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ORIGINAL ARTICLE

Feasibility of a quantitative polymerase chain reaction assay for diagnosing pneumococcal pneumonia using oropharyngeal swabs

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Abstract

Streptococcus pneumoniae is the most important pathogen causing community-acquired pneumonia (CAP). The current diagnostic microbial standard detects *S. pneumoniae* in less than 30% of CAP cases. A quantitative polymerase chain reaction (PCR) targeting autolysin (*lytA*) is able to increase the rate of detection. The aim of this study is validation of this quantitative PCR in vitro using different available strains and in vivo using clinical samples (oropharyngeal swabs). The PCR autolysin (*lytA*) was validated by testing the intra- and inter-run variability. Also, the in vitro specificity and sensitivity, including the lower limit of detection was determined. In addition, a pilot-study was performed using samples from patients (n = 28) with pneumococcal pneumonia and patients (n = 28) with a pneumonia without detection of *S. pneumoniae* with the current diagnostic microbial standard, but with detection of either a viral and or another bacterial pathogen to validate this test further. The intra- and inter-run variability were relatively low (SD's ranging from 0.08 to 0.96 cycle thresholds). The lower limit of detection turned out to be 1–10 DNA copies/reaction. In-vitro sensitivity and specificity of the tested specimens (8 strains carrying *lytA* and 6 strains negative for *lytA*) were both 100%. In patients with pneumococcal and non-pneumococcal pneumonia a cut-off value of 6.000 copies/mL would lead to a sensitivity of 57.1% and a specificity of 85.7%. We were able to develop a quantitative PCR targeting *lytA* with good in-vitro test characteristics.

Keywords *Streptococcus pneumoniae* · Quantitative PCR · Pneumonia · *LytA* · Community-acquired pneumonia

Introduction

Streptococcus pneumoniae is the most important pathogen causing community-acquired pneumonia (CAP) [1–3]. The current diagnostic standard, comprised of blood cultures, sputum cultures and the urinary antigen test (UAT), is only able to detect *S. pneumoniae* in less than 30% of CAP cases [4, 5]. Furthermore, it takes up to several days to yield a positive result and antibiotic therapy can be narrowed [6]. The UAT is currently the test with the highest sensitivity, ranging from 59 to 87% and specificity of 94%, increasing the detection of *S. pneumoniae* in patients with CAP from 14.0 to 27.0% [7]. Detecting *S. pneumoniae* before or after the start of antibiotic treatment requires a target. Different genes of *S. pneumoniae* have been used in research as a target, including spn9802, pneumolysin (*ply*), wzg (*cpsA*), and autolysin (*lytA*) by PCR [4, 5, 8–18]. A target gene should be specific for *S. pneumoniae* and be absent in the other non-pneumococcal streptococci such as *Streptococcus mitis, Streptococcus oralis* and the recently discovered

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Streptococcus pseudopneumoniae [18]. Remarkably, *ply* is believed to be less specific for *S. pneumoniae* than *lytA* [19, 20]. The *ply* and *lytA* gene have both been found in *S. mitis* strains. The isolates containing these genes were all associated with respiratory disease [21]. One recent study by Albrich et al. [5] showed that quantitative polymerase chain reaction (qPCR) tested on nasopharyngeal (NP) samples targeting the *lytA* gene in a study population that consisted mainly of HIV-infected adults detected *S. pneumoniae* in 52.5% of CAP cases. The diagnostic standard (blood culture, sputum Gram stain or culture or UAT) detected *S. pneumoniae* in only 27.1% of CAP cases. The combination of target genes has been suggested to improve the reliability of the qPCR. The target gene *piaB* has been used next to *lytA* to increase the specificity. A recent study by Simoes et al. [22] used both *lytA* and *piaB* to identify *S. pneumoniae* and the addition of *piaB* led to the discovery of two pneumococcal strains that were not identified by *lytA* alone. However, the authors mention that *piaB* is not present in some non-encapsulated pneumococci and some non-typeable pneumococci. An earlier study also combined *lytA* and *piaA* for the detection of colonization of the nasopharynx by *S. pneumoniae* [23]. A strain was considered to be a *S. pneumoniae* species when both genes were present. Four strains did not include the *piaA* gene, but turned out to be *S. pneumoniae* species.

