Original Article

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Microbiology Section

Development of Gene-based Conventional PCR Assay for Diagnostic Application of *Streptococcus pneumoniae* Infections as an Alternative to Conventional Culture: A Cross-sectional Study

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ABSTRACT

Introduction: *Streptococcus pneumoniae (S. pneumoniae)*, an opportunistic pathogen, is the primary cause of pneumonia and is classified as a high-priority bacterial pathogen. However, it is often underdiagnosed by conventional microbiological tests. The present study was conducted to develop a simple, rapid and reliable molecular method for the early and accurate diagnosis of *S. pneumoniae* using the Duplex Polymerase Chain Reaction (PCR) Assay. Among various genes responsible for the pathogenesis of pneumococci, the *LytA* gene and the *CpsA* gene were targeted for the molecular identification of *S. pneumoniae*.

Aim: To evaluate the developed duplex PCR assay alongside the microbial culture of the *S. pneumoniae* pathogen.

Materials and Methods: This study was carried out at the microbiology department of Surat Municipal Institute of Medical Education and Research (SMIMER), Surat, Gujarat, India, from March 2020 to March 2022. A molecular method for the detection of *S. pneumoniae* present in various clinical samples by PCR was developed. The optimised Duplex PCR assay employs primers specific for the *LytA* and *CpsA* genes of *S. pneumoniae*. The method involves the extraction of bacterial genomic DNA, PCR amplification and agarose gel electrophoresis to analyse the amplification products. The performance of the newly developed Duplex PCR assay was evaluated and compared its results with previously described assays, such as monoplex PCR assays targeting a single gene and conventional microbiological tests. Different clinical samples (n=75) from patients with pneumonia-like symptoms were used for PCR analysis and culture. The sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of the PCR assay were determined in comparison with the gold standard microbial culture test. The level of significance was assessed using Fisher's exact test, with tests considered significant at a p-value <0.05.

Results: The duplex PCR assay demonstrated the best performance in terms of sensitivity and specificity, with no misidentifications found among non-pneumococcal strains for the *CpsA* and *LytA* genes. The assay was capable of detecting 6 CFU/mL of *S. pneumoniae* American Type Culture Collection (ATCC)® 49619™. The specificity of the developed Duplex PCR assay was 100% for both *LytA* and *CpsA*-specific genes. In total, 20% (n=15/75) of different clinical samples yielded positive results using the culture method. *S. pneumoniae* was isolated from 7 (9.3%) of sputum samples, 4 (5.3%) of blood cultures, 3 (4%) of cerebrospinal fluid (CSF), and 1 (1.3%) of pleural fluid samples, respectively, by conventional microbiological culture methods. Using the *S. pneumoniae*-specific Duplex PCR assay, 23 (30.7%) of samples tested positive for *S. pneumoniae*, as these samples exhibited amplification for both the *LytA* and *CpsA* genes. Of these, *S. pneumoniae* was detected in 9 (12%) of nasopharyngeal/oropharyngeal (NP/OP) swabs, 6 (8%) of sputum samples, 4 (5.3%) of blood cultures, 3 (4%) of CSF samples, and 1 (1.3%) of pleural fluid samples, respectively. The sensitivity, specificity, PPV and NPV of PCR for detecting *S. pneumoniae* in comparison with the culture method were 100%, 96.7%, 88% and 100%, respectively.

Conclusion: The developed and optimised *S. pneumoniae*-specific Duplex PCR assay targeting the *CpsA* and *LytA* genes, as described in this study, is a powerful strategy that resulted in no false identifications. This significantly increases the sensitivity and specificity of pneumococcal identification compared to standard microbial culture and monoplex PCR assays targeting only a single gene.

Keywords: Molecular identification, Polymerase chain reaction, Pneumococcal infection

INTRODUCTION

Streptococcus pneumoniae is a commensal of the upper respiratory tract within the mitis group. It is a major cause of bacteraemia, pneumonia, meningitis and acute otitis media in children, leading to high morbidity and mortality in developing countries [1-6]. The World Health Organisation (WHO) estimates that *S. pneumoniae* causes the annual death of over 300,000 children under the age of five worldwide [7]. Older adults with underlying co-morbidities are primarily affected by the invasive form of the disease [8,9]. Despite the effectiveness of conventional antimicrobial treatments, there has been an alarming rise in pneumococcal resistance to critical antimicrobials over the past few decades, presenting a significant global concern [7,10,11].

Other closely related species within the mitis group, such as *S. pseudopneumoniae*, *S. mitis* and *S. oralis*, have also been linked to human disease [11-13]. Thus, for early and accurate diagnosis and improved treatment, it is crucial to identify these species accurately. Misidentifying causative agents affects disease surveillance and falsely inflates pneumococcal antimicrobial drug resistance, as closely related species like *S. mitis* have a high prevalence of penicillin and other multidrug resistance [11,14-16].

For early pneumococcal disease diagnosis and effective antibiotic therapy, sensitive and specific laboratory tests are required. However, no gold standard method exists for diagnosing *S. pneumoniae*. For pneumococcal detection, growth-based assays, colony characteristics,

Optochin (Op) susceptibility, bile or deoxycholate solubility and serological tests can be used, but they are time-consuming, less sensitive and often produce equivocal results. All these challenges can be addressed by using Nucleic Acid Amplification Tests (NAAT) as diagnostic tools. Molecular assays are valuable because they have higher sensitivity and specificity than culture methods, can be applied to various specimen types and do not require viable bacteria [2,17-22].

Molecular methods like DNA-DNA hybridisation techniques are difficult to implement in routine clinical laboratories for distinguishing *S. pneumoniae* from other streptococci in the mitis group. Multilocus Sequence Typing (MLST) has been recommended as an alternative for identifying pneumococci [23]. However, most clinical laboratories cannot routinely use MLST because it is costly and requires specialised expertise [24]. Thus, PCR serves as a simple, cost-effective and user-friendly alternative for rapid and accurate molecular identification of pneumococci. Researchers have utilised PCR amplification of several pneumococcal-specific genes, such as *Lyt*A, *Ply*, *Cps*A, *Psa*A, *Spn*9828, *Rec*A and 16S rRNA, to distinguish true pneumococci from closely related non pneumococci [24-27].

