DNA sequencing and phylogeny of *Pseudomonas aeruginosa* isolated from nosocomial infections' in Iraq

Abstract

PCR product of *16SrRNA* gene was used for partial sequencing, and registration in gene bank-NCBI and phylogeny. Ten accession numbers were obtained from registration of ten sequences of*16srRNA* gene bank-NCBI: The accession numbers were: KX963356.1, KX963357.1, KX963358.1, KX963359.1, KX963360.1, KX963361.1, KX963362.1, KX963363.1, KX963364.1 and KX963365.1.

The phylogenetic analysis of local isolates of *P. aeruginosa* showed a close related to NCBI-BLAST *P. aeruginosa* strain(KR81540.1) except the Iraq isolates 2 (burns source) and Iraq isolates 8 from (ear source) which showed genetically differences as unique isolates.

Based on the update local literatures, this is the first study in Iraq which employed sequencing, registration of sequences in gene bank-NCBI, and carrying out phylogeny of local, clinical isolates and world strains of *P. aeruginosa*, in addition to that, a new records of two strains of local isolates from ear (KX963362.1) and burns (KX963357.1) are established in Gene bank locus.

introduction

Pseudomonas aeruginosa is a Gram's negative opportunistic pathogen has emerged as one of the most problematic of the nosocomial pathogens; it is considered Multi-resistant infections in both communal and hospital settings. It is an opportunistic pathogen that causes infections in immunocompromised, cancer, burn, urinary

tract, surgical wound, eye, blood, ear infection, sepsis cystic fibrosis, and intensive care unit (Tille, 2014).

DNA sequencing is the process of determining the precise order of nucleotides within a DNA molecule. It includes any method or technology that is used to determine the order of the four bases—adenine, guanine, cytosine, and thymine—in a strand of DNA. The advent of rapid DNA sequencing methods has greatly accelerated biological and medical research and discovery. Knowledge of DNA sequences has become indispensable for basic biological research, And in numerous applied fields such as diagnostic, biotechnology, forensic biology, and biological systematics. (Clyde, 2007).

The rapid speed of sequencing attained with modern DNA sequencing technology has been instrumental in the sequencing of complete DNA sequences, or genomes of numerous types and species of life, including the human genome and other complete DNA sequences of many animal, plant, and microbial species **(**Pettersson *et al*., 2009**)**. Fifteen years elapsed between the discovery of the DNA double helix in 1953 and the first experimental determination of a DNA sequence (Reinert and Huson, 2007).

Whole genome sequencing, facilitated by the Advent of high-through put approaches, brings the promise of single-base-pair resolution between isolates, making it the ultimate molecular typing method for bacteria. Several recent studies have shown that analysis of single nucleotide polymorphisms (SNPs) in bacterial genomes provides a means of determining relatedness between epidemiologically linked isolates and tracking bacterial evolution over periods of months to years(Eppinger *et al*., 2011).

Snyder *et al*., 2013 Studied of epidemiological investigation of *P. aeruginosa* isolate from a six-year-long hospital outbreak using

high –through put whole genom sequencing, In that study they demonstrated that the single base resolution of whole genome sequencing is a powerful tool in analysis of outbreak isolates that can not only show strain similarity, but also evolution over time and potential adaptation through gene sequence change.

In Iraq, there are several studies were conducted during the last five years on phenotypic and genotypic characterization of *P. aeruginosa* (Abdullah, 2012; Fadhel, 2013; Al Doory,2012; AL-Obaidi, 2013), but the present study focused on the causes of the continuous antibiotic resistance by *P. aeruginosa* that prevalent in hospitals especially that caused the nosocomial or hospitalized infections based on the identify the genetic variation between the isolates collected from different clinical samples by using the DNA sequencing and phylogeny analysis.

The study was aimed to identify some microbial factors (phenotypic and genotypic) that support the antibiotic resistance and prevalence of *Pseudomonas aeruginosa* that causes hospitalized infection. **2.2.**

Method

Five hundred specimens from urine, wounds, burns, sputum, ear swab and diabetic foot were collected from inpatients of many hospitals and Central Public Health Laboratory during November, 2015 to April, 2016 in Baghdad city. All Specimens were collected by clean sterilized cotton swabs or containers under supervision of a clinical consultant physicians. The time between samples collection and bacteriological exam never exceeded 1-2 hours. The swabs were primary cultivated on suitable incubation at 37C° for 18-24 hour under aerobic condition. Positive culture media represented by Blood agar and MacConkey agar, then culture samples re-cultured on selective media besides performing morphological characteristic, biochemical tests followed by confirmative diagnostic methods (phenotypic and genotypic method).

. Isolating Genomic DNA from *P. aeruginosa*

The following procedure was done according to the instructions of manufacturing company:

- A volume 1ml of an overnight culture was added to a 1.5ml microcentrifuge tube, Centrifuged at 16,000 × *g* for 2 minutes to pellet the cells and Removed the supernatant.
- A volum 600µl of Nuclei Lysis Solution was added. Gently pipet until the cells are suspended, Incubated at 80°C for 5 minutes to lyse the cells and then cooled to room temperature.
- A volum 3µl of RNase solution was added to the cell lysate. Invert the tube 2–5 times to mix. Incubated at 37°C for 30 minutes. Cooled the sample to room temperature, A volume 200µl of Protein Precipitation Solution was added to the RNase-treated cell lysate Vortex vigorously at high speed for 20 seconds to mixed the Protein Precipitation Solution with the cell lysate.
- The sample was Incubated on ice for 5 minutes, Centrifuged at 13,000–16,000 × *g* for 3 minutes and then transfered the supernatant containing the DNA to a clean 1.5ml microcentrifuge tube which contained on 600µl of isopropanol at room temperature then Gently mixed by inversion until the thread-like strands of DNA form a visible Mass then Centrifuge at 13,000–16,000 × *g* for 2 minutes.
- Carefully the supernatant was poured off and drained the tube on clean absorbent paper, A volum 600µl of room temperature 70% ethanol was added and gently inverted the tube several times to wash the DNA pellet then Centrifuged at 13,000–16,000 × *g* for 2 minutes and Drained the tube on clean absorbent paper and allow the pellet to air-dry for10–15 minutes.
- A, volume 100µl of DNA rehydration solution was added to the tube and rehydrate the DNA by incubating at 65°C for 1 hour. Was

periodically mixed the solution by gently tapping the tube. Alternatively, rehydrate the DNA by incubating the solution overnight at room temperature or at 4°C and then stored the DNA at 2–8°C.

2.2.11.2. Isolation of Plasmid DNA from *p. aeruginosa*

The following procedure was performed at room temperature, to instructions according of Manufactory Company:

- A total of 600µl was transferred of bacterial culture grown in Lauria bertani broth medium to a 1.5ml micro centrifuge tube. A 100µl of Cell Lysis Buffer was added, and mixed by inverting the tube 6 times. The solutions should change from opaque to lysis clear blue, indicating complete

- A ,volum 350µl of cold (4–8°C) neutralization solution was used and mixed thoroughly by inverting the tube the sample will turn yellow when neutralization is completed, and will form a yellow precipitate, inverted the sample an additional 3 times to ensure completed neutralization, Centrifuged at maximum speed in a microcentrifuge for 3 minutes andTransfered the supernatant (~900µl) to a PureYield™ Minicolumn.Do not disturb the cell debris pellet. For maximum yield, transfer the supernatant with a pipette.

- The minicolumn was placed into a PureYield™ Collection tube, and centrifuged at maximum speed in a microcentrifuge for 15 seconds then discarded the flowthrough, and placed the minicolumn into the same PureYield™ Collection Tube and A volume 200µl of endotoxin removal washed to the minicolumn centrifuged at maximum speed in a microcentrifuge for 15 seconds. It is not necessary to empty the PureYield™ Collection Tube.

- A, volume of 400µl of column wash solution was added to the minicolumn, centrifuged at maximum speed in a micro centrifuge for

30 seconds then transferred the minicolumn to a clean 1.5ml micro centrifuge tube, then added30µl of Elution buffer directly to the minicolumn matrix, let stand for1 minute at room temperature. And then Centrifuged at maximum speed in a micro centrifuge for 15 seconds to elute the plasmid DNA. sealed the micro centrifuge tube, and stored eluted plasmid DNA at –20°C

2.2.12. Estimation of DNA yield and purity

The extracted genomic DNA was checked by using Nano drop spectrophotometer to estimate the concentration and extracted purity of DNA through reading the absorbance in at (260 /280 nm).

2.2.13. Detection of specific genes for *P. aeruginosa* by PCR method.

2.2.13.1. Preparing the Primers:

Oligonucleotide primers were prepared depending on manufacturer's instruction by dissolving the lyophilized product with Nuclease - free water after rotating down briefly. Working primer tube was prepared by diluting with Nuclease - free water. The final picomoles depended on the procedure of each primer.

Table (2-13): PCR amplification program of *16SrRNA* gene used for confirmatory identification of *P. aeruginosa.*

Stage		Steps	Temperature (Cº)	Time	No. of cycles
First		Initial Denaturation	95ºC	120sec	1
Second	I	Denaturation	95ºC	20sec	25
	II	Annealing	58ºC	20sec	
	III	Extension	72ºC	40sec	
Third		Final Extension	72ºC	60sec	1

Table (2-14): PCR amplification program of *16SrRNA* gene used for DNA sequencing in *P. aeruginosa.*

Stage		Steps	Temperature (C˚)	Time (sec)	No. of cycles
First		Initial Denaturation	95˚C	4min	1
Second	I	Denaturation	95˚C	30sec	30
	II	Annealing	55˚C	30sec	
	III	Extension	72˚C	90sec	
Third		Final Extension	72˚C	9min	1

2.2.14. Agarose gel electrophoresis:

This agarose gel was prepared by dissolving 1 g of agarose powder in 100 ml of (1X) TBE buffer (pH 8) on hot plate with magnetic stirrer and magnetic capsule was added, left until boiling and becoming clear, allowed to cool to 50˚C, and 5 µl Ethidium Bromide was added(Sambrook and Russell, 2001).