Using two target genes leads to the difficult situation of interpreting a strain which encompasses one gene, but lacks the other gene. Adding *piaB* will lead to a lower sensitivity. This means that some patients will be withheld narrow-spectrum antibiotics.

LytA encodes for an autolysin that is activated in the presence of antibiotics such as penicillin and detergents such as deoxycholate [24]. It has also been considered to be a virulence factor, which means that it enables *S. pneumoniae* to enter the cells of its host, replicate inside these cells and persist in them [25]. *LytA* is a stable or conserved gene, which is a favorable target for detection [13]. In 2001 McAvin et al. [13] found that in vitro the *lytA* gene showed promising results with a sensitivity and specificity of 100% for *S. pneumoniae*. A more recent study in which clinical samples were used also found a specificity of 100%, but a much lower sensitivity of 53% [26]. Other research stated that *lytA* is not specific enough to differentiate between *S. pneumoniae* and some strains of *S. mitis, S. pseudopneumoniae* and *S. oralis* [18, 27, 28]. However, there are studies that claim that *lytA* can rarely be found in non-pneumococcal bacteria [14, 15].

Streptococcus pneumoniae is a pathogen capable of colonization of the upper respiratory tract [23, 29]. Differentiating between colonization and infection is necessary to detect the patients with true pneumococcal pneumonia. Setting a cut-off value using a qPCR could potentially deal with this problem.

The aim of our study is to set up and validate a quantitative PCR assay targeting the *lytA* gene for detection of *S. pneumoniae* in adult patients with CAP. First, we validated the assay by examining the quality and reproducibility. Subsequently, the sensitivity and the lower limit of detection (LLOD) of the assay, as well as the specificity of the PCR was tested. After validation, we performed a pilot-study with clinical samples in patients with pneumonia caused by different pathogens.

Materials and methods

Study outline

The study was performed in the *Regional Laboratory for Public Health Kennemerland* in Haarlem between the 1st of September and the 8th of December 2015.

Amplification of a part of the bacterial DNA (the amplicon) using PCR leads to extremely high levels of amplicons after the experiment, in contrast to relatively low levels before the start of the amplification cycles. To check for possible inaccuracy the qPCR assay was compared with one other method of quantification: quantification using universal 16S ribosomal RNA primers. The concentration of the sample that was used containing a quality control strain of *S. pneumoniae* (*S. pneumoniae* American Type Culture Collection (ATCC) 49619) was calculated with PicoGreen quantification and 16S rDNA quantification. This calculated concentration was used to asses a standard curve for a *lytA* qPCR using primers/probe constructed by Carvalho et al. [26]; forward primer (560 nM): 5′-ACGCAATCTA GCA GATGAAG CA-3′; reverse primer (2800 nM): 5′-TCGTGC GTTT TAATTCCAGC T-3′; probe (700 nM): 5′-FAM-GCC GAAAACG CTTGATACAG GGAG-3′-BHQ1. The standard curve enabled calculation of concentrations from other samples of *S. pneumoniae* and other non-pneumococcal streptococci (provided by the Department of Paediatric Immunology and Infectious Diseases, Wilhelmina's Children Hospital, University Medical Centre Utrecht, Utrecht, The Netherlands). The concentrations of the standard curve were compared to those calculated using 16S rDNA quantification performed by aforementioned samples.