Studies have found that PCR-based amplification of an internal *Lyt*A fragment accurately identified *S. pneumoniae* [24,28,29]. The *Lyt*A gene encodes N-acetylmuramoyl L-alanine amidase (NAM-amidase). With 1% bile or deoxycholate, *Lyt*A NAM-amidase solubilises pneumococci. All human pneumococci that are solubilised by 1% bile or deoxycholate possess an active *Lyt*A gene. The limited allelic variation of the *Lyt*A gene makes it an ideal target for epidemiological and clinical studies [30,31]. This *Lyt*A gene can differentiate pneumococci from human oral commensals, such as *S. mitis* and *S. oralis*. However, recently, organisms that are phenotypically and genetically related to *S. mitis* have been found to possess autolysin and pneumolysin genes, which were previously associated only with *S. pneumoniae*. All of these observations suggest that the presence of the *Lyt*A gene in various *S. mitis* group isolates, such as *S. oralis*, *S. mitis* and *S. pseudopneumoniae*, may lead to false positive results and, consequently, misidentification in PCR amplification experiments [12,24,26,31-33]. This may overestimate the burden of *S. pneumoniae*.

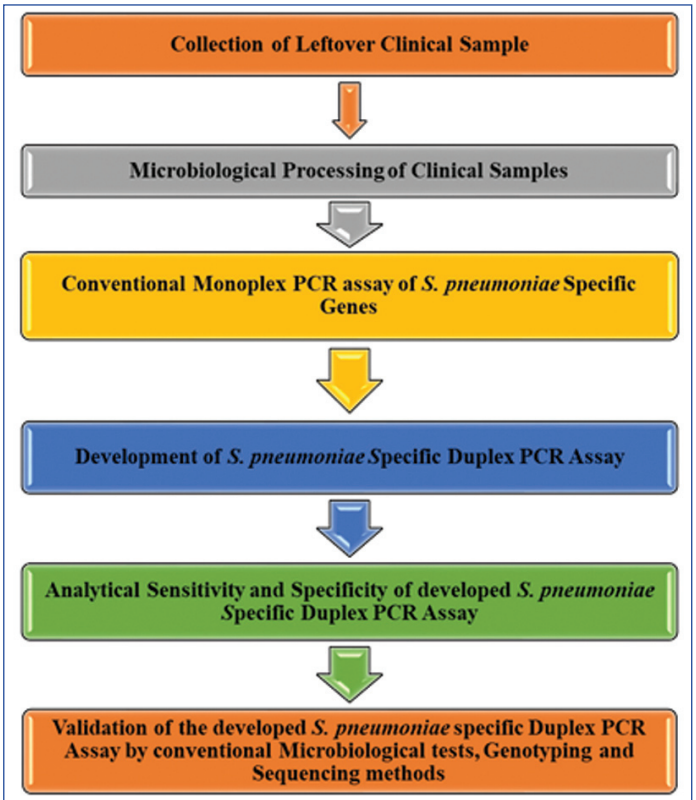
Hence, to overcome false positive results from detecting the WHO-recommended *Lyt*A gene target alone, it is advantageous to target more genes specific to *S. pneumoniae* [30,34]. One such potential target gene for the specific detection of *S. pneumoniae* is the *Cps*A gene, which is responsible for capsular polysaccharide biosynthesis. The *Cps*A gene was discovered as a novel genomic marker unique to pneumococci, allowing for a precise distinction between *S. pneumoniae* and the closely related viridans group streptococci [26,35]. Both the *Lyt*A and *Cps*A genes are required for pathogenesis and have been identified as virulence markers for *S. pneumoniae*. Thus, the combination of these two genes is the most promising approach for the accurate detection of *S. pneumoniae* [26,36].

In the pursuit of high diagnostic specificity and sensitivity, present study used the most promising combination of *S. pneumoniae*-specific *Lyt*A and *Cps*A genes to be amplified by PCR assay. The goal was to develop a rapid, highly sensitive and pneumococcal species-specific PCR-based DNA amplification method. The current study evaluated the duplex PCR assay alongside the microbial culture of the *S. pneumoniae* pathogen to enhance non culture pneumococcal surveillance methods.

MATERIALS AND METHODS

The present study was a cross-sectional study which was carried out at the microbiology department of Surat Municipal Institute of Medical Education and Research (SMIMER), Surat, Gujarat, India, from March 2020 to March 2022. The study was approved by the Institutional Ethics Committee (IEC) with letter no. VRK/ICMR/Ethical Approval/2018/91.

A brief description of the current study methodology is shown in the following flow chart [Table/Fig-1]. Data for all studies were collected scientifically for tabulation and analysis.



[Table/Fig-1]: A brief description of the present study methodology.

Bacterial isolates and clinical samples: *S. pneumoniae* ATCC® 49619™ (type strain) standard culture was procured from the ATCC (Lot No: 947-117-2, Ref: 0947U) for the development of monoplex and duplex PCR assays and was used as a positive control. A panel of well-characterised bacteria commonly isolated from pneumonia patients was also included in the study to determine the specificity of the developed assay [Table/Fig-2]. All samples investigated in the present study were either leftover clinical samples or samples obtained after written consent was taken from patients or individuals who attended government and private hospitals and diagnostic laboratories located in Valsad and Surat districts, as well as sickle cell camps organised by the Comprehensive Sickle Cell Clinic (CSCC), Valsad Raktdan Kendra, Valsad, Gujarat, India, from March 2020 to March 2022.

S. No.	Bacterial culture	No. of culture
1	*Streptococcus pneumoniae* ATCC® 49619™	01
2	*Staphylococcus aureus* ATCC® 25923™	01
3	*Klebsiella pneumoniae* ATCC® 13883™	01
4	*Pseudomonas aeruginosa* ATCC® 27853™	01
5	*Escherichia coli* ATCC® 25922™	01
6	*Streptococcus pneumoniae* clinical isolates	20
7	*Streptococcus mitis* clinical isolates	03
8	*Streptococcus dysgalactiae* subsp. *equisimilis* clinical isolate	01
9	*Klebsiella pneumoniae* clinical isolates	04
10	*Pseudomonas aeruginosa* clinical isolates	03
11	*Staphylococcus aureus* clinical isolates	02
12	*Escherichia coli* clinical isolates	02
Total		40

[Table/Fig-2]: Bacterial DNA sample panel for specificity test.

Inclusion criteria: Untreated patients with signs and symptoms of Lower Respiratory Tract Infection (LRTI) or pneumonia-like symptoms were included in this study.

Exclusion criteria: Patients who were undergoing treatment with antibiotics or had been treated seven days prior to sample collection, as well as TB-confirmed patients, were excluded from the study.

Microbiological processing of clinical samples: All the samples were processed for isolation and identification of bacteriological agents of pneumonia by following standard microbiological procedures [37-40].

DNA extraction: For genotypic analysis of *S. pneumoniae*, DNA was extracted from *S. pneumoniae* ATCC® 49619™ (type strain), other bacterial culture isolates, as well as from Todd Hewitt Broth (HiMedia-M313) inoculated with different clinical samples. DNA extraction was performed using a silica column-based DNA extraction kit (HiMedia). All extracted DNA samples were stored at -20°C for further use.

