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Designing and Optimizing a Multiplex PCR Method for the Simultaneous Identification of Respiratory Pathogens from Patients in Selected Hospitals of Mashhad

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Abstract

Background and Objective: Pneumonia is considered one of the most serious and fatal diseases among children and adults worldwide. *Streptococcus pneumoniae* and *Haemophilus influenzae* are pathogenic bacteria causing community-acquired pneumonia. *Mycoplasma pneumoniae*, *Chlamydia pneumonia*e, and *Legionella pneumophila* are another group of acute pathogens. The present study aims to identify the mentioned infectious agents through multiplex PCR (Multiplex Polymerase Chain Reaction).

Materials and Methods: This experimental and developmental study used the multiplex PCR method to identify the specific sequences of the genome of prominent pneumonia agents simultaneously. The specific genes selected in this study are LytA for *Streptococcus pneumoniae*, BexA for *Haemophilus influenzae*, P1 for *Mycoplasma pneumoniae*, Mip for *Legionella pneumophila*, and ompA for *Chlamydia pneumonia*e. Also, the 16s ribosomal gene was used as a positive control in this study.

Results: In this study, 180 patients with respiratory infections who were referred or hospitalized at Mashhad Hospital were sampled. The results showed that 13 samples were positive. The frequency distribution of *Streptococcus pneumoniae* with 5 people (2.8%) was the most bacteria isolated. Additionally, *Legionella pneumophila* bacteria were isolated in two people, *and Mycoplasma pneumoniae* bacteria and *Haemophilus influenzae* bacteria were isolated in three people (1.6%). *Chlamydia pneumonia*e was not isolated. The results revealed that simultaneous identification of *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae* is possible in one PCR reaction.

Conclusion: This method can be used in the direct and simultaneous identification of these factors in respiratory samples.
Keywords: Multiplex PCR, Respiratory infections, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Legionella pneumophila*

Introduction

Pneumonia is considered one of the primary causes of morbidity and mortality among children worldwide. The annual incidence of pneumonia worldwide is estimated to be about 120 million in children aged below 5 years. Among them, 1.3 million cases lead to death (1). The severe pneumonia mortality rate worldwide is estimated at

8.7%. Most deaths occur in the younger age group. Specifically, 81% of all pneumonia deaths occur in children less than 2 years of age (2). It replaced the TaqMan-based fluorescent probe techniques due to the limitations of conventional PCR, which required post-PCR modifications and lacked sensitivity and quantification. The commercial and independent, laboratory-developed, sequence-specific, proliferation-based, multiplex real-time PCR assays have grown rapidly in recent years (3). These are available for more efficient identification of selected respiratory pathogens.

Using PCR is limited in diagnostic laboratories due to cost and sometimes the availability of sufficient test sample volume. Multiplex PCR is described to overcome these shortcomings and increase the diagnostic capacity of PCR. More than one target sequence can be proliferated by including more than one pair of primers in the reaction in multiplex PCR. Multiplex PCR can save significant time and effort in the laboratory without compromising the experiment instrument. Multiplex PCR has been successfully used since its introduction in many areas of nucleic acid detection, including gene deletion analysis, mutation, and polymorphism analysis (4, 5), quantitative analysis, and RNA detection. This technique has been proven to be a valuable method for identifying viruses, bacteria, fungi, and parasites in infectious diseases (6-10). The multiplex PCR optimization aims to minimize or reduce such non-specific interactions. Empirical test and a trial-and-error approach may be used when testing multiple primer pairs since there is no means for predicting the performance characteristics of a selected primer pair even among those that meet the general primer design parameters. However, special attention should be paid to primer design parameters such as the primers' homology with the target nucleic acid sequence, their length, GC content, and their concentration.

All primer pairs in a multiplex PCR should provide similar proliferation efficiency for their relevant target. This goal may be achieved using primers with almost the same optimal annealing temperatures (a primer length of 18–30 bp or more and a GC content of 35–60% is satisfactory) and should not show significant homology with each other (11-16). Multiplex PCR should avoid using nested primers that require a second-round reinforcement, if possible. The second one greatly contributes to false positive results due to transitional pollution. However, anti-pollution protocols including PCR controls (reaction controls and sample extraction) should be implemented in all PCR-based protocols. Similarly, precautious and methodological measures should be taken to prevent false negative results caused by reaction failure. Multiplex PCRs, which reinforce target sequences with the presence of target nucleic acids, have been developed to show the reaction failure (17-120).

Jiang et al. (2022) conducted a study titled "Development of the Multiplex PCR method to simultaneously identify 9 microorganisms causing acute respiratory infection". The results revealed that all positive strains were detected and the detection constraints were between 250 and 500 copies per milliliter (1-25-2.5) (21). Alikhani et al. (2022) identified pathogenic agents in nasopharynx secretions in pneumonia and non-pneumonia patients with Multiplex RT-PCR. The results of examining 60 patients revealed the mean and the standard deviation of 52.13± 16.84 (22). Lim et al. (2021) conducted a study titled "Multiplex Real-Time PCR to simultaneously diagnose four pneumonia bacterial pathogens". Multiplex Real-Time PCR is a reliable tool that helps to evaluate decision-making better than other standard methods (23). Lapa et al (2021) conducted a study titled "Development of Multiplex RT-PCR with motionless primers to detect human pneumonia infectious pathogens. The results showed that this design was appropriate for preventing cross-contamination and for simultaneously testing many experimental samples. It can also detect several pneumonia pathogens (24).

Chio et al. (2021) conducted a study titled "Respiratory pathogens using Multiplex PCR Panel during the early Coveid-19 epidemic in a Taiwan hospital". The results revealed that out of 253 respiratory samples, 135 (53.4%) belonged to adults and 118 (46.6%) belonged to children. The total positive rate of 33.9% was found at 21.48% in adults and 48.31% in children (25). Jiang et al. (2020) conducted a study titled "Development and use of a method for detecting 27 respiratory pathogens using Multiplex RT-PCR and MassARRAY technology. The results indicated that ten pathogens were detected in this measurement. Among them, Rhinovirus, Adenovirus, and the A PDMH1N1 influenza virus were the most common ones (26).