Bacterial strains

Streptococcus pneumoniae ATCC 49619 was used to compare methods and for optimization of the quantitative PCR targeting *lytA* as well as assessing a standard curve. A collection of strains was used to test the specificity and sensitivity of the assay. *S. pneumoniae* strains with known concentrations (OK-2-816; OK-2-1213; OK-2-1214;OK-2-077) and unknown concentrations (serotype 8; serotype 14; serotype

19A; strain 406) were used to test the sensitivity. *S. pseudopneumoniae* strains (k221; ILI42; OK-3-VE-224; 2120942), as well as a *S. mitis* (*S. mitis* SK579 (b1019)) and a *S. oralis* strain (2021933), all *lytA* negative, were used to test the specificity. The strains were provided by the Department of Paediatric Immunology and Infectious Diseases, Wilhelmina's Children Hospital, University Medical Centre Utrecht, Utrecht, The Netherlands. Concentrations and characteristics are available from the supplementary appendix.

Clinical samples

Samples were prospectively collected from patients with CAP (REDUCE study; clintrials.gov database NCT01964495). For this present (pilot-)study oropharyngeal (OP) swabs were used. All oropharyngeal swabs were obtained by rolling the swabs on the tonsils and posterior wall of the oropharynx with enough pressure to dislodge cells from the mucosal surface. The oropharyngeal swabs used in this study are eSwab™ with liquid Amies medium as preservation medium (Copan Italia SpA, Brescia, Italy). Viral pathogens could be identified using a PCR performed on these OP swabs. Of every swab 5 µL liquid was added to the primer/probe mix. The patient characteristics are shown in Table 1.

Isolation of bacterial DNA

DNA of *S. pneumoniae* ATCC 49619 was isolated using the Highpure PCR template preparation kit (Roche Diagnostics Nederland BV, Almere, The Netherlands). Bacterial DNA from clinical respiratory samples were obtained by total DNA extraction using a NucliSENS EasyMag total nucleic acid extractor (bioMérieux, Marcy l'Etoile, France). The total nucleic acid component of the sample (200 µL) was eluted in a final volume of 40 µL.

Molecular quantification of bacterial DNA of the positive control (*S. pneumoniae* ATCC 49619)

For quantification of a positive control (*S. pneumoniae* ATCC 49619) we used the Quant-IT PicoGreen dsDNA assay kit (Life Technologies, Bleiswijk, The Netherlands). The fluorescence was measured using a LightCycler® 480II real-time PCR analyser (Roche, Almere, The Netherlands). To convert from the concentration in ng/µL to the number of genome copies per µL the genome size, approximately 2.1 million base pairs was estimated [30].

As a comparison for the Picogreen quantification method, quantification of *S. pneumoniae* ATCC 49619 was performed using a 16S PCR targeted by universal primers [31].

Table 1 Patient characteristics

	Patient characteristics			
	S. pneumoniae (n = 28)		Other pathogens (= 28)	
Age (year)	67.38 ± 16.218 (Range 24–92)		67.54 ± 13.226 (Range 44–94)	
Male	16	57.1%	16	57.1%
Female	12	42.9%	12	42.9%
Current smoker	9	32.1%	6	21.4%
Previous smoker	13	46.4%	16	57.1%
CURB-65				
0	5	17.9%	8	28.6%
1	5	17.9%	11	39.3%
2	10	35.7%	6	21.4%
3	8	28.6%	2	7.1%
4	0	–	1	3.6%
5	0	–	0	–
COPD	11	39.3%	14	50%
Pre-treatment with AB	2	7.1%	7	25%
Positive blood culture	12	42.9%	3	10.7%
Positive sputum culture	10	35.7%	11	39.3%
Positive urinary antigen test	13	46.4%	1[a]	3.6%
Positive pharyngeal swab (viral pathogens)			20	71.4%

The patient characteristics of the two groups (infected with *S. pneumoniae* or other viral/bacterial pathogens) of patients admitted with CAP. *AB* antibiotics. Pharyngeal swabs were only tested for viral pathogens at time of admittance

[a]Positive for *Legionella pneumophila*

The quantitative PCRs were run on LightCycler 1.5 or 2.0 (Roche, Almere, the Netherlands). LightCycler software (Version 4.1) resulted in the calculation of the number of *S. pneumoniae* DNA copies of the positive control.

