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RESEARCH ARTICLE

Sequencing of 16S rRNA Gene and Phylogenetic Analysis of *Pseudomonas aeruginosa* isolated from well water in Mosul city/Iraq.

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Abstract

Pseudomonas aeruginosa is a gram negative bacterium isolated from well water. The isolate was cultured in a MacConkey agar and nutrient agar at 37ºC for 24 hours. For species identification, Pseudomonas like organisms creates lot of problems when identified with the help of morphological and biochemical characters. However, sequencing of 16S rRNA region is a suitable technique for species identification. The amplified product of 16S rRNA was submitted to NCBI database. Amplification of 16S rRNA gene region, and new sets of primer pairs were designed by NCBI database search tool. To study phylogenetic relationship between various strains of *Pseudomonas aeruginosa* have often been based on sequencing of 16S rRNA gene region. Distance tree was constructed to find out genetic similarity between the organisms. Hence gene sequencing of 16S rRNA region was a suitable technique to identify *Pseudomonas aeruginosa* at molecular level.

Introduction:-

Water well is an artificial opening or artificially altered natural opening, however made, by which groundwater is sought, or flows under natural pressure, or is artificially withdrawn or injected. Examples would include holes drilled, bored, dug or jetted into the ground to reach water. Wells are usually held open by a pipe, well casing or a liner and can provide drinking water or can be used for non-potable uses such as irrigating and washing. A well is private or domestic if it serves water for no more than three households for drinking, culinary or household uses and is not used as a public water supply (OHA, 2015).

Pseudomonas aeruginosa is a Gram-negative bacteria belong to the family *Pseudomonaceae* , motile with a polar flagellum, non-spore forming, obligate aerobes that grows in a wide range of temperatures (10-44 °C) and the optimum thermal temperature is 35°C, widespread in nature, it is considered an opportunistic pathogen, causing acute and chronic infections in patients who are in hospital, especially in patients with burns, its grows well when cultured on simple media , It has the ability to produce two kinds of pigment green- blue (pyocyanin) and yellowish green pigment (pyoverdin)(Ochoa *et al*., 2013).

16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria in clinical microbiology laboratories. For bacterial identification, 16S rDNA sequencing is particularly important in the case of bacteria with unusual phenotypic profiles, rare bacteria, slow growing bacteria, uncultivable bacteria and culture-negative infections. Not only has it provided insights into a etiologies of infectious

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disease, but it also helps clinicians in choosing antibiotics and in determining the duration of treatment and infection control procedures. (Woo *et al*., 2008).

Material and methods:-

Pseudomonas aeruginosa was isolated from the wells water collected at Mosul city/ Iraq. It was cultured in two types of media (nutrient agar and MacConkey agar) and incubated at 37ºC for 24 hours. The isolate was identified based on the morphological and biochemical characters by using Vitek 2 compact for identification (Collins *et al*., 2009).For species identification sequencing of 16S rRNA gene region was carried out.

2.1 DNA Extraction

The extracted DNA kit from Jena Bioscience GmbH was used to isolate the DNA from *Pseudomonas aeruginosa*. After extraction of the DNA sample the DNA purity and concentration of isolate was measured by Nanodrop 2000 with a purity of 1.89 and a concentration of 730 ng / µl and the DNA sample was run on 1% agarose gel at a constant voltage of 80V. The gel was examined on UV Tran illuminator. (Pitcher *et al*., 2008)

2.2 PCR amplification and sequencing of 16S rRNA

16S rRNA gene region was amplified with the universal primers. For setting up PCR, the following reaction mixtures were added into the PCR tube. The reaction mixtures were 1µl of template, Primers: 1 µl of Forward primer- 27F (5' AGAGTTTGATCCTGGCTCAG 3'), 1 µl of Reverse primer- 1492R (5' TACCTTGTTACGACTT 3') (Yang *et al*.,2010).10 µl of assay buffer, 0.5 µl of Taq DNA polymerase (Bioline, UK), The amplification was carried out in a thermal cycler using the following reaction conditions, initial denaturation of DNA at 95ºC for 5 minutes followed by 30 amplification cycles of 95 °C for 30 sec for denaturation, primer annealing at 55 ºC for 60 seconds and primer extension at 72 ºC for 1 minute. The amplified PCR product was mixed with 5 µl of gel loading buffer. 1.5% agarose gel was casted. The samples were loaded along with 5 µl of 1kb hyperladder (Bioline, UK) as a DNA marker. The gel was run and examined on UV-trans illuminator (Syngene, UK) to visualize the bands. PCR products were purified by using the ISOLATE II PCR and Gel kit for DNA extraction from agarose gel (Bioline, UK) following the manufacturer's instructions and it was sequenced with an ABI Prism 3730XL automatic DNA sequencer the same primers as used for PCR amplification were used for this purpose.

