Partial 16SrRNA gene sequencing of *Legionella pneumophila* Isolated from COVID-19 Iraqi Patients

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Abstract

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 occur 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. The current study aimed to detect *Legionella pneumophila* and identification of DNA sequencing with Studying the phylogenetic tree . Sixty samples of sputum were obtained from qRT - PCR confirmed COVID-19 patients .DNA extracted using a specific kit, and PCR product for *16SrRNA* gene were sequenced in Macrogen Company of korea ,Sample were sent through Company of Iraq biotechnology . From sixty patients investigated for COVID-19 infection ,it was found that4\60(6.6 %) had co-infection with *legionella pneumophila* where local *legionella pneumophila* strains were diagnosis that recorded under accession number ID; ON399095, ON399096 ON399097and ON399098with NCBI-Blast *legionella pneumophila* by using (MEGA X program).

Keywords : COVID-19 , DNA Sequencer analysis, *Legionella pneumophila* , ,phylogenetic tree

Introduction

During the COVID-19 pandemic, patients are at risk for *L. pneumophila* infection in community and healthcare settings. Because periods of water system disuse can permit Legionella to grow and increase risk for infection (*Chalker et al., 2021*). Pandemic measures, such as temporary closure and reopening of buildings, could increase risk for Legionella exposure. Healthcare facilities should follow national guidance for managing Legionella during the COVID-19 pandemic and consider publications from the European Society for Clinical Microbiology and Infectious Disease European Study Group for Legionella Infections (Palazzolo *et al* ., 2020) Hospital-acquired *L. pneumophila* cases and outbreaks can have higher fatality rates than community-acquired single cases .Recent data indicates bacterial co-infection in SARS-CoV-2 cases is uncommon in patients newly admitted to the hospital , however, effects of *L. pneumophila* co-infection on COVID-19 mortality rates is not yet known. Large outbreaks might be missed because of reduced testing or less consideration for *L. pneumophila* infection in differential diagnosis. Clinicians should maintain Legionella testing and conduct patient investigations where clinically indicated during the pandemic (*Chalker et al., 2021*) *Legionella pneumophila* is the etiologic agent

responsible for most Legionnaires disease (LD) with other species identified less frequently causing severe pneumonia and the less-studied Pontiac fever (an acute, but generally milder set of cold-like signs and symptoms). The first recognized outbreak of LD, caused by *L. pneumophila*, occurred in 1976.in the United States (US), LD prevalence has increased significantly since 2000, and in 2018 there were approximately 10 000 reported cases.(Dowdell *et al.,* 2019) The diseases associated with *legionella pneumophila* consider paramount public health due to difficulty assessment and control in water because the *legionella spp.* survive in the biofilm and amoebae host.(*Dowdell et al., 2019)*

Material and Methods

Sample collection :

Sixty sputum sample collected from qRT- PCR confirmed COVID-19 patients (28male and 32 female)age ranging 18-80 years in period from 1novomber 2021 to 1 march 2022, which collected from Al-Diwanyiah Technical Hospital, The City of Medicine in Baghdad, and AL-Amal Specialized Hospital in Najaf City

Sample processing : In the laboratory prior to DNA extraction , sputum samples homogenized by adding an equals volume of mucolytic agent (2-mercaptoethanol 0.1M) and vortex vigorously .after that incubation for 30 min at room temperature and vortexing was done .Then ,the solution was centrifuged at 1000g\min for 10 min and the supernatant was removed .the pellet was re suspended in 100 ml of saline water. the pellet was re suspended in 100 ml of saline water.(Hassan *et al .,* 2013)

Bacterial Genomic DNA extraction : Bacteria genomic DNA was extracted directly from the 60 sputum sample using a specific extraction Kit from Geneaid Company USA (NO GBB101) and following the manufacturer's instructions

primer **:**The primers were designed by using primer software provided by IDI company in Candia are illustrated in the table (1) for the detection of *Legionella pneumophila* .

Gel electrophoresis :

Analyze the amplification product by a 2% agarose gel with 1X TBE and dissolved it in a water bath at 100 °C for 15 min, after which, it was left to cool at 50 °C, PCR products were visualized using the Gel Documentation System.

Bacterial genes sequencing:

1- PCR products for *16SrRNA* were sequenced in Macrogen Company in South Korea, samples were sent through company of Iraq biotechnology and according to requirement of the company include 15µl for each forward and reverse of PCR products and 50 µl (10pmoles/ µl) for each forward and reverse of each primes, each samples labeled with a number and name identical to the number that sent to company and samples were sent in a cool box containing cool gel pack.

2- PCR products for *16SrRNA* genes of *Legionella pneumophila* confrmation and study variation between its were sequenced in Macrogen Company in South Korea, samples were sent by the same company to sequence in same company .

Analysis of the sequencing results :

The *16 SrRNA* gene sequencing results were viewed for each forward and reverse by using BLASTN algorithm of NCBI.Then was selected the treatment sequence from text file to applied in the box of Blast Nucleotide Sequence program from NCBI, the name of the bacterial species will appear directly with the percentage of its compared sequence .

