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Original Research

Co-infection of COVID-19 patients with atypical bacteria: A study based in Jordan

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Abstract

Objective: The aim of this work was to know the prevalence of *Chlamydophila pneumoniae* and *Mycoplasma pneumoniae* in coronavirus disease 2019 (COVID-19) patients in Jordan. Also, to assess a TaqMan real-time polymerase chain reaction (PCR) assay in detecting these two bacteria. **Methods:** This is a retrospective study performed over the last five months of the 2021. All nasopharyngeal specimens from COVID-19 patients were tested for *C. pneumonia*, and *M. pneumoniae*. The *C. pneumoniae* Pst-1 gene and *M. pneumoniae* P1 cytadhesin protein gene were the targets. **Results:** In this study, 14 out of 175 individuals with confirmed severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection (8.0%) were co-infected with *C. pneumoniae* or *M. pneumoniae*. Co-infection with SARS-CoV-2 and *C. pneumoniae* was reported in 5 (2.9%) patients, while 9 (5.1%) patients had *M. pneumoniae* and SARS-CoV-2 co-infection. The mean (± std) of the correlation coefficient of the calibration curve for real-time PCR analysis was –0.993 (± 0.001) for *C. pneumoniae* and –0.994 (± 0.003) for *M. pneumoniae*. The mean amplification efficiencies of *C. pneumoniae* and *M. Pneumoniae* were 187.62% and 136.86%, respectively. **Conclusion:** In this first study based in Jordan, patients infected with COVID-19 have a low rate of atypical bacterial co-infection. However, clinicians should suspect co-infections with both common and uncommon bacteria in COVID-19 patients. Large prospective investigations are needed to give additional insight on the true prevalence of these co-infections and their impact on the clinical course of COVID-19 patients.

Keywords: *Chlamydophila pneumoniae*; coronavirus; Jordan; *Mycoplasma pneumoniae*; polymerase chain reaction

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INTRODUCTION

The current universal pandemic of coronavirus disease in 2019 (COVID-19) is being triggered by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). It has spread quickly, causing varying degrees of illness and creating a public health risk throughout the world.[1,2] While clinical care is heavily focused on the diagnosis and/or management of COVID-19, there is emerging evidence that patients with COVID-19 are at risk of developing concurrent pulmonary infections caused by bacteria and other microorganisms.[3-7] This co-infection is critical in COVID-19, as it can exacerbate diagnostic and treatment difficulties, as well as increase disease symptoms and mortality.[1] Other respiratory viruses are also associated with bacteria to varying degrees. For instance, "influenza-related bacterial infections may account for up to 30% of community-acquired pneumonia (CAP) cases," according to one study.[8] Additionally, co-infection has been related to worse outcomes during the pandemic and seasonal influenza outbreaks.[8] As a result, a clinical need exists for a thorough examination of co-infection in COVID-19 patients.

Chlamydophila pneumoniae and *Mycoplasma pneumoniae are* both capable of infecting the lungs and causing chronic respiratory illnesses.[9,10] However, elucidating the consequence of these atypical bacteria is difficult due to difficulty in cultivating them from respiratory tract samples. Additionally, there is substantial variation in how researchers interpret the results of serological assays. There are also insufficient conclusive investigations utilizing real-time polymerase chain reaction (PCR) to determine the detection and involvement of

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these atypical bacteria in COVID-19.

Using rapid molecular methods, such as real-time PCR, should increase the yield of microbial detection and provide a better insight of the microbial association with COVID-19. The real-time PCR can detect minute amounts of nucleic acids from the target pathogens; it does not rely on the viability of the pathogen; and it is probably less affected by previous antibiotic treatment compared with the culture-based approaches.[11]

In different countries other than Jordan, SARS-CoV-2 co-infection with *M. pneumoniae* alone[12-16] or with *C. pneumoniae*[17] have been investigated. The present work was therefore aimed to know the prevalence of *C. pneumoniae and M. pneumoniae* in COVID-19 patients in Jordan. Also, to assess a method of TaqMan real-time PCR assay in detecting *C. pneumoniae,* and *M. pneumoniae* from nasopharyngeal samples.

METHODS

Study design and participants

Between first of August and 20th of December 2021, we retrospectively examined all nasopharyngeal samples from adults with confirmed SARS-CoV-2 infection who did not require hospitalization.

