Molecular Determination of Mycoplasma Pneumoniae, Chlamydia Pneumoniae and Legionella Pneumophila among Iraqi COVID-19 Patients

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Abstract
Background: Coronavirus disease 2019 (COVID-19) is associated with a high rate of morbidity and mortality. SARS-CoV-2, the virus that causes COVID-19, is transmitted primarily through respiratory droplets from symptomatic, asymptomatic, or pre-symptomatic individuals. Patients who died from COVID-19 were found to have various bacterial co-infections, complicating their hospitalization and prognosis, according to the studies. More than that, these concomitant infections are known to worsen overall clinical severity by increasing mortality, ICU admissions and the need for aggressive respiratory support including mechanical ventilation, all of which are factors in increased LOS in hospitals. Aim of study: The current study aim to investigate atypical bacteria (Mycoplasma pneumonia, Chlamydia pneumoniae and legionella pneumophila) co -infection of (120) COVID-19 confirmed patients and determined if it's affected severity of infection. Material and Methods: 120 samples of sputum were obtained from qRT - PCR confirmed COVID-19 patients, DNA extracted using a specific kit, and PCR performed Results: From the 120 qRT-PCR confirmed COVID-19 patients (65 male and 55 Female) it was found that 6\120(5%) infected with Mycoplasma pneumonia, 4\120(3.3%) infected with Chlamydia pneumonia and 4\120(3.3%) infected with Legionella pneumophila while two patient infected by both Chlamydia pneumonia and Mycoplasma pneumonia and co-infection contribute with severity of infection. Conclusion: Molecular method is more specific and rapid used for detection atypical bacteria (Mycoplasma pneumonia, Chlamydia pneumonia and Legionella pneumophila) causes co-infection in COVID-19 patient.
Keywords: COVID-19, Chlamydia pneumoniae Mycoplasma pneumoniae and Legionella pneumophila

1. Introduction

Coronavirus disease 2019 (COVID-19) is associated with a high rate of morbidity and mortality. According to current research, SARS-CoV-2, the causative agent of COVID-19, is transmitted primarily through symptomatic, asymptomatic or presymptomatic individuals' respiratory droplets (1). The SARS-CoV-2 spike protein, which has a functional polybasic cleavage site and enters the lungs, heart, kidney, and gastrointestinal cells via fusion with the human angiotensin-converting enzyme 2 (ACE2) receptor, is critical to the infection (2).Age, immune system status, and the underlying disease of the patient all play a role in the onset of symptoms, which can range from mild symptomatic emersion to acute respiratory failure with high mortality. There was a correlation between increased biomarker and inflammatory cytokine levels in some SARS-CoV-2-infected patients and co-infection with bacteria. Secondary bacterial infections are common in older patients who have other respiratory pathogens, such as influenza (3). Some studies have found evidence of SARS-CoV-2 super infections, which occur when a patient who already has SARS-CoV-2 infection also has a co-infection, and others have found evidence of super infections, which are when additional respiratory pathogens are recovered during treatment for SARS-CoV-2 infection. (1&4).

A few studies have found that people can be infected with both SARS-CoV-2 and bacteria that are not grown in labs, such as Legionella pneumophila, Mycoplasma pneumoniae, and Chlamydia pneumoniae. It is hard to tell the difference between SARS-CoV-2 and bacteria that cause atypical pneumonia because their symptoms and appearances are similar. Aside from this, it's not clear if co-infection with atypical bacteria can make COVID-19 patients' health worse (5).

Mycoplasma pneumoniae one of the most important unique prokaryotes is genus Mycoplasma, belong to the class Mollicutes. Mycoplasmas are the tiniest known organism have ability to replicate independently, despite the fact that leaking of many genes of the genome that participate in many biosynthetic activities (6). In addition to respiratory tract infection Mycoplasma pneumonia responsible extra pulomanory infection such as pharyngitis, encephalitis, steven –johnson syndrome, septic,

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arthritis pericarditis and other manifestations and autoimmune pneumonia, that progress to sever life threating pneumonia(7) Legionella pneumophila gram-negative, aerobic bacilli that do not form spores, are capsule-free, and possess the enzymes catalase and oxidase .belong to genus legionella which single genus in legonellacea family the genus legionella consist of 53 spices and 70 serogroup. Legionella ubiquite and inhabits nature water such as rivers and lacks and artificial water also moist soil. Infection occurs when human inhaled aerosol contained pathogen or aspiration. Person to person not transmitted after inhaled bacteria invade alveolar macrophage and other cells such as monocytes by process know coiling phagocytosis. Legionella pneumophila is the cause of most Legionnaires disease (LD).

