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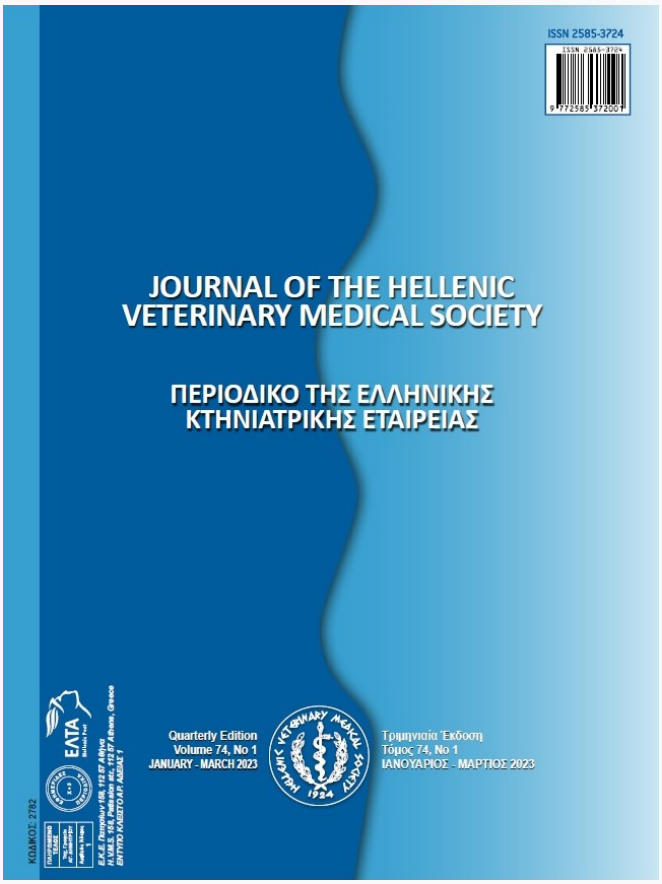

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Research article
Ερευνητικό άρθρο

Molecular identification and sequencing of *Pseudomonas aeruginosa* 16S rRNA gene isolated from a variety of raw cow milk samplesin Iraq

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ABSTRACT: *Pseudomonas aeruginosa* is a gram-negative bacterium that is notably known as a pathogen in humans, animals, and plants. Suspect raw milk samples were analyzed to amplify the 16S rRNA gene and confirm Pseudomonas aeruginosa strains. This study was conducted at different farmland in diwaniya and al-Najaf., a total of 70 samples were collected (28 from mastitic cows and 42 from apparently healthy cows)the detection of the 16S rRNA gene in this study revealed 11 (positive isolates of Pseudomonas were identified from samples after culture on cetrimid agar. New sets of primer pairs were designed using the NCBI database search tool. The phylogenetic relationship between different strains of Pseudomonas aeruginosa has been studied through the use of 16S rRNA gene region sequencing. A distance tree was constructed to determine the genetic similarity between species. As a result of the 9 isolate gene sequencing, it was determined, and submitted under GenBank accession numbers (MZ799357, MZ817077, MZ820788, MZ820878, MZ821016, MZ823355, MZ823356 MZ823387, MZ823389). In conclusion, PCR is a reliable technique that identifies Pseudomonas aeruginosa at the molecular level.

Keywords: *Pseudomonas spp,* milk, sequencing, 16S rRNA gene, phylogenetic.

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INTRODUCTION

In many countries, cow, milk production is an important industry, with the highest percentage in Asian countries (Araujo et al.,2012). The milk industry is the principal economic activity, particularly cheeses manufactured entirely from cow milk. (DeBuyser et al., 2001), (Garedew et al., 2012). The pathogenic microorganism may be present in raw milk as a direct consequence of udder disease or can serve as an efficient agent. Foodborne illnesses, particularly bacteria negatives, have a human transmission vector because they are widely dispersed in the environment (Lendenbach et al., 2009). Food like milk could be contaminated by a variety of microorganisms that originate from the ground, water, skin, hair, and milk producers (Nikbin et al., 2012). Spoilage bacteria in refrigerated raw milk are mostly from the Pseudomonadoceae family (Aysel, et al., 2015: Wargo, et al., 2011).They are called psychroths because they can grow and survive in low temperatures (0-150 C). The nutritional needs of Pseudomonas spp. are minimal and can be found in a variety of natural environments, including soil, freshwater, and marine environments. Psychotropic pseudomonas have been regarded as major degradation bacteria for several decades (Stover et al., 2000).Because of their extracellular thermo-tolerant lipolytic and proteolytic enzymes, which are used to determine the quality of protein and lipid-rich foods that have been stored. (Vela, 1997). This bacterium is naturally common among humans. (Radostitis et al., 2000). It has a relatively large genome, which is likely to promote survival in various environments, with a variety of gene-regulatory activities to facilitate adaptation to new environmental conditions (Mcphee and Griffith, 2011). During the storage of raw milk, *Pseudomonas spp*. plays a significant role in milk spoilage by reducing both the quality and shelf life of processed milk (Abdalhamed et al., 2016). recently Several techniques for identifying bacteria in foods have been tested. Molecular methods such as polymerase chain reaction (PCR) have been broadly applied for the detection and characterization of bacteria in foods like dairy and meat products (13). However, these tests employed selective enrichment techniques to recover bacteria in food samples, and it takes 48-72 hours to determine the identity of bacteria. In this study *Pseudomonas* spp spoilage in milk was studied using both PCR and conventional culture- Phenotypic techniques have proved effective for detecting and characterizing microorganisms.

