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Prevalence and Virulence Gene Analysis of *Mycoplasma pneumoniae* Co-infection in Hospitalized COVID-19 Patients: A Case-Control Study in Iran

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Abstract

Background: The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has highlighted the need to investigate bacterial co-infections in patients with respiratory illnesses. *Mycoplasma pneumoniae* has been identified as a significant pathogen in COVID-19 patients. Understanding its prevalence and molecular characteristics is crucial for improving clinical management.

Objectives: To molecularly identify virulence genes of *M. pneumoniae* as a secondary infection in COVID-19 patients hospitalized in Kerman, Iran.

Methods: A descriptive analysis was performed on 200 COVID-19 patients and 200 non-COVID-19 individuals. Respiratory samples from both acute and chronic lung and pharyngeal infections were collected. DNA extraction and PCR were used to detect *Mycoplasma* and the virulence genes p1, p40, and p90 of *M. pneumoniae*. Statistical analysis assessed the infection frequency and factors affecting bacterial co-infections.

Results: Eleven percent of the samples were positive for *Mycoplasma*, with a slightly higher prevalence in COVID-19 patients. Among individuals with chronic lung damage, *M. pneumoniae* infection was more frequent compared to those with acute lung damage. No significant difference was observed in the virulence genes between COVID-19 patients and controls. The highest frequency (23.8%) was associated with the p1 gene, while p90 was not detected in either group.

Conclusions: The study suggests that *M. pneumoniae* is a common co-infecting pathogen in COVID-19 patients, particularly in those with chronic respiratory conditions. Targeted monitoring and treatment of bacterial co-infections could improve patient outcomes in COVID-19. Further research is needed to explore the molecular mechanisms of *M. pneumoniae* virulence and its interaction with SARS-CoV-2.

Keywords: Virulence Genes, Molecular Identification, COVID-19, *Mycoplasma pneumoniae*, Respiratory Co-infection

1. Background

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has emerged as a global pandemic since its first reported case in December 2019 in Wuhan, China (1). This novel respiratory illness presents with symptoms ranging from mild flu-like symptoms to severe pneumonia, leading to critical illness and death in some cases (2). As the virus rapidly spread across the globe, it became evident that co-infections with various pathogens, including bacteria, are common in patients with COVID-19 (3). Among the bacterial co-infections observed in COVID-19 patients, *Mycoplasma pneumoniae* has been identified as a significant pathogen associated with the disease (4, 5). *Mycoplasma pneumoniae* is one of the major causative pathogens of community-acquired pneumonia (CAP) and the most frequent pathogen in

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atypical pneumonia infections (6). Several studies reported that *M. pneumoniae* is responsible for around 10 - 30% of CAP cases (7-9). In severe cases, *M. pneumoniae* infection can lead to extrapulmonary manifestations such as myocarditis, nephritis, and hemolytic anemia (10). The CAP caused by atypical pathogens can have symptoms and presentations similar to COVID-19, making it difficult to differentiate between the two based on clinical observations or imaging results (11).

The pathogenesis of *M. pneumoniae* involves adherence to the host's respiratory tract cells, leading to damage to the respiratory cilia and destruction of the epithelium (12). One of the key adhesins involved in this process is the P1 protein, which, along with P40/P90, forms the transmembrane adhesion complex at the tip of the attachment organelle (13). Understanding the molecular mechanisms of *M. pneumoniae* virulence is crucial for elucidating its role in co-infections with SARS-CoV-2 and the pathogenesis of COVID-19 (14, 15). Current research indicates that individuals with COVID-19 who are also infected with other respiratory pathogens may experience more severe symptoms and higher mortality rates (16).

Therefore, it is important to conduct studies to identify these co-infections and assess how they impact the overall clinical outcome of COVID-19 patients. In this study, by investigating the co-infection of *M. pneumoniae* with SARS-CoV-2, we seek to elucidate the clinical implications and risks associated with this bacterial pathogen in COVID-19 patients. Specifically, this study focuses on the molecular characterization of the key virulence genes p1, p40, and p90, which play a critical role in the pathogenesis of *M. pneumoniae* respiratory infections.

