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Rapid Identification of some typical and atypical Pneumonia co-infections associated with COVID-19 patients by a real -time PCR assay

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Abstract: Bacterial co-pathogens are commonly identified in viral respiratory tract infections. Co-infections with other pulmonary pathogens arisen in COVID-19 patients with the patient population evaluated had laboratory-confirmed corona virus infection. All extracted nucleic acid of nasopharyngeal swabs and sputum specimens which positive for COVID-19 diagnostic assays were submitted to morphological, cultural, biochemical and comparison the results with molecular test (Real time PCR technique) for detection of association of *Streptococcus pneumoniae* and *Mycoplasma pneumoniae* with COVID-19 patients. Cultural analysis showed S. pneumoniae and *M. pneumoniae* infection revealed an incidence of 20% and 10% percentage, respectively from 100 patients, while the molecular test were 33%, 13% respectively. Also, the strength of the correlation between age, sex, symptoms vaccinated and unvaccinated patients and its outcomes. From 100 positive COVID -19, medium patient's age was 55 years, 42% were women, and 58% were male. Cough was the most common submitting a complainant to all age bands (20–39, 40–59,60-79, and +80) 10%, 43%, 43%, and 4%, respectively ($p<0.001$). Diagnostic tests for known lung pathogens have limits. It was concluded that, despite positive cultural and biochemical not for all typical and atypical pathogens, in an environment where clinical suspiciousness for Corona virus is rise, specific analyses are being conducted for it. So, we are being performed RT-PCR to confirm a diagnosis. The most widespread signs are more presented in the older patients, and more infected with bacterial microorganism *S. pneumoniae* followed with *M. pneumoniae*, and with unvaccinated patients higher than vaccinated patients with 87%.

Key words: Pneumonia Co-infections, COVID-19, *Streptococcus pneumonia*, *Mycoplasma pneumoniae*, RT-PCR.

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Introduction

Bacterial infections are widespread with pulmonary viral pathogens. These infections can increase the significance of mortality and morbidity. The SARS-CoV-2 pandemic, is affected more 15 million persons universally with over 0.5 a million deaths. However, there is some studies on the current SARS-CoV-2 pandemic and associated coinfections, which announced a different infection averages among 1% and 8% based on several cross-sectional researches. The microorganisms that cause coinfections were as follows up: *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Streptococcus pneumoniae*, and *Chlamydia pneumoniae* (1). There are no specific and direct results for co-infections in COVID-19 on morbidity and mortality. Some studies have reported more acute and difficult disease courses in states of co-infection (2). *Streptococcus pneumoniae* is commonly the main bacterial agent cause the increment the illness and mortality in virus-associated pneumonia (3). Historically, the most

culprit bacterium causing community-acquired pneumonia worldwide was *S. pneumoniae* which is a lancet-shaped, gram-positive, facultative anaerobic microorganism (4). Although pneumococcal pneumonia can infect all people from childhood to adulthood, patients older than 65 years are the common age; those who smoke, abuse alcohol, and have asthma or Chronic obstructive pulmonary disease.(5). While, *Mycoplasma pneumoniae* is the most significant pathogen of community-acquired pneumonia (CAP). This is a popular respiratory pathogen that causes diseases of different acuteness reaching from mild to acute atypical pneumonia (6). This microorganism is also in charge of causing a broad spectrum of non-pulmonary disorders, like neurological, hepatic, cardiac diseases, hemolytic anemia, arthritis and erythema multiform(7). The traditional identification methods as cultural and serological assay lack sensitivity. Molecular techniques as PCR give a better process for rapid diagnosis but are apt to load more impurities through amplification of specific genes. Quantitative real-time PCR (qRT-PCR) method offers an excellent alternate way for diagnosing pathogens (8). In the current work, qRT-PCR and cultural methods were used to detect *S. pneumoniae* and *M. pneumoniae* bacteria So,we aimed to define some associated bacterial infections by rapid molecular tests compared with cultural and biochemical tests and study some clinical factors related with (COVID-19) patient s in Baghdad city.