Conventional PCR assay of *S. pneumoniae* specific genes: Specific primers were selected for the molecular detection of *S. pneumoniae*. Monoplex and duplex PCR assays were developed and standardised using DNA from the *S. pneumoniae* ATCC®49619™ control strain [36,41]. This developed and optimised duplex PCR protocol was used for the molecular detection of *S. pneumoniae* from clinical samples. At the end of the PCR, amplicons were sent for sequencing at Eurofins Genomic India Pvt., Ltd., Bengaluru, Karnataka, India.

Primers: An extensive literature review was conducted for the selection of primer sequences for the amplification of the *Cps*A and *Lyt*A genes [1,2,11-18,36,41]. Based on the results of NCBI Nucleotide BLAST Analysis, the primers listed in [Table/Fig-3] [36,41] were selected for the *S. pneumoniae* specific *Cps*A and *Lyt*A gene amplification.

Target gene	Primer sequence	Product size	Reference
CpsA	Forward: AGTGGTAACTGCGTTAGTCCTA	653 bp	[36]
	Reverse: GTGGCGTTGTGGTCAAGAG		
LytA	Forward: GGA GTA GAA TAT GGA AAT TAA TGT	263 bp	[41]
	Reverse: GCT GCA TAG GTC TCA GCA TTC CAA		

[Table/Fig-3]: Primers used for *S. pneumoniae* specific *CpsA* and *LytA* gene amplification [36,41].

Monoplex PCR for *S. pneumoniae* specific *CpsA* and *LytA* gene amplification: The oligonucleotide sequences of PCR primers used in this study are listed in [Table/Fig-3] [36,41]. The oligonucleotide primers for the amplification of *Cps*A and *Lyt*A genes, previously described by Luo YC et al., and Gillespie SH et al., respectively, were synthesised by Eurofins Genomic India Pvt., Ltd., [36,41]. After DNA extraction, PCR-based molecular techniques were used to detect *S. pneumoniae* with a set of primers designed for the recognition and amplification of the *S. pneumoniae* specific *Cps*A and *Lyt*A genes.

For PCR amplification of the *CpsA* and *Lyt*A genes, 10 pmol/µL forward and reverse oligonucleotide primers were used. The final PCR reaction mixture was prepared in a total volume of 25 µL. For optimisation of the monoplex PCR, different concentrations of primers and DNA samples were tested. The PCR reaction mixture contained PCR master mix (EmeraldAmp GT PCR Mix-2X Premix from TaKaRa Bio, Inc.), forward and reverse primers (concentrations ranging from 0.04, 0.2, to 0.4 pmol/µL), and bacterial genomic DNA template (between 25 to 50 ng). Amplification was performed using a PCR thermal cycler (HiMedia Prima 96). PCR conditions included an initial denaturation at 95°C for 15 minutes, followed by 30 cycles consisting of denaturation at 95°C for 30 seconds, primer annealing (52°C for the *Lyt*A gene and 58°C for the *Cps*A gene, for 30 seconds), extension at 72°C for 1 minute, and a final elongation at 72°C for 10 minutes. PCR amplicons were analysed on a 2% agarose gel and visualised by UV transillumination.

Based on the results of the optimisation of the monoplex PCR assays, the optimised *Cps*A and *Lyt*A gene-specific monoplex PCR reaction preparation included 1 µL (0.4 pmol/µL) each of forward and reverse primers, 12.5 µL of PCR master mix, and 10 µL (5 ng/µL) of DNA template in a total reaction volume of 25 µL. The PCR cycling conditions were the same as described above.

Development of *S. pneumoniae* specific duplex PCR assay: Based on the optimised monoplex PCR amplification assay for the *Cps*A and *Lyt*A genes, duplex PCR experiments were conducted to observe the simultaneous amplification of the *Cps*A and *Lyt*A genes in a single PCR reaction tube. Amplification was performed using a PCR thermal cycler (HiMedia Prima 96) with an annealing temperature gradient ranging from 48°C to 55°C and from 54°C to 68°C. PCR conditions included an initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing with a temperature gradient from 48°C to 68°C for 90 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes. PCR amplicons were analysed on a 2% agarose gel and visualized by UV transillumination.

Based on the results of the temperature gradient PCR experiment, the developed and optimized *Cps*A and *Lyt*A gene-specific duplex PCR assay reaction preparation included 1 µL (0.4 pmol/µL) each of forward and reverse primers specific for the *Cps*A gene, 1 µL (0.4 pmol/µL) each of forward and reverse primers specific for the *Lyt*A gene, 12.5 µL of PCR master mix, and 8.5 µL (5 ng/µL) of DNA template in a total reaction volume of 25 µL. Optimised PCR cycling conditions included an initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 30 seconds, primer annealing at 54°C for 90 seconds, extension at 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

Analytical specificity of developed *S. pneumoniae* specific duplex PCR: To determine the specificity of the *S. pneumoniae* specific duplex PCR assay, a DNA sample panel was prepared from various ATCC quality control bacterial strains as well as morphologically and biochemically well-characterised clinical isolates, as shown in [Table/Fig-2]. Twenty clinical strains of *S. pneumoniae* were isolated from different clinical samples, including sputum, cerebrospinal fluid (CSF), pleural fluid and blood culture samples. Phenotypic identification of pneumococcus was performed using the WHO-recommended algorithm (which includes optochin susceptibility, bile solubility and serotyping of cultured α-hemolytic colonies) to ensure the identification of the *S. pneumoniae* isolate, confirmed by using the Vitek 2 Compact Machine with the VITEK 2 GP IP Card (Reference No. 21342-Biomerieux), which consists of various test panels. From these different bacterial isolates, DNA was extracted using a silica column-based kit procured from HiMedia. This DNA sample panel was used for specificity measurement. A non template control (NTC) was included in all experimental setups, and the experiments were repeated three times. The protocol for the *S. pneumoniae* specific duplex PCR (for *Cps*A and *Lyt*A genes) as described above was followed using these different bacterial DNA templates.