Phylogenetic tree:

The phylogenetic tree for the *L.pneumophila* isolates was drawn by using the Rooted Neighbour-Joining phylogenetic tree, this tree constructed from concatenated sequences for each strain derived from an alignment of *16SrRNA* gene sequences then produced from an analysis conducted in MEGA X program. This tree showing the distribution and phylogenetic relationships between *Legionella pneumophila* in the current study and reference strains

Discussion :

The result of PCR show 4(6.6%) patients had Co-infection of *legionella pneumophila* with COVUD-19. figure (1) this result near to result of study done by Soltani, *et al .,* (2021) from which also used molecular assay to investigated *L. pneumophila* cases co-infection . However other studies used the serology assay to detect *L. pneumophila* 7, 8but molecular methods used (PCR technical) because are more specific in diagnostic from other method (culture and serology) Furthermore, the method's price, the high degree of technical skill required, and the time it takes to get results are all important drawbacks(Malhotra, *et al .,* 2013). Multiple sequence alignment analysis of the partial sequence in *16 SrRNA* gene for local *Legionella pneumophila* strain ON399095,ON933096,ON399097and ON399098 with NCBI-Blast *Legionella pneumonphila* using (MEGA X program) that showed nucleotide similarity (*) and mutation in *16SRNA* gene nucleotide sequences as figure(2) The phylogenetic tree for the *Legionella pneumophila* isolates was drawn by using the Rooted Neighbor-Joining phylogenetic tree, this tree constructed from concatenated sequences for each strain derived from an alignment of*16Sr RNA gene* sequences then produced from an analysis conducted in MEGA X program. This tree showing the distribution and phylogenetic relationships between *Legionella pneumophila* in the current study and reference strains figure(3)and Table (4) .The present study revealed that the genetic *strains of Legionella pneumophila* from Iraq patients that recorded under accession number IDs (ON 399095 ,ON399096,ON399097 and ON399098) also had strongly related to strain from Germany, China and Turkey (NCBI- BANK) . The results can be explained by the wars suffered by Iraq and the presence of the occupation armies as well as travelers and the movement of Iraq traders .

Conclusion :

Legionella pneumophila cases co-infection with SARS-COV2 patients and DNA sequencing for local *L. pneumophila* strains which diagnosed within COVID-19 patients recorded under accession number ON 399095 ,ON399096,ON399097 and ON399098 However when comparing the sequencing alignment with standard strain reference at gene bank that had matching many strains from Germany, China and Turkey with identify ranged between (99.13 to 99.81%)

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Table (1) specific primer used in the present study for the detection of *Legionella pneumophila*

Bacterial target gene	**Primer sequences**		**product(bp)**	**Reference**
16SrRNA gene	F	AGGGTTGATAGGTTAAGAGC	386 bp	Sheehan *et al* ., 2005
	R	CCAACAGCTAGTTGACATCG		

Table (2): PCR components used to identify *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 *L. pneumophila*

Legionella pneumophila				
Genes	**Step**	**T.M**	**Time**	**Cycle**
16 SrRNA	**Initial denaturation**	**94.0C**	**5min**	**1**
	Denaturation	94.0C	45 sec	40
	Annealing	57.0C	45 sec	
	Extension	72.0C	45 sec	
	Final Extension	72.0C	10 min	1

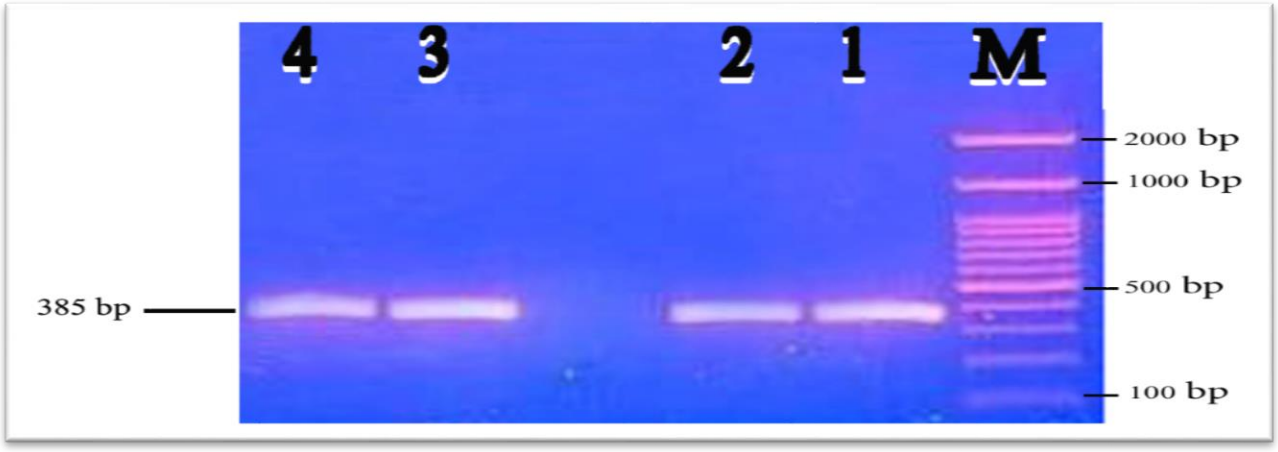

Fig.(1) Positive samples of *Legionella pneumophila* on agarose gel . Stain with Ethidium bromid .Electrophoresis of the product PCR was carried out on the agarose gel at concentration of (2%) at 70 volt for 80 minutes . M(marker ladder 2000-100bp). Lane (1-4) positive *legionella pneumophila* sample at 386bp PCR product size