Material and methods

Patients and the clinical specimens

Between November 2021 to March 2022, total of 80 of sputum and 20 of nasopharyngeal swab specimens collection were from outpatients and inpatients whom suffer from SARS-CoV-2 attendance to the chest unit in several hospitals in Baghdad with clinic signs of respiratory infection such as weakness and lethargy, fatigue, persistent headache and dry cough, shortness of breath, diarrhea, sputum, muscle pain. The patient's gender involved both sexes (male and female) and the age range (24 - 85 years).

Molecular identification

Bacterial infections were detected based on a molecular examination through direct extraction of bacterial DNA from sputum and nasopharyngeal swab samples directly in order to compare the efficiency of rapid molecular diagnosis compared to waiting for several days when conducting bacteriological and biochemical tests. Genomic DNA was extracted from hundred specimens of sputa and nasopharyngeal swab by use (of ABIO pure Extraction, USA) A 1ml of sample was placed in 1.5ml micro centrifuge tube and 20µl of Proteinase K solution (20 mg/ml) and 200µl of Buffer BL was added to sample then the tube was mixed vigorously using vortex and incubated at 56℃ for 30 min. Then, the extraction was done according to company instruction. After that, the extracted DNA was checked through Quantus fluorometer, and then stored in -20C at refrigerator until perform Real-Time PCR.

Detection of *S. pneumoniae and M. pneumoniae by* Real-Time PCR Assay

It was performed by use Syber green dye based amplification of *16SrRNA* gene *S. pneumoniae* and *23SrRNA* gene *M. pneumoniae*. The primers were mentioned in table (1). After confirming the sensitivity and

specificity of the primers by NCBI primer BLAST, The Real-Time PCR amplification reaction was done by using (GoTag qPCR Master Mix, Nuclease Free Water, Promega, USA) and the qPCR master mix reaction components: qPCR master mix total volume 7 µL , Genomic DNA template 3 µL, 2X Green star master mix 5µL, primer Forward primer (10pmol) 0.5 µL Primer Reverse primer (10pmol) 0.5vµL Nuclease Free Water 1 µL was placed in sterile white qPCR strip tubes and then transferred into vortex centrifuge for 3minutes, Real-Time PCR system and applied the following thermo cycler conditions as shown in the following Table(2).

Table (1): Primer used in this study

Primer	Sequence 5---3	Products size (bps)	Temp.	Ref.
16SrRNA (S. pneumoniae)	F" AGCGATAGCTTTCTCCAAGTGG" R" CTTAGCCAACAAATCGTTTACCG"	75	50	(9)
23SrRNA (M. pneumoniae)	F"ATGTACTATCAGCAAAAGCTCAGTATGG" R" CCACATACCGCTTTAAGTTAGCAA"	82	60	(10)

Table (2): RT- PCR step and thermo cycler conditions

qPCR steps	Temperature(°C)	Time (min.)	Cycles
Initial Denaturation	95	5	1
Denaturation	95	0.5	40
Annealing	50 , 60	0.5	
Extension	72	0.5	

Bacterial isolation and phenotypic identification

The sputum and nasopharyngeal swabs were transported to microbiology lab by transport medium, and then inoculated on to Blood agar and PPLO agar. The phenotyping characteristics of *Streptococcus pneumoniae* was described through using gram staining, catalase test and optochin test plated on 5% sheep blood agar was streaked with an inoculum from a pure isolate to be tested, then an optochin disc was placed in the center of the inoculum and incubated for 24-48 hrs. at 37 C°, then observation of zones of growth inhibition larger than 14 mm surrounded the disc which means appositive results. This test was utilized to distinguish *S. pneumoniae* (sensitive) from *Viridans streptococci* (resistance) (11).

There are many methods we used to diagnosis *Mycoplasma pneumoniae* infected as culture, microscopic examination and RT-PCR (12). *M. pneumonia* was identified via culture, biochemical tests, such as fermentation glucose by a new protocol to culture *M. pneumoniae* was formulated by adding animal serum and yeast extraction into the specific commercial medium according to Chang (2014), which involved 1% Glucose 20% calf serum and phenol red indicator after incubation at CO2 5 - 10% at 35°C from 2 – 3days) (13). Once the broths changed color, which indicated growth of *M. pneumonia*. In addition to identification by growth on PPLO agar PPLO agar with blue methylene dye (specific medium for *M. pneumonia*) and examined under 4X, 10X lens of light microscope and observation a fried egg shaped of colony (14).