Quantitative PCR targeting lytA

The *S. pneumoniae*-quantitative PCR uses primers targeting the *lytA* gene as described by Carvalho et al. [26]. Roche LightCycler® 480 Probes Master mix was used for all PCR reactions. PCR ran the following program using the LightCycler® 480 (Roche): 10 min at 95 °C, followed by 45 cycles that are comprised of 15 s at 95 °C and 1 min at 60 °C. A standard curve was assessed for the quantitative assay by using the *S. pneumoniae* ATCC 49619 strain. Standard curves (three standard curves, calculated with three different experiments, the average of these curves was used as a final standard curve) were validated using strains with known concentrations (OK-2-816; OK-2-1213; OK-2-1214; OK-2-077), kindly provided by the Department of Paediatric Immunology and Infectious Diseases, Wilhelmina's Children Hospital, University Medical Centre Utrecht, Utrecht, the Netherlands. Inter and intra-run variability were established by determination of triplicate serial dilutions in three independent runs.

Sensitivity of the lytA PCR

LLOD of the quantitative PCR was determined by multiple serial dilutions of purified DNA from *S. pneumoniae* ATCC 49619 equivalent to from 17,000 to 0.17 copies per µL.

Specificity of the lytA PCR

Specificity of the *lytA* real-time PCR was defined by testing purified DNA from eight pneumococcal strains. These strains include 4 strains that were non-typeable by culture (OK-2-816; OK-2-1213; OK-2-1214; OK-2-077) as well as serotype 8, serotype 14, serotype 19A and strain 406.

Further determination of the specificity was performed by using 6 strains including 4 strains of *S. pseudopneumoniae* (k221; ILI42; OK3-VE-224; 2120942), 1 strain of *S. mitis* and 1 strain of *S. oralis*. None of these strains encloses the *lytA* gene.

Statistical analysis

The 2 different methods (the concentrations calculated with the *lytA* qPCR and 16S rDNA quantification) were compared using the Bland–Altman-method [32]. Inter-run variability was calculated by one-way analysis of variance (ANOVA) [33, 34]. Intra-run variability was also tested by one-way analysis of variance. SPSS statistical software (SPSS version 23 for Windows, Chicago, IL, USA) was used to perform the statistical tests mentioned above. A p-value < 0.05 was considered as statistically significant.

Results

Molecular quantification of the positive control (*S. pneumoniae* ATCC 49619)

The first step in the validation was molecular quantification of the positive control (*S. pneumoniae* ATCC 49619). Concentrations calculated with PicoGreen had and 16S rDNA experiments resulted in an average concentration of 1.70×10^6 DNA copies/µL.

Validation of the lytA quantitative PCR

The calculated average concentration of *S. pneumoniae* (ATCC 49619) was used to assess a standard curve. This standard curve had a slope of approximately −3.4 and an efficiency of 95.1%. Four *lytA* positive strains with known concentrations were used to validate our standard curve.

Intra- and inter-run variability

To examine the feasibility of the qPCR as a diagnostic tool for CAP, the specificity and sensitivity characteristics are determined. Serial dilutions of *S. pneumoniae* (ATCC 49619) were used to account for intra- and interrun variability. For each step dilution, the standard deviation was calculated. Standard deviations ranged from 0.08 Ct-value for the samples with the highest concentration to an average of 0.96 Ct-values for the lowest concentration (0.17 DNA copies/µL or 170 DNA copies/mL). No significant differences were found when testing the inter-run variability with a one-way analysis of variance (ANOVA; p-value ranging from 0.426 to 0.929).

In vitro performance of the lytA quantitative PCR

In total 6 *lytA*-negative strains were tested. Four strains showed fluorescence, while 2 other strains showed no fluorescence after 45 cycles (supplementary appendix). The 4 *lytA*-negative strains that did show fluorescence appeared as multiple groups of DNA fragments (shorter than the amplicon of 75 base pairs), meaning they contained an accumulation of waste products. Dilutions of *S. pneumoniae* (ATCC 49619) were used to establish the lower limits of detection (LLOD) of the qPCR targeting *lytA*. The LLOD ranged from approximately 0.85 (SD 0.96 Ct) DNA copy to approximately 8.5 (SD 0.36 Ct) DNA copies per well.