Other species have been found to cause severe pneumonia and Pontiac fever, which has been studied less (an acute, but generally milder set of cold-like signs and symptoms). (8) Chlamydia pneumoniae is a pathogen that can only live inside cells and spreads through the air. It is a common cause of respiratory diseases in people, and pneumonia and bronchitis are the most common symptoms. It causes 10% of all cases of pneumonia in the community and 5% of bronchitis, pharyngitis, and sinusitis (9). Chlamydia pneumoniae is likely to come back or stay in the body for a long time. The importance of chlamydial diseases is linked to the immune pathogenesis of persistent or repeated infection, which can slowly cause severe effects in the host. Chronic C. pneumoniae infection has been linked to a number of long-term health problems, such as obstructive pulmonary disease, asthma, coronary heart disease, and even lung cancer (10).

2. Material and Methods

A120 sample (sputum and Blood) collected from RT-PCR confirmed COVID-19 patients (65male and 55female age ranging 18-80 years, from Al-Diwanyiah Technical Hospital, The City of Medicine in Baghdad, and and AL-Amal Specialized Hospital in Najaf City . In period from 1novomber 2021 to 1 March 2022.The inclusion criteria are mild moderate, severe symptoms and patients with acute respiratory distress syndrome (ADRS)

Sample processing

Sputum sample

Sputum samples were mixed together before DNA was extracted by adding an equal amount of a mucolytic agent (2-mercaptoethanol 0.1M) and shaking them vigorously. After 30 minutes at room temperature, the samples were stirred again, and the solution was centrifuged at 1000g/min for 10 minutes. The supernatant was thrown away, and the pellet was resuspended in 100 ml of saline water. The pellet was mixed back into 100 ml of salty water (11).

BLOOD Sample

To measure D-dimer and C-reactive protein in the blood, a vein blood sample was taken and placed in the test tube. Blood was placed on the gel tub, allowed to sit at room temperature for 15 to 2 minutes, and then centrifuged at a speed of 3,000 revolutions per minute for 5 minutes. Pipetting the top part allowed for the isolation of the serum. The serum was refrigerated after being centrifuged.

C-reactive protein test

Measurement the C - reactive protein level in blood based on fluorescence immunoassay technology using ichroma™ for the quantitative determination of CRP.

D-dimer test

Measurement the D-dimer level in blood-based fluorescence immunoassay technology using ichroma™ for the quantitative determination of D-dimer.

Bacterial genomic DNA extraction

By strictly adhering to the manufacturer's instructions and using a specific extraction kit from Geneaid Company USA (NO GBB101), bacteria genomic DNA was directly extracted from the 120-sputum sample.

Primers

The primers were used for the detection of Mycoplasma pneumonia, Chlamydia pneumonia and Legionella pneumophila according to IDI Company in Candia.

Table (1) Primer for detection M. pneumonia, C. pneumonia and L. pneumophila

Bacterial target gene	Primer sequences		product(bp)	Reference
M. pneumonia P1adhesion gene	F	CAAGCCAAACACGAGCTCCGGCC	542bp	Chaudhry,et al ., 2017
	R	CAGTGTCAGCTGTTTGTCCTTCCCC		
C. pneumoniae 16SrRNA Gene	F	TGACAACTGTAGAATACAGC	465 bp	Jantos, et al.,1998
	R	CGCCTCTCTCCTATAAAT		
L. pneumophila 16SrRNA gene	F	AGGGTTGATAGGTTAAGAGC	386bp	Sheehan et al., 2005
	R	CCAACAGCTAGTTGACATCG		

Table (2): PCR components used to identify chlamydia pneumoniae, mycoplasma pneumoniae and legionella pneumophila

Component	Volume
PCR Master mix	12.5 µl
DNA template 50ng	4 µl
Forward primer (10pmol)	1.5 µl
Reveres primer (10pmol)	1.5 µl
Nuclease free water	5.5 µl
Total volume	25 µl