Statistical analysis

The Statistical Analysis System-SAS (2012) program was used to detect the effect of difference factors in study parameters. Chi-square test was used to significant compare between percentage (0.05 and 0.01 probability) in this study(15).

Results and discussion

Isolation and identification

The infections of respiratory tract are main reasons of universal morbidity and mortality. *S. pneumoniae* synchronous contagion is familiar to be accountable for the increment gravity of H1N1 pandemic influenza (16). While, *M. pneumonia* atypical pathogens cause community-acquired pneumonia (CAP) with progression of nonspecific signs are increased from the top respiratory tract to the lowest one. (17). Fast and precise identification is an agent that decreases the time of diagnosis and costs of infectious diseases (18).

As the results in the table (3 & 4) shows that there is no significant results between media and biochemical test for diagnosis of *S. pneumoniae* via the blood agar plates for determining the bacterial growth and positive plates (α-hemolysis) and pin point colony shape add to sensitive to optochin disc as specific tests for *S. pneumoniae* with percentage 20 % positive isolates. On other hand, among 100 specimens collected 17 yielded positive cultures results by PPLO agar. While, the new culture method (PPLO broth with glucose 1%) gave only 10 positive isolates. The average incubation time for isolation *M. pneumoniae* in this new method was 2 days. The incubation periods using PPLO agar shorter than the new protocol was significantly shorter than the other culture method, which usually took more than one week to yield *M. pneumoniae*. There is a high significant value between the growth of *M. pneumonia* on PPLO agar compared with the growth on PPLO broth with glucose and PPLO agar with methylene blue. The morphological property as a fried egg shaped of colony on PPLO agar and color change on PPLO broth with 1% glucose and growth on methylene PPLO agar for diagnosis of *M. pneumoniae*.

Table (3): Biochemical test for *Streptococcus pneumonia* from 100 patients

No.	Biochemical tests	Result	No. of +ve isolates	%
1	**PPLO agar (Microscopic examination)**	Positive, Round shape on 4,10 X	17	100
2	**Methylene blue PPLO media**	Positive	10	58.82
3	**Glucose fermentation test**	Positive	10	58.82
4	**Urea hydrolysis test**	Negative	17	100%
	Chi-Square (χ^2)	---	---	7.85 **
**** (P≤0.01).**				

Table (4): Biochemical test for *Mycoplasma pneumonia*e from 100 patients

No.	Biochemical tests	Result	No. of +ve isolates	%
1	Blood agar	α-hemolysis and pin point colony shape	20	20.0
2	Gram Stain	Gram positive	20	20.0
3	Microscopic shape	Strip shape	20	20.0
4	Catalase production	Negative	20	20.0
5	Optochin test	Positive	20	20.0
	Chi-Square (χ^2)	---	---	0.0 NS
(P≤0.01).				

In the present study, all collected specimens were confirmed the identification of bacteria by RT-PCR technique that was done by using particular primers for *S. pneumoniae* and *M. pneumonia*e. Subsequently, direct extraction of DNA of sputum and nasopharyngeal specimens. The existence of *16SrRNA* and *23SrRNA* genes of *S. pneumonia*e, *M. pneumonia*e were confirmed only in 33(33%) and 13(13%) COVID-19 patients respectively as showed in in figures (1&2). This will aid in rapid identification of these bacteria especially *M. pneumonia*e infection. Also we found that there is a viral co-infection was detected in our cohort of critically ill COVID-19 patients While, we report only a 9% rate of bacterial mix infection (*S. pneumoniae* & *M. pneumoniae*) in the same patients with severe SARSCoV-2 pneumonia. This confirms the effectiveness and speed of molecular diagnosis compared to culture and biochemical assays.