In addition, an attempt was made to identify 8 different *lytA*-positive *S. pneumoniae* strains. These different strains were all identified with Ct values ranging from 18 to 25 cycles. Only one strain had a positive result after approximately 36 amplification cycles (Serotype 19A). Testing the *lytA*-positive and *lytA*-negative strains resulted in an in-vitro sensitivity and specificity of both 100%. The LLOD was 1–10 copies/reaction.

Pilot-study of in vivo specimens

OP samples from 28 patients with CAP caused by *S. pneumoniae* and 28 patients with a viral pneumonia or pneumonia with another bacterial pathogen, identified by a positive blood, sputum culture or UAT result were used for this pilot study. Concentrations in the OP swabs tested in the group with *S. pneumoniae* ranged from 0 to 1190 DNA copies/µL; 5 patients had a negative result. Concentrations in the group with other pathogens ranged from 0 to 210 DNA copies/µL; 18 patients had a negative result. The largest Area Under the Curve (AUC) was found for a cut-off value of 6.000 DNA copies/mL (AUC 0.714 with a sensitivity of 57.1% and a specificity of 85.7%) (Figs. 1, 2) with a positive predictive value of 80% and a negative predictive value of 66.7%.

Eleven patients in the *S. pneumoniae* group and 13 patients in the group with other pathogens had a complete composite diagnostic microbial standard (blood culture, sputum culture and UAT). The range of the *S. pneumoniae* group (11 patients) was 0–145 DNA copies/µL with one negative result and the concentrations in the group with other pathogens (13 patients) ranged from 0 to 211 DNA copies/µL, with eight negative results. The AUC for this second comparison was also highest with a cut-off value of 6.000 DNA copies/mL (AUC 0.787, with a sensitivity of 72.7% and a specificity of 84.6%). The positive and negative predictive value were 80% and 78.6% respectively.