2.2.14.1. Casting of the horizontal Agarose gel:

The tape was placed across the end of the gel tray. The comb was fixed at one end of the tray for making wells used for loading DNA samples. The agarose was poured gently into the tray, and allowed to solidify at room temperature for 30 minutes. Then, the comb was removed gently from the tray and the tape was also removed from the

ends of the tray. The agarose gel was fixed in electrophoresis chamber which was filled with TBE buffer (1X) that had covered the surface of the gel (Sambrook and Russell, 2001).

2.2.14.2. Loading and running DNA in agarose gel:

Five µl of each DNA template was transferred to eppendorf tube, 3µl of loading dye was added to the tube and the mixture was loaded into the wells in agarose gel with the addition of loading buffer and DNA Ladder (100bp) as standard in electrophoresis. The electric current was allowed at 100 volt for 10mintes and then 80 volt for 1 hour to detected the 16srRNA gene (956 base pairs amplicon), DNA extraction and plasmid extraction while used90 min at 100 volt/50 mAmp to detected, 16srRNA gene(1504base pairs amplicon) and exotoxine A. After complete time read the reselts on UV transilluminater was used for the observation of DNA bands, and the gel was photographed.

2.2.1.4.6. Analysis of PCR results:

PCR product was analyzed by gel electrophoresis in 1-2% agarose gels containing Ethidium Bromide Staining Solution. 5 µl from amplified sample was directly loaded in agarose gel with the addition of loading buffer and DNA Ladder (100 base pairs) as standard in electrophoresis and the gel was run at 100 Volt for10 mintes and 80 volt for 1hour. The products were visualized with UV illuminator and photographed.

2.2.15. DNA sequencing:

DNA sequencing method was performed for Phylogenetic tree analysis study of local *P. aeruginosa* isolates based on *16srRNA* gene1504bp. DNA capillary sequencing was performed by using their

ABI 3730xl genetic analyzer (Applied Bio systems, US), (Larkin *etal*., 2013).

As the following steps:

1. The specific PCR product was excised from the gel by clean, sharp scalpel. Then, transferred into a 1.5mL micro centrifuge tube.
2. 400µl Binding Buffer II was added to gel fragment. Then, incubated at 60ºC for 10 minutes and shaked until the agarose gel is completely dissolved.
3. Add the above mixture to the EZ-10 column and let stand for 2 minutes. Centrifuge at 10,000rpm for 2 minutes and discard the flow-through in the tube.
4. 750µl Wash Solution was added to each tube and centrifuged at 10000rpm for one minute. Then, solution discarded.
5. After that, the step 4 was repeated. Then, centrifuged at 10000rpm for an additional minute to remove any residual wash Buffer.
6. The column was placed in a clean 1.5ml micro centirfuge tube and added 30µl of Elution Buffer to the center of the column and incubated at room temperature for 2 minutes. Then, the tube was centrifuged at 10000rpm for 2 minutes to elute PCR product and store at -20ºC. (Tamura *etal*., 2013)

Confirmative Detection of *P. aeruginosa* isolates by conventional PCR by using *16SrRNA* gene.

3.1.2.3.1. Extraction chromosomal DNA

The DNA from 60 isolates of *P.aeruginosa* was extracted and purified using genomic DNA kit. The results were detected by gel electrophoresis process using 1%agarose gel and then examined under UV. Light in which the DNA appeared as clear compact band in Figure (3-4).

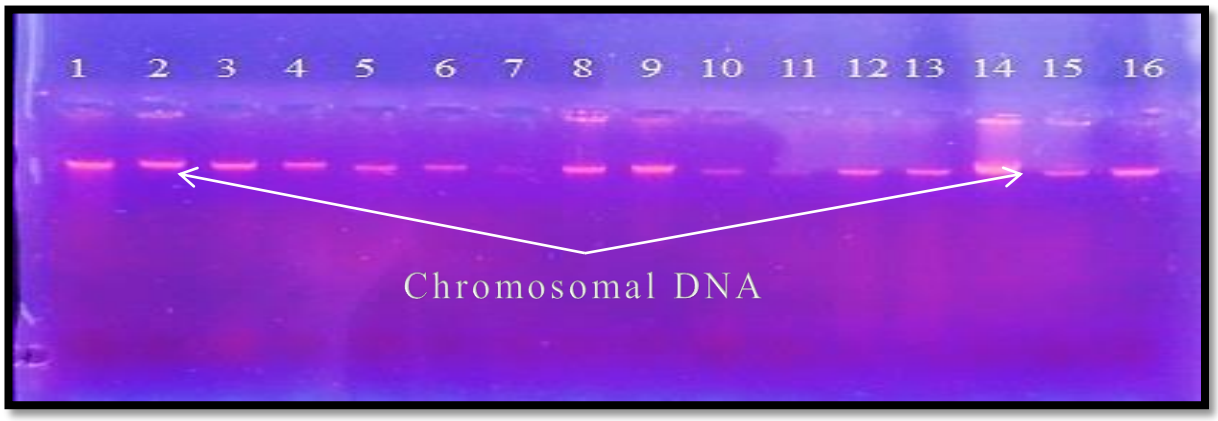


Figure (3-4): Ethidium bromide stained Agarose gel electrophoresis of extracted DNA from *P. aeruginosa* isolates using 1% Agarose, 90 min at 100 volt/50 mAmp.

3.1.2.3.2. Total DNA concentration and purity

Total **DNA** concentrations and purity that extracted from different clinical samples were measured by Nano drop spectrophotometer. Results (Mean) concentrations exhibited as ng/µl. The purity of the extracted **DNA** was estimated by measuring the ratio of A_{260}/A_{280}. It gave an optimal concentration of DNA for amplification process ranged from 35.3-39.8 ng/µl. While optimal purity amplification process ranged from 1.6-2.01 ng/µl. (Table 3-3).

Clinical samples	No. of isolates	Concentration of DNA (ng/µl) Mean	Purity A_{260}/A_{280} Mean
Diabetic foot	2	38.35	1.8

Sputum	8	35.3	1.8
Ear	13	38.5	2.01
Urines	5	35.6	1.6
Burns	16	38.4	1.96
Wounds	16	39.8	2.0

Table 3.3: Values of extracted DNA concentration and purity of selected sample of Extracted DNA.

Based on the standard values of DNA concentration for amplification, the values of the present study are considered an efficient values and suitable for the establishment of the DNA extracted with target primers or sequences amplification.

Sequencing and phylogeny analysis of *16SrRNA* gene

The sequencing *16srRNA* gene based on the PCR product of the nucleotide sequence with 1054 base pairs (fig.3-13) of 10 isolates *P. aeruginosa* were identify the genetic variation and phylogeny of these isolates after compared with their accession numbers and their definition of another global sequence data of two standard strains in NCBI-BLAST the 10 sequences were recognized as *P. aeruginosa* as follow with their accession number and their definition (fig3-13 to fig 3-22).

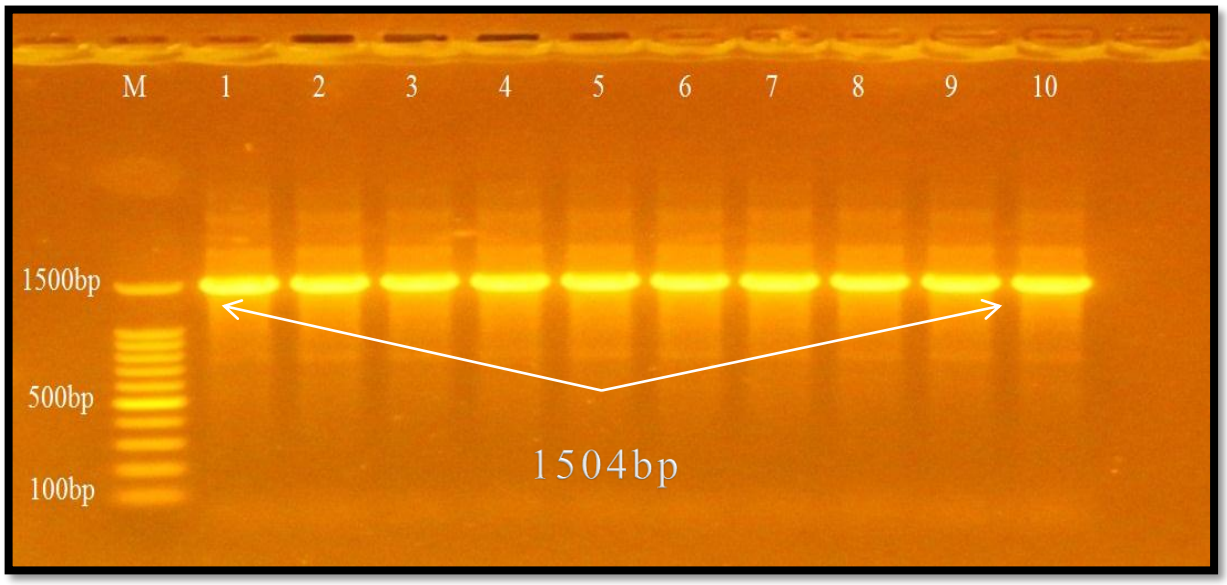


Figure (3-13): Ethidium bromide stained gel electrophoresis of PCR product~1504base pairs of *P. aeruginosa* isolates using (1%) Agarose for 90 minutes at 100 volt positive PCR product~1504b p size Lane (M) DNA marker 100bp. Lanes (1-10) show positive result to*16SrRNA* gene.

Sequencing1: Results of 16S *Ribosomal* RNA gene, partial sequences forward of *P. aeruginosa* isolates from Diabetic foot, showed the identities percent 74% with world strains when examined in Gene bank of NCBI (fig.3-12).

Sequencing2: Results of 16S *Ribosomal* RNA gene, partial sequences forward of *P. aeruginosa* isolates from burns, showed the identities percent 77% with world strains when examined in Gene bank of NCBI (fig.3-).