In the present study, a multiplex method was developed, standardized, and comprehensively approved to achieve the simultaneous and rapid detection of selected pathogens. They include common bacterial pathogens that often cause acute respiratory infections in clinical procedures. The primer pair used in this study has been designed to

optimize the reinforcement. In this primer pair, elements such as primer homology with target nucleic acid sequences, G+C content, melting point, and product length have been theoretically optimized. Also, using the PCR technique to achieve the precise quantity of pathogenic nucleic acid molecules used to build standard curves was one of the innovative methodological features of this study. It facilitated linear validation and minimum reliable calculation.

Materials and Methods

This is a descriptive and cross-sectional study. It was conducted from the beginning of October 2021 to October 2022 for 1 year at the Mashhad School of Mashhad Medical Branch with the best facilities. The inclusion criteria in this study included all the people referred to the selected hospital in Mashhad suffering from respiratory infections. In this study, sampling was done among all patients in the age range of 3 to 80 years old who were referred to the selected hospital in Mashhad due to respiratory infections or were hospitalized with the physician's detection.

In this study, to prepare appropriate samples and isolate bacteria causing respiratory infections, nasopharyngeal swabs, throat swabs, and nasopharyngeal aspirates were used. Then, the samples were transferred to the microbiology research lab in the transitional environment. The samples were kept for research after transmitting to the laboratory at 4 C° or 70 for research investigations. Cochran statistical formula was used to calculate the sample size and it was determined to be 100. McCoy cellular medium, Broth PPLO medium (BCYE), Brain Heart Infusion medium, and agar medium containing 5 % sheep blood were used.

Experiment method

1. The bile salt solution was prepared in the following order. First,10% Sodium deoxycholate solution was prepared in the distilled water and kept at room temperature.

2. A separate colony was selected in a reader or chocolate agar plate and a drop of bile salt solution was poured directly on it.

3. The plate was placed on a very flat surface. This prevents the washing of the non-pneumococcal colonies leading to false positive reactions. Then, the plate was dried. Note: Placing the plate in the incubator accelerates the reaction speed.

4. When the reagent was dried (about 15 minutes), the area was examined for the disappearance or spread of the initial chlorine that indicates the solubility in the bile.

Primer design for proliferation through the polymerase chain reaction

To perform the polymerase chain reaction, the sequence of the primers was extracted from the articles and then examined on the NCBI site in the Blastn program, and the most appropriate prime sequences were selected. As stated, the primers used in this test related to the fragments of genes, including MIP, OMPA, P1, LYT, and BEX, which synthesized a fragment with different base pairs, as shown in Table (1).

Table 1: The sequence of primers used in this study

Gene	)’5-’3(primer sequence	Size
MIP	Forward GGTGCCGATTTGGGGAAGAA Reverse CAACAACGCCTGGCTTGTTT	253 bp
OMPA	Forward TGTAGGTAGTTTTGGCTGAGCA Reverse GTTGCTTTCCCCTTGCCAAC	201 bp
P1	Forward GAACGCCCAAGTGAAACCAC Reverse ATCCGGACGATTGGCTCATC	695 bp
Lyt	Forward GGTAGTACCAGCCTGTTCCG Reverse CACAGACGGCAACTGGTACT	215 bp

Bex	Forward AGTGGTGTTGAGCCTCCAAC Reverse TCATCGCACTCGGGCTATGT	400 bp
16S	GCAAACAGGATTAGATACCC Forward CGTCATCCCCACCTTCCTCC Reverse	427 bp

Performing Multiplex PCR technique

Small micro-tubes inside the rack were arranged samples based on the number of samples. All samples were numbered.

2- First, a mixture of Forward and Reverse primers (1µ) was poured into each microtube.

3. From each DNA, 4 µ were poured according to the microtube numbers.

4- From the Master mix solution, 10 µl (half the total volume) was poured into each microtube.

5. 4 µl of water was poured into each microtube, and the final volume of 20 µl was obtained.

6. After obtaining the final volume, all the samples were transferred to the spin after making sure they were closed.

7. After spinning for thirty seconds, the samples were transferred to the PCR device (the same was done for all 3 markers).

Table 2: Materials required for reproduction reaction for the research genes

Chemicals	Value of each reaction
Master mix	10 µl
Forward Primer	1 µl
Reverse Primer	10 µl
Template DNA	4 µl
H2O	4 µl
Total Volume	20 µl

Table 3 presents the proliferation reaction program of time and temperature.

Table 3: Polymerase chain reaction steps for the research genes

Number of cycles	Steps of polymerase chain reaction	Time (in seconds)	Temperature (in Celsius)
	Initial denaturation	300	95
35	denaturation annealing Elongation	30 30 60	95 59 72
	Final elongation	600	72

The process was performed in the thermocycler device in 35 cycles. The data from this study will be analyzed in SPSS software using the chi-square test and the ANOVA test. P-value≤0.05 will be considered a significant level.

Results

For qualitative observation of the extracted DNA, 5 µl of it was 1 % of agarose gel was electrophoresed, and the presence of DNA and its non-break was found based on the obtained band (Figure 1).