The Research Ethics Board of Applied Science Private University (ASU), Amman, Jordan (2021-PHA-35) and Al-Rayhan Medical Center approved the study (no. 2021-IRB-8-1). Informed consents are available for all of the participants.

Nucleic acid extraction and detection of SARS-CoV-2

Nucleic acid (NA) extraction was carried out utilizing the BIOBASE extraction kit (Biobase Bioindustry (Shandong) Co., Ltd). After extraction, SARS-CoV-2 was detected using the eluted NA samples. The CDC-recommended SARS-CoV-2 reverse transcriptase (RT)-qPCR assay was carried out using the TIANLONG: Real-Time qPCR System with 96-well block equipment and the LiliFTM COVID-19 Multi Real-time qRT-PCR Kit. A SARS-CoV-2 assay result was classified as positive when the ribonuclease P (RNP) gene was detected along with either the N1 or N2 gene, and as negative if just the RNP gene was detected.

C. pneumoniae and M. pneumoniae detection

All nasopharyngeal specimens were tested for *C. pneumoniae* and *M. pneumoniae*. *C. pneumoniae* Pst-1 gene and *M. pneumoniae* P1 cytadhesin protein gene were the targets for *C. pneumonia* and *M. pneumoniae*, respectively.

For real-time qPCR analysis of the calibration data, *C. pneumoniae* and *M. pneumoniae* pure DNA templates of cell-conditioned media from cultivated primary epithelial cells were employed. All real-time qPCR tests used TaqMan approach. The primers and probes sequences utilized in this study are available in Table 1 (Eurofins, UK).

To create the standard curve for the researched atypical bacteria, ten-fold serial dilutions of four concentrations of standard DNA (from 1000 to 1 copy/L) were prepared in three replicates. For each replicate of the four concentrations, we utilized 9-µl of master-mix (Roche Diagnostics, UK) and a 6-µl DNA template, and this final process was repeated twice, resulting in a total of 40 wells, each with 15- µl. The samples were analyzed using absolute quantification in a manner similar to that described in a recent article.[18]

Six microliters of each patient's specimen were pipetted into a 96-well plate following the dispensing of nine microliters of the master-mix (Table 2). Plates were sealed with sealing foil and centrifuged at 1500 rotations per minute (rpm) before being loaded into the TIANLONG: Real-Time PCR System. The TaqMan PCR program comprised of 15 minutes at 50°C, 5 minutes at 95°C, 45 cycles at 95°C for 10 seconds, then 60°C for 1 minute, followed by 15 seconds at 40°C.

Table 1. Primer and probe sequences of C*. pneumoniae* and M. *pneumoniae* targets

Target Primers and Probe	**sequence**
Chlamydophila pneumoniae Pst-1 gene	CAACGGCTAGAAATCAATTATAAGACTGAAGTTGAGCATATTCGTGAGGGAGATGCAGATTTAGATCATG-GTGTCATTCGCCAAGTTAAAGTCTACGTTGCCTCTAAGAGAAAACTTCAAGTTGGAGATAAAATGGCTG-GACGACACGGAAATAAAGGTGTTGTTTCCAAAATCGTTCCCGAAGCGGATATGCCATATCTCTCT
Mycoplasma pneumoniae P1 cytad-hesin protein gene	AACCTGATCAAGATACCCAACCAAACAACAACGTTCAGGTCAATCCGAATAACGGTGACTTCTTACCACT-GTTAACGGCCTCCAGTCAAGGTCCCCAAACCTTGTTTAGTCCGTTTAACCAGTGACCTGATTACGT

Table 2. Master mix preparation for *Chlamydophila pnemoniae*, and *Mycoplasma pneumoni*ae

Reagent	**Volume**
PCR grade water	5.61µl
DNA BASE MIX X5	3µl
MPN 4A @ 200µM (final 0.4µM)	0.03µl
MPN 4B @ 200µM (final 0.4µM)	0.03µl
MPN 4P1@ 100µM (final 0.2µM) CY5-BHQ	0.03µl
CPN 6A @ 200µM (final 0.5µM)	0.0375µl
CPN 6B @ 200µM (final 0.5µM)	0.0375µl
CPN 6P1 @ 100µM (final 0.3µM) YAK-BHQ	0.045µl

MPN: *Mycoplasma pneumoniae*; CPN: *Chlamydophila pnemoniae*

Statistical analysis

Data were analysed using SPSS Statistics v.25 (IBM Corporation, New York, NY, USA). All data are presented as mean (± standard deviation) or number (percentage) unless otherwise specified.

