pneumococcal DNA. Agarose gel electrophoresis of the products showed that all samples with a \( C_q \) value >40 contained a product of the expected size, indicating that the detection for both assays was indeed specific (results not shown).

To determine the diagnostic specificity, the assay was also validated on a control group of sera from 51 patients that were blood culture positive for other bacteria (\( n=33 \) Gram-positive; \( n=18 \) Gram-negative) (Table 2). One serum sample (blood culture positive for *E. coli*) was

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**Fig. 2.** Intraspecies coverage of \( \text{comX} \) on clinical isolates. Coverage of \( \text{comX} \) and \( \text{lytA-CDC} \) of clinical isolates of different pneumococcal serotypes using 10 \( \mu g \) template DNA per PCR. Neg, Negative control.

**Fig. 3.** Detection of pneumococcal DNA in sera of culture-proven pneumococcal bacteraemia patients. DNA was isolated from the serum of 30 patients, obtained prior to antibiotic treatment. Real-time PCR with the \( \text{comX} \) (\( \triangle \)) and \( \text{lytA-CDC} \) (\( \circ \)) assays was performed with an input equivalent to 10 \( \mu l \) serum. \( \triangle \), Samples that were detected with \( \text{comX} \) but not with \( \text{lytA-CDC} \); \( \circ \), samples that were detected with \( \text{lytA-CDC} \) but not with \( \text{comX} \). The dotted line indicates the detection limit (number of amplification cycles of the PCR). Horizontal bars indicate the mean of samples with detectable \( C_q \) values, statistics was performed with a paired \( t \)-test. ***\( P<0.001 \).
Table 2. Clinical sera blood culture positive for non-pneumococcal strains

A panel of 51 different clinical sera, which were blood culture positive for different non-pneumococcal strains, were assayed for both comX and lytA-CDC. One serum sample (blood culture positive for E. coli) was positive for lytA-CDC, while none was positive for comX.

<table>
<thead>
<tr>
<th>Species</th>
<th>No. clinical samples tested</th>
</tr>
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<tbody>
<tr>
<td>Staphylococcus epidermidis</td>
<td>11</td>
</tr>
<tr>
<td>Staphylococcus hominis</td>
<td>2</td>
</tr>
<tr>
<td>Staphylococcus sp.</td>
<td>2</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>5</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>2</td>
</tr>
<tr>
<td>Streptococcus pyogenes</td>
<td>1</td>
</tr>
<tr>
<td>Enterococcus faecium</td>
<td>1</td>
</tr>
<tr>
<td>Aerococcus urinae</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus gordonii</td>
<td>2</td>
</tr>
<tr>
<td>Streptococcus mitis</td>
<td>3</td>
</tr>
<tr>
<td>Streptococcus mutans</td>
<td>1</td>
</tr>
<tr>
<td>Streptococcus oralis</td>
<td>2</td>
</tr>
<tr>
<td>Streptococcus salivarius</td>
<td>2</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>8</td>
</tr>
<tr>
<td>Acinetobacter hwoffii</td>
<td>1</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>3</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
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</tr>
<tr>
<td>Klebsiella oxytoca</td>
<td>1</td>
</tr>
<tr>
<td>Acinetobacter species</td>
<td>1</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>1</td>
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<td>Proteus mirabilis</td>
<td>1</td>
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<tr>
<td>Bacteroides fragilis</td>
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positive for lytA (C_q value 43.9), while none of the control samples was positive for comX, resulting in a diagnostic specificity of 98.2 % and 100 % respectively.

**DISCUSSION**

In this study, we developed a novel qPCR assay for the detection of *Streptococcus pneumoniae*. The comX assay had a higher analytical and a comparable clinical sensitivity compared with the current gold standard for qPCR detection of *Streptococcus pneumoniae* in the pre-clinical setting, i.e. the lytA assay described by Carvalho et al. (2007). The current gold standard for detection of the causative agent in septic patients is blood culture. Typically, blood culture takes 24–48 h, while targeted treatment within the first few hours after admission to the intensive care unit is preferred. In order to switch from blood culture to qPCR-based detection of pathogens without a pre-culture step, it is important to be able to detect the pathogen with high analytical sensitivity without compromising the specificity. This may be particularly useful to identify *Streptococcus pneumoniae* in the blood of patients with low pneumococcal load.