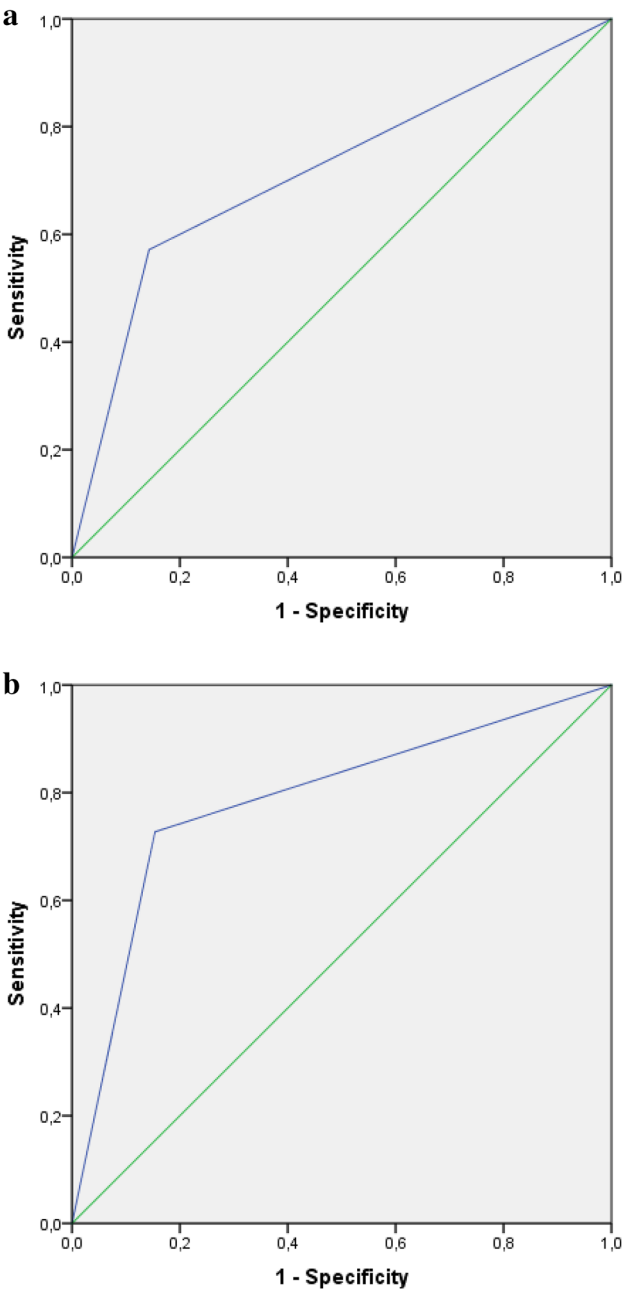

Fig. 1 **a** ROC-curve with cut-off value 6.000 copies/mL. Sensitivity is 57.1% and specificity is 85.7%. AUC is 0.714. **b** ROC-curve with a cut-off value of 6.000 copies/mL. Only samples from patients with a complete composite diagnostic standard (blood culture, sputum culture and urinary antigen tested) performed were used for this curve. Sensitivity is 72.7% and specificity is 84.6%. AUC is 0.787

Discussion

The present study shows that the *lytA* quantitative PCR is a reliable test in order to detect *S. pneumoniae* in vitro and has the potential to be a reliable test in vivo. In vitro sensitivity and specificity are both 100%. More important the test shows promising results in differentiating between infection and colonization. When tested on a small sample of patients, with a complete diagnostic work-up, a sensitivity and specificity of 72.7 and 84.6% respectively were reached using a cutoff value of 6.000 copies/mL. One would expect a low number of DNA copies in patients with colonization without infection. With a sensitive test, which can detect a low number of DNA copies per microliter and makes it possible to set a low cut-off value when this hypothesis is true. The in-vitro LLOD turned out to be between approximately 1 and 10 copies/µL, which is similar to the LLOD's found by others varying from < 10 copies per reaction to 4.3 copies per reaction [12, 15, 26]. This LLOD makes the differentiation between colonization and infection possible. The standard deviations of our standard curve illustrate the reproducibility of our test.

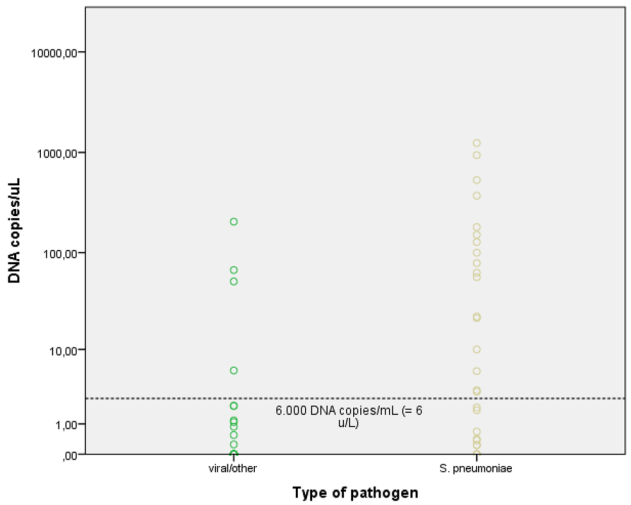

Fig. 2 Pneumococcal load in oropharyngeal swabs. Number of DNA copies/microliter in oropharyngeal swabs in patients with confirmed pneumococcal pneumonia (n = 28) or viral/other pathogens (n = 28). The dotted line represents the cut-off value of 6.0000 DNA copies/mL

The specificity and sensitivity are based on a total number of just 14 strains, which is a drawback of our study. In other studies a much larger numbers of pneumococcal strains and controls were tested [13–15]. An in-vitro specificity and sensitivity of 100% in the first study was found using 70 positive controls and 9 non-pneumococcal streptococci (including 2 *S. mitis* strains) [13]. This 100% specificity was confirmed by another study using 23 non-pneumococcal streptococci (including three that closely resemble *S. pneumoniae;* 2 *S. oralis* strains and 1 *S. mitis* strain) [14]. The largest study tested a total of 257 strains belonging to 37 different species including 30 *S. mitis* strains, with no false negative results and only one false positive result out of 30 *S. mitis* strains. This sample was also tested positive by two rapid antigen tests (Wellcogen and Phadebact) [15].