Table(3)Thermo-cycler and the PCR cycling program conditions for M. pneumonia, C pneumonia and L.pneumophila which changed according the primer				
Mycoplasma pneumonia				
Genes	Step	T.M	Time	Cycle
P1 adhesion	Initial denaturation	94 ° C	5min	1
	Denaturation	94° C	30 sec	35
	Annealing	53° C	30 sec	
	Extension	72° C	1min	
	Final Extension	72° C	10 min	1
Chlamydia pneumonia				
Genes	Step	T.M	Time	Cycle
16 SrRNA	Initial denaturation	94° C	5min	1
	Denaturation	94 °C	30sec	40
	Annealing	53° C	30 sec	
	Extension	72° C	45 sec	
	Final Extension	72° C	10min	1
Legionella pneumophila				
Genes	Step	T.M	Time	Cycle
16 SrRNA	Initial denaturation	94° C	5min	1
	Denaturation	94 °C	45 sec	40
	Annealing	57° C	45 sec	
	Extension	72° C	45 sec	

Gel electrophoresis

The amplification product was examined using a 2 percent agarose gel with 1X TBE and then dissolved in a water bath at 100 °C for 15 min. PCR products were then seen using the Gel Documentation System.

3. Statistical Analysis

Statistical Package for Social Sciences (SPSS) version (27) also Microsoft Excel (2010) are used to analyze the study's data using the statistical data analysis techniques listed below:

1. Descriptive data analysis

A- Tables (frequencies and percentages).
B- Brief statistical tables including: mean, Standard deviation, chi-square test, one way ANOVA and Person correlation. P Values less than or equal to 0.05 were considered statistically significant (12)

4. Results

Age ranged from (18-80) years for the 120 patients in this study, 65 of whom were male and 55 of whom were female (there are no significant differences between gender). The most prevalent symptoms in all of the patients were reported to be fever and headache, which were followed by myalgia, which appeared in 116–120 patients (96.6%), dyspnea (41.6%), chills (60%) and diarrhea (33.3%). Additionally, the study took into account the comorbidities of COVID-19 patients. The findings indicated that (25%) of patients had diabetes, and (35%) of patients had hypertension, asthma, and renal disease, among other comorbidities. Strong correlation between CRP and D-dimer levels and infection severity is shown in tables (4) and table (5)

Table (4) Association between disease severity and C – reactive level.	
Disease severity	Mean ± SD
Mild	6.50 ± 2.57
Moderate	23.57 ± 7.61
sever	63.02 ± 14.8
critical	110.9 ± 25.8
P value	<0.001*

Table (5)Association between disease severity and D dimer level	
Disease severity	Mean ±SD
Mild	251.5±51.4
Moderate	358±97
sever	652.3±83.6
critical	863.3±53.8
P-value	<0.001*

DNA was extracted from 60 sample of sputum from COVID-19 patients to detect Mycoplasma pneumonia using the PCR technique and specific target sequences primers (p1 adhesion gene 542 bp) the results show that only 6(5%)samples give positive result for Mycoplasma pneumonia .Figure(1).

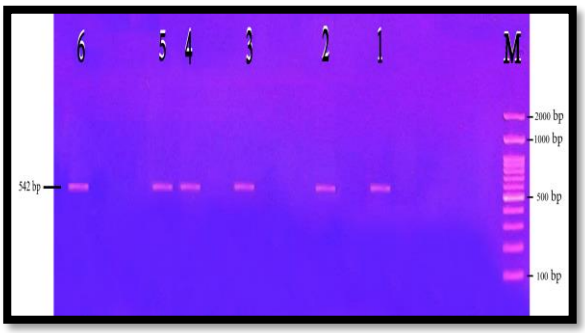

Fig. (1) Image for positive samples of Mycoplasma pneumonia on agarose gel. Stain with Ethidium bromid Electrophoresis of the product PCR was carried out on the agarose gel at concentraction of (2%) at 70 volit for 80 minutes. M (marker ladder 2000-100bp). Lane (1-6) positive Mycoplasma pneumonia sample at 542 bp PCR product size