RESULTS

C. pneumoniae, and M. pneumoniae calibration data

The means of the C_t values with standard deviation were calculated for all the concentrations and replicates of *C. pneumoniae* and *M. pneumoniae* calibration data (Table 3). The mean (± standard deviation) of the correlation coefficient (r^2) was −0.993 (± 0.001) for *C. pneumoniae* and –0.994 (± 0.003) for *M. pneumoniae* (Table 3).

By plotting the log of target DNA concentrations against C_t values, the standard curve was established; linear regressions for each of the replicate dilution series were applied. Figure 1 depicts the calibration curves for *C. pneumoniae* and *M. Pneumoniae*. The amplification efficiency (E) of the absolute quantification is the mean efficiency derived by $E = (10^{-Slope} – 1) \times 100$. The results indicated that the mean efficiencies of *C. pneumoniae* and *M. Pneumoniae* were 187.62% and 136.86%, respectively.

The prevalence of C. pneumoniae and M. pneumoniae

Among 175 patients with laboratory-confirmed COVID-19, 14 (8.0%) were co-infected with *C. pneumoniae* or *M. pneumoniae*. Co-infection with SARS-CoV-2 and *C. pneumoniae* was reported in 5 (2.9%) patients, while 9 (5.1%) patients had *M. pneumoniae* and SARS-CoV-2 co-infection (Table 4). There was no detected amplification of the negative control samples.

DISCUSSION

Bacterial co-infections have been a main cause of death during past influenza pandemics. The study's goal was to find out how often atypical bacterial co-infections were among COVID-19 patients in Jordan. In this study, 14/175 COVID-19 confirmed cases (8.0%) were co-infected with *C. pneumoniae* or *M. pneumoniae*. Co-infection with SARS-CoV-2 and *C. pneumoniae* was reported in 5 (2.9%) patients, while 9 (5.1%) patients had *M. pneumoniae* and SARS-CoV-2 co-infection.

Table 3. *C. pneumoniae* and *M. pneumoniae* calibration data

Log10 copies/reaction	C. pneumoniae		M. Pneumoniae	
	A*	B	A	B
Correlation (r^2)	-0.992	-0.996	-0.991	-0.998
Mean of r^2	-0.993		-0.994	
S.D of r^2	0.001		0.003	
Mean of slopes (gradient)	-2.180		-2.670	
The qPCR Efficiency (E): E = (10^(-1/slope) –1) x100	187.62%		136.86%	

*A and B represent duplicate reactions.

Table 4. C. *pneumoniae* and M. *pneumoniae* detection rates

Target	Positive sputum samples no.	% of total positive (n= 175)
C. pneumoniae	5	2.9%
M. pneumoniae	9	5.1%
Total	14	8.0%