MATERIALS AND METHODS

Isolation Characterization and of Pseudomonas *spp*.

A total of 70 raw milk samples were obtained from healthy and mastitis cows in different locations across Al-Diwaniayia and al -Najaf city. Each sample was collected in sterile containers and transferred to cooled containers. The samples were serially diluted 10-3 and 10-4 times for bacteriological examination according to (Du et al., 2010)Suspected isolates were confirmed by a series of biochemical identifications according to (Stoeckel, et al., 2016). The Pseudomonas isolation was submitted to the IMViC test. The results of the test were used to cultivate isolated colonies on nutrient agar medium. and on cetrimide agar plates. The results of the test were taken care of and tallied.

DNA extraction of isolated *P. aeruginosa*

G-spin genomic kit (iNtRON Biotechnology, Seongnam-Si, South Korea)was used for DNA extraction from bacterial isolates. Transfer 1-2ml from overnight cultured fresh single colony bacteria in to 2ml tube., then pelleted by centrifugation for 1min at 13000rpm and discarded supernatant. 200µl of buffer CL, 20µl and 5µl RNase solution were added to the sample tube and vigorously mixed. The lysate was incubated at 56 C° in a water bath for (10-30) minutes, then 200µl of buffer BL was added to the sample and thoroughly mixed before being incubated at 70 C° for 5 minutes. The supernatant was transferred into a new 1.5 ml tube and centrifuged at 13000 rpm for 5 minutes to remove un-lysed tissue particles. To the lysate, add 200 l of absolute ethanol and thoroughly mix with a vortex. After discarding the filtrate and placing the column in a new 2 ml collection tube, I transferred the mixture to a spin column (in a 2 ml collection tube)and centrifuged for 1 minute at 13000 rpm. 700µ l of buffer WA was applied to the spin column, and the column was centrifuged for 1 minute at 13000 rpm, discarding the flow through and reusing the collection tube. In a new 1.5ml tube, I placed the spin column and 50 l of warmed buffer CE directly onto the membrane, incubated for 1 minute at room temperature, and then centrifuged at 13000rpm.

Primer design. The universal primer of 16S ribosomal RNA gene sequences available in the GenBank database were species-specific primers were designed, (Singh etal., 2012)as in Table (1)

Table 1: Universal primer of 16S ribosomal RNA gene sequences

gene	Primer sequences (5˚-3˚)		Product size (bp)	Reference
16SrRNA	F	AGAGTTTGATCCTGGCTCAG	**1157**	(Gomila, et al.,2015)
	R	GGTTACCTTGTTACGACTT		

F- forward; R - reverse

Table (2-): Program of PCR Genes Step

Genes	Step	Temperature	Time	Cycle
16SrRNA	Initial denaturation	95.0 ^{0}C	5 min	1
	Denaturation	95.0 ^{0}C	30 sec	35
	Annealing	55.0 ^{0}C	1 min	
	Extension	72.0 ^{0}C	1 min	
	Final Extension	72.0 ^{0}C	10 min	1
	Hold	4.0 ^{0}C	forever	

RESULTS

Polymerase chain reaction PCR

Pseudomonas aeruginosa is one of the most important causes of mastitis, leading to high numbers of economic losses. According to the study findings, the number of *Pseudomonas aeruginosa* isolates was determined by detecting the 16S rRNA gene. About 11/70 (15.70 %)remarkably positive by using conventional polymerase chain reaction PCR (Fig. 1). The molecular methods have been reported to be superior to the phenotypic methods for for identifying of *Pseudomonas aeruginosa* (Cousin et al., 2001), by designing PCR assay based on 16S rRNA gene for molecular detection of *Pseudomonas aeruginosa*, that showed the specificity and sensitivity of the PCR assay were 100% (Singh et al., 2012).