2.3 Nucleotide sequence accession number and BLAST analysis

The nucleotide sequence 16S rRNA gene region data was submitted to NCBI nucleotide sequence database. Using BLAST tool, phylogenetic tree, primer pairs were designed from NCBI database search tool.

Results and Discussion:-

Pseudomonas aeruginosa was isolated from well water sample purely and the image (1) shows the bacteria under the microscope and image (2) shows the colonies of bacteria on nutrient and MacConkey agar.

Image (1):- gram's stain of *Pseudomonas aeruginosa* under microscope (1000X)

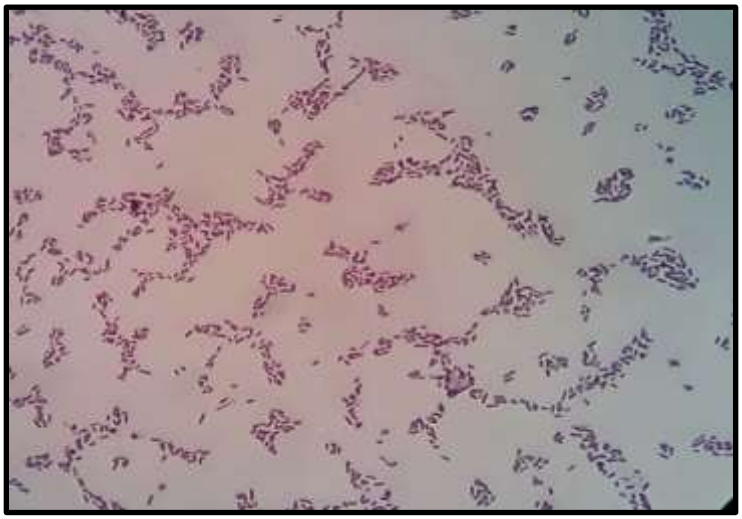

Image (2):- colonies of *Pseudomonas aeruginosa* on nutrient agar (A) and MacConkey agar (B).

The PCR technique was performed to confirm its molecular diagnosis by detecting the 16S rRNA gene by investigating the results of the DNA replication of the gene. And when the gel electrophoresis is performed for DNA screening of isolates, the results show the appearance of bands of 1500 base pairs as shown in image. 3 as S3.

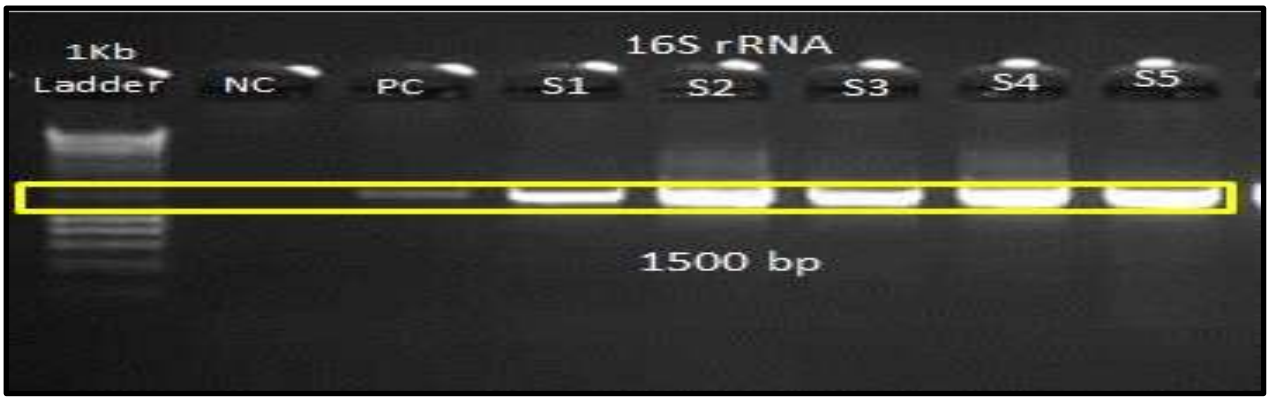

Image (3):- bands of 16S rRNA gene (S1-S5) the bands from the left indicate hyperladder, negative control (NC) and positive control (PC, *S. coelicolor* M145) and (S3) represent *Pseudomonas aeruginosa*.