To detect Chlamydia pneumonia using the PCR technique using specific target sequences primers for the (16SrRNA gene 465 bp) the result show only4(3.3%) samples give positive result for C. pneumonia Figure (2)

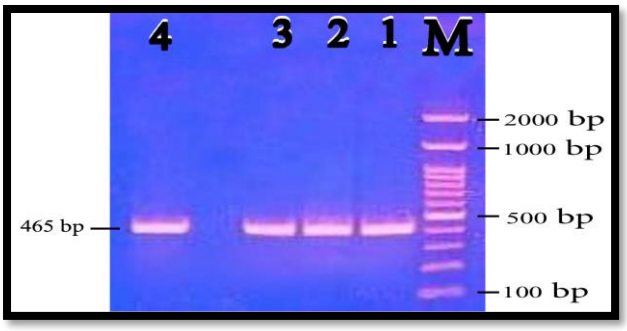

Fig. (2) Positive samples of Chlamydia pneumonia on agarose gel. Stain with Ethidium bromide. Electrophoresis of the product PCR was carried out on the agarose gel at concentration of (2%) at 70 volt for 80 minutes. M (marker ladder 2000-100bp). Lane (1-4) positive Chlamydia pneumonia sample at 465 bp PCR product size

In the current study specific target sequences primers for the (16SrRNA gene 386 bp) to detect Legionella pneumophila where the result show that only 4 (3.3%) samples give positive result for L. pneumonphila figure (3)

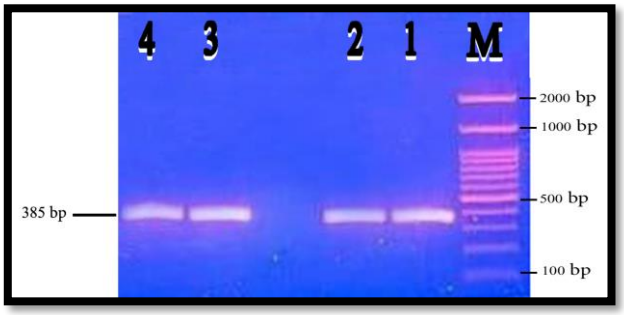

Fig.(3) Positive samples of Legionella pneumophila on 2% agarose gel . Stain with Ethidium bromid. Electrophoresis of the product PCR was carried out on the agarose gel at concentration of (2%) at 70 volt for 80 minutes. M (marker ladder 2000-100bp). Lane (1-4) positive legionella pneumophila sample at 385bp PCR product size.

In this study correlation between severity of infection and investigated bacteria .M. pneumonia registered in 6 patients (3 critical and 3 sever) and C. pneumonia reported in 4 patients (2critical and2 sever) while L. pneumophila identified in 4 patients (2 critical and 2sever).Table (6)

Table (6) Association between disease severity and Causes infection

Disease severity	No.	M. pneumonia	L. pneumophila	C. pneumoniae	Mixed infection C. pneumoniae M. pneumonia
Mild	28	0(0)	0(0)	0(0)	0(0)
Moderate	40	0(0)	0(0)	0(0)	0(0)
severe	36	3(8.33)	2(5.55)	2(5.55)	1(2.77)
critical	16	3(18.75)	2(12.5)	2(12.5)	1(6.25)
Total	120	6(5)	4(3.33)	4(3.33)	2(1.66)
X2		10.78	7.06	7.06	3.47
P value		0.013*	0.07	0.07	0.324