Analytical sensitivity of developed *S. pneumoniae* specific duplex PCR: The analytical sensitivity of the *S. pneumoniae* specific duplex PCR assay was determined by using different concentrations of *S. pneumoniae* ATCC® 49619™ DNA. A bacterial suspension of *S. pneumoniae* ATCC® 49619™ was prepared in sterile nuclease-free water and the optical density was adjusted to 2.0 McFarland standard using the DensiCHEK™ PLUS VITEK Optical Density Meter (BioMerieux), which corresponds to 6×10^8 CFU/mL. Serial 10-fold dilutions from this 6×10^8 CFU/mL suspension were carried out to estimate the lower limit of detection for *Cps*A and *Lyt*A gene amplification. A 100 µL aliquot of the 2.0 McFarland turbidity-adjusted *S. pneumoniae* suspension was transferred into a 1.5 mL centrifuge tube containing 900 µL of sterile nuclease-free water and the process was repeated until 10 dilutions were prepared. These 10-fold serially diluted bacterial suspensions were used for colony-forming unit (CFU) enumeration and for DNA extraction

using the HiMedia kit. The 10-fold serially diluted *S. pneumoniae* DNA samples (42.5 ng, 4.25 ng, 0.425 ng, to 0.0425 fg) were then used to determine the lower limits of detection of the developed and optimised *S. pneumoniae* specific duplex PCR assay [Table/Fig-4]. After PCR, amplicons were electrophoresed on a 2% agarose gel and visualised under UV light by EtBr fluorescence.

Tube/Lane no.	Corresponding CFU/mL	Corresponding DNA concentration (ng/µL)
1	6×10^{8}	425×10^{-1}
2	6×10^{7}	425×10^{-2}
3	6×10^{6}	425×10^{-3}
4	6×10^{5}	425×10^{-4}
5	6×10^{4}	425×10^{-5}
6	6×10^{3}	425×10^{-6}
7	6×10^{2}	425×10^{-7}
8	6×10^{1}	425×10^{-8}
9	6	425×10^{-9}
10	0.6	425×10^{-10}

[Table/Fig-4]: 10-fold serial dilution of 2.0 McFarland turbidity matched *S. pneumoniae* ATCC® 49619™ suspension and corresponding CFU/mL.

Validation of developed *S. pneumoniae* specific duplex PCR assay: The developed and optimised *S. pneumoniae* specific duplex PCR assay was validated by testing DNA samples extracted from pure culture isolates, spiked samples and clinical samples. The duplex PCR amplified products obtained from different *S. pneumoniae* clinical isolates, as well as from clinical samples, were purified using a PCR product purification kit (HiMedia-MB512). Purified PCR products were sent to Eurofins Genomics India Pvt., Ltd., for standard Sanger sequencing purposes. The same primers as described in [Table/Fig-3] were used for this purpose.

Pure culture: Bacterial genomic DNA extracted from pure clinical culture isolates of *S. pneumoniae*, *Klebsiella pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* and *S. mitis* were subjected to the *S. pneumoniae* specific duplex PCR assay.

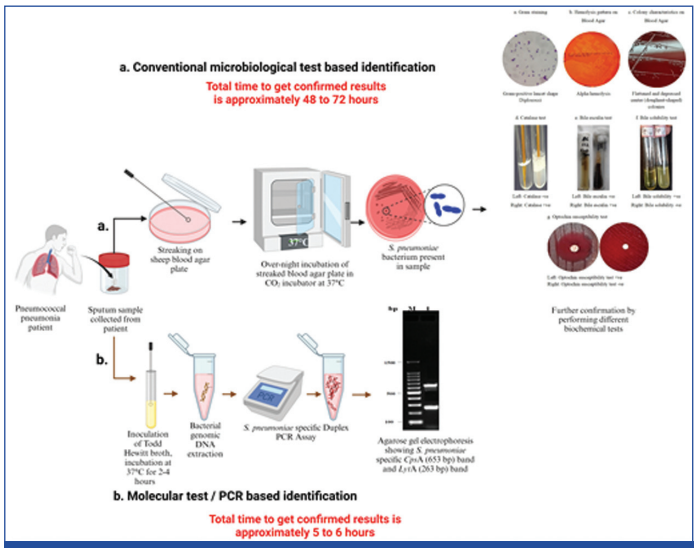
Spiked samples: Pure culture of *S. pneumoniae* was spiked/mixed with pure cultures of *Klebsiella pneumoniae*, *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* and *S. mitis*. DNA was extracted from these spiked samples and used for the *S. pneumoniae* specific duplex PCR assay to assess the interference caused by the presence of other bacterial DNA.

Clinical samples: Clinical samples like sputum, blood culture, nasopharyngeal (NP) or oropharyngeal (OP) swabs, CSF and pleural fluid from pneumonia patients were used. A loopful of the clinical sample was inoculated in Todd Hewitt Broth (HiMedia-M313) and incubated for four hours at 37°C in a candle jar for the cultivation of Streptococci prior to DNA extraction. After four hours of incubation, a loopful of culture was streaked on a sheep blood agar plate (HiMedia-MP1301) and incubated overnight at 37°C in a candle jar to observe the growth of *S. pneumoniae*. A bacterial cell pellet was prepared from 1 mL of Todd Hewitt broth culture in a sterile 2 mL tube by centrifugation at 2250 × g for 10 minutes at room temperature. This pellet was subjected to DNA extraction using a silica column-based bacterial genomic DNA extraction kit (HiMedia-MB505). The extracted DNA was subsequently used for the detection of *S. pneumoniae* specific *Cps*A and *Lyt*A genes using the optimised and standardised *S. pneumoniae* specific duplex PCR protocol as described above.

After overnight incubation, sheep blood agar plates were analysed for the growth of *S. pneumoniae*. Any suspected colonies of *S. pneumoniae* were confirmed phenotypically by traditional microbiological tests, such as colony characteristics, bile solubility test and optochin susceptibility test. Phenotypic identification to ensure the *S. pneumoniae* isolate was confirmed using the Vitek 2 Compact Machine with the VITEK® 2 GP ID Card (Reference No. 21342-Biomerieux), which consists of various test panels.

Sequence similarities analysis: From the sequencing data of the *Cps*A and *Lyt*A gene products, sequence contig assembly was carried out using BioEdit. Using the NCBI Basic Local Alignment Search Tool (BLAST), available at https://blast.ncbi.nlm.nih.gov/blast.cgi, homology searching for sequences of *S. pneumoniae* *Cps*A and *Lyt*A gene products against references of *S. pneumoniae* in genomic databases was performed. These results were analysed to calculate the identities, similarities and differences among the sequenced queries. Gene sequence data were submitted to NCBI.

The total turnaround time comparison between conventional microbiological test-based identification and molecular test-based identification of *S. pneumoniae* from clinical samples is briefly illustrated in [Table/Fig-5].



[Table/Fig-5]: Total turnaround time comparison of: a. Conventional Microbiological test based identification; and b. Molecular test/PCR based identification of *S. pneumoniae* from clinical sample (Created with BioRender.com).