A recent study using the same positive control (ATCC 49619), primers and probe, tested 23 *S. pneumoniae* strains and 29 negative controls (including six non-pneumococcal species, one being *S. mitis*) [35]. The six negative controls used in the present study are all six closely related to *S. pneumoniae*. Testing these non-pneumococcal strains makes for a valuable contribution to previous trials because they generate signals reported specific to *S. pneumoniae*, in terms of optochin susceptibility, bile solubility, and Quellung testing, the classic methods used to identify pneumococci. These signals make it difficult to discriminate them from pneumococcal strains when performing these tests on blood cultures. However, our PCR was able to discriminate between these strains and *S. pneumoniae*. The small number of strains tested might overestimate the true specificity. The specificity could be improved by adding a *piaB* confirmation-PCR, which can be used for the samples tested positive for *lytA*.

Our pilot-study consisted of a only small number of patients admitted with either pneumococcal pneumonia or CAP caused by another pathogen. The very small number of samples is a clear limitation of our study. This pilot-study was conducted to perform a preliminary in vivo validation of the qPCR and was not designed as a full clinical trial. A larger population could have resulted in a proper cut-off value, which could be used in further studies or in clinical practice. Although the use of this limited number of OP samples was not intended to define a proper cut-off value, preliminary results are promising: best AUC of 0.714 with a sensitivity of 57.1% and specificity of 85.7% with a cut-off value of 6.000 copies/mL. The AUC was even higher when only using the samples of patients with a complete diagnostic workup; a sensitivity of 72.7% and a specificity of 84.6% using a cut-off value of 6.000 copies/mL (AUC 0.787). Choosing a different cut-off value to achieve the highest sensitivity may implicate a lower specificity. In clinical practice, in patients who are colonized with *S. pneumoniae* and infected with another bacterial pathogen, the test may be considered as (false) positive and consequently these patients would be treated with narrow-spectrum antibiotic therapy.

Recent research suggested a cut-off value of 8.000 copies/mL of the *lytA* gene when using NP swabs, which led to a sensitivity and specificity of the qPCR of 82.2% and 92% respectively [5]. The authors claim that this cut-off value is capable of differentiating between asymptomatic colonization and infection in HIV-infected patients. Another recent study used a much lower cut-off value of 10^2 copies/mL [4]. Other researchers, who used the *Spn9802* target gene found a similar cut-off value of 4.000–8.000 [10].

A very recent investigation by Blake et al. used a *lytA* rt-PCR on whole blood samples to identify *S. pneumoniae* in patients with CAP in Togo. The cut-off value was set at a Ct-value instead of the number of DNA copies/mL. The cut-off was set on a Ct-value of 35 [36]. The sensitivity of the *lytA* rt-PCR was significantly higher than blood culture, 17.1% versus 12.9%, but has a much lower sensitivity compared to the *lytA* qPCR we tested on OP swabs. The specificity of the rt-PCR on blood samples was 97.4%. The authors consider this a possible consequence of cross-reactivity with *S. mitis* among other bacteria. This limitation of the *lytA* PCR has been described in other research as well. One study tested 11 streptococcal isolates that showed conflicting or previously unknown patterns when using optochin susceptibility, bile solubility, *lytA* PCR and multilocus sequence analysis and discovered that three strains were misidentified with the *lytA* rt-PCR (one false-negative result and two *S. pseudopneumoniae* strains led to a false-positive result) [22]. In three patients without detection of *S. pneumoniae*

using the current diagnostic standard, but with detection of a virus (two coronaviruses and one influenza A virus) concentrations of *S. pneumoniae* above 40.000 copies/mL were detected, which limits the specificity of our test in this experiment. A possible explanation for these high concentrations of DNA copies/mL is false-negative results of the current pneumococcal tests. Only one of these patients was pretreated with antibiotics and they all had a favorable outcome with amoxicillin. Given the high DNA concentrations above the cut-off values for colonization and the favorable response to therapy an underlying pneumococcal infection seems very likely.