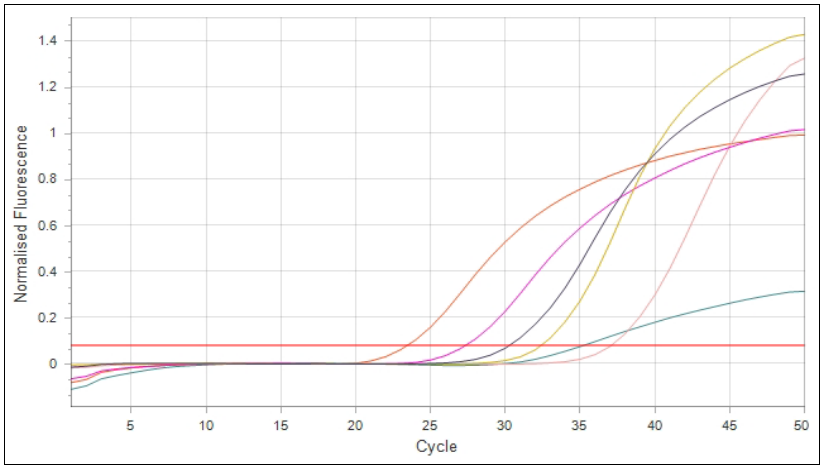

Figure (1): RT-PCR detection of some samples *S. pneumoniae* by using *16SrRNA gene.*

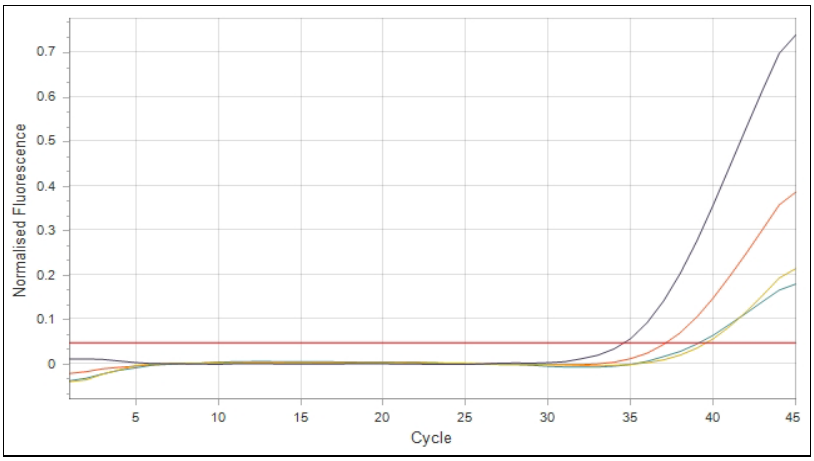

Figure (2): RT-PCR detection of some samples *M. pneumoniae* by using *23SrRNA gene*.

The consequences of our current study showed that higher percentages of *S. pneumoniae* were obtained by molecular tests in comparison with the results of cultures and biochemical examinations, which confirms the

importance of special principles for rapid diagnosis and ensuring the administration of antibiotics in a faster time, especially for hospitalized Corona virus patients.

On the other hand, we note that the molecular examinations gave results in lower percentages than the culture and biochemical tests, and this indicates that the PPLO medium is a special medium for diagnosing many species of the genus *Mycoplasma. Spp*. in general and not specifically for the species *M. pneumoniae*, in contrast using the Real time PCR technique based on bacterial DNA within clinical samples directly.

The increment of bacterial infection related to the injury of ciliated cells can moreover be determined in correlation with viral respiratory infection; it can result in clearance and retro gradation of mucous layer , add to increment the adhesion of pathogens to mucins and, encouraged colonize of bacterium cell to the respiratory route. Furthermore, new adhesion receptors for bacteria can protrude after the virus-induced death (19&20). Subsequent of the acute inflammatory reaction and pulmonary tissue injury induced via viral infections, a repairing phase of the lung tissue was occurred, add to the different immune responses in persons, this stage may lead an induction sensitivity of respiratory bacterial infections. Therefore, bacterial super infection can happen following a viral infection, which in turn might due to increment illness and death (21).