Sequencing 3: The results of 16S *Ribosomal* RNA gene, partial sequences forward of *P. aeruginosa* isolates from urine, showed the identities percent 99% with world strains when examined in gene bank of NCBI in (fig.3-).

Sequencing4: The results of *16S Ribosomal* RNA gene, partial sequences forward, *P. aeruginosa* isolates from sputum, showed the

identities percent 95% with world strains when examined in Gene bank of NCBI (fig.3.19).

Sequencing 5: The results of *16S Ribosomal* RNA gene, partial sequences forward, *P. aeruginosa* isolates burns, showed the identities percent 99% with world strains when examined in Gene bank of NCBI fig 3-20.

Sequencing 6: Results of *16S Ribosomal* RNA gene, partial sequences forward, *P. aeruginosa* isolates sputum, showed the identities percent 99%with world strains when examined Gene bank of NCBI (fig 3-21).

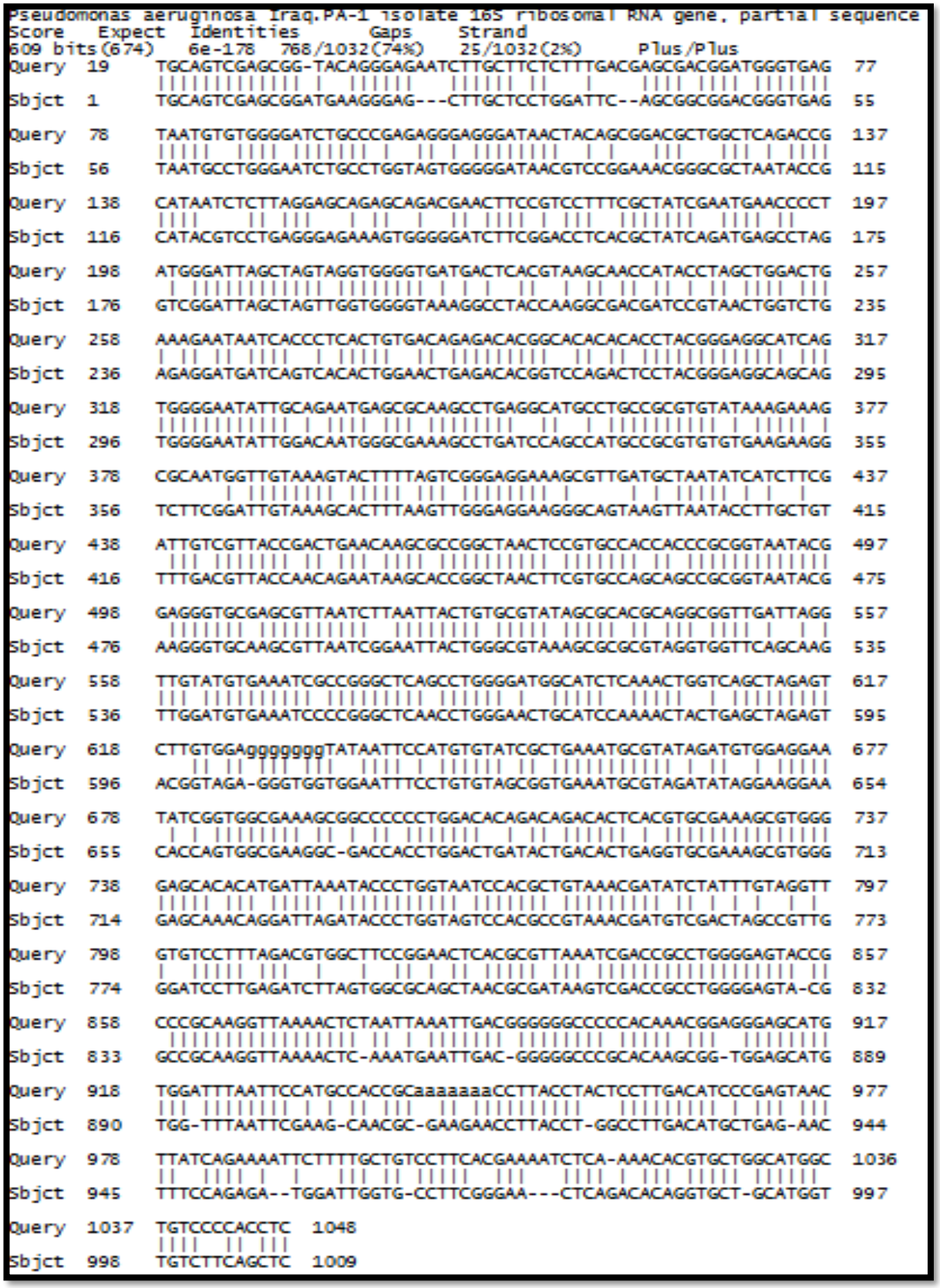
Sequencing7: The results of *16S Ribosomal* RNA gene, partial sequences forward, *P. aeruginosa* isolates wound showed the identities percent 81% with world strains when examined in Gene bank of NCBI (fig.3-22)**.**

Sequencing 8: The results of *16S Ribosomal* RNA gene, partial sequences forward, *P. aeruginosa* isolates ear, showed the identities percent 89% with world strains when examined in Gene bank of NCBI (fig.3-23).

Sequencing 9: Results of *16S Ribosomal* RNA gene, partial sequences forward, *P. aeruginosa* isolates wound, showed the identities percent 99% with world strains when examined in Gene bank of NCBI fig. (3-24).

Sequencing 10: The results of *16S Ribosomal* RNA gene, partial sequences forward, *P. aeruginosa* isolates wound showed the

identities percent 98% with world strains when examined in Gene bank of NCBI (fig.3-25)**.**



```
Pseudomonas aeruginosa Iraq.PA-1 isolate 16S ribosomal RNA gene, partial sequence
Score    Expect   Identities      Gaps     Strand
609 bits(674)    6e-178   768/1032(74%)    25/1032(2%)     Plus/Plus
Query  19    TGCAGTCGAGCGG-TACAGGGAGAATCTTGCTTCTCTTTGACGAGCGACGGATGGGTGAG  77
             ||||||||||||| | ||||||   |||||| ||    |    |||| |||| |||||||
Sbjct  1     TGCAGTCGAGCGGATGAAGGGAG---CTTGCTCCTGGATTC--AGCGGCGGACGGGTGAG  55

Query  78    TAATGTGTGGGGATCTGCCCGAGAGGGAGGGATAACTACAGCGGACGCTGGCTCAGACCG  137
              |||||  |||| ||||||| |  || | ||||||||  | |   |||   ||| | ||||
Sbjct  56    TAATGCCTGGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCG  115

Query  138   CATAATCTCTTAGGAGCAGAGCAGACGAACTTCCGTCCTTTCGCTATCGAATGAACCCCT  197
              ||||     || |||    | ||   |  || |||| | |||  ||||||||  |||| ||
Sbjct  116   CATACGTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAG  175

Query  198   ATGGGATTAGCTAGTAGGTGGGGTGATGACTCACGTAAGCAACCATACCTAGCTGGACTG  257
               | ||||||||||| |||||||| | | |   ||  | || || || | || ||||  |||
Sbjct  176   GTCGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTG  235

Query  258   AAAGAATAATCACCCTCACTGTGACAGAGACACGGCACACACACCTACGGGAGGCATCAG  317
              | || || ||||   | |||||  || |||||||||  || || ||||||||||||| |||
Sbjct  236   AGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAG  295

Query  318   TGGGGAATATTGCACAATGAGCGCAAGCCTGAGGCATGCCTGCCGCGTGTATAAAGAAAG  377
              ||||||||||| | |||| ||| ||||||||  ||   | |||||||||| | |||||  |
Sbjct  296   TGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG  355

Query  378   CGCAATGGTTGTAAAGTACTTTTAGTCGGGAGGAAAGCGTTGATGCTAATATCATCTTCG  437
                 | |||||||| ||||| ||| |||||||| |     | | |||||  | |   |
Sbjct  356   TCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGT  415

Query  438   ATTGTCGTTACCGACTGAACAAGCGCCGGCTAACTCCGTGCCACCACCCGCGGTAATACG  497
               ||| ||||||| || ||| ||||| |||||||||| ||||||| || ||||||||||||
Sbjct  416   TTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACG  475

Query  498   GAGGGTGCGAGCGTTAATCTTAATTACTGTGCGTATAGCGCACGCAGGCGGTTGATTAGG  557
               ||||||| ||||||||||  ||||||||| ||||| ||||| || ||| |||| |   | |
Sbjct  476   AAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAG  535

Query  558   TTGTATGTGAAATCGCCGGGCTCAGCCTGGGGATGGCATCTCAAACTGGTCAGCTAGAGT  617
              ||| |||||||||| |||||||| |||||| |  |||||  |||||  | |||||||||
Sbjct  536   TTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGT  595

Query  618   CTTGTGGAgggggggTATAATTCCATGTGTATCGCTGAAATGCGTATAGATGTGGAGGAA  677
               || || ||| |||  |||| | |||||| || |||||||||| | ||  | |||||
Sbjct  596   ACGGTAGA-GGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAA  654

Query  678   TATCGGTGGCGAAAGCGGCCCCCCTGGACACAGACAGACACTCACGTGCGAAAGCGTGGG  737
               | | |||||||| || | || ||||||||  | || |||||| | ||||||||||||||||
Sbjct  655   CACCAGTGGCGAAGGC-GACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGG  713

Query  738   GAGCACACATGATTAAATACCCTGGTAATCCACGCTGTAAACGATATCTATTTGTAGGTT  797
              ||||| ||| ||||| |||||||||||| ||||||| ||||||||| || | | |   | |
Sbjct  714   GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTG  773

Query  798   GTGTCCTTTAGACGTGGCTTCCGGAACTCACGCGTTAAATCGACCGCCTGGGGAGTACCG  857
              |  ||||| ||| |   |  || | || ||||| ||| ||||||||||||||||| ||
Sbjct  774   GGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTA-CG  832

Query  858   CCCGCAAGGTTAAAACTCTAATTAAATTGACGGGGGGCCCCCACAAACGGAGGGAGCATG  917
               |||||||||||||||| || | |||||||| |||||||| ||||| |||  |||||||||
Sbjct  833   GCCGCAAGGTTAAAACTC-AAATGAATTGAC-GGGGGCCCGCACAAGCGG-TGGAGCATG  889

Query  918   TGGATTTAATTCCATGCCACCGCaaaaaaaCCTTACCTACTCCTTGACATCCCGAGTAAC  977
              ||| |||||||| | | || |||  || ||||||||||   |||||||| | ||| |||
Sbjct  890   TGG-TTTAATTCGAAG-CAACGC-GAAGAACCTTACCT-GGCCTTGACATGCTGAG-AAC  944

Query  978   TTATCAGAAAATTCTTTTGCTGTCCTTCACGAAAATCTCA-AAACACGTGCTGGCATGGC  1036
              ||  |||| |  |   ||| || |||||  |||   |||| | ||| ||||| ||||||
Sbjct  945   TTTCCAGAGA--TGGATTGGTG-CCTTCGGGAA---CTCAGACACAGGTGCT-GCATGGT  997

Query  1037  TGTCCCCACCTC  1048
              ||||  || |||
Sbjct  998   TGTCTTCAGCTC  1009
```

Fig. (3-13): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from diabetic foot.