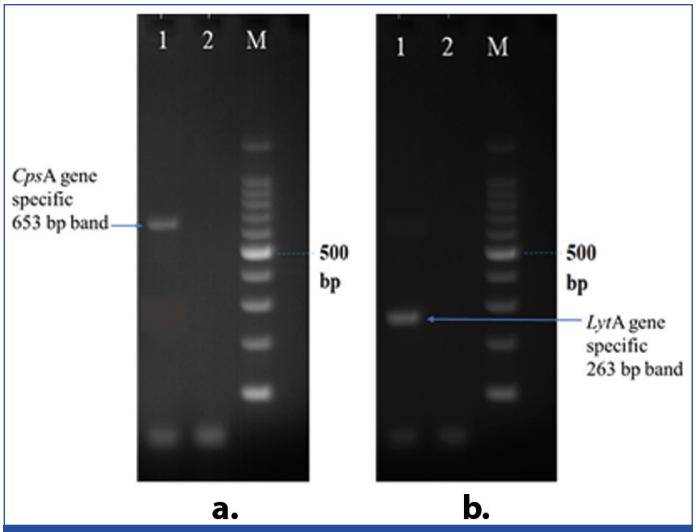
STATISTICAL ANALYSIS

All tests were performed in duplicates. Data from all studies were collected scientifically for tabulation and analysis. Sensitivity, specificity, PPV and NPV were calculated using GraphPad version 10.0.0 (153) software. The level of significance was determined through Fisher's exact test (GraphPad online tool, https://www.graphpad.com/quickcalcs/contingency1/). Tests were considered significant at a p-value of <0.05.

RESULTS

Results of optimisation of monoplex PCR for *CpsA* and *LytA* gene amplification: Two specific genes, *Cps*A and *Lyt*A, were selected for the molecular detection of *S. pneumoniae*. Initially, a monoplex PCR was developed and optimised, followed by the duplex PCR assay for the diagnosis of *S. pneumoniae*. The monoplex PCR assay for the amplification of the *Cps*A and *Lyt*A genes was carried out in a total of 25 µL of PCR reaction mixture. Different concentrations of PCR reagents were tested. In these experiments, a dark, intense band of the *Cps*A (653 bp) and *Lyt*A (263 bp) genes was obtained with 10 µL (5 ng/µL) of DNA template from *S. pneumoniae*, 1 µL (0.4 pmol/µL) each of forward and reverse primers and an annealing temperature of 52°C for the *Lyt*A gene and 58°C for the *Cps*A gene, as shown in [Table/Fig-6].

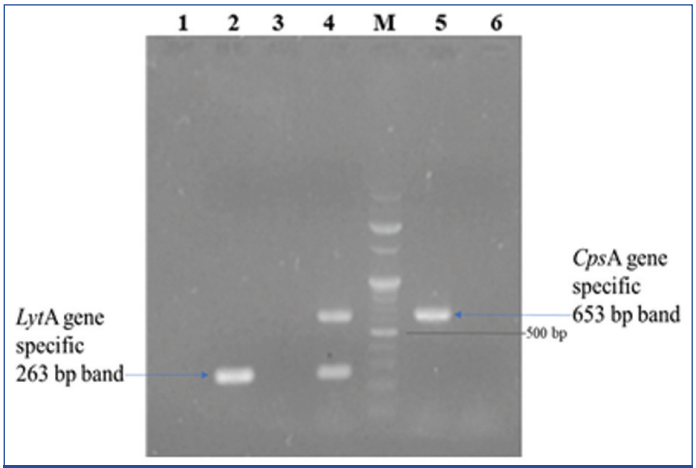
Results of optimisation of duplex PCR for *CpsA* and *LytA* gene amplification: After optimising the monoplex PCR assay for the *Cps*A and *Lyt*A genes, the optimisation of the duplex PCR was carried out by performing a temperature gradient experiment within the range of 48°C to 68°C. The maximum intensity for both genes was observed at an annealing temperature of 54°C. Therefore, 54°C



[Table/Fig-6]: Monoplex PCR Optimisation for *CpsA* gene and *LytA* gene.
a. Lane 1: *CpsA* gene specific 653 bp band, Lane 2: Non Template Control (NTC); b. Lane 1: *LytA* gene specific 263 bp band, Lane 2: Non Template Control (NTC). Lane M: DNA marker (100 bp) (TaKaRa Bio. Inc. _Cat. No.3422A)

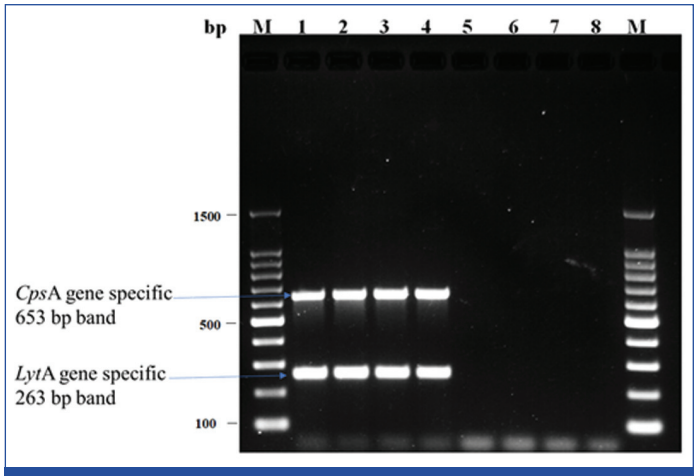
was selected as the ideal primer annealing temperature for further optimisation and validation of the protocol.

Results of optimisation of duplex PCR: After finalising the optimum primer annealing temperature, the *Cps*A and *Lyt*A gene-specific duplex PCR was performed using DNA extracted from *S. pneumoniae* ATCC® 49619™, resulting in the desired amplification of 653 bp and 263 bp amplicons, respectively, as shown in [Table/Fig-7].



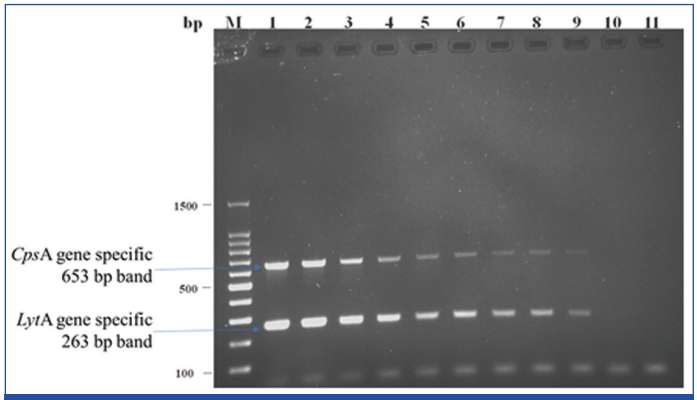
[Table/Fig-7]: Monoplex and duplex PCR for *CpsA* and *LytA* gene.
Lane 1 and 2: Non Template Control (NTC) and Test band (263 bp) respectively by *LytA* gene specific Monoplex PCR Assay, Lane 3 and 4: Non Template Control (NTC) and Test bands (653 and 263 bp) respectively by *CpsA* and *LytA* genes specific Duplex PCR Assay, Lane 5 and 6: Test band (653 bp) and Non Template Control (NTC) respectively by *CpsA* gene specific Monoplex PCR Assay; Lane M: DNA marker (100 bp) (TaKaRa Bio. Inc.)