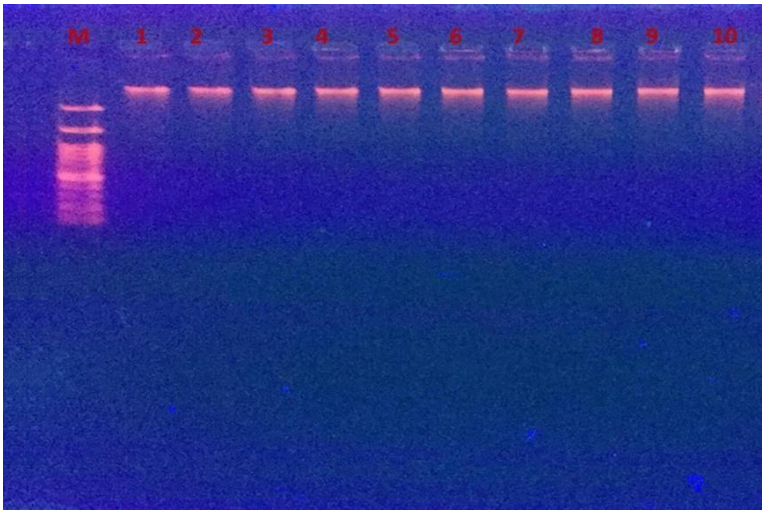

Figure 1: DNA product gel image, samples 1 to 10 examined, and m: Molecular marker 100 bp

To measure DNA quantitatively, the absorption rate was measured in the spectrophotometer with 1:49 and in the OD: 260 nm. Accordingly, the OD result of all samples for 260/280 and 260/230 relative to DNA showed normal values, indicating low protein and polysaccharide.

Results of Lyta gene proliferation of *Streptococcus pneumoniae* bacterium

As shown in Figure (2), the expected band that was the result of the proliferation of a part of the LyTA genes synthesized a fragment with a length of 215 bp from the DNA. The presence of this band in this range confirms the reaction validity.

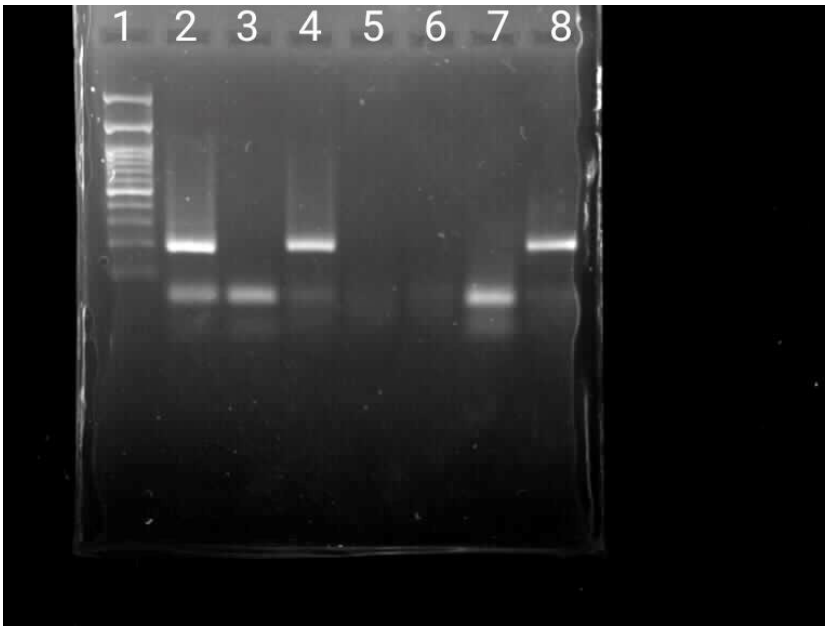

Figure 2: Electrophoresis Gel, specific PCR products of *Streptococcus pneumoniae* (length of 215 bp). Column (1): 100 bp ladder of DNA, column (2) Positive control of standard strain (ATCC6305) of *Streptococcus Pneumococcus* (length of 215 bp). Column (3): Negative control, columns 4 and 8 related to clinically isolated samples

As shown in Figure 3, the expected band that is a part of the proliferation of a fragment of a specific primer of LytA reproduced gene synthesized the 215 bp of DNA. The presence of this band in this range confirms the validity of the reaction specifications. PCR positive on the genomic DNA of *Streptococcus pneumoniae* and PCR negative on the genomic DNA of other bacteria were observed.

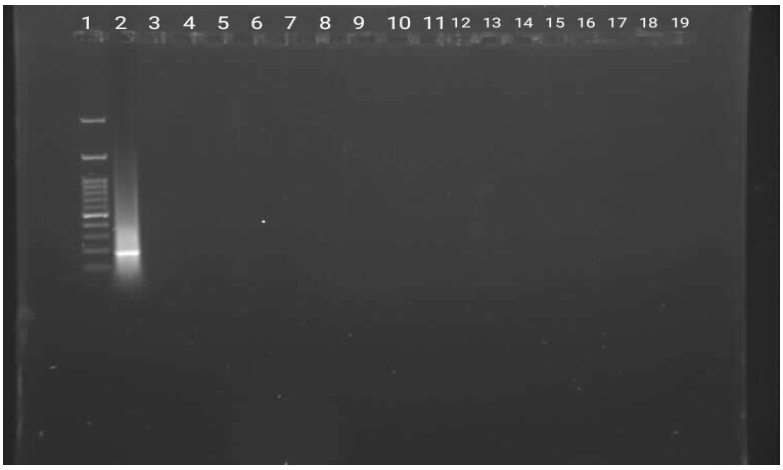

Figure 3: Determining the specificity of optimized PCR. Column (1): DNA100bp ladder, Column 2) PCR Product for genomic DNA of *Streptococcus Pneumococcus*, Column 3) Negative Control. Columns 4 to 18 are related to DNA bacteria: Column 4) genomic DNA of *Mycobacterium tuberculosis*, Column 5) Genomic DNA of *Haemophilus influenzae*, Column 6). Genomic DNA of *Mycoplasma pneumoniae*, Column 7) Genomic DNA of *Klebsiella pneumoniae*, Column 8) Genomic DNA of *Staphylococcus aureus*, Column 9) Genomic DNA of *Enterococcus*, Column 10) Genomic DNA of *Enterobacter aerogenes*, Column 11) Genomic DNA of *Legionella pneumophila*, Column 12) Genomic DNA of Nocardia, Column 13) Genomic DNA of *E.coli*, Column 14) Genomic DNA of *Streptococcus pyogenes*, Column 15) Genomic DNA of *Moraxella catarrhalis*, Column 16) Genomic DNA of Pseudomonas aeruginosa, Column 17) Genomic DNA of *Citrobacter*

Results of MIP gene sequence proliferation of *Legionella pneumophila*

As shown in Figure 4, the expected band, which was the result of the proliferation of a part of the *Legionella pneumophila* MIP gene, synthesized a fragment with a length of 235 bp from DNA. The presence of this band in this range confirms the reaction validity.