Previously the usefulness of the qPCR has been questioned in patients who were pre-treated with antibiotics [26, 37]. The total number of patients who have been pre-treated with antibiotics in the present study is rather low (16.1%) and no reliable conclusion can be made on this topic. We believe this is an important issue not only with qPCR but with any microbiological test, so further studies should address this question [12].

A recent study showed that it is possible to detect 26 respiratory bacteria and viruses with one single test. 85% of the patients tested had been pre-treated with antibiotic therapy, and still in 78% of these patients a bacterial pathogen was detected, where only 32% of cultures were positive [38]. A bacterial pathogen was found in 71.5% of cases. No blood cultures or urinary antigen tests were included and only mucopurulent sputum was used. *S. pneumoniae* was detected in 35.6% of cases.

A possible explanation for the low sensitivity is the cut-off value, which at this point is based on a low number of patients, as mentioned before. When counting all positive results (every patient with a DNA copy number of more than zero) the sensitivity of the qPCR is 82% (23 out of 28 patients). Another possible explanation for the low sensitivity compared to the in-vitro sensitivity is the sample technique or sample site. Some studies use sputum samples which are difficult to obtain, whereas others use NP swabs instead of OP swabs [4, 5, 19]. One of these studies compared trans-nasal and trans-oral sampling, and concluded that the nasopharynx is the main reservoir for *S. pneumoniae* [23], but data on the best sampling technique is limited and unclear about which technique is superior. According to the WHO Pneumococcal Carriage Working Group NP samples have a slightly higher sensitivity in detecting colonization with *S. pneumoniae* in healthy adults and children. A combination of NP and OP samples is recommended for detection of *S. pneumoniae* carriage in adults. There are no current recommendations about molecular diagnostics and detection of *S. pneumoniae* in patients with CAP [39, 40]. Recent research showed that in healthy adults and adults with influenza-like-symptoms the qPCR targeting *lytA* and *piaA* or *piaB* yielded more positive results than cultures (carriage in healthy adults 20% using the qPCR vs. 5% detection using cultures). The detection rate of *S. pneumoniae* in adults with influenza-like-illness was highest in saliva samples (28%) followed by OP swabs (11%), cultures (10%) and NP swabs (5%) [41, 42].

Primarily, the test will have to be validated in a larger collection of clinical samples so a distinction between colonization and infection can be made. Another important question is the performance of the qPCR in patients pre-treated with antibiotics. Furthermore, the additional value needs to be determined to see if the qPCR will increase microbiological yield and leads to changes in antibiotic regimes.

In conclusion, we were able validate a quantitative PCR targeting *lytA* with good in-vitro test characteristics. One to 10 DNA copies per reaction could be detected with an in-vitro sensitivity and specificity of 100%. The results of the in-vivo tests are promising with a sensitivity of 57.1% and a specificity of 85.7%.

We believe the qPCR targeting *lytA* could be a rapid and reliable tool for diagnosing pneumococcal CAP, but further research with larger groups is necessary.

Author contributions RD, WvdR, WR, MvS and WB were involved in the conception and design of the study. WvdR, RJ and MvS were involved in method development and molecular testing for the study. MvS, RD, WvdR, NP and WB participated in the drafting of the manuscript. All authors interpreted the data and revised the manuscript critically for important intellectual content. All authors approved the final manuscript.

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Data availability This study represents a subset of the data collected for the REDUCE study and can be accessed by academic researchers with permission from the principal investigator, WB (w.boersma@NWZ.nl).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval For the REDUCE study ethical approval was obtained through the METC Noord-Holland (Postbus 501, 1800 AM, Alkmaar, The Netherlands) which is now part of the METC of the Amsterdam University Medical Centres.

Informed consent For the REDUCE study informed consent was obtained for taking oropharyngeal swabs.

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