Results of analytical specificity: A DNA sample panel from 40 different bacterial isolates [Table/Fig-2] was used to check the analytical specificity of the *S. pneumoniae* specific duplex PCR assay. As shown in [Table/Fig-8], *Cps*A and *Lyt*A specific bands were obtained only with the *S. pneumoniae* isolates (including quality control strains and clinical isolates with different serotypes, such as 1, 3, 6A, 6C, 10A, 14, 19A, 19F, 23B). No PCR amplification products were seen with either primer pair in the DNA from *S. aureus* ATCC® 25923™, *K. pneumoniae* ATCC® 13883™, *E. coli* ATCC® 25922™, *Pseudomonas aeruginosa* ATCC® 27853™ quality control strains, as well as clinical isolates of the same species. Additionally, no amplification was observed with clinical isolates of *S. mitis*, *S. aureus* and *Streptococcus dysgalactiae* subsp. *equisimilis* on the agarose gel. Thus, the optimised and standardised *S. pneumoniae* duplex PCR assay demonstrated 100% specificity. No false amplification was observed with bacterial isolates other than *S. pneumoniae* used for the specificity test of the duplex PCR assay.



[Table/Fig-8]: Analytical specificity of *S. pneumoniae* specific duplex PCR assay.
Lane 1. Positive control strain *S. pneumoniae* ATCC® 49619™ 2. *S. pneumoniae* (Clinical Isolate 1 - Serotype 19A), 3. *S. pneumoniae* (Clinical Isolate 2 - Serotype 6A), 4. *S. pneumoniae* (Clinical Isolate 3 - Serotype 14), 5. *S. mitis* (Clinical Isolate), 6. *S. aureus* ATCC® 25923™, 7. *Klebsiella pneumoniae* ATCC® 13883™, 8. Non Template Control (NTC), Lane M: DNA marker (100 bp) (TaKaRa Bio. Inc.). 653 bp band of *CpsA* gene and 263 bp band of *LytA* gene

Results of analytical sensitivity: The developed and optimised *S. pneumoniae* specific duplex PCR assay showed an analytical sensitivity/limit of detection of 6 CFU/mL. DNA extracted from the 9th tube containing 6 CFU/mL (0.425 fg/µL) was the minimum amount of DNA [Table/Fig-4] needed to detect both the *Cps*A and *Lyt*A genes, as shown in [Table/Fig-9].



[Table/Fig-9]: Analytical Sensitivity/Lower Limit of Detection (LLoDs) of *S. pneumoniae* specific Duplex PCR assay.
Lane 1: 6×10^{8} CFU/mL, 2: 6×10^{7} CFU/mL, 3: 6×10^{6} CFU/mL, 4: 6×10^{5} CFU/mL, 5: 6×10^{4} CFU/mL, 6: 6×10^{3} CFU/mL, 7: 6×10^{2} CFU/mL, 8: 6×10^{1} CFU/mL, 9: 6 CFU/mL, 10: 0.6 CFU/mL, 11: Non Template Control (NTC). Lane M: DNA marker (100 bp) (TaKaRa Bio. Inc.)

Results of validation of *S. pneumoniae* specific duplex PCR assay:

Pure culture isolates: *Cps*A (653 bp) and *Lyt*A (263 bp) gene-specific bands were obtained only with *S. pneumoniae* clinical isolates. No false positive results were found with other pure bacterial culture isolates.

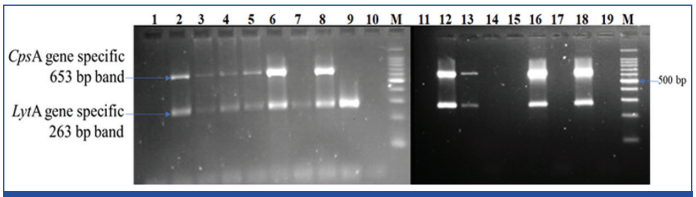
Spiked samples: There was no interference observed from the presence of other bacterial DNA, as intense *Cps*A and *Lyt*A gene-specific bands were obtained with the spiked samples.

Clinical samples: DNA samples extracted from four hours of incubation in Todd Hewitt broth, inoculated with 75 different clinical specimens [Table/Fig-10], were subsequently tested by the *S. pneumoniae* specific duplex PCR assay for the detection of both the *Cps*A and *Lyt*A specific genes. The *Cps*A and *Lyt*A gene-specific PCR amplicons were purified and sent to Eurofins Genomics India Pvt. Ltd. for standard Sanger sequencing purposes. The *Lyt*A and *Cps*A gene sequences were compared for similarity with references of *S. pneumoniae* in genomic database banks using the NCBI BLAST. Gene sequence data were submitted to NCBI, and a GeneBank accession number (OQ290840.1) was received.

Sample type	Total number of samples tested (n=75)	Phenotypically identified by traditional microbiology tests (n=15)	Genotypically identified by Duplex PCR assay		
			Only *LytA* gene amplification occurred in Duplex PCR assay (n=3)	Only *CpsA* gene amplification occurred in Duplex PCR assay (n=0)	Both *LytA*+*CpsA* genes amplification occurred in Duplex PCR assay (n=23)
Nasopharyngeal/Oropharyngeal (NP/OP) swab	24	0	2*	0	9
Sputum	12	7	1**	0	6
Blood culture	22	4	0	0	4
CSF	3	3	0	0	3
Pleural fluid	7	1	0	0	1
Other samples	7	0	0	0	0
Total	75	15 (20%)	3 (4%)	0	23 (31%)

[Table/Fig-10]: Results of comparing the phenotypic and genotypic identification of *S. pneumoniae*.
*Sanger sequencing data of these two *LytA* gene specific PCR amplicons revealed that the organisms are non-pneumococcal species (*Streptococcus pseudopneumoniae* IS7493). Phenotypically also not identified by traditional microbiology tests. **Sanger sequencing data of this one *LytA* gene specific PCR amplicon revealed that the organism is *Pneumococci*. Phenotypically also identified by traditional microbiology tests that the isolated organism is *Streptococcus pneumoniae* having mutated *CpsA* gene

As shown in [Table/Fig-10], out of 75 clinical samples, 15 (20%) showed typical growth of *S. pneumoniae* and were confirmed phenotypically by traditional microbiological tests (colony characteristics, bile solubility test and optochin susceptibility test), as well as by the Vitek 2 machine. Among these 15 (20%) phenotypically identified *S. pneumoniae* isolates, 14 pneumococcal isolates harbored the *Cps*A and *Lyt*A genes, irrespective of the source of isolation [Table/Fig-11].