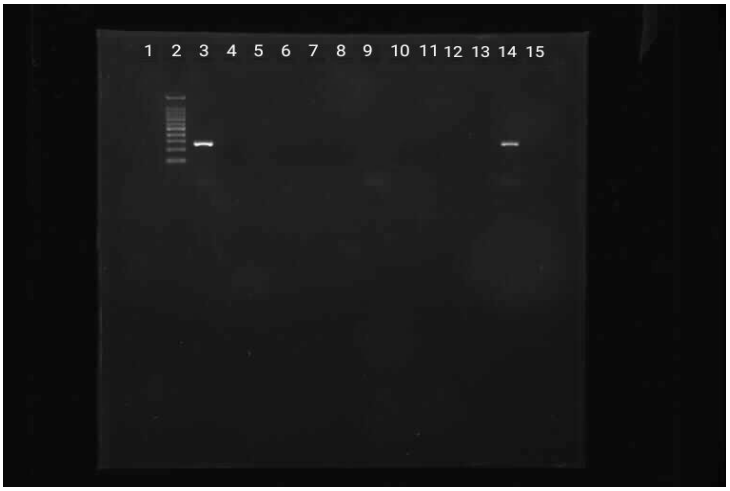

Figure 4: Electrophoresis gel of PCR products, the specific gene of *Legionella Pneumophila* (a length of 235 bp). Column (1): Negative Control, Column 2) DNA 100 bp ladder Column (3) Positive Control related to standard strain of *Legionella Pneumophila* Bacteria (235 bp). Column (4): Related to the clinically isolated sample.

As shown in Figure 5, the expected band that was a result of proliferation of a part of the specific primer of the MIP gene synthesized a fragment with a length of 235 bp of DNA. The presence of this band in this range confirms the validity of the reaction specificity. PCR positive on the genomic DNA of *legionella pneumophila* and PCR negative on the genomic DNA of other bacteria were observed.

Figure 5: Determining the specificity of optimized PCR Test. Column (1): Negative Control, Column 2) DNA100 bp ladder, Column 3) PCR product related to genomic DNA of *Legionella Pneumophila*, Column 4) Genomic DNA of *mycobacterium tuberculosis*, Column 5), Genomic DNA of *Haemophilus influenzae*, Column 6) Genomic DNA of Pneumonia Bacteria, Column 7) Genomic DNA of *Klebsiella pneumoniae*, Column 8) Genomic DNA of *Staphylococcus aureus*, Column 9) Genomic DNA of *Enterococcus*, Column 10) genomic DNA of *Enterobacter aerogenes*, Column 12) Genomic DNA of *Streptococcus Pneumococcus*, Column 13) Genomic DNA of Nocardia, Column 14) Genomic DNA of *E.coli*, Column 15) Genomic DNA of Genomic DNA of *Streptococcus pyogenes*, Column 16) Genomic DNA of *Moraxella*. Column 17) Genomic DNA of *Moraxella catarrhalis*, and Column 18) Genomic DNA of *Citrobacter*

The results of the BexA gene sequence proliferation of *Haemophilus influenzae*

After optimizing the reaction conditions and performing the PCR using the standard genome, the product was electrophoresed on 1% agarose gel. As shown in Figure 6, the expected band that was the result of a proliferation of a part of the BexA gene of *Haemophilus influenzae* synthesized a fragment of 400 bp of DNA. The presence of this band in this range confirms the reaction validity.

Figure 6: Electrophoresis gel of PCR products of BexA gene of *Haemophilus influenzae* (a length of 400 bp). Column (1): DNA100 bp ladder Column (2) Positive control related to standard strain (ATCC9006) of *Haemophilus influenzae* (400 bp). Column (3): Negative Control, Column 4 is related to clinically isolated sample

As shown in Figure (7), the expected band that was the result of a proliferation of a part of BexA gene synthesized a fragment of 400 bp of the DNA. The presence of this band in this range confirms the validity of the reaction sensitivity.

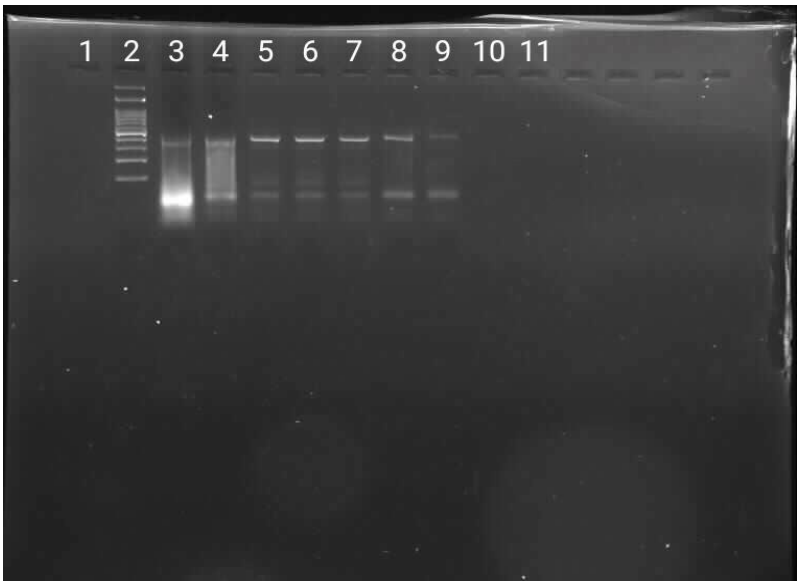

Figure 7: Determining the sensitivity of the optimized PCR test using different DNA dilutions from standard *Haemophilus influenzae*. Column (2): DNA 100 bp ladder, columns 3 to 11 are related to different dilutions of *Haemophilus influenzae* DNA. Column 2: Negative control

Results of P1 gene sequence proliferation of *Mycoplasma pneumoniae*

After optimizing the reaction conditions and performing the PCR using the standard genome, the product was 1 electrophoresed on 1% agarose gel. As shown in Figure 8, the expected band that was the result of the proliferation of a part of the P1 gene of *Mycoplasma pneumoniae* synthesized the 695 bp of DNA. The presence of this band in this range confirms the reaction validity.