2. Objectives

By advancing our understanding of the interactions between *M. pneumoniae* and SARS-CoV-2, this study aims to provide insights into the potential implications of bacterial co-infections in COVID-19 patients and the development of targeted therapies for improved patient outcomes.

3. Methods

3.1. Study Design and Sampling

In this study, we conducted a descriptive study to investigate the prevalence of *M. pneumoniae* and virulence genes in admitted patients with SARS-CoV-2 infection. The study was carried out in three hospitals in Kerman, Iran, from January 2021 to June 2021. The study included a total of 200 patients with COVID-19 (patient group) and 200 healthy individuals who tested negative for SARS-CoV-2 (control group). The age and blood pressure levels of the participants in both groups were studied as dependent variables, with gender, occupation, body weight, and other confounding variables controlled for. The number of women and men selected was equal. None of the participants had a high-risk occupation related to respiratory diseases, and their Body Mass Index (BMI) ranged from 18.5 to 25, which is considered the normal weight range.

Samples were collected from individuals who visited the hospitals over six months in 2021. The age and blood pressure of the patients in both groups were also recorded. The confirmation of positive and negative results in both groups was conducted by the hospital laboratory, followed by repeat real-time PCR testing. The respiratory secretion samples collected from patients included samples from both the upper respiratory tract (pharynx) and lower respiratory tract (lungs). The study included both acute and chronic cases of infection in both the patient and control groups. Clinical symptoms observed in patients ranged from fever, headache, cough, weakness, and lethargy to allergies, diarrhea, and muscle pain. The details and number of collected samples are presented in Table 1 and Appendix 1 in Supplementary File.

3.2. Polymerase Chain Reaction Analysis

3.2.1. DNA Extraction

The DNA extraction process involved isolating the DNA from each sample using the CinnaPure-DNA purification kit (CinnaGen, Iran) according to the manufacturer's instructions and was used as the template for PCR amplification.

3.2.2. Specific Primer Design and Gene Targets

The specific primers to amplify target genes for the identification of the *Mycoplasma* genus, as well as the p1, p40, and p90 virulence genes of *M. pneumoniae*, were analyzed and designed according to gene information

Table 1. Number and Source Sampling in Case and Control Groups

Groups	Number of Samples	Sample Source	
Case group (patients with coronavirus)			
Acute	100	Lung (n = 50)	Pharynx (n = 50)
Chronic	100	Lung (n = 50)	Pharynx (n = 50)
Control group (non-coronavirus patients)			
Acute	100	Lung (n = 50)	Pharynx (n = 50)
Chronic	100	Lung (n = 50	Pharynx (n = 50)
Total	400		

Table 2. Nucleotide Sequences and Primers Used in the Detection of the *Mycoplasma* Genus and Virulence Genes p1, p40, and p90 of *Mycoplasma pneumoniae* by PCR [a]

Primer	Sequence (5'-3')	Length (bp)
MH1, MH2	F: TGAAAGGCGCTGTAAGGCGC; R: GTCTGCAATCATTTCCTATTCCAAA	277
MG37	F: AGTTGATGAAACCTTAACCCCTTGG; R: CCGTTGAGGGGTTTTCCATTTTTGC	163
P1	F: TTATTGAAATACGATACAGCTCATGGA; R: CGTTGGCAATAGGAGCTAAACAG	233
P40	F: TAAGAGTTTGATCCTGGCTCAG; R: GGACTACCAGGGTATCTAATCC	397
P90	F: TGCAATCTGCTCGTGAAGTATTAC; R: CGAAACGACGTCCATAAGCAACT	518

[a] Target gene: 16s rRNA

in the GenBank database using the MEGA5 software (Table 2).