Therefore, real-time PCR technology is one of the most efficient techniques for rapid detection of associated bacterial infections, which speeds up the administration of antibiotics, increases recovery and reduces deaths.

Patients distribution according to their age groups, sex and symptoms.

From 100 COVID- 19 patients 58 were male and 42 females. The age and sex distribution of cases is shown in Table (5). The highest minimum age of cases (86%) were aged between (40–79) years, followed by the age (20-29) and (<80) groups 10(10) % and 4(4) % respectively. Significant differences were found in the distribution by age and sex under P≤ 0.01. Our finding agreed with a study in Duhok from 1012 Covid patients 60.2% were male and 39.8% were female (22).

Table (5): Patients distribution according to age groups and sex.

sex	Age categories				Total	%	χ^2
	(20-39)y	(40–59) y	(60–79)y	(< 80)y			
Male	4	26	24	4	58	58%	11.43 **
Female	6	17	19	0	42	42 %	10.51 **
Total	10 (10%)	43 (43%)	43 (43%)	4 (4%)	100	100	10.65 **
χ^2	0.782 NS	4.07 *	1.08 NS	2.59 NS	2.91 NS	5.26*	---
* (P≤0.05), ** (P≤0.01).							

The results in figure (3) showed the common symptoms associated with patients that were fever and head ache was the most common presenting symptom with 100%, followed by fatigue, hypoxia and lethargy. While dry cough, loss of appetite, bronchitis, nausea was the least visible, this findings have a significant value under (P≤0.01) reached 26.097. Our finding agreed with some clinical manifestations of a study in Wuhan,

China from 99 Covid patients 83% fever, cough 82%were male (23).

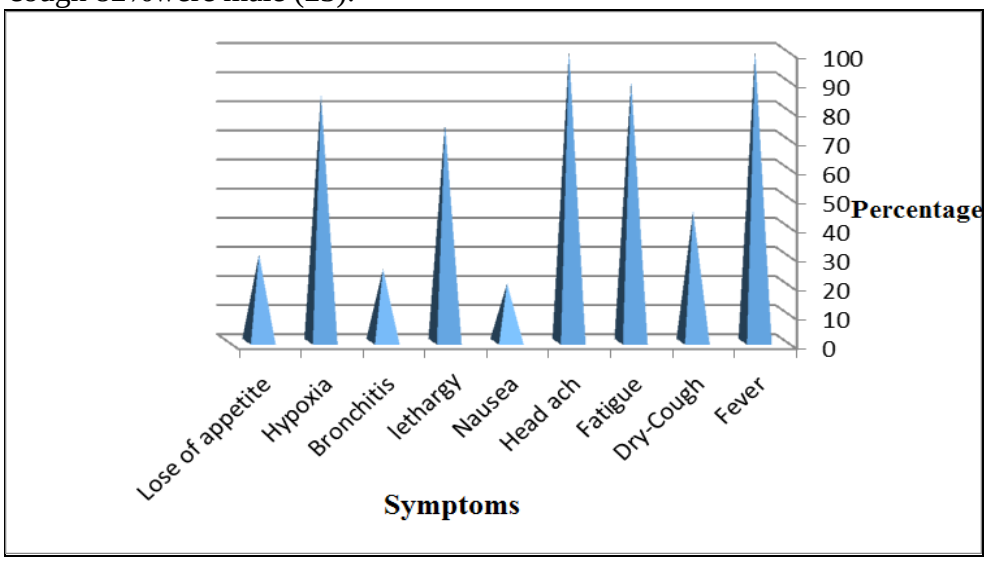

Figure (3): The percentage of clinical symptoms associated with COVD-19 patients.

We occur some typical signs, including cough, fever, and shortness of breath were more shared in patients with COVID-19, while older patients had higher rates of altered of clinical symptoms.

The primary symptoms were fever (100%) and head ach (100%) in all population, while other symptoms were fatigue (90%), hypoxia (85%), lethargy (75%), dry-cough (45%), loss of appetite (30%), Bronchitis (25%), and nausea (20%) respectively, the most severity patients were the oldest who had further systemic diseases in comparison with other patients.