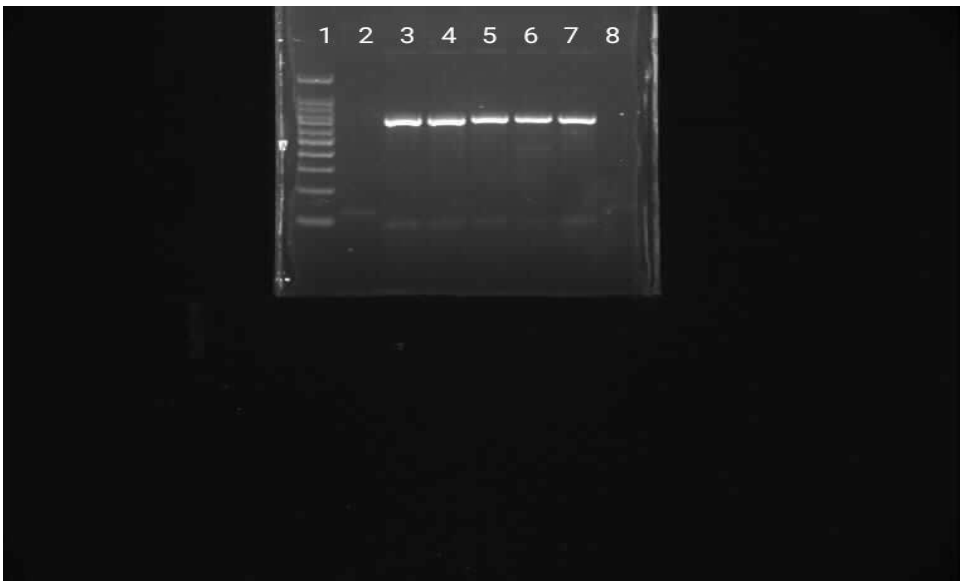

Figure 8: Electrophoresis Gel of PCR products of *Mycoplasma pneumoniae* P1 gene (a fragment length of 695 bp). Column (1): DNA100 bp ladder Column (2) Negative Control, columns 7, 6, 5, 4, and 3 are respectively related to the positive results of the clinical sample in PCR Gradient annealing temperature of 60, 59, 58, 57, and 56 from the P1 gene (695 bp).

As shown in Figure 9, the expected band that was the result of the proliferation of a part of the P1 gene synthesized a fragment with a length of 695 bp of DNA. The presence of this band in this range confirms the reaction sensitivity validity.

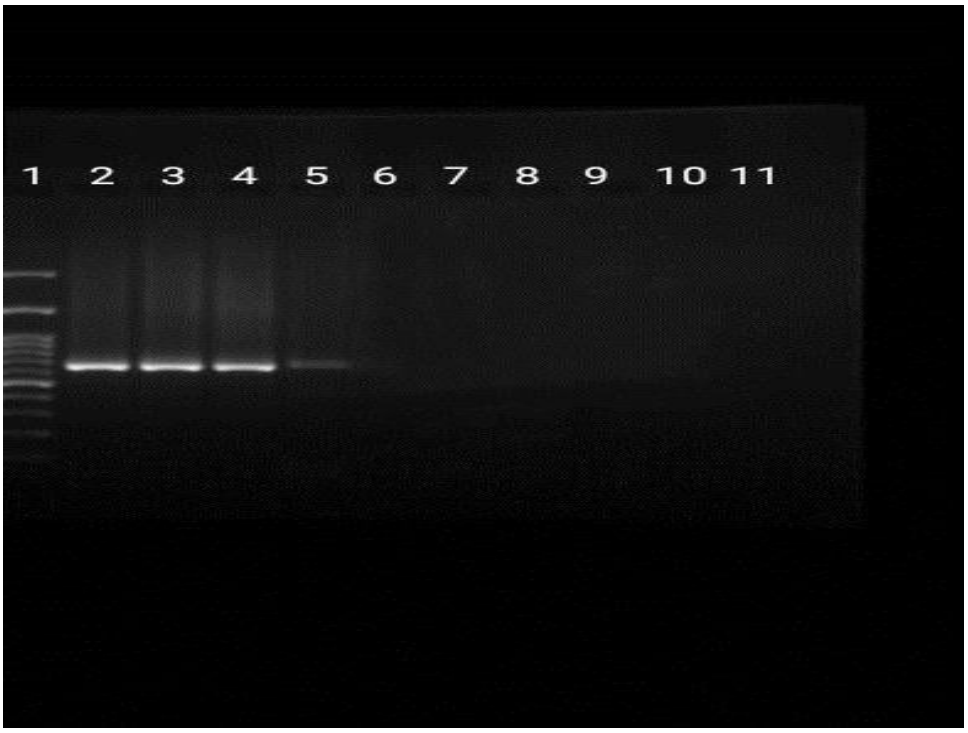

Figure 9: Determining the sensitivity of optimized PCR test using different DNA dilutions from the standard *Mycoplasma pneumoniae*. Column (1): DNA 100 bp, columns 2 to 10 are related to different dilutions of *Mycoplasma pneumoniae* DNA. Column 11: Negative control

To determine the minimum detectable concentration, the DNA was extracted from all 4 samples. Then, the concentration of extracted DNAs of each sample was read with the Nanodrop.