DNA sequencing method

A distance tree was constructed to determine the genetic similarity between species. As a result of the 9 isolate gene sequencing, it was determined and submitted under GenBank accession numbers (MZ799357, MZ817077, MZ820788, MZ820878, MZ821016, MZ823355, MZ823356 MZ823387, MZ823389).

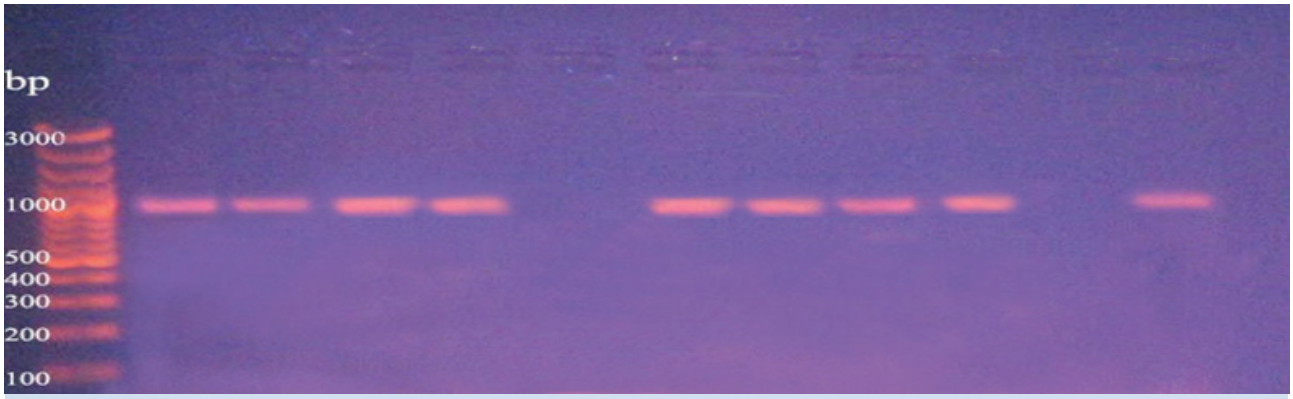

Fig. 1. Typical amplicon of the gene 16S rRNA product of *Pseudomonas aeruginosa* isolates on agarose gel electrophoresis (1.5%) showing the Lane (M) DNA marker (3000 bp), Lane (1-10) represent positive isolates