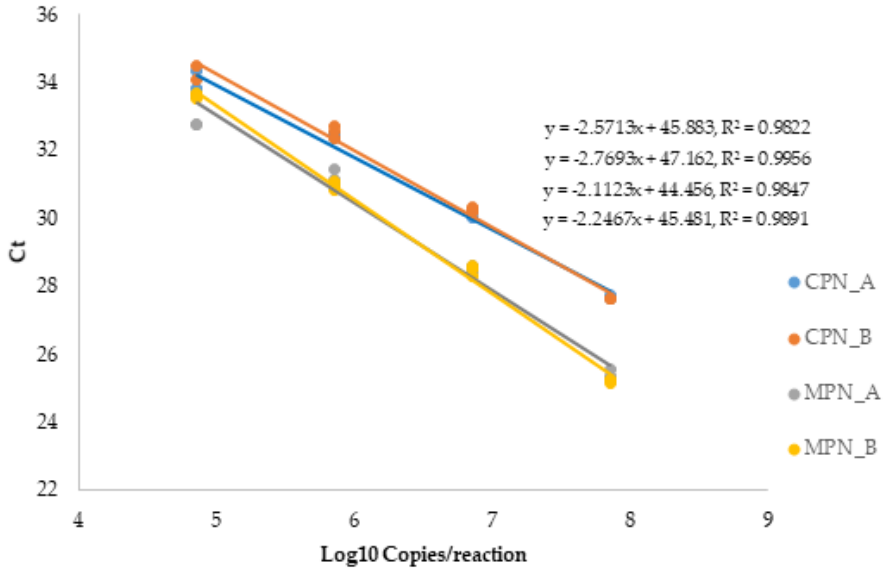

Figure 1. The calibration curve for *C. pneumoniae* and *M. pneumoniae*. Ct: cycle threshold; MPN: *Mycoplasma pneumoniae*; CPN: *Chlamydophila pnemoniae*. A and B represent duplicate reactions

Almost similar to our finding, among 194 patients from India, 17 (8.8%) COVID-19 cases were co-infected with *C. pneumoniae* or *M. pneumoniae*.[17] However, slightly a higher rate of *M. pneumoniae* co-infection was observed in our study compared to other recent studies. For instance, 8/209 (3.8%) *M. pneumoniae* was reported from a study conducted in India[12] and 6/350 (1.7%) from a study conducted in the United Kingdom [13]. In contrast, a study from China, with a small sample size, stated that almost half of COVID-19 cases were co-infected with *M. pneumoniae*; 47.2% (25/53).[15] A group of seven COVID-19 patients from Italy were documented, and co-infection with *C*. pneumoniae (n = 5) or M. pneumoniae (n = 2) and SARS-CoV-2 was identified in a large teaching hospital.[19]

Studies of hospitalized COVID-19 patients have shown that antibiotics are used empirically in the vast majority of these cases.[20-22] However, elevated procalcitonin and C-reactive protein, typically associated with bacterial infection, may show up in COVID-19 cases even when there is no accompanying infection.[23, 24] Acute respiratory distress syndrome (ARDS) caused by M. *pneumoniae* was recently misconstrued as a COVID-19 case in a case report. This emphasizes the critical nature of following established treatment guidelines, evaluating treatment techniques, and not overlooking other possible causes of ARDS or severe pneumonia.[16]

The findings of the assay, used in this study, targeting *C. pneumoniae* and *M. pneumoniae* were highly linear throughout replicated dilution series (as determined by r^2 values), and the data from replicates were highly repeatable.

In earlier studies, serological evidence of *C. pneumoniae* and *M. pneumoniae* has been reported. However, variability between researchers exists in the interpretation of the serological assays' findings. Although it is challenge to state broad conclusions depending on the results of our study, the present findings provide indirect evidence against the clinical practice of prescribing antibiotics directed at these atypical bacteria in COVID-19 patients.[25] However, prospective controlled trials are required to confirm the suggested conclusion.

There are a few limitations to our study. None of the COVID-19 verified samples were examined concurrently for atypical bacteria. As a result, the true prevalence of co-infection remains unknown. Although a lower respiratory tract specimen is appropriate, invasive respiratory samples were not collected from COVID-19 patients to avoid aerosol-generating procedures that represent a considerable risk to health care staff and patients. This pilot investigation, however, provides baseline data on the incidence of atypical bacteria co-infection with SARSCoV-2.

Clinicians should examine the presence of other respiratory infections, such as atypical bacteria, while managing COVID-19 patients. Co-existing microorganisms can be identified in time to provide focused therapy and prevent tragic consequences for individuals during the present pandemic.

CONCLUSION

Patients infected with COVID-19, in this first study based in Jordan, have a low rate of atypical bacterial co-infection, which is significantly lower than in previous pandemics. These findings run contrary to the common usage of antibiotics in the treatment of proven COVID-19 infection. However, co-infections with *C. pneumoniae*, *M. pneumoniae*, and SARS-CoV-2 may occur in patients with pneumonia. Clinicians should suspect co-infections with both common and uncommon bacterial pathogens in COVID-19 patients. In severe COVID-19 cases, additional testing for possible respiratory pathogens should be performed to rule out other concurrent infections. Finally, early identification of co-existing respiratory pathogens may allow for more precise antimicrobial treatment, potentially saving patient lives during the ongoing COVID-19 outbreak.

Large prospective investigations are needed to give additional insight on the true prevalence of these co-infections and their impact on the clinical course of COVID19 patients.

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