5. Discussion

Age and sex of patients were found to be the most important factors in the spread of COVID-19 infection in the study. According to the results of the current study, men are more likely to be infected with the diseases than women. Males are more likely than women to be exposed to social gatherings and mixing because of their jobs, as well as secondary factors like smoking and drinking that affect the immune system (13). Infection by coronavirus effect on a biomarker of body, as the level of CRP and D-dimer .In the current study elevated level of CRP in the patient's serum COVID-19, which are significantly high and there are positive correlation between level of CRP and severity of infection, also in the study found positive correlation between level of D-dimer in the patients serum COVID-19, many studies support the current study which demonstrated an increased level of CRP and D-dimer in the patient's serum COVID-19 (14,15,16,17). The molecular results show 12\120 (10%) patients had co-infection with un-cultivated bacteria 6(5%) Mycoplasma pneumoniae 4(3.3%) figure (1) 4(3.3%) had infection with Chlamydia pneumonia figure (2) and 4(3.3%) Legionella pneumophila figure (3) also in the study two patients had infection with both Chlamydia pneumonia and Mycoplasma pneumonia there are few studies similar to this study (2,11) In this study using the molecular assay to detected atypical bacteria cases co-infection with SARS-COV2 infection . However in this study correlation between severity of infection and investigated bacteria M. pneumonia registered in 6 patients (3 critical and 3 sever) and C. pneumonia reported in 4 patients (critical and sever) while L. pneumophila identified in 4 patients (critical and sever).Table (6) Many studies had result similar current result that found co-infection with atypical bacteria (M. pneumonia, L. pneumophila and C. peumoniae increased severity of infection also can lead to mortality (19,20,21,22) In this study also found the signs and symptoms of patients had co-infection with Mycoplasma pneumonia, Chlamydia pneumonia and Legionella pneumophila and COVID-19 similar including fever, cough, myalgia and dyspnea and most of patients had underlying diseases which consider potential factor for community –acquired bacteria pneumonia(23), moreover 10\12 patients had co-infection of atypical bacteria with COVID-19 needed to mechanical ventilation and emergency intubation .The patients with mechanical ventilation are at

increased risk for pneumonia because of impairment in mucocciliary clearance caused by the endotracheal tube also are often on brod-spectrum antibiotic and therefore are at greater risk for infection s with resistance organisms which lead to increases severity of infection(24&25) and all the patients had co-infection were no received vaccine against COVID-19 infection

Table (7) Charactereristic of patients with SARS-COV-2 and Chlamydia pneumonia (n-=4), Mycoplasma pneumoniae (n=6) and Legionella pneumophila (n=4) co-infection

Patent	Age \ sex	Comorbidities	Clinical presentation	Laboratory biomarker	Type of co-infection	Oxygen delivery	ICU	Vaccine
Pt1	34\F	Diabetes	Fever, cough, dyspnea	CRP 97.8 D-dimer 688	M. pneumonia	Yes	Yes	No
Pt2	65\M	Diabetes Hypertension	Fever, cough dyspnea ,myalgia	CRP 100 D-dimer 992	M.pneumonia C. pneumonia	Yes	Yes	No
Pt3	68\M	Hypertension	Fever, dyspnea	CRP 90.4 D-dimer 908	M. pneumonia C. pneumonia	Yes	Yes	No
Pt4	48\F	Hypertension Diabetes	Fever, cough dyspnea	CRP 89.8 D-dimer 945	L. pneumophila	Yes	Yes	No
Pt5	55\M	No	Fever, cough Dyspnea, myalgia	CRP 66.9 D-dimer 809	M. pneumonia	Yes	Yes	No
Pt6	69\M	Hypertension Diabetes	Fever, dyspnea, myalgia	CRP 93.7 D-dimer 769	L. pneumophila	Yes	Yes	No
Pt7	58\M	Renal diseases Diabetes	Fever, cough Dyspnea	CRP 103 D-dimer 911	C. pneumonia	Yes	Yes	No
Pt8	79\M	Hypertension	Fever, myalgia	CRP 76.8 D-dimer 567	L.pneumophil	Yes	Yes	No
Pt9	77\M	Hypertension	Fever, cough myalgia	CRP 69.6 D-dimer 789	M. pneumonia	NO	Yes	No
Pt10	79\M	Hypertension Diabetes	Fever, Dyspnea	CRP 85.9 D-dimer 718.	C. pneumonia	Yes	Yes	No
Pt11	76\F	Asthma	Fever, cough	CRP 91.0 D-dimer 807	M. pneumonia	No	Yes	No
Pt12	46\F	Diabetes Asthma	Fever, cough Dyspnea. myalgia	CRP 71.2 D-dimer 950	L. pneumophila	Yes	Yes	No

6. Conclusion

Atypical bacteria (Mycoplasma pneumonia, Chlamydia pneumonia and Legionella pneumophila) causes Co-infection with COVID-19 and contribute in severity of infection, also other factor such as age and gender paly important role in the prevalence of COVID-19infection and that infection causes an increase in both the effective protein level c-reactive protein and D-dimer.

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