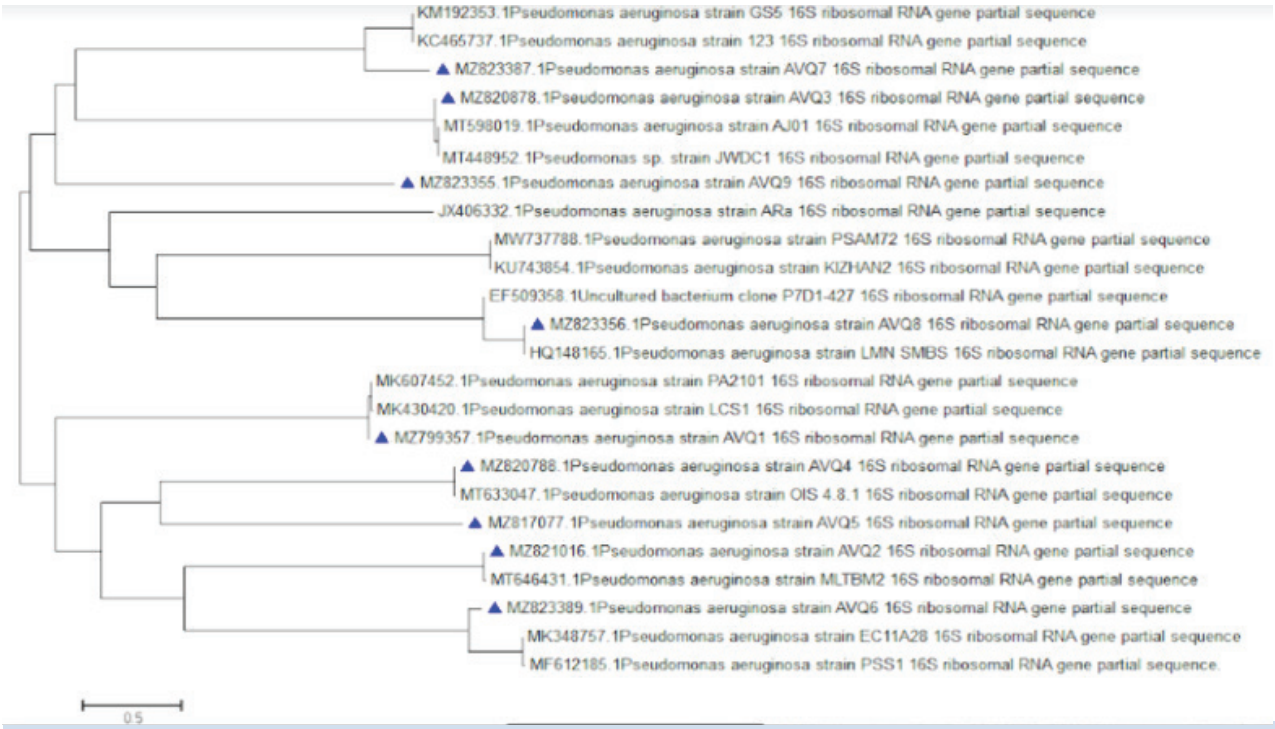

Fig. 2. Phylogenetic tree analysis based on the 16S rRNA gene partial sequence that used for nine isolates of *Pseudomonas aeruginosa*

DISCUSSION

As shown by thephylogenetic tree, our isolate of *Pseudomonas aeruginosa* (MZ823387)was closely similar in its 16S rRNA gene, for the specified sequenced genetic-piece, to the isolate, KC465737, an isolate from India, as shown by the phylogenetic tree. This Indian sequence for this bacterium was identified from the cucumber rhizosphere. This could mean that this infectious agent may cause mastitis due to coming into direct contact with contaminated soil. This could be true, especially when looking at the second phylogenetic tree neighbor, KM192353, which is also an isolate strain from India, detected from samples of petrochemical contaminated soil. This can be reflected in our results when we found infected cows with this strain of the bacterium, indicating an infection that might have arisen from contaminated soil. The literature indicates that one of the main sources of *Pseudomonas aeruginosa* infection is contaminated soil (Kelly and Wilson 2016).

Our isolates could be highly infectious. In this regard, (Pedersen et al.,2021)mentioned that 17 mastitis isolates of this bacterium had previously been identified to produce biofilm, a virulent activity of many pathogenic bacteria. Another question that may arise is what the connection is between our strain and the Indian isolates mentioned above.The answer can be relatively generous. However, the first and easiest response is that Iraq, for many years, has considered India as one of its main sources for cattle imports. Bringing in live animals can introduce new strains of different infectious agents, especially bacteria, and over time, these strains may suffer evolutionary genetic modifications to get to the local sequence level that was detected for our strain in this study ((Murato et al., 2021).The second in our isolate set of *Pseudomonas aeruginosa* (MZ820878) and (MZ823355) were similar in their 16S rRNA gene nucleotide sequences, to MT598019, an isolate from Indonesia, as revealed by the phylogenetic tree. This sequence for this bacterium was identified from *Psidium gajava* plant roots. This study isolate was also a close match with the strain, MT448952, an isolate from samples of petroleum contaminated soil in China. The link between our isolate, here, and these global strains is that Iraq, after a long period of limited goods exchange with these countries, the last two decades were a burst for the trade that covered all life aspects, including the animal import sector, leading to the introduction of new bacterial strains that may, later, be exposed to genetic alteration to have the local sequence picture that is detected in the current study .Moreover, HQ148165, is an Indian isolate that was detected at the wastewater treatment plant in India. This sequence

showed a similar identity via sequencing to one of our isolates, MZ823356. MZ799357, an Iraqi strain detected in the present study, was also similar to a strain from China, MK430420, identified from the soil. The Russian isolate, MT633047, is on the list of similar strains to our isolates, MZ820788 and MZ817077. Sometimes, trade with Iraq in a direct manner is not possible due to trade preferences with other countries. In this way, genetic connections between local strains and global isolates could be indirectly leading to introducing the strains of the countries which are not on the trade list of animals via the intermediate country. Thus, Iraq may have been introduced to the Russian strain through this method (Rush et al., 2021). Moreover, our strain, MZ823389, was similar to the global strain, MK348757, isolated from Sunbird cloacal fluid in Israel. Analysis of sequencing of the 16S rRNA gene is considered an important method to assess the phylogenetic relationship between strains.

CONCLUSION

The PCR assay based on 16S rRNA sequencing is a highly sensitive, rapid, and effective technique for identifying phylogenetically relevant Pseudomonas species. Phylogenetic and taxonomic relationships between strains were investigated using DNA sequencing of the 16S rRNA gene. These worldwide strains have genetic connections with our local strains, which were identified in the present study, and these connections were preserved via physical contact with the country of origin via dairy animal trading and genetic evolution of the incoming strains.

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CONFLICT OF INTEREST

None declared.

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