The sequence was submitted to NCBI database and the accession number is BAMA0100031. By using BLAST analysis, which showed a high similarity of isolation with 100% which confirmed the biochemical diagnosis of isolate and the table (1) shows the rate of convergence in the diagnosis with the nearest 15 reference isolates, based on identity.

Table (1):- ratios of convergence of genetic diagnosis of *Pseudomonas aeruginosa* with reference isolates

No.	Top-hit taxon name	Top-hit strain	Similarity (%)	Accession No.
1	*P. aeruginosa*	JCM 5962	100	BAMA0100031
2	*P. otitidis*	MCC10330	99.47	AY953147
3	*P. alcaligenes*	NBRC14159	99.05	BATI0100007
4	*P. resinovorans*	LMG 2274	98.40	Z76668
5	*P. mendocina*	NBRC 14162	98.21	BBQC0100001
6	*P. anguilliseptica*	NCIMB 1949	98.10	X99540
7	*P. guguanensis*	CC-G9A	97.89	JQ864237
8	*P. composti*	CCUG 59231	97.89	FOWP0100002
9	*P. alcaliphila*	JCM 10630	97.89	FNAE01000025
10	*P. peli*	R-20805	97.89	AM114534

11	*P. toyotomiensis*	HT-3	97.89	AB453701
12	*P. indoloxydans*	IPL-1	97.89	DQ916277
13	*P. oleovorans*	RS1	97.89	DQ842018
14	*P. chengduensis*	MBR	97.78	EU307111
15	*P. glareae*	KMM 9500	97.68	LC011944

The results were consistent with those of Kim *et al* (2014) when isolates were used from clinical specimens. Dependence on two pairs of primer the first was universal primer and the second was a specific type of PCR primer and they proved the results were 100% consistent Which confirms the accuracy of diagnosis using the universal primer in giving accurate results at a very high rate compared to the specific PCR primer.

The results were also consistent with the results of the researchers Amutha and Kokila (2014), isolating the bacteria from the soil and it was about the sequence of the whole genome of the bacteria genetically close to our isolate , confirming the stability of bacterial genome sequences by geographical location.

The results were consistent with the results of Jaysree *et al* (2015). They isolated the bacteria from various environmental sources such as soil, water and salt pans and proved that the changing of environmental conditions ,Salt concentration does not affect the bacterial gene sequence, This supports that the genome of the bacteria is not affected by the changing circumstances surrounding the bacteria.

Whiteley *et al* (2001) have shown that free-living bacteria, which contribute to the formation of biofilm, have the same DNA and express the same genes, and there is little change in the exposure of free-living bacteria to the antibiotics that are resistant to those found in the biofilm Prove that their gene expression is similar.

Alignment of the nitrogen bases of isolation with the nearest isolates was done as shown in Fig. (3). the genetic tree of isolation was also performed using the Molecular Evolutionary Genetics Analysis (MEGA6) using the algorithm and the technique of the neighbor joining as shown in Fig. (4).