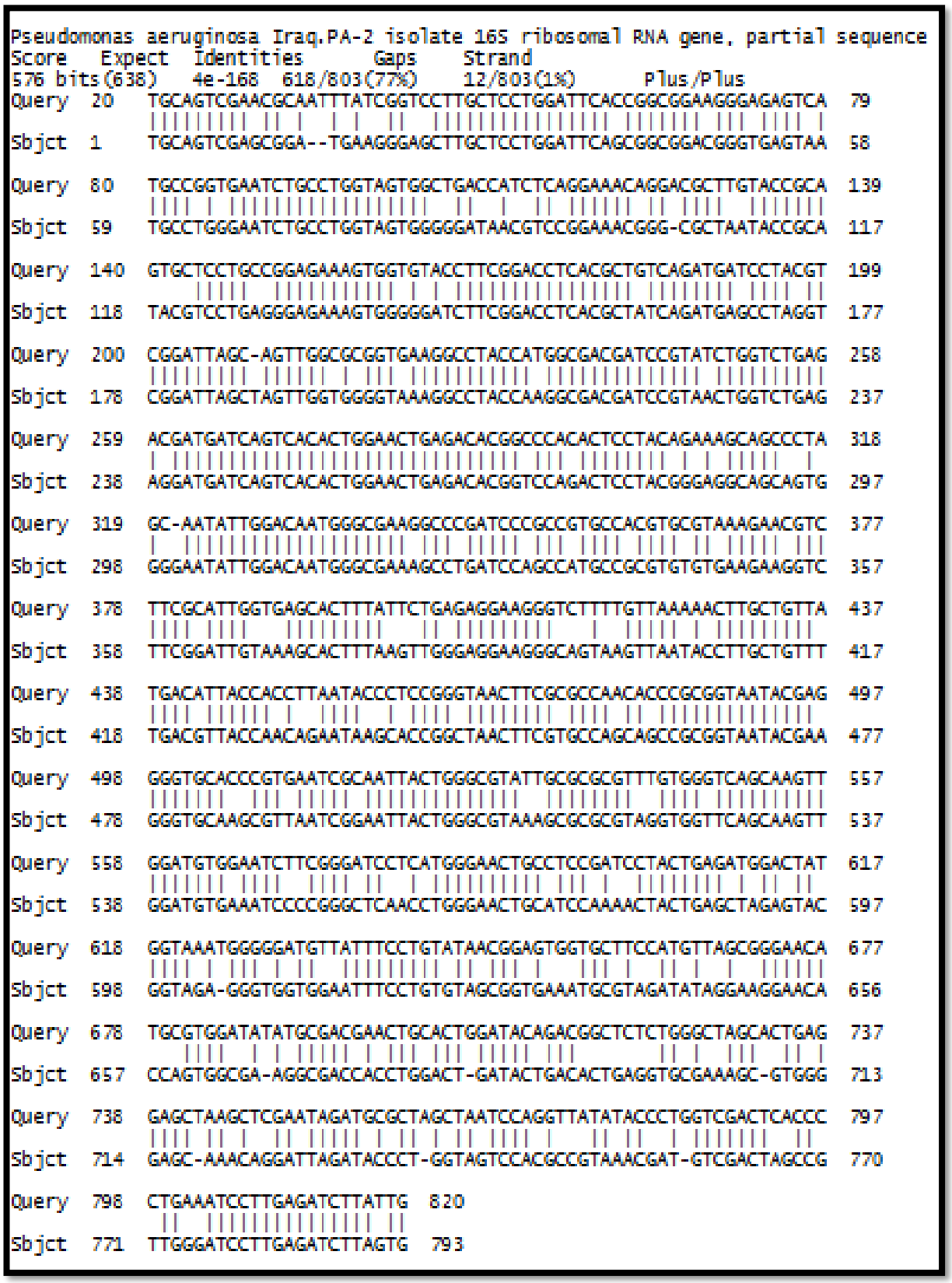


Fig. (3-14): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from burns.

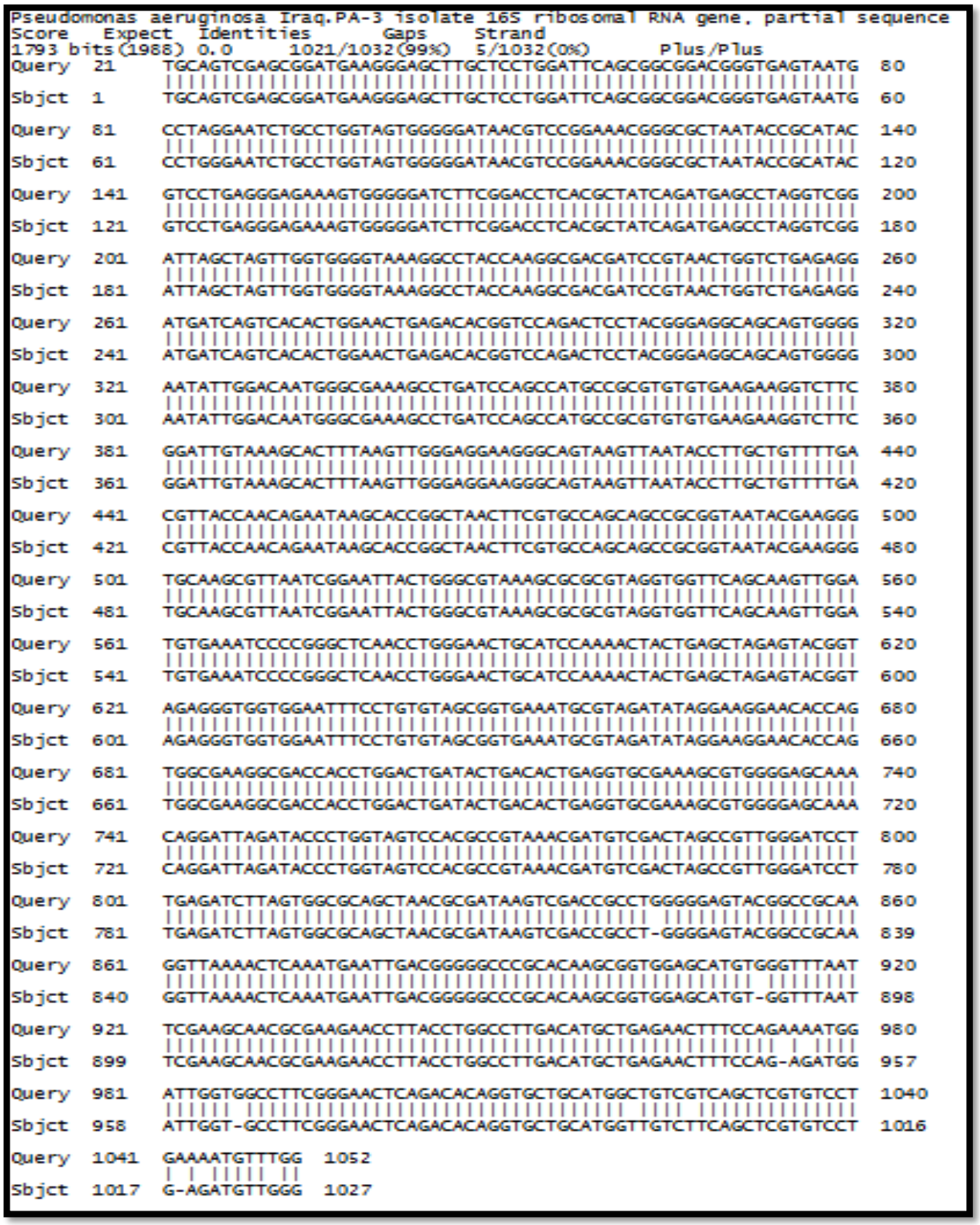


Fig. (3-15): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from urine.