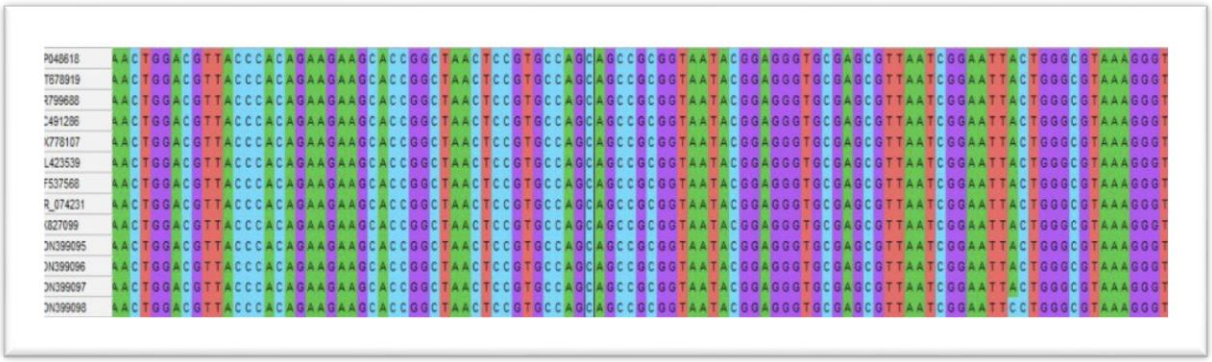

Fig. (2) Multiple sequence alignment analysis of the partial sequence in *16SRNA gene* for local *L. pneumophila*

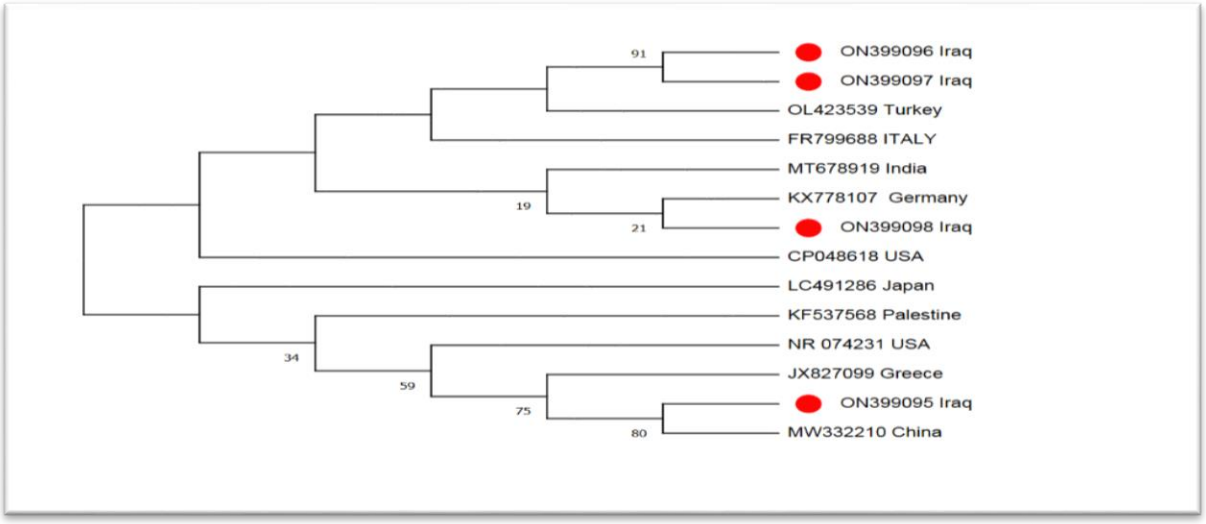

Fig (3) Phylogenetic tree analysis based on *16SRNA gene* partial sequence that used for local *Legionella pneumophila* isolates ON399095 Iraq , ON399096 Iraq , ON399097 Iraq ,and ON399098 Iraq , genetic relationship analysis .

Table (4) NCBI-Blast Homology sequence identity between local *Legionella pneumophila* and NCBI BLAST Identical *Legionella pneumophila* .

NCBL –Blast Legionella pneumophila NO.	NCBL Gene bank Accession number	NCBL Identical clones	Region	Identify (%)
1-	ON399095	MW332210	China	99.13%
2-	ON399096	OL423539	Turkey	99.81%
3-	ON399097	OL423539	Turkey	99.32%
4-	ON399098	KX778107	Germany	99.65%