3.2.3. PCR Amplification

The PCR method was conducted on total volumes of 25 µL using 12 µL of mixed master mix (Sinaclon, Iran), 2 µL of DNA, 1 µL of each primer (20 pmol/µL), and 9 µL of double distilled water (DDW). The amplification conditions for the *Mycoplasma* genus and species using specific primers were processed by initial heating at 94°C for 7 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a 5-minute extension at 72°C. In addition, p1, p40, and p90 virulence genes detection was carried out by initial heating at 35°C for 5 minutes, 35 cycles of denaturation at 95°C for 45 seconds, annealing at 55°C for 40 seconds, and extension at 72°C for 40 seconds, followed by a 5-minute extension at 72°C. PCR products were analyzed by electrophoresis (50 V, 2 h) in 1% agarose gels stained with 0.5 µL/mL of ethidium bromide. After that, PCR products were visualized and photographed using a GEL Imaging System (Bio-Rad Laboratories, CA, USA).

3.3. Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 10.0 for Windows). The chi-square test was used with a significance level (α) of 0.05. The confidence interval was considered to be 95%.

4. Results

4.1. PCR Amplification

In total, 400 samples of respiratory secretions were used in the current study, of which 200 patients were positive for genome targets of SARS-CoV-2 by real-time PCR, and 200 patients were negative. All samples were subjected to PCR amplification for the *Mycoplasma* genus and virulence genes of *M. pneumoniae* in both case and control groups. A total of 44 (11%) and 35 (8.75%) samples were positive for the *Mycoplasma* genus and *M. pneumoniae*, respectively, according to the PCR analysis in both groups. The PCR analysis results are shown in Figures 1 and 2.

4.2. Frequency of Mycoplasma Genus in Chronic and Acute Samples of Lung and Pharynx

Based on the data analysis, the study found that there was no significant difference in the proportion of *Mycoplasma* genus infection rates between the control

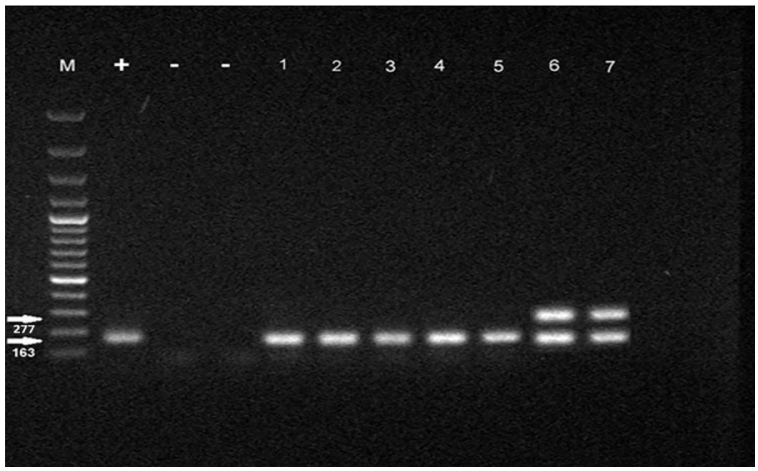

Figure 1. Electrophoresis analysis of PCR product with approximately 277 and 163 bp for evaluating the presence of *Mycoplasma* genus using species-specific primers; M, marker 100bp; + positive control; - negative control; and 1 to 7 lane: *Mycoplasma* genus.