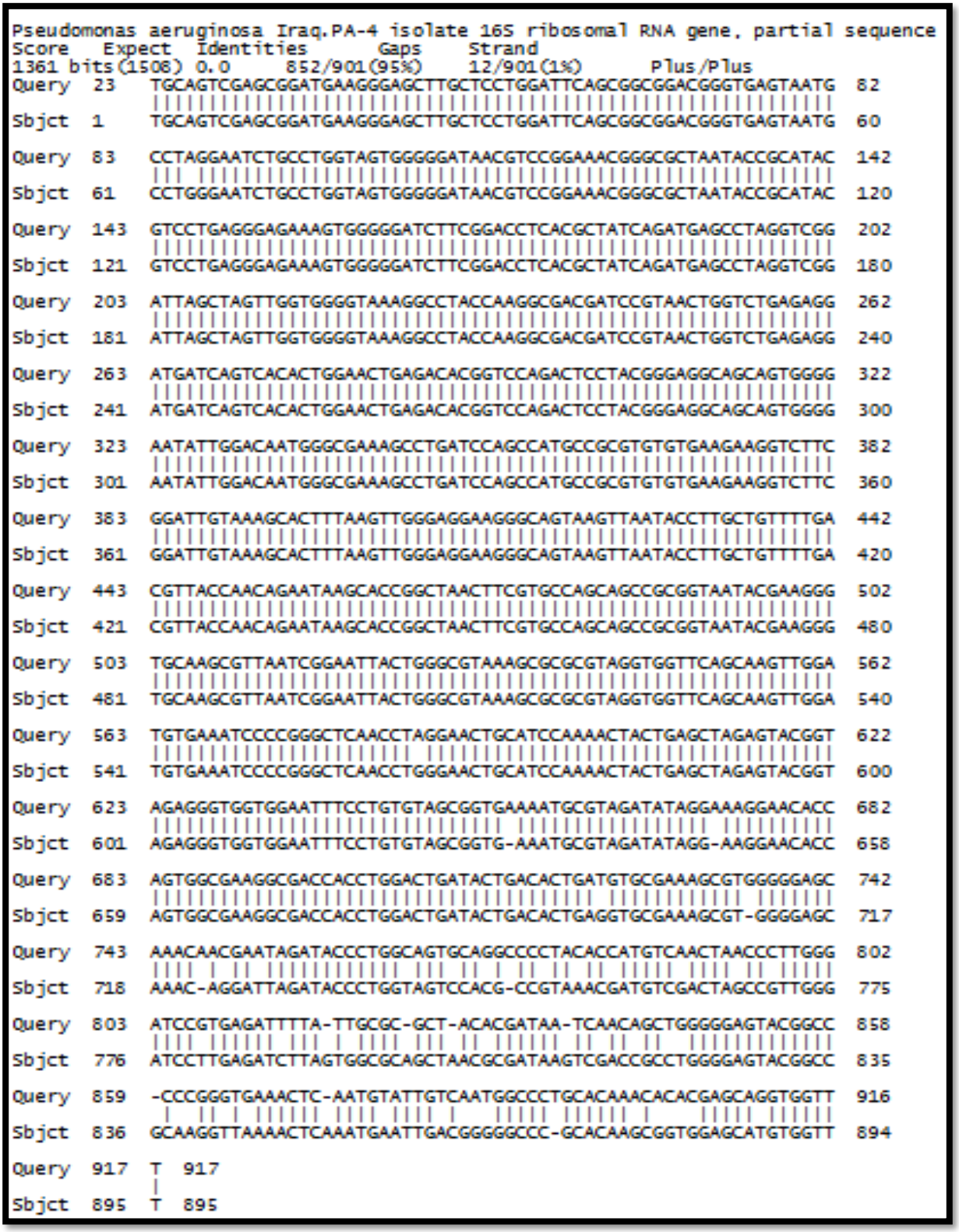


Fig. (3-16): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from sputum.

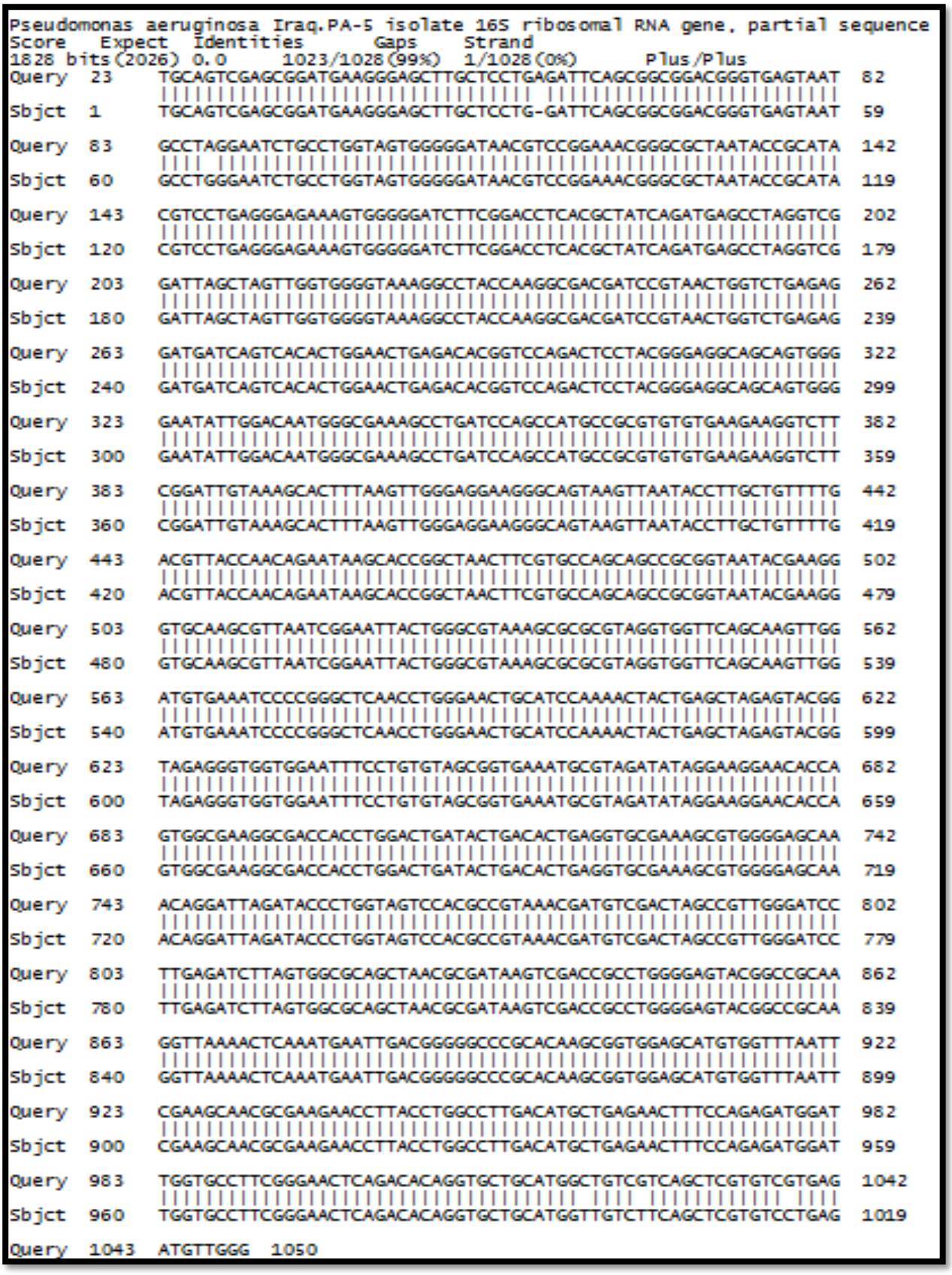


Fig. (3-17): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from sputum.

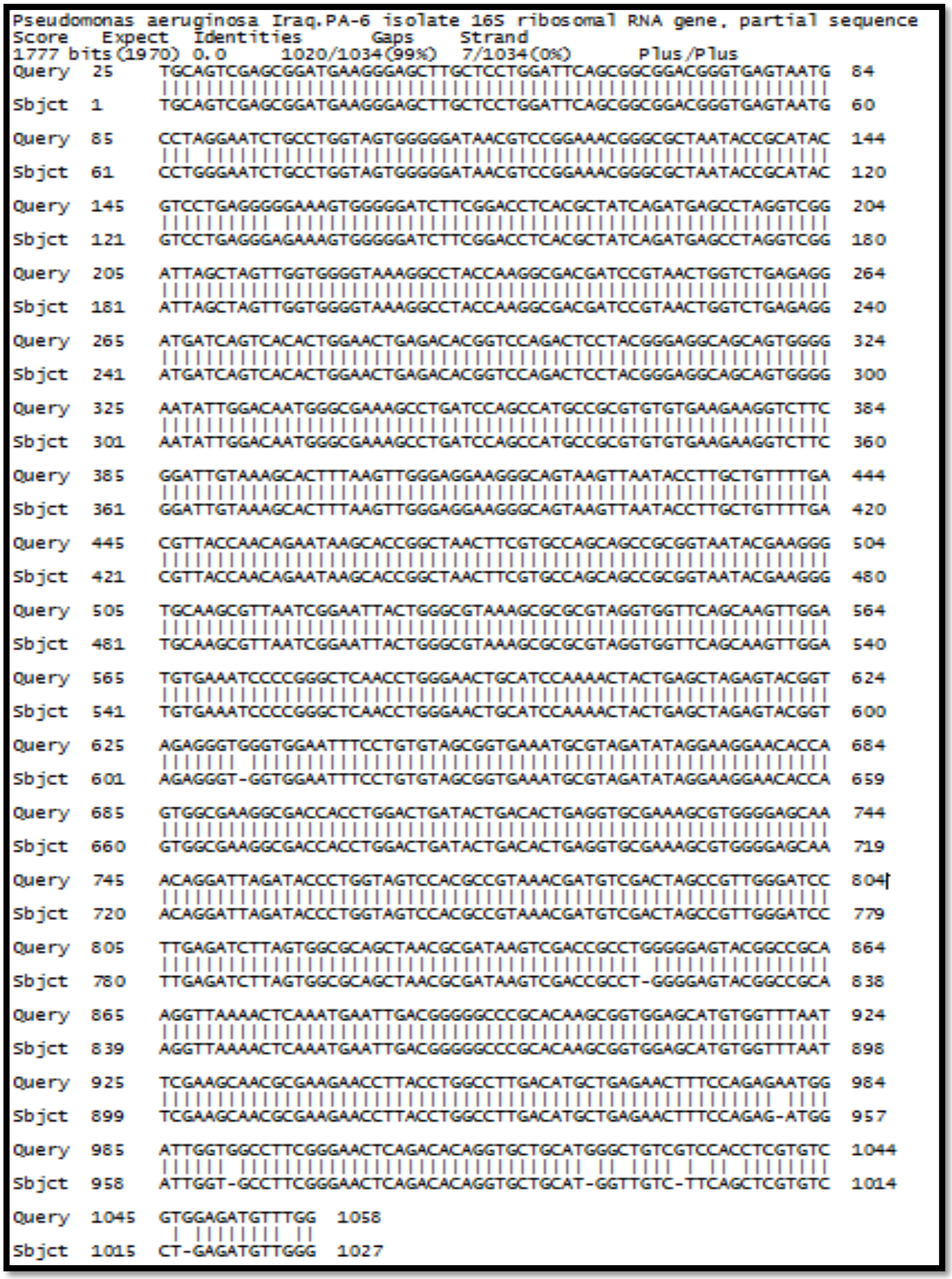


Fig. (3-18): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from sputum.