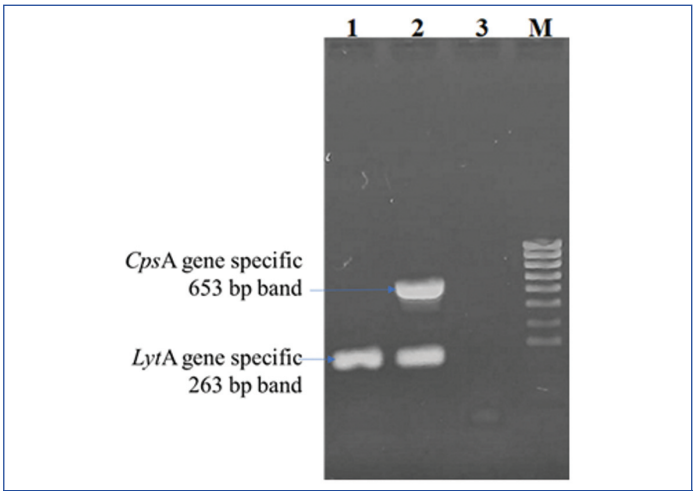
[Table/Fig-11]: Results of analysis of clinical samples by *S. pneumoniae* specific duplex PCR.
Lane 1 to 17: DNA from clinical samples, Lane 1, 10, 11, 14, 15, and 17: No *LytA* and *CpsA* specific bands, Lane 2-6, 8, 12, 13, and 16: *LytA* and *CpsA* specific bands, Lane 18: *S. pneumoniae* ATCC® 49619™ DNA as a positive control, Lane 19: Non Template Control (NTC), Lane 7 and 9: Only *LytA* specific band, no *CpsA* specific band; Lane M: DNA marker (100 bp) (TaKaRa Bio. Inc.)

However, one *S. pneumoniae* isolate from a sputum sample of a 63-year-old male patient suffering from lobar pneumonia showed amplification of only the *Lyt*A gene; the *Cps*A gene was not amplified by the developed duplex PCR assay. This sample was retested to confirm the absence of the *Cps*A gene [Table/Fig-12]. The *S. pneumoniae* strain showed a 6A serotype by the Quellung reaction and sequencing data of this *Lyt*A gene confirmed the presence of *S. pneumoniae*.

DNA templates from two nasopharyngeal/oropharyngeal (NP/OP) swab samples enriched in Todd Hewitt broth amplified only the *Lyt*A gene and did not yield a *Cps*A gene-specific band [Table/Fig-10,11,13] (Lane 7 and 9). These two samples were culture-negative for *S. pneumoniae*. Sequencing data from these two *Lyt*A genes confirmed the presence of *Streptococcus pseudopneumoniae* IS7493.

DNA extracted from four hours of incubation in Todd Hewitt broth inoculated with NP/OP swabs (n=24) produced significantly more *Cps*A and *Lyt*A genes, showing a 12% (n=9) increase compared to other clinical samples [Table/Fig-10,11,13]. Sequencing data of these *Cps*A and *Lyt*A genes confirmed the presence of *S. pneumoniae*. Notably, these 9 (12%) NP/OP swab samples were culture-negative for *S. pneumoniae*.

A total of 15 (20%) of different clinical samples showed positive results by the conventional microbiological culture method. *S. pneumoniae* was isolated from 7 (9.3%) of sputum, 4 (5.3%) of blood cultures, 3 (4%) of Cerebrospinal Fluid (CSF), and 1 (1.3%) of pleural fluid clinical samples, respectively. Using the *S. pneumoniae* specific duplex PCR assay, 23 (30.7%) of samples tested positive for *S. pneumoniae*, as these samples exhibited amplification for both the



[Table/Fig-12]: *S. pneumoniae* Specific Duplex PCR result: Lane 1. *S. pneumoniae* (Serotype 6A) isolated from sputum sample gave only *LytA* band and no *CpsA* gene specific band; 2: *S. pneumoniae* ATCC® 49619™; 3: Non Template Control (NTC); Lane M: DNA marker (100 bp) (TaKaRa Bio. Inc.).

	Culture +Ve	Culture -Ve	Total
PCR +ve	14	9	23
PCR -ve	1	51	52
Total	15	60	75

[Table/Fig-13]: Association between culture test and PCR.
Fisher's exact test
The two-tailed p-value is less than 0.0001
The association between rows (groups) and columns (outcomes) is considered to be extremely statistically significant

*Lyt*A and *Cps*A genes. Of these, *S. pneumoniae* was detected in 9 (12%) of NP/OP swabs, 6 (8%) of sputum, 4 (5.3%) of blood cultures, 3 (4%) of CSF, and 1 (1.3%) of pleural fluid samples.

The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the developed duplex PCR assay for the detection of *S. pneumoniae* in comparison with the culture method were 100%, 96.7%, 88%, and 100%, respectively [Table/Fig-13].

DISCUSSION

Differentiating *S. pneumoniae* from its close commensal relative is essential for understanding its clinical importance, pathogenic features and epidemiology in clinical microbiology and research. Several phenotypic features were once thought to be distinct to *S. pneumoniae*; however, new data show that these features are also present in a large proportion of *Streptococcus mitis* isolates [11,21,42,43]. Currently, a confirmative diagnosis of *S. pneumoniae* infection requires the isolation of pneumococci from clinical samples. This isolation method provides the most precise diagnosis, but culture is positive in less than 30% of pneumococcal

pneumonia patients. The culture may be negative if the patient has been treated with antibiotics before the sample collection, and most importantly, it may take two to three days to obtain definitive results [11,21,44-47]. Thus, diagnosis and treatment are delayed if only the conventional culture method is employed. An alternative to the culture method is the detection of antigens using various fluids, such as serum and urine. However, this method has low sensitivity and specificity, even with samples collected from culture-confirmed pneumococcal bacteremic pneumonia patients. Another alternative assay, based on the immunological detection of pneumococcal capsular polysaccharide, requires pneumococcal serotype-specific antisera and viable bacteria for serotyping. The cost of antisera and the requirement for viable bacterial cultures limit their use in routine diagnostic laboratories [11,21,46].