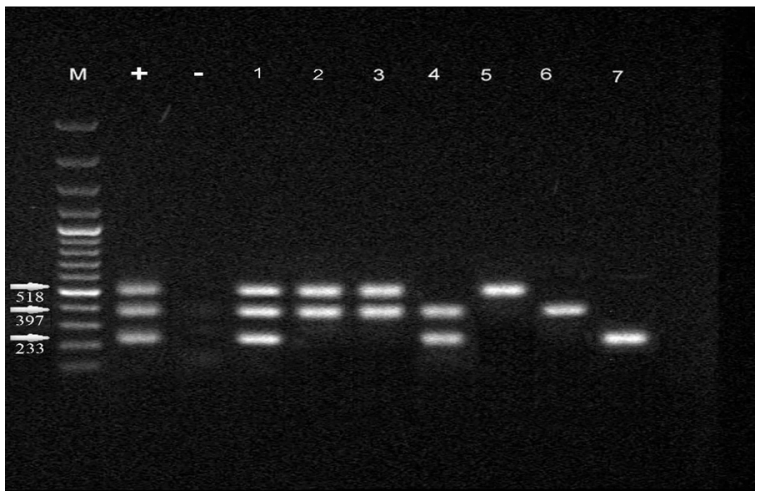

Figure 2. Electrophoresis analysis of PCR product with approximately 233, 397, and 518 bp for evaluating the presence of virulence genes p1, p40, and p90 of *Mycoplasma pneumoniae*; M, marker 100bp; + positive control; - negative control; and 1 to 7 lane: *M. pneumonia*.

groups with acute and chronic lung infection and those infected with coronavirus (P = 0.734). In the control group, 6% of individuals with acute lung damage and 26% of individuals with chronic lung damage were infected with the *Mycoplasma* genus. Among individuals infected with coronavirus, 12% of those with acute lung damage and 22% with chronic lung damage were infected with the *Mycoplasma* genus. Additionally, the proportion of individuals infected with the *Mycoplasma* genus was relatively consistent in both the lung and pharyngeal samples across the different groups. Comparing the number of *Mycoplasma* in lung samples from control subjects with acute and chronic lung injury revealed a significant difference between the two groups. Specifically, samples from control subjects without exposure to COVID-19 showed a higher

Table 3. The Infection Rate of Patients with *Mycoplasma* Genus in Control and Case Groups [a]

Infection	Case Group	Control Group
Acute lung	6 (12)	3 (6)
Chronic lung	11 (22)	13 (26)
Acute pharyngeal	3 (6)	3 (6)
Chronic pharyngeal	3 (6)	2 (4)

[a] Values are expressed as No. (%).

Table 4. The Infection Rate of Patients with *Mycoplasma pneumoniae* in Control and Case Groups [a]

Infection	Case Group	Control Group
Acute lung	6 (12)	2 (4)
Chronic lung	10 (20)	12 (24)
Acute pharyngeal	3 (6)	0 (0)
Chronic pharyngeal	2 (4)	0 (0)

[a] Values are expressed as No. (%).

percentage of *Mycoplasma* species in samples with chronic lung damage compared to those with acute lung damage (P = 0.012). The results of *Mycoplasma* genus isolation in the control and case groups are presented in Table 3 and Appendix 1 in Supplementary File.

4.3. Frequency of Mycoplasma pneumoniae in Chronic and Acute Samples of Lung and Pharynx

Based on the prevalence of the p1, p40, and p90 virulence genes among the control samples, it was found that a higher proportion of individuals with chronic lung damage were infected with *M. pneumoniae* compared to those with acute lung damage. However, there was no significant difference in the prevalence of infection between the control and coronavirus groups. Further analysis comparing the presence of *M. pneumoniae* in lung samples from control subjects with acute and chronic injury revealed a significant difference between the two groups.

In individuals without exposure to COVID-19, samples showing chronic lung damage exhibited a higher percentage of *M. pneumoniae* compared to samples with acute lung damage (P = 0.008). Notably, *M. pneumoniae* was not detected in pharyngeal samples from control subjects with acute and chronic disease. Additional details regarding these findings can be found in Table 4. The difference in the p virulence gene in both control and case groups showed that there is no significant difference in the distribution of these genes between the two groups (P = 0.708) (Figure 3).