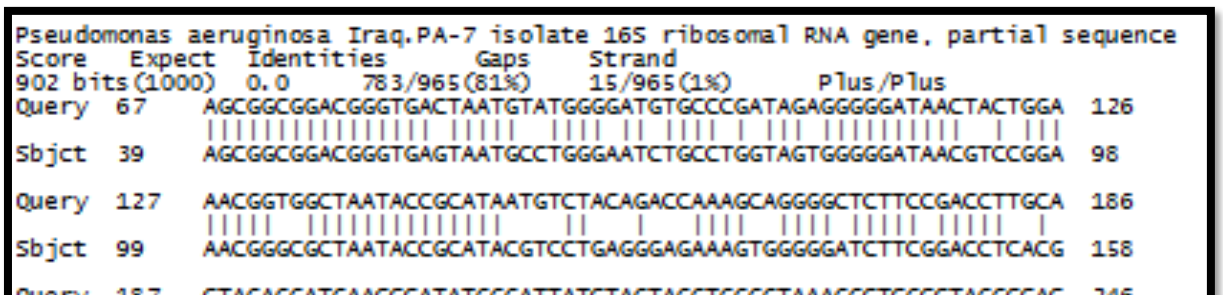


Fig. (3-19): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from wound.

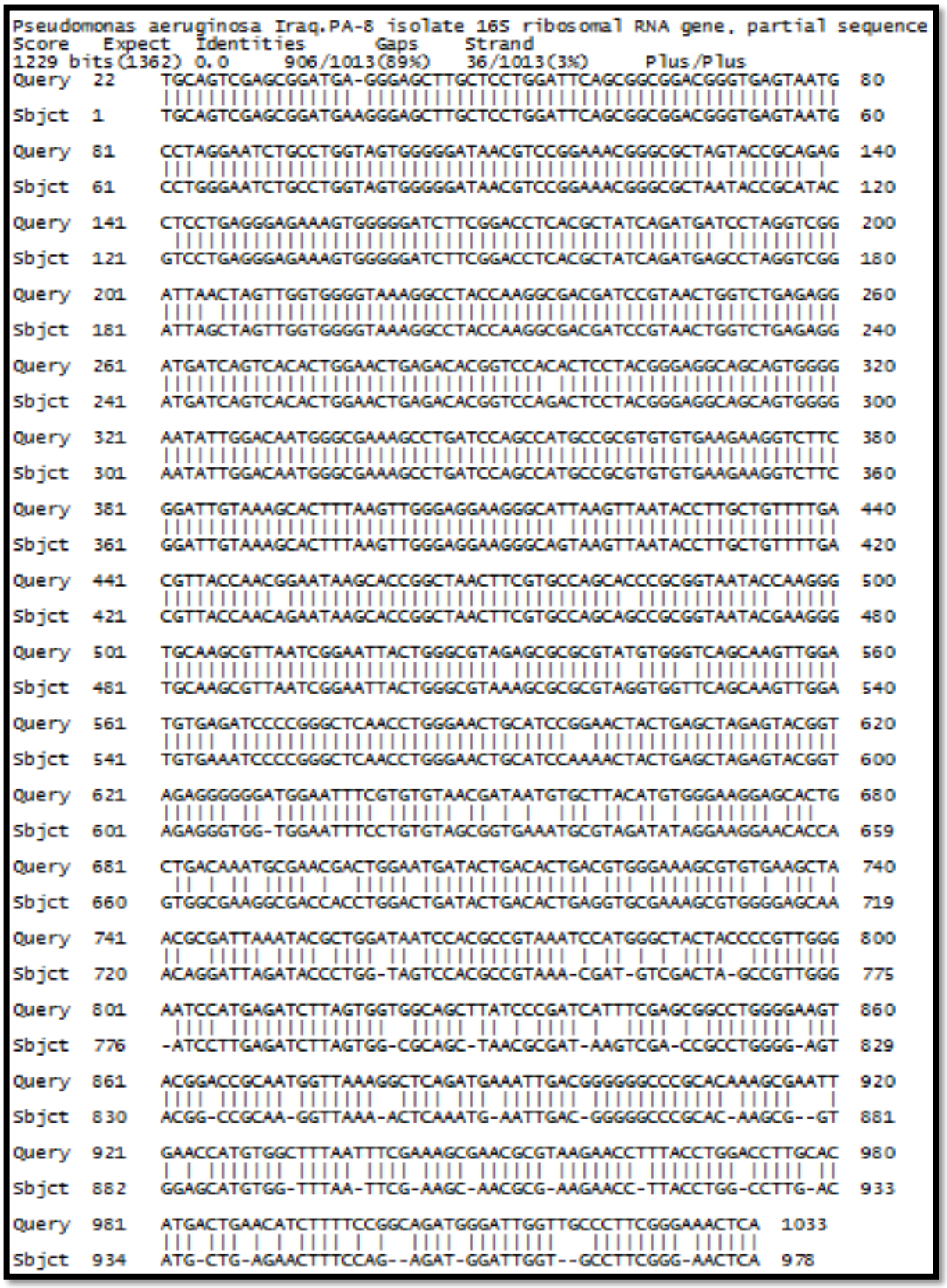


Fig. (3-20): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from ear.

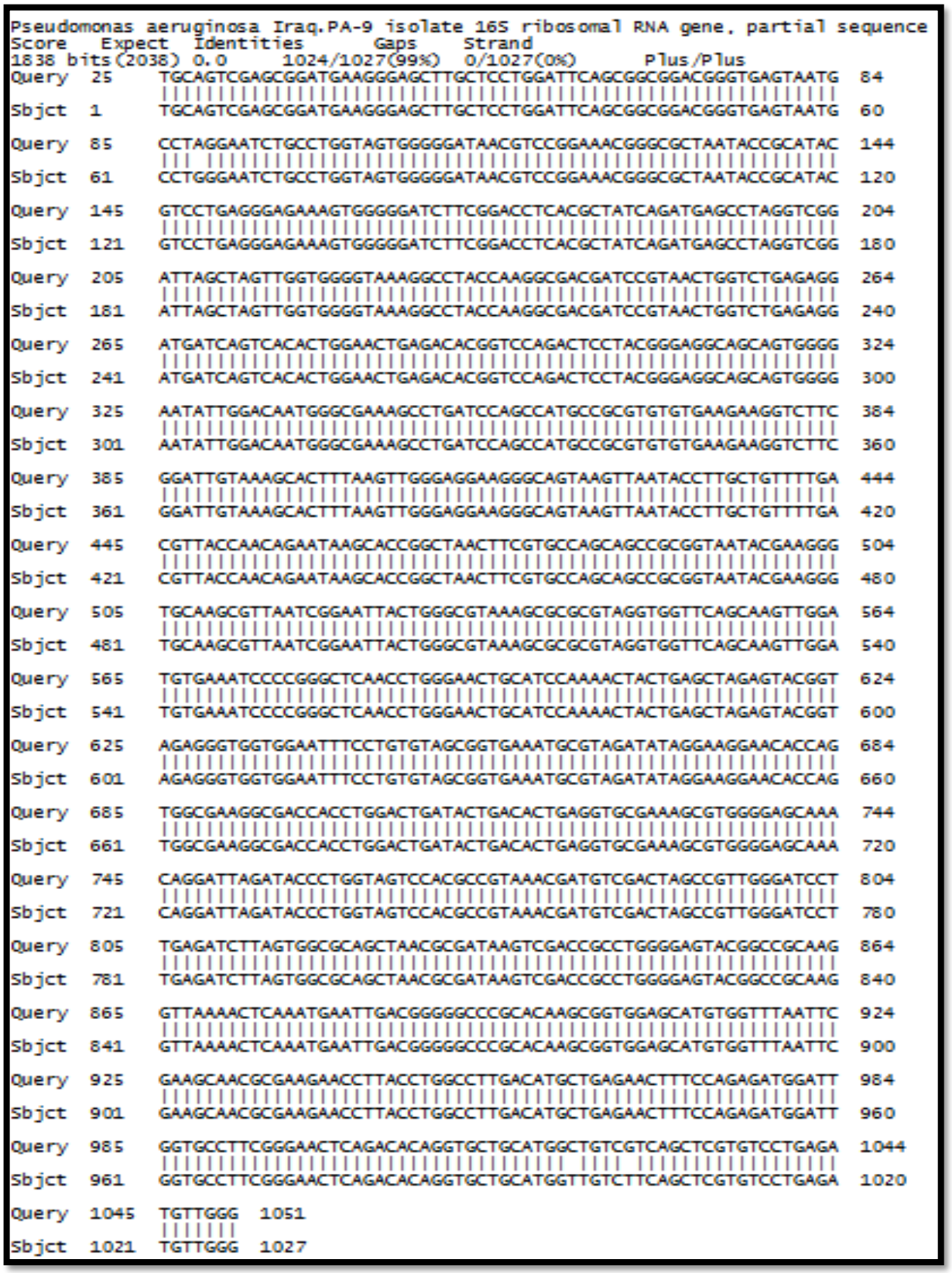
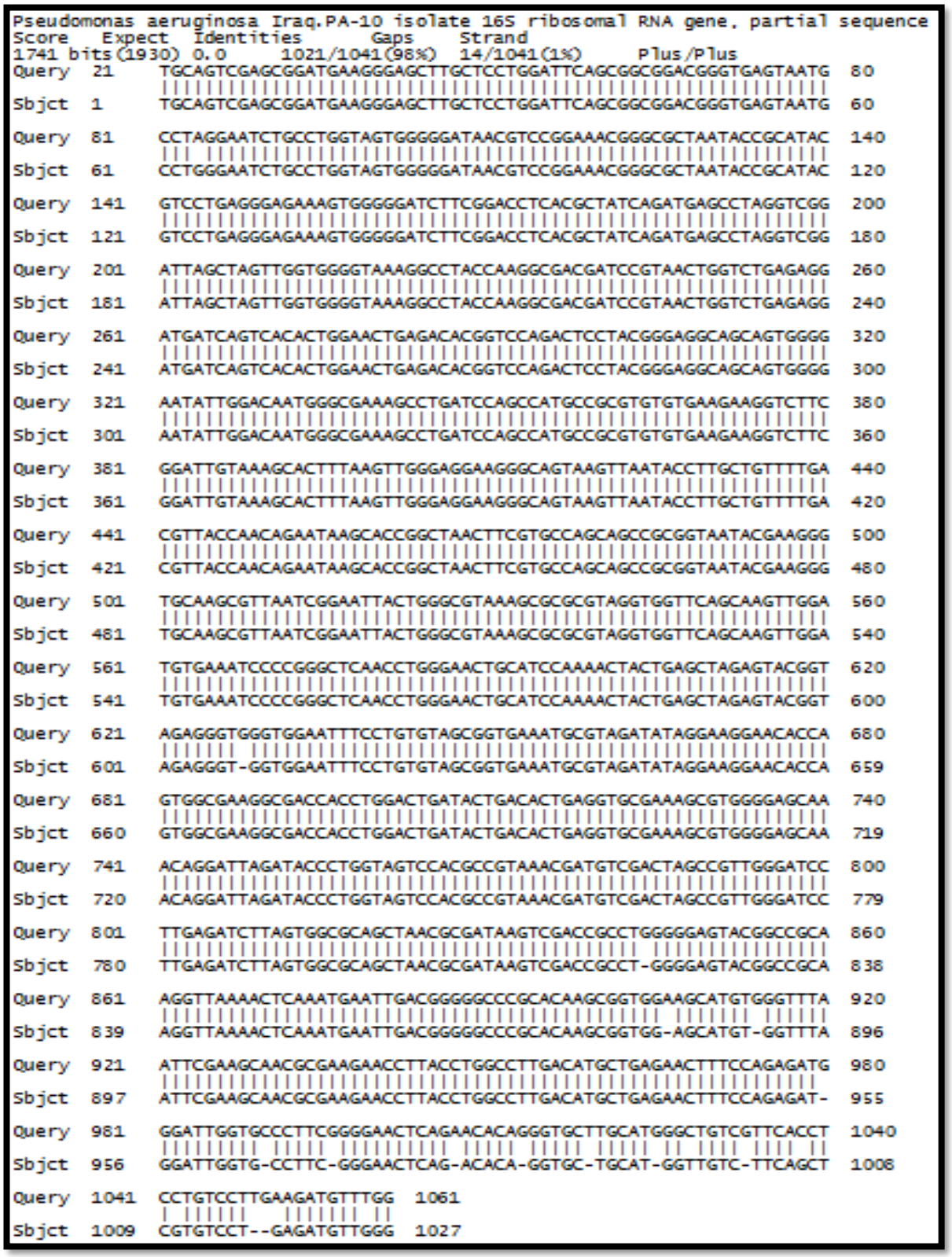