In the last decade, significant progress has been made in the development of molecular diagnostic methods for detecting the aetiological agents of infectious diseases. Recently, PCR has enabled the detection of various infectious pathogens by selectively amplifying specific microbial genes or DNA fragments. Thus, PCR has significant potential for enhancing clinicians' capacity to diagnose infectious diseases caused by fastidious pathogens, such as *S. pneumoniae* [25,46]. The growth of pneumococci necessitates the use of sheep blood agar and a CO_2-enriched environment (a CO_2 incubator), both of which are typically unavailable in routine diagnostic laboratories in developing countries [48]. In recent years, for the rapid, easy and early diagnosis of pneumococci, several different *S. pneumoniae*-specific genes have been targeted for amplification by PCR. Based on findings from various researchers, the *Cps*A and *Lyt*A genes are the most reliable candidates for accurately identifying pneumococci [11,24,26-27,41,46].

A study conducted by Suzuki N et al., concluded that the most effective primers for detecting *S. pneumoniae* are the *Lyt*A primers. The *Lyt*A gene has been identified as a reliable target for *S. pneumoniae* detection [41,49,50]. Compared to other genes, such as the *PLY* gene, the *Lyt*A gene reportedly exhibits superior specificity [51]. However, present study found two samples that amplified only the *Lyt*A gene using the developed *S. pneumoniae* specific duplex PCR assay. The BLAST analysis of these samples indicated the presence of *S. pseudopneumoniae*. Present study finding aligns with Seki M et al., which noted that organisms sharing a genotype with *S. pseudopneumoniae* or *S. mitis* can harbor the *Lyt*A gene typically associated with pneumococci [31]. Thus, a single *Lyt*A gene-based PCR approach may be sufficient for cost-effective screening for the presumptive identification of *S. pneumoniae*. Still, given that other species besides pneumococci have been discovered [27,33], leading to false positive results, targeting an additional gene is advantageous for the accurate identification of pneumococci.

Examination of all pneumococcal cps loci showed that the *Cps*A and *CpsB* genes have a high degree of conservation, suggesting that they likely descended from a common ancestor [52]. In the present study, the *Cps*A gene was selected as an additional target gene for the accurate identification of pneumococci. The *Cps*A gene-specific primers used in this study amplify an extremely conserved region that exists in all capsular loci of *S. pneumoniae*. In contrast, many researchers have found that the lack of amplification of the *Cps*A gene may result from *S. pneumoniae* having mutated or distinct capsular genes, or a complete absence of the capsular locus. Serotyping of these isolates with antisera yielded no positive results. Few studies found that approximately 3.7% of such non typeable isolates were present [53,54]. Therefore, any PCR assay targeting only a single *Cps*A gene may result in false-negative results. In addition to the *Cps*A gene, another promising target gene recommended by the WHO for precise identification of pneumococci is the *Lyt*A gene. However, members of the mitis group of Streptococci may also yield false positive amplification for the *Lyt*A gene [11,55].

As a result of these findings, it is advantageous to use a combination of at least two genes for the molecular-based detection of *S. pneumoniae*. Consequently, the present study aimed to evaluate the feasibility of a Duplex PCR assay targeting the *Cps*A and *Lyt*A genes for the detection of pneumococci from clinical samples. The goal of this study was to establish a highly sensitive and species-specific PCR-based pneumococcal DNA amplification test and to determine its reliability in discriminating among species.

The developed and optimised duplex PCR assay showed 100% specificity with different *S. pneumoniae* isolates (including serotypes 1, 3, 6A, 6C, 10A, 14, 19A, 19F, and 23B) as well as closely related bacterial cultures like *S. mitis* and *Streptococcus dysgalactiae* subsp. *equisimilis* clinical isolates. No false positive results were found with different gram-positive and gram-negative bacterial strains isolated from clinical cultures and ATCC type strains.

Present study found that one *S. pneumoniae* isolate from a sputum sample of a 63-year-old male patient suffering from pneumonia showed no amplification of the *Cps*A gene. This isolate was optochin-susceptible, bile-soluble and susceptible to the penicillin antibiotic. The Quellung reaction for this *Pneumococcus* revealed the 6A serotype. When compared for similarity with references for *S. pneumoniae* in genomic database banks using the NCBI BLAST, the Sanger sequencing data of the *Lyt*A-specific band obtained from this *Pneumococcus* showed a 97.74% match. Thus, the detection of at least two genes in the case of bacteria like *S. pneumoniae*, where mutation and horizontal gene transfer are common, is highly recommended and advantageous compared to targeting a single gene.

In the present study, two NP/OP swab samples yielded only the *Lyt*A gene-specific band and no *Cps*A band. These results support previous findings indicating that the *Lyt*A gene is present in viridans group Streptococci, such as *S. pseudopneumoniae*. This again reinforces our view that a single gene should not be targeted for the detection of *S. pneumoniae*.

Direct analysis of Todd Hewitt broth inoculated with clinical samples using the *S. pneumoniae*-specific Duplex PCR assay showed that 9 NP/OP swabs produced both *Cps*A and *Lyt*A-specific bands. However, the same samples did not yield *S. pneumoniae* growth on sheep blood agar plates. The sequencing data of these PCR amplicons confirmed the presence of *S. pneumoniae* when compared with NCBI BLAST. This is due to the assay described here having very high analytical sensitivity/limit of detection (6 CFU/mL), requiring only a small amount of DNA (0.425 fg/µL) to detect both the *Cps*A and *Lyt*A genes. Thus, the developed and optimised Duplex PCR assay is more sensitive for detecting *S. pneumoniae* compared to the conventional culture method. Present study findings are also supported by other researchers [22].

Limitation(s)

The data presented here also indicate that the *S. pneumoniae*-specific Duplex PCR assay described in this study cannot distinguish colonisation from infection in respiratory tract samples. Further studies should be carried out with a larger number of clinical samples.

The developed and optimised *S. pneumoniae*-specific Duplex PCR assay has demonstrated adequate sensitivity and specificity to warrant further clinical development. This test could serve as the foundation for a diagnostic battery alongside other tests for accurate and specific diagnosis of pneumococcal disease.

CONCLUSION(S)

Present study developed a Duplex PCR assay for the selective amplification of *S. pneumoniae* DNA based on the *Cps*A and *Lyt*A genes. Authors determined its reliability in species-specific discrimination to be exceptionally high. The combination of *S. pneumoniae*-specific

*Cps*A and *LytA* genes described in the present study facilitates a more specific, sensitive, user-friendly and reliable detection of *S. pneumoniae* from clinical samples. The culture-independent Duplex PCR analysis of the enriched clinical samples shown here is potentially advantageous for facilitating quick, accurate routine diagnoses and provides valuable data on *S. pneumoniae* infections that may be missed by traditional microbiological techniques.

Authors' contribution: The article covers the original findings presented in the study. Additional inquiries, including sequencing data, may be sent to the corresponding author.

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