4.4. Age and Blood Pressure Effect

The chi-square test analysis of the blood pressure of individuals in both the control and patient groups revealed no significant difference in the prevalence of high blood pressure (P = 0.695). Additionally, the age distribution of people in both control and patient groups was analyzed by the chi-square test. The result of the test showed that there is a significant difference in terms of age distribution between the two groups (P = 0.004). Based on the results, it was observed that in the patient group, the age group of 40-50 years had a higher percentage of *Mycoplasma* isolation compared to the control (Figure 4).

5. Discussion

The emergence of the COVID-19 pandemic has brought significant challenges to the healthcare system worldwide (17). Understanding the impact of bacterial co-infections in COVID-19 patients is crucial for determining optimal treatment strategies and improving patient outcomes (18). To the best of our knowledge, there are few molecularly documented cases of co-infection with both SARS-CoV-2 and *M. pneumoniae* in the medical literature, and co-infection

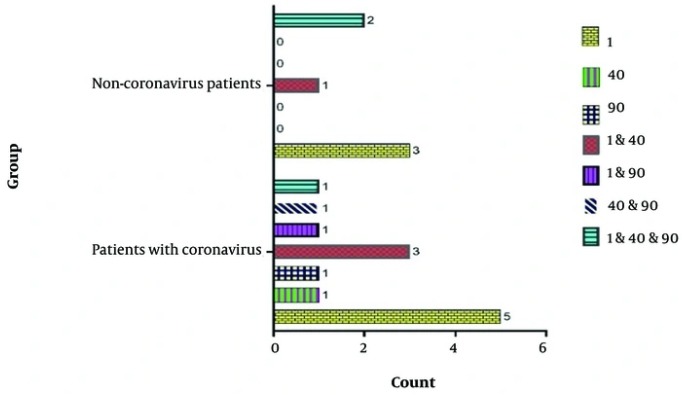

Figure 3. Frequency of p1, p40, and p90 genes of *Mycoplasma pneumoniae* in the case and control groups

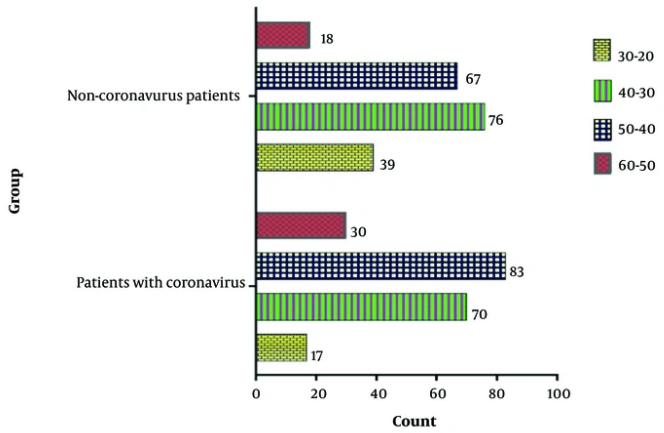

Figure 4. The age distribution in case and control groups with *Mycoplasma* isolation

of *Mycoplasma* with SARS-CoV usually has been found in serological tests (4, 19). In this study, we investigated the co-infection of *Mycoplasma* and virulence p genes in COVID-19 and non-COVID-19 patients. The results of our study revealed that approximately 11% of respiratory secretion samples from both COVID-19 patients and non-coronavirus individuals were positive for the *Mycoplasma* genus, with a slightly higher prevalence of *M. pneumoniae* in the case group. This finding highlights the potential role of *M. pneumoniae* as a common co-infecting pathogen in individuals with COVID-19, which could exacerbate respiratory symptoms and lead to more severe clinical outcomes (20). High rates of COVID-19-bacterial co-infections identified among COVID-19 patients have also been reported by other researchers (7, 21-23).