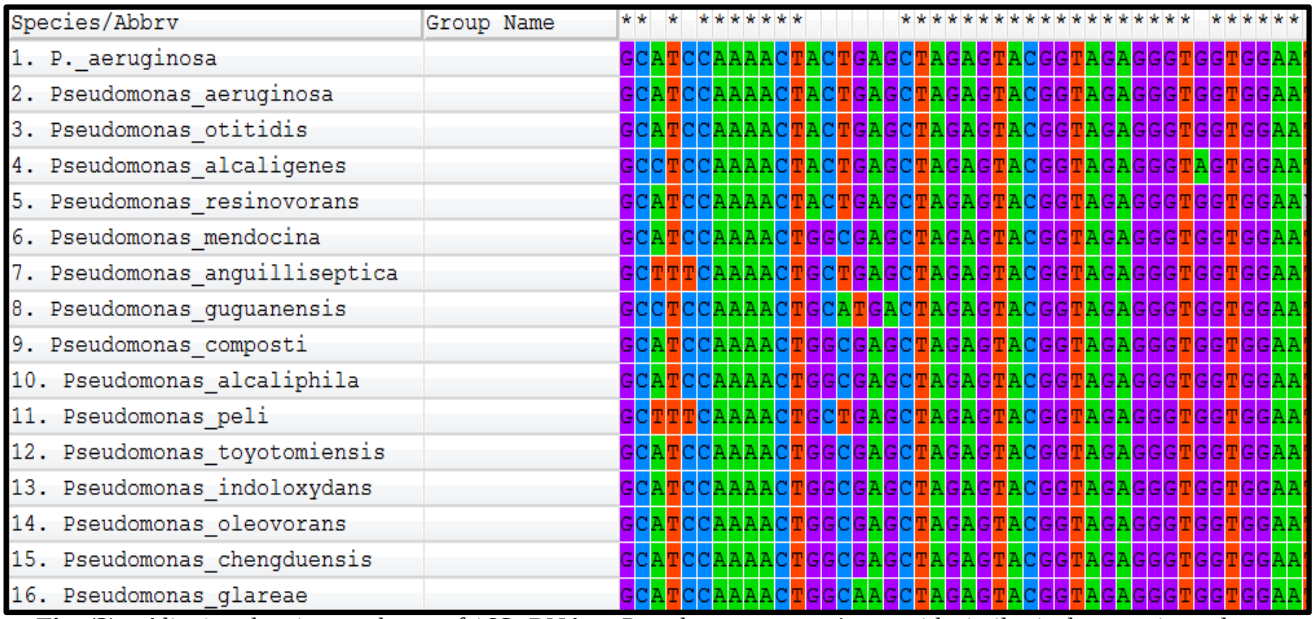

Fig. (3):- Aligning the nitrogen bases of 16S rRNA to *Pseudomonas aeruginosa* with similar isolates registered at NCBI-Genbank.

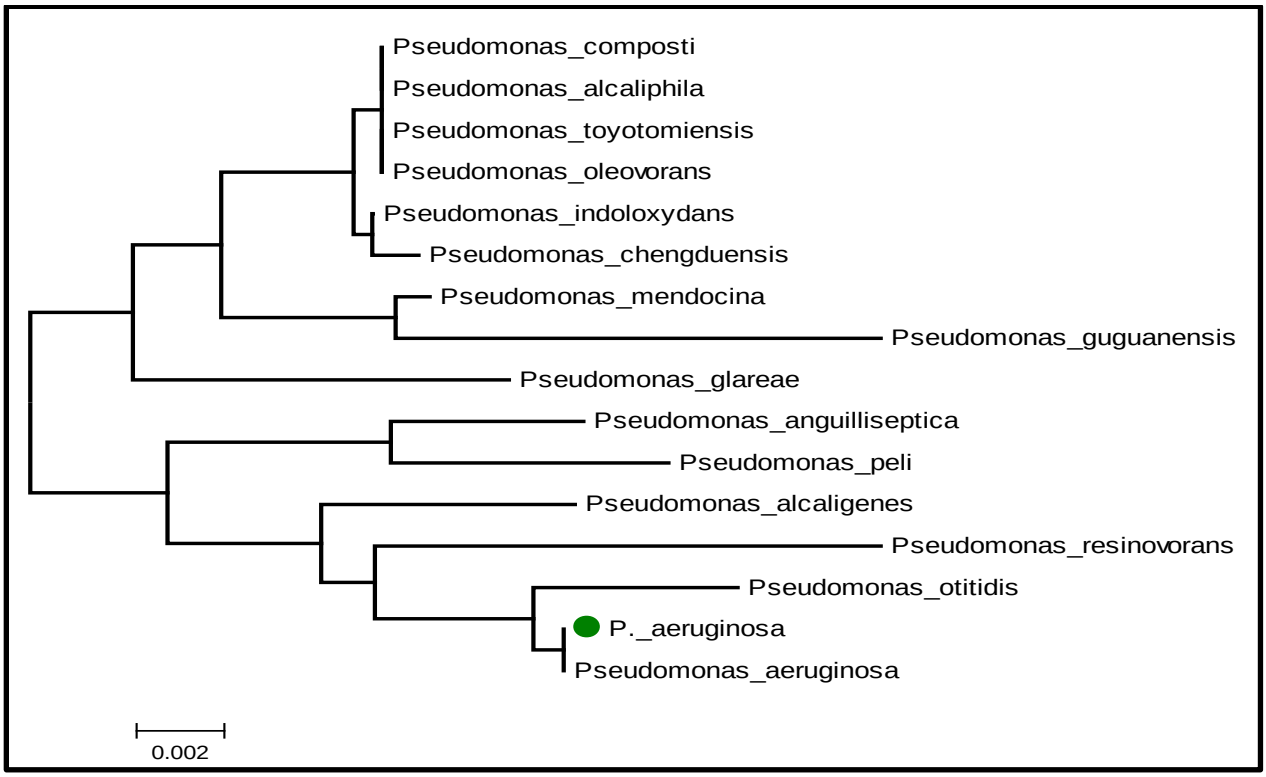

Fig.(4):- The genetic tree of the identified *Pseudomonas aeruginosa* with the bacteria that have been adopted by NCBI.

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