Effect of taking vaccine on the prevalence of co- infections.

The results that is shown in the figure (4) appeared the high risk of bacterial pneumonia infections associated with non-vaccinated persons compared with unvaccinated patients especially Pfizer-BioNtech. Type with the least percentage of infections with a significant value about 181.06 under ($P \leq 0.01$). COVID-19 vaccines are highly efficient in prevention severe disease, hospitalization, and death from COVID-19 especially Pfizer vaccine (24) .This results agreed with Uzun *et al.* (2022) who identified 1401 corona patients, of which 529 (37.7%) were admitted to intensive care units. Nearly half (47.8%) of the patients of which 529 (37.7%) were admitted to intense care units. about half (47.8%) of the patients were not vaccinated, and those with two doses of Sinovac vaccine the second biggest group (32.9%). Hospitalizations decreased in the patients that extradited two doses of Sinovac and a booster dosage of BioNTech than in the group that accepted three doses of Sinovac(25).

The people immunity after having COVID-19 can be high. Though, the sort of immunity that's established afterward infection differs from person to another, creating it fewer expectable than protection after vaccination.

According to the WHO COVID-19 vaccines have been safely assumed to millions of individuals. It is more harmless to take vaccine than it is to danger getting COVID-19 (26).

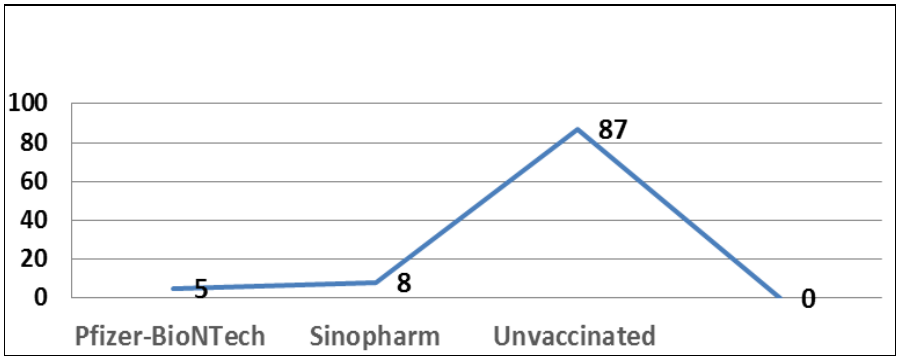

Figure (4): The percentage of vaccinated and unvaccinated hospitalized patient with type of vaccine.

Commonly, the COVID-19 vaccines are real way for avoiding and reducing serious sickness, hospitalization and death from all present virus variants. Vaccines are less effective at protecting you against infection and mild disease than they were for earlier virus variants; but if you do become infected after being vaccinated; your symptoms are more expected to be mild. COVID-19 vaccines accepted by WHO are extremely active at decreasing the danger of developed severe sickness and mortality, no vaccine with 100% efficient. (27).

The appraisal power checks some typical and atypical Pneumonia by using qPCR. Because the real-time PCR assay through high sensitivity that can quickly identify the target pathogenic bacteria in a wide variety of samples, Confirmed performance, Ready-to-use convenience, reduced risk of contamination (28 & 29). In this study use power check *S. pneumoniae and M. pneumoniae* real-time PCR kit to identified specific sequence of pathogen specific primers. We agreed with Chaudhry (2013) who found a higher sensitivity of molecular tests in comparison with culture and/or biochemical tests for the detection of *S. pneumoniae* and *M. pneumoniae* from samples of patients by using real time PCR (30).

The emergency of fast, susceptible, and wide tests to identify respiratory pathogens, including *S. pneumoniae*, which is the most popular bacteria viral-pneumonia associated. The COVD-19 vaccine could assist to prevention a part of a secondary infection, particularly in high-hazard cases (31).

Conclusions

The importance of utilization RT-PCR to confirm the diagnosis for some pneumonial infectious bacteria for getting treatment under specific and fast time, especially COVID - 19 patients.

Conflicts of Interest

Authors announce that there is no conflict of interest.

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