Furthermore, this study found that individuals with chronic lung damage, both in the COVID-19 patient group and the control group, had a higher prevalence of *M. pneumoniae* infection compared to those with acute lung damage. This suggests that chronic respiratory conditions may predispose individuals to bacterial co-infections, including *M. pneumoniae*, which could

further complicate the course of COVID-19 (24, 25). Moeller et al. suggested that children with bronchopulmonary dysplasia (BPD) and other respiratory conditions were at higher risk of requiring ventilatory support, such as supplemental oxygen or invasive ventilation, if infected with the virus (26). The presence of *M. pneumoniae* in chronic lung damage samples highlights the need for targeted monitoring and treatment of bacterial pathogens in individuals with underlying conditions. COVID-19 can cause inflammation and damage to the lungs, making it harder for individuals with pre-existing lung conditions to breathe properly. This can lead to a higher risk of developing respiratory failure, pneumonia, and acute respiratory distress syndrome (ARDS) (27).

The analysis of the virulence genes p1, p40, and p90 of *M. pneumoniae* in our study did not reveal a significant difference in the distribution of these genes between the COVID-19 patient group and the control group. However, the higher prevalence of *M. pneumoniae* in chronic lung damage samples suggests that the expression of these virulence genes may contribute to the pathogenesis of the disease in individuals with pre-existing respiratory conditions. These findings are consistent with previous studies by Shi et al. (28) and Vizarraga et al. (13). Further studies focusing on the molecular mechanisms of *M. pneumoniae* virulence and its interaction with SARS-CoV-2 are warranted to elucidate the role of this bacterium in COVID-19 pathogenesis.

The age distribution analysis in the study revealed a higher percentage of *Mycoplasma* isolation in the age group of 40 - 50 years in the patient group compared to the control group. This finding highlights the potential susceptibility of middle-aged individuals to *M. pneumoniae* infections, particularly in the context of COVID-19. However, Li et al. found that the positive detection rate of *M. pneumoniae* was highest among children aged 5 to 7 years old in the outbreak of COVID-19 (22). Age-related factors, such as immune function and comorbidities, may influence the susceptibility to bacterial co-infections in COVID-19 patients and should be considered in clinical management strategies (29, 30).

5.1. Conclusions

This study provides important insights into the prevalence of *M. pneumoniae* and its virulence genes in COVID-19 patients and highlights the potential clinical implications of bacterial co-infections in individuals with COVID-19. Targeted monitoring and treatment of bacterial pathogens, particularly in individuals with chronic respiratory conditions, may help improve patient outcomes and reduce the burden of co-infections in COVID-19. Further research on the molecular mechanisms of *M. pneumoniae* virulence and its interactions with SARS-CoV-2 is necessary to advance our understanding of bacterial co-infections in COVID-19 and develop targeted therapeutic interventions for improved patient care. Changes in treatment and care protocols, history of patients' respiratory diseases, and change of seasons were some of the limitations of this research.

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Supplementary Material

Supplementary material(s) is available here [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Authors' Contribution: B. K. and E. H. conceived the presented idea, designed the model and the computational framework, also planned the experiments and analyzed the data. E. H. developed the theory and performed the computations. B. K. and F. R. verified the analytical methods. B. K. encouraged E. H. to investigate [a specific aspect] and supervised the findings of this work and the whole project. E. H., B. K., and F. R. contributed to sample preparation and interpretation of the results. E. H. and B. K. provides the study materials and equipment and also carried out the experiment. E. H. performed the analytic calculations and the numerical simulations and wrote the manuscript with support from B. K.'s supervision and F. R. helped to consultation of the project. Both E. H. and B.

K. participated in presenting the last draft. All authors provided critical feedback furtherance the research.

Conflict of Interests Statement: The authors declare no conflict of interest.

Data Availability: The data presented in this study are uploaded during submission as a supplementary file and are openly available for readers upon request.

Ethical Approval: The consent of each participant was declared by filling out the informed consent form and the code of National Committee of Ethics in Kerman Branch, Islamic Azad University, Kerman, Iran (IR.IAU.KERMAN.REC.1401.040).

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Informed Consent: Written informed consent was obtained from all participants before sample collection.

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