Fig. (3-21): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from wound.



```
Pseudomonas aeruginosa Iraq.PA-10 isolate 16S ribosomal RNA gene, partial sequence
Score    Expect  Identities      Gaps    Strand
1741 bits(1930) 0.0     1021/1041(98%)  14/1041(1%)     Plus/Plus
Query  21    TGCAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATG  80
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1     TGCAGTCGAGCGGATGAAGGGAGCTTGCTCCTGGATTCAGCGGCGGACGGGTGAGTAATG  60

Query  81    CCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATAC  140
             ||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61    CCTGGGAATCTGCCTGGTAGTGGGGGATAACGTCCGGAAACGGGCGCTAATACCGCATAC  120

Query  141   GTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGG  200
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121   GTCCTGAGGGAGAAAGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGG  180

Query  201   ATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGG  260
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181   ATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGG  240

Query  261   ATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG  320
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241   ATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG  300

Query  321   AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC  380
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  301   AATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC  360

Query  381   GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGA  440
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361   GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGA  420

Query  441   CGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG  500
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421   CGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGG  480

Query  501   TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGA  560
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481   TGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGA  540

Query  561   TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGT  620
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541   TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGT  600

Query  621   AGAGGGTGGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCA  680
             ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601   AGAGGGT-GGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCA  659

Query  681   GTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAA  740
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  660   GTGGCGAAGGCGACCACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAA  719

Query  741   ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCC  800
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  720   ACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCC  779

Query  801   TTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGGAGTACGGCCGCA  860
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
Sbjct  780   TTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCT-GGGGAGTACGGCCGCA  838

Query  861   AGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGGTTTA  920
             |||||||||||||||||||||||||||||||||||||||||||| ||||||| |||||||
Sbjct  839   AGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG-AGCATGT-GGTTTA  896

Query  921   ATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATG  980
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  897   ATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGAT-  955

Query  981   GGATTGGTGCCCTTCGGGGAACTCAGAACACAGGGTGCTTGCATGGGCTGTCGTTCACCT  1040
             ||||||||| ||||| |||||||||| ||||| ||||| ||||| || |||| |||| ||
Sbjct  956   GGATTGGTG-CCTTC-GGGAACTCAG-ACACA-GGTGC-TGCAT-GGTTGTC-TTCAGCT  1006

Query  1041  CCTGTCCTTGAAGATGTTTGG  1061
             | ||||||   ||||||| ||
Sbjct  1009  CGTGTCCT--GAGATGTTGGG  1027
```

Fig. (3-22): Partial sequences of *16S* RNA gene of *P. aeruginosa* isolates from wound.

The results of DNA sequencing should be firstly examined to confirm the nucleotide sequences and closed relationships with others world strain, test used to confirm was through NCBI-BLAST-query-

nucleotide –online, it was perfect program and gave the exact results of identity percentage with other world strains and they were ranged from (97%-100%).

It has been demonstrated that *16srRNA* gene sequence data on an individual strain with a nearest neighbor exhibiting a similarity score <97% represent a new species, the meaning of similarity scores of >97% is not as clear (Petti, 2007).

Table. (3-8), shows the Evolutionary Divergence between studied sequences of *P. aeruginosa* based on the number of base substitutions per site between sequences. The differences in comparison among bases sequences were considered in evolutionary. The analysis involved 11 nucleotide sequences 10 local isolates and one stranded strain: KR815840.1. All ambiguous positions were removed for each sequence pair. There were a total of 1606 base positions in the final dataset. The results revealed a genetic variation as base substitutions in the nucleotide sequences of involved local isolates when compared with standard world. The evolutionary divergence between involved sequences ranged from 0.005between local isolates No.5 burns sample) and Standard world isolates to 0.332 between local isolates No.1 and standard World strain (KR815840.1) based on the sequences analysis of *16srRNA* gene.

No. of isolate in Sequence and source	**NCBI Accession Number**	**NCBI-BLAST Homology sequence identity**

		Sequence Identity (%)	Genetic variation (base substitutions)
1.Diabetic foot	KX963356	74%	0.332
2.burn	KX963357	77%	0.350
3.urine	KX963358	99%	0.006
4.sputum	KX963359	95%	0.117
5.burn	KX963360	99%	0.005
6.treach sputum	KX963361	99%	0.013
7.wound	KX963361	81%	0.255
8.ear	KX963362	89%	0.148
9.wound	KX963363	99%	0.003
10.wound	KX963365	98%	0.017

Table (3-8): Homology sequence identity of *P. aeruginosa* compared with *P. aeruginosa* Standard strain by using NCBI-Blast Gen bank Database:

	1	2	3	4	5	6	7	8	9	10	11
1. P.aeruginosa iraq-1 isolate 16S ribosomal RNA gene											
2. P.aeruginosa iraq-2 isolate 16S ribosomal RNA gene	0.688										
3. P.aeruginosa iraq-3 isolate 16S ribosomal RNA gene	0.387	0.406									
4. P.aeruginosa iraq-4 isolate 16S ribosomal RNA gene	0.422	0.417	0.121								
5. P.aeruginosa iraq-5 isolate 16S ribosomal RNA gene	0.397	0.413	0.037	0.121							
6. P.aeruginosa iraq-6 isolate 16S ribosomal RNA gene	0.373	0.419	0.079	0.128	0.073						
7. P.aeruginosa iraq-7 isolate 16S ribosomal RNA gene	0.270	0.621	0.290	0.339	0.289	0.292					
8. P.aeruginosa iraq-8 isolate 16S ribosomal RNA gene	0.398	0.394	0.197	0.218	0.189	0.204	0.382				
9. P.aeruginosa iraq-9 isolate 16S ribosomal RNA gene	0.405	0.421	0.041	0.127	0.054	0.087	0.294	0.209			
10. P.aeruginosa iraq-10 isolate 16S ribosomal RNA gene	0.347	0.382	0.051	0.121	0.049	0.065	0.294	0.184	0.053		
11. Pseudomonas aeruginosa 16S ribosomal RNA gene (KR815840.1)	0.332	0.350	0.006	0.117	0.005	0.013	0.255	0.148	0.003	0.017	

The present study showed that showed that sequences identity (%) of *P. aeruginosa* (10 isolates) when using DNA sequencing of *16srRNA* gene ranged from 74% -99% according to type of isolates.(Table 3-8) when compred with world strain (KR815840.1). There are 5/10 of local isolates of *P. aeruginosa* gave more than 97% of sequence identity which mean these isolates belong to same species or strain while the remaining isolates (n=5) had a similarity score less than 97%, which mean these isolates belong to other new strains of *P. aeruginosa*.