Table 4: The concentration of DNAs extracted with nanodrop

Name of organism	ng/µl
Streptococcus pneumoniae	2.2
Haemophilus influenzae	5.2
Legionella pneumophila	8.4
Mycoplasma pneumoniae	3.3

Then, the dilution of different serials from the DNA of each bacterium was prepared as shown below.

Table 5: DNA dilution for each of the highest dilution (1) to the least dilution (9)

1	2	3	4	5	6	7	8	9
5.1	10.1	100.1	10000.1	5000.1	25000.1	125000.1	625000.1	3125000.1

For each dilution, simple PCR and Multiplex PCR from the DNA mixture of binary bacteria (Streptococcus pneumonia and Haemophilus Influenza) and triple bacteria (Streptococcus pneumonia and hemophilus infiltrated pneumophila) were performed. The products were run on the electrophorese gel and the lowest dilution in each three repetitions was considered the minimum detection limit. Accordingly, we calculated the final concentration of DNA in this dilution based on the below formula.

Concentration of DNA = Input DNA in PCR × OD DNA (NG/µl) ÷ Dilution Factor

Table 6: Results of the detection limit of the studied organisms

Name of organism	Input DNA(µl)	ng/µl	Dilution factor	LOD
Streptococcus pneumoniae	2	2.2	1/125000	35.2 fg
Haemophilus influenzae	2	5.2	1/125000	83.3 fg
Legionella pneumophila	2	8.4	1/25000	0.672 pg
Mycoplasma pneumoniae	2	3.3	1/1000	6.6 pg

PCR results of sequences derived from extracted DNA

After optimizing the reaction conditions and performing the PCR using the standard genome, the product was electrophoresed on 1% agarose gel. As shown in Figure 10, the expected band that was the result of the proliferation of a part of the specific genes of bacterial samples. The presence of these bands in this range confirms the reaction validity.

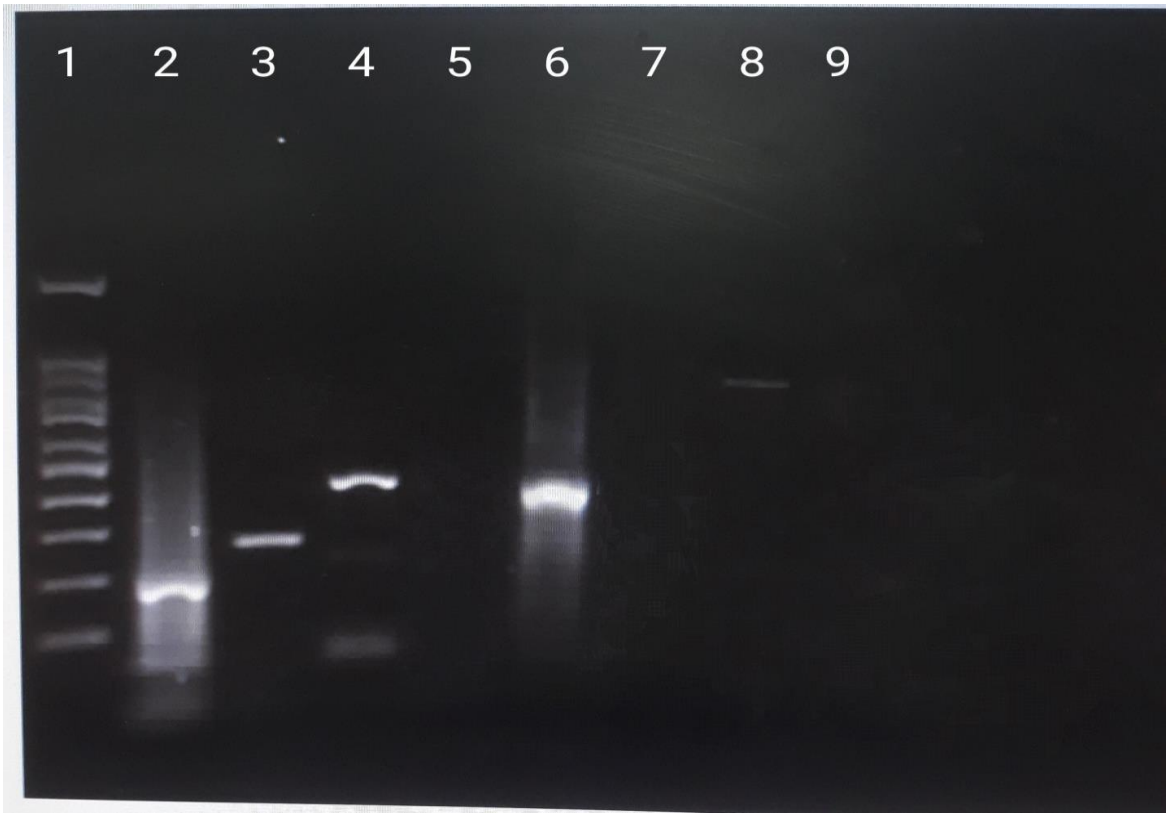

Figure 10: Electrophoresis Gel of PCR products. Column 1) DNA 100 bp ladder, bands 2 to 8 bands are respectively related to Streptococcus Pneumonia (215 bp), *Legionella Pneumophila* (215 bp), Universal (427 bp), Haemophilus Influenzae (400 bp), Mycoplasma Pneumonia (695 bp).

Multiplex PCR technique

The results of the proliferation of the specific gene sequence using the Multiplex PCR technique

Figures 11 and 12 show the results of the proliferation of the specific gene sequence of the bacteria studied in this study using the Multiplex PCR technique.