The novel target comX encodes an alternative sigma factor that regulates a set of late competence genes (Lee & Morrison, 1999). The comX gene is present in duplicate in all pneumococcal genomes that have been fully sequenced to date and is highly conserved, an important property of any target to identify pathogens. In addition, we performed the qPCR on 10 pneumococcal strains representing different serotypes, which all gave a positive PCR result. Although the serotypes that we examined in this study represent clinically relevant pneumococcal serotypes, it should be noted that in total more than 90 different pneumococcal serotypes have been identified. Testing of multiple isolates belonging to all serotypes should be performed to fully establish the analytical specificity of this target. We tested genomic DNA of 18 non-pneumococcal strains that are prevalent in sepsis. Some of these species were selected based on a BLAST search (*Streptococcus pseudopneumoniae* and *Streptococcus mitis*). Additionally, we used 51 sera of patients that were blood culture positive for in total 21 distinct non-pneumococcal bacterial species. Although none of these control samples returned a positive PCR result, for a more extensive future validation additional non-pneumococcal strains should be tested.

As the comX gene has two copies per genome instead of a single copy, as for lytA, comX should theoretically have a twofold increased sensitivity, corresponding to 1 C_q. However, we found that the comX PCR detected *Streptococcus pneumoniae* at ~3.5 C_q lower than the lytA-CDC assay, i.e. more than expected. This cannot be attributed to differences in primer efficiency, as this was very similar between the two assays. The difference also could not be attributed to suboptimal magnesium concentrations or suboptimal buffer conditions. However, we did notice that the threshold for the lytA qPCR had to be set much lower than for the comX qPCR. The lytA-CDC probe has been designed with a guanine nucleic acid at its 5’ end, which is not recommended in the TaqMan guidelines (Primer Express Software Version 3.0 Getting Started Guide; Applied Biosystems) and could possibly play a role in the lower performance of the lytA assay in terms of C_q values. Another potential explanation for the unexpected C_q difference may be that one of the two copies of comX is located very close (14 kb) to the putative origin of replication, compared with a distance of 1729 kb for lytA-CDC. The presence of multiple replication forks in an actively growing cell (Lemon & Grossman, 1998) could result in a higher copy number at any given time of the genes that are close to the ori, compared with genes that are located more distantly from the ori. Preliminary data from our laboratory suggest that the difference between cycle threshold values of the comX and lytA assay is predominantly present in actively growing cells, and not in cells that are in the stationary phase.

We showed that, in a clinical setting, our assay performed equally as well as the lytA-CDC assay in terms of overall detection rates in blood-culture-confirmed *Streptococcus pneumoniae* infections, but at significantly lower C_q values. Although the template DNA was isolated from an equivalent of 10 μl serum per PCR, we were still able to detect...
pneumococcal DNA in almost half of the samples that were blood culture positive for *Streptococcus pneumoniae*. In comparison, the blood culture results were obtained using two sets of blood cultures that each required a volume of 10–15 ml blood, while bacterial loads can be as low as 10 c.f.u. ml\(^{-1}\) during sepsis. Increasing the amount of serum used for the PCR is therefore likely to improve detection rates. Differences between samples that had a positive signal with comX but not with lytA-CDC, and vice versa, were restricted to samples with high \(C_q\) values (>40), suggesting that these may represent patients with a low bacterial load at or below the LLOD. Increasing the input volume of blood from these patients is expected to improve the clinical sensitivity of the qPCR. Several papers have been published recently on increasing the input blood volume for PCR applications, up to an input volume of 5–10 ml whole blood (Boardman et al., 2015; Loonen et al., 2013). The development of these methodologies is key in the advancement of qPCR-based diagnostic assays. qPCR using comX on genomic DNA at the LLOD showed enhanced analytical sensitivity and detection rates of *Streptococcus pneumoniae* as compared with lytA-CDC, suggesting that comX may particularly have added value for the detection of *Streptococcus pneumoniae* in the blood of patients with a low bacterial load. The small sample size of the clinical cohort used in this study does not allow us to draw solid conclusions on whether using comX as a qPCR target would increase the detection rate. In follow-up studies, it may therefore be useful to increase the number of patient samples and the blood volume used in the PCR. This study described the development of a qPCR assay that only detects *Streptococcus pneumoniae*. However, the development of a qPCR assay that in addition to *Streptococcus pneumoniae* would also be able to detect multiple other bacterial and/or fungal causative agents of sepsis and/or bacteraemic pneumonia, would have significantly more clinical relevance. Ongoing research efforts in our laboratory are therefore focusing on the incorporation of this PCR assay in such a multiplex PCR assay.

Although translation into diagnostic use requires further validation studies, in this study we have shown that comX represents an attractive novel multicopy target for qPCR detection of *Streptococcus pneumoniae*. The development and inclusion of comX into a robust, automated test for rapid diagnosis of sepsis may facilitate a more targeted treatment and as such has the potential to improve patient outcome and reduce the use of broad-spectrum antibiotics.

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