Open reading frame (ORF) program was a perfect tool which used for reading each nucleotide sequences to 4-6 segments of genetic codes with its translation to amino acids within six ORF three ORF in the direction 5-3 and other 3 ORF in the direction 3-5, one genetic code or more consider proper code which start by methionine and end by stop codon. Also, ORF provide high information about amino acid translations for each sequence. The translated protein by ORF program then submitted to NCBI-Blast by using this protein query for searching on translated database, and results of the ORF were important for searching on other sequences on NCBI, these sequences were collected and download, then added with local sequences of this

study, then submitted for MEGA6 software for alignment and phylogeny.

ORF also provide information used in the submission data of this study to gene bank database information for recording and publishing isolates of this study. The Molecular Evolutionary Genetics Analysis (MEGA) software is a desktop application designed for either from multi gene families or from different species with a special emphasis on different evolutionary relationship and patterns of DNA and protein evolution.

The*16srRNA* gene sequence analysis is the most commonly used method for identifying bacteria or for constructing bacterial phylogenetic relationships (Wang *et al*., 2008).

In addition to the tools for statistical analysis of data MEGA6 provides many convenient facilities for the assembly of sequence data sets from files or web based repositories, and it includes tools for visual presentation of the results obtained in the form of interactive phylogenetic trees and evolutionary distance matrices (Kumar *et al*., 2008).

First step of analysis by alignment of all sequences of this study with other world selected reference by using (clustal W) program step in MEGA6. This program demonstrated had accurate degree of identity with all world sequences including sequences of this study.

These results with (clustal W) were important because they used directly in the phylogeny tree constriction.

The present used study nucleotide maximum likelihood method for detection the close relationship of world and local sequences which was better method of the nucleotide sequences in MEGA6. Also the phylogenetic tree was constructed using unweight pair group method with arithmetic mean (UPGMA Tree) in MEGA6 version. In the*16srRNA* gene phylogeny of present study introduced for sequencing.

The *16srRNA* gene sequences in formation have an expanding role in the identification of bacteria in clinical or public health settings. This may due to several reasons include (i) its presence in almost all bacteria often existing as a multi gene family, or operons; (ii) the function of *16srRNA* gene over time has not change, suggestion the random sequences change are a more accurate measure of time (evolution); and (iii) the*16srRNA* gene (1500bp) is large enough for informatics purpose (Janda and Abbott, 2007).

3.5. Phylogenetic analysis of local strains:

The present study used the*16srRNA* gene for the phylogeny whem submitted (10 sequences belong to the local isolates and 5 sequences belong to globial strains downloaded from NCBI to analysis by using MAGA6 software programe to find nucleotide maximum likehood between sequencing and obtaining Phylogenetic relationship among local and global strains sequences.

The results showed that the local isolates of these bacteria were closed related to NCBI-Blast *P. aeruginosa* (KR815840.1) except the Iraq-2 isolate (Burn sources) and Iraq-8 isolate (ear sources) showed genetically difference as unique isolates. Whereas the NCBI-Blast *P.aeruginosa* isolates showed different and out of tree. These results means that isolates is considered as a new strains record of *P. eruginosa* in Iraq and world fig. (3-23).

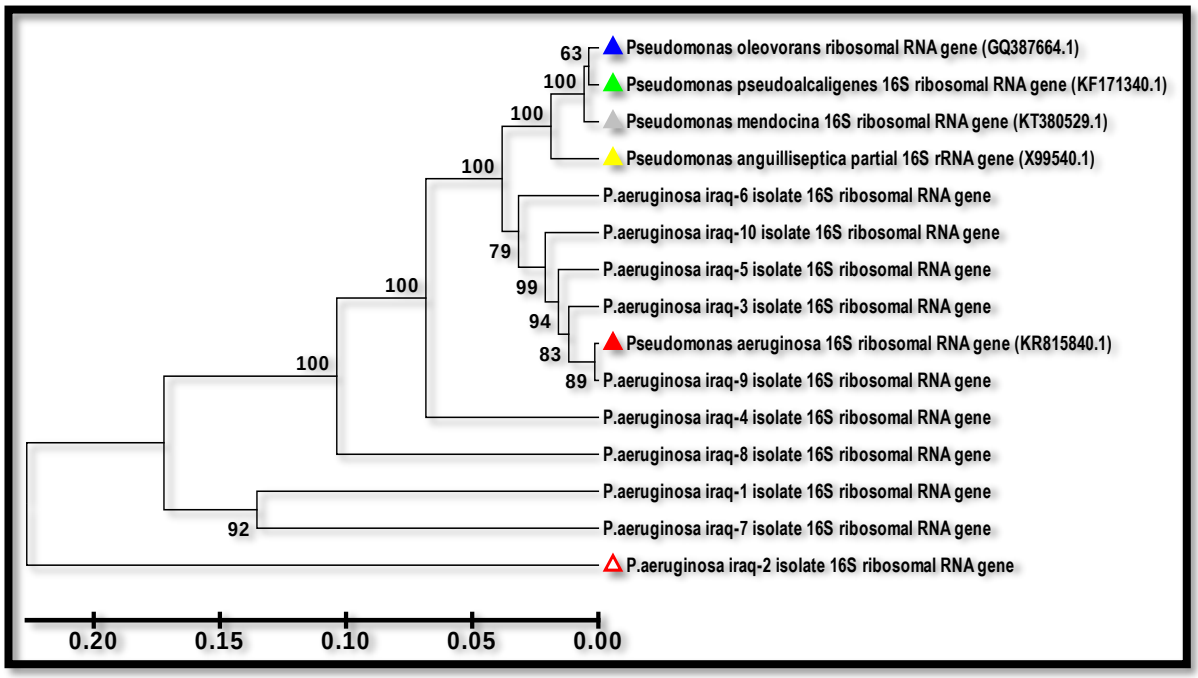


Figure (3- 23): Shows the Phylogenetic tree analysis based on *16S ribosomal RNA* gene partial sequence of 10 *P. aeruginosa* isolates.

P. aeruginosa according to clinical samples that collected from hospitals the results indicated that the sources of 9 isolate with the same origin which No.3 represent (urine), No.7, 9, 10(wounds) No. 5, (burns), No.6 (Trachea) No.4 (sputum), No.1 (Diabetic foot) while the isolate No.8 from ear and isolate No. 2 from burns that collected show different origin or sources (unique isolate) fig 3-

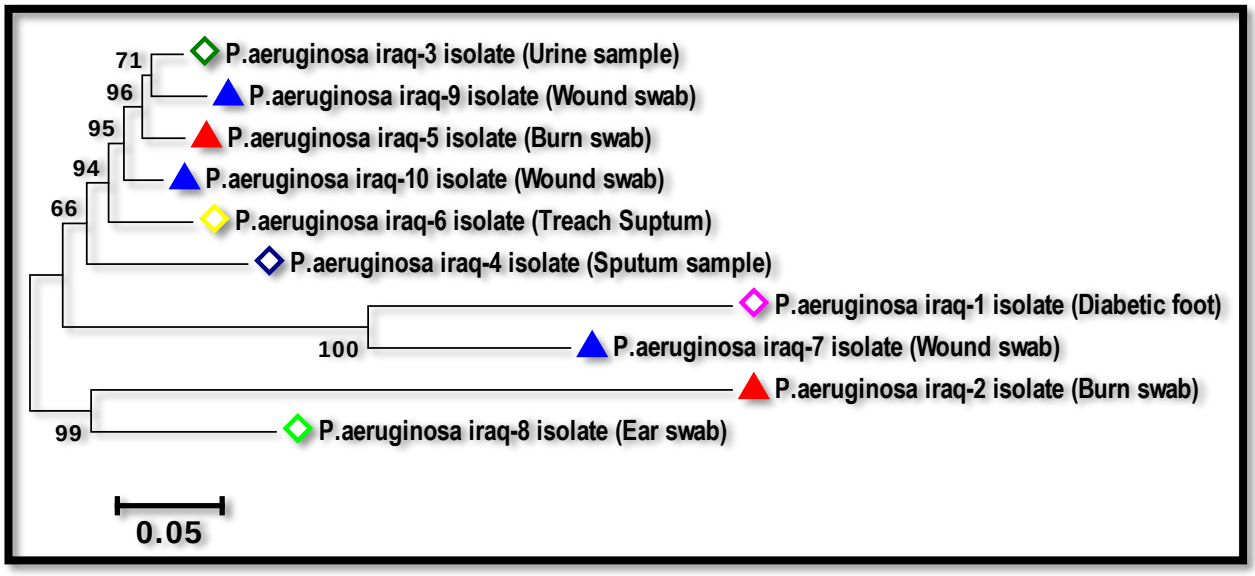


Figure (3-24): Shows the Phylogenetic tree analysis based on 16S ribosomal RNA gene partial sequence 10 isolates.

3.6. Recording Iraqi *P. aeruginosa* isolates in gene bank-NCBI:

Ten sequences of *P. aeruginosa* were isolated from human sources in Baghdad city and each sequence has a symbol code (Iraq.PA-1isolate, Iraq.PA-2isolate,Iraq.PA-3isolate, Iraq.PA-1isolate4, Iraq. PA-5isolate, Iraq.PA-6isolate, Iraq.PA-7isolate, Iraq.PA-8isolate, Iraq.PA-9isolate, Iraq.PA-10isolate. *16srRNA* gene sequences submitted to Gen Bank- Ban kit under submission code BankIt11959593.The results of these sequences were analyzed and examined by professional staff in gene bank in two working days. All these sequences accepted in gene bank and each sequence take accession number (KX963356, KX963357, KX963358, KX963359, KX963360, KX963361, KX963362 KX963363, and KX963364 KX963365).(appendices. -10) Version number codes after two months (KP420229.1, KP420230.1, KP420231.1, KP420232.1, KP420233.1, KP420234.1, KP420235.1, and KP420236.1) in the pobset number: 798546704. Appendix (III -) Accession number of Iraqi isolates of *P. aeruginosa* in Gene bank.