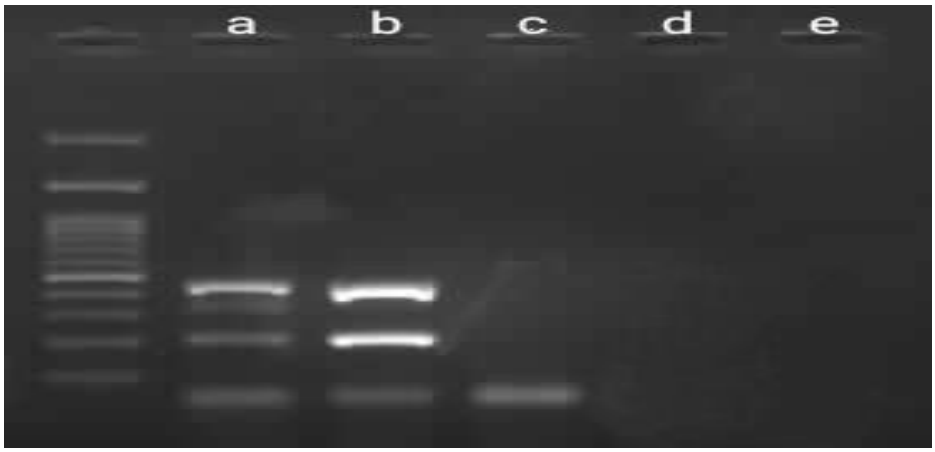

Figure 11: Multiplex PCR results of group A bacteria: pneumococcus (215 bp) and *Haemophilus influenzae* (400 bp) from bottom to top

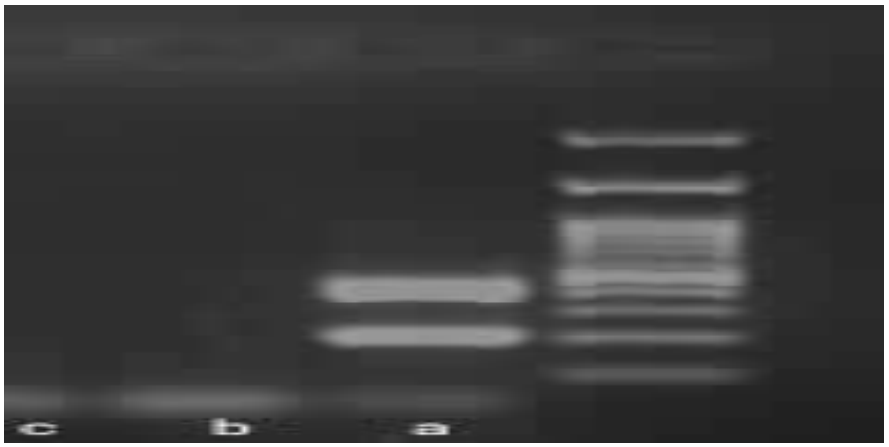

Figure 12: Multiplex PCR results of group B bacteria: pneumococcus (215 bp), *Haemophilus influenzae* (400 bp), and universal (427bp)

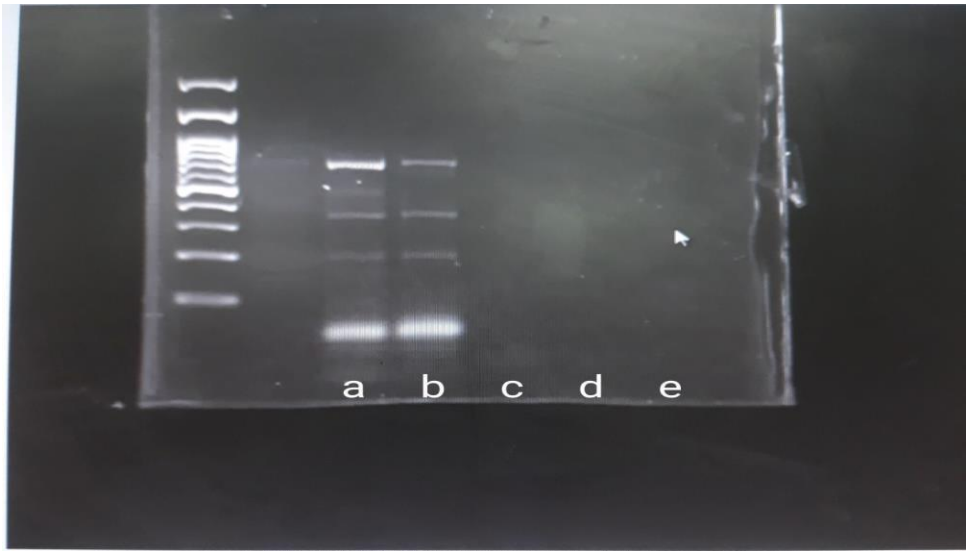

Figure 13: Multiplex PCR results of group C bacteria: Pneumococcus (215 bp), *Haemophilus influenzae* (400 bp), and *Mycoplasma pneumoniae* (695 bp)

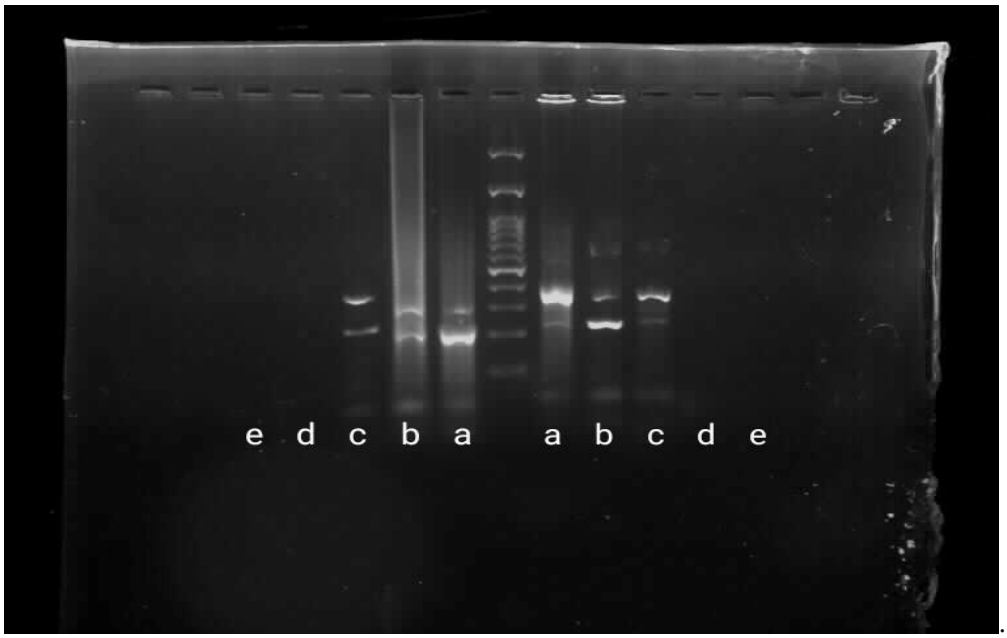

Figure 14: Multiplex PCR results of group D bacteria: *Legionella pneumophila* (215bp), *Haemophilus influenzae* (400bp), Universal (427bp), and *Mycoplasma pneumoniae* (695bp) in order from bottom to top (right side) and left from bottom to top. *Streptococcus pneumoniae* (215bp), *Legionella pneumophila* (235bp)

In this study, 180 patients with respiratory infections who were referred or hospitalized in Mashhad Hospital were sampled. The results indicated that 15 samples were positive. As shown in Table 7, the frequency distribution of *Streptococcus pneumoniae* with 5 people (2.8%) was the most isolated bacteria. Also, *Legionella pneumophila* bacteria was not isolated in 2 people, and *Chlamydia pneumonia*e was not isolated. Also, *Mycoplasma pneumoniae* bacteria and *Haemophilus influenzae* bacteria were isolated from 3 people (1.6%).

Table 7: Frequency distribution of bacteria isolated from patients with respiratory infections in a selected hospital in Mashhad

Bacterium	N	%
Legionella pneumophila	2	11%
Mycoplasma pneumoniae	3	16%
Chlamydia pneumonia	0	0
Streptococcus pneumoniae	5	28%
Haemophilus influenzae	3	16%
sum	15	100
Total sample	180	

Regarding gender distribution in this study, 60% were male and 40% were female. The age range in 15 positive isolates was between 15 and 71 years.

Discussion

Evaluating whether the multiplex method can be a rapid screening tool in a clinical setting was one of the goals of this study. Thus, nucleotide sequencing was used to confirm pathogenic sequences in each clinical sample as a reference method to validate what was detected by the PCR method. Unlike the direct immunofluorescence method, designed to detect 7 common viruses, the significant characteristic of the new multiplex real-time RT-PCR is the coverage of not only 4 viruses but also 5 types of bacteria, mycoplasma, and chlamydia. Such a design to target different pathogens enables the competence of the assay to provide comprehensive information of diagnostic value considering the current situation where mixed viral-bacterial infections are studied poorly.

Alikhani et al. (2022) examined 60 patients suspected of pneumonia in the age range of 19 to 87 years, with a mean, and a standard deviation of 52.13 ± 16.84. PCR results detected 56.7% of viruses and 7.26% of bacteria, and no agent was detected in 16.7%. Thus, the Multiplex RT-PCR test has high sensitivity and specificity, it is simple, and the cost of performing it is not low. However, it can increase the effectiveness of treatment and reduce drug resistance in the future due to the identification of various pathogens and the prevention of incorrect treatments (22).

Lim et al. (2021) showed that multiplex real-time PCR detected all target pathogens from sputum samples. Multiplex real-time PCR method showed faster time and higher performance than reference methods. Multiplex real-time PCR assay is a reliable tool that helps decision-making evaluation and performs better than other standard methods (23). Lapa et al. (2021) indicated that this design is appropriate for avoiding cross-contamination and for simultaneously testing large numbers of test samples. It can also detect the presence of several pneumonia pathogens in one sample and provides the possibility of expanding the range of detectable pathogenic bacteria and viruses (24). Zhao et al. (2021) showed that 100 copies per microliter was of high specificity and there was a high degree of agreement between the test results of the two methods. Thus, the detection system of 27 respiratory pathogens based on MassARRAY technology has high sensitivity and specificity, high throughput, and simple operation. It provides clinical diagnostic value of respiratory pathogens and is vital in the screening of respiratory pathogens (25).

Based on a thorough literature review, this study is the first reported trial of a one-step multiplex PCR reaction system that can detect multiple respiratory pathogens, including DNA- and RNA-based viruses and Chlamydia and mycoplasma bacteria. The cost-effectiveness of the assay is another significant advantage of this technique in addition to its wide application. The cost of each sample was about 3000000 Rials, which is half of the cost of the multiplex assay for pathogen detection if nine separate tests were performed. This is a cost-effective technique compared to commercially available multipathogen screening panels that require specialized instruments to perform.

Additionally, with a closed-tube design, this method is designed to minimize the possibility of cross-contamination while allowing for sensitive detection of co-infection. Given the similarities between this study and the previous report in some aspects such as patient's age, hospitalization seasons, sample collection method, and pathogen detection technique, there are supportive views confirming the results of the present study. Similarly, the results of the quantification of the infected pathogen load in the relevant samples with detected Ct values have not been presented here due to the lack of correlation with the relevant clinical data. Further analysis of these data is required. Further sputum culture of *Haemophilus influenzae* and *Streptococcus pneumoniae* is recommended if detected by this multiplex PCR due to the need to distinguish between nasopharyngeal colonization and pulmonary infection. However, this was not possible in this study due to the simultaneous performing of the assay with the collection of clinical samples. Additionally, DNA values determined by real-time PCR are mostly correlated with bacterial load, which can indicate where a "true" infection exists.

Conclusion

It was shown that the developed multiplex real-time PCR method has excellent performance characteristics to provide clinicians with accurate genetic information on causative pathogens. This method can be used for rapid detection leading to rapid initiation of treatment, which may significantly and positively affect the patient outcome. Real-time PCR data using established commercial methods is the primary limitation of this study due to the limited volume of initial samples. Integrating more clinical data to enable a comprehensive analysis provides useful information to assist clinicians in aspects of treatment strategies and prognosis prediction.

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