Isolation and Molecular Identification of *proteus mirabilis* isolated from hospitals in the capital *Baghdad*

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Abstract

Proteus mirabilis a Gram-negative bacteria belonging the *Enterobacteriaceae* family and that causes several infections to patients such as urinary tract infections, middle ear infections, wounds and burns, in addition to other infections. This study aimed to isolate and diagnose *Proteus mirabilis* bacteria collected from different samples of infection sources from patients present in many hospitals using traditional diagnostic like morphological and biochemical tests as well as molecular methods. The Identification system for Gram negative bacteria GN24 KIT had used to detect *Proteus mirabilis* species . The results of using this kit were confirmed by the molecular diagnosis of bacteria through the PCR technique of the *16S* rRNA gene, where the confirmation rate was 100%. The PCR sample was analyzed in Macrogen Corporation–Korea for Sanger sequencing using ABI3730XL, an automated DNA sequences. the findings were obtained and analyzed via genius software in order to Synthesis of the phylogenetic tree of the isolated strains that resulted from the discovery of a new global strain of *Proteus mirabilis* bacteria and was recorded in NCBI genebank with accession number (MN 700085) and name (M.K.84).

Keywords: *Health; Genebank; toxicity; sequencing;* **proteus mirabilis**

Introduction

Proteus spp . considered one of Gram-negative bacteria and they are members of the family *Enterobacteriaceae,* as well as belong to gastrointestinal microorganisms [1]. *Proteus mirabilis* is a rod formed bacteria, it is commonly documented by its urease generation and recognizable capability to differentiate to elongated swarm cells and the distinctive bull-eye style of motility on agar media plates [2]. *P. mirabilis* return to the Gammaproteobacteria class, and had been for a long time regarded as an individual of the *Enterobacteriales* Order, *Enterobacteriaceae* family, Besides that, lately, one group suggested re-classification of the *Enterobacteriales* order to put *Proteus* in a new position within the *Morganellaceae* family based on the recreating phylogenetic tree depending on four multilocus sequence analysis proteins, ribosomal proteins , shared core proteins [3]. *Proteus mirabilis* was of medicinal significance and is typically responsible for most serious bacterial infections in hospitals including such urinary tract infections, wound, ear infections and other infections [4]. In healthy hosts P. *mirabilis* is not considered a major cause of urinary tract infection , on the contrary , *P. mirabilis* is detected in complicated UTIs comparatively repeatedly like Patients with functional or physiological disorders, in particular patients with urolithiasis or chronic urinary catheter [5]. *Proteus mirabilis* ureolytic activity in the catheterized urinary tract results in ammonia production and an elevation of urinary pH. Under these alkaline environment, magnesium ammonium phosphate (struvite) and calcium phosphate (hydroxyapatite) crystals are usually begin to form by normally soluble urine constituents precipitation [6].

16S rRNA genes encoding a small subunit of rRNA in prokaryotes have been widely used in taxonomic classification and determination of phylogenetic relationships. The 16S rRNA gene sequence is used to detect bacterial species in natural specimens and to

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establish phylogenetic relationships between them . This is made possible by the fact that all bacterial species contain the 16S rRNA gene, which has highly conserved regions on which to design universal primers, as well as hypervariable regions that are useful in distinguishing species. The 16S rRNA gene has hypervariable regions which are an indication of divergence over evolutionary time[7] . For addition reasons the use of *16S* rRNA gene sequences to study bacterial phylogeny and taxonomy was by far the most common genetic marker used , these reasons include (i) its existence in almost all bacteria, often identified as a multigene family or operons; (ii) the function of the *16S* rRNA gene has not changed over time, indicating that alterations in the sequence are a more accurate measure and (iii) the *16S* rRNA gene (1500 bp) is fairly large for informatics purposes[8].

Genotypic bacterial analysis starts with the PCR product's nucleotide sequence analysis of the respective gene(s) followed by a comparison of such sequences with the identified sequences in the database [9].

Materials and Methods

One hundred and fifty swab samples were collected from different sources of infections (urinary tract infections, Otitis media swab, Stool , Wound swab, Vaginal swab) that were taken from patients suffering from various diseases, which were eighty samples from females and seventy samples of males (With age range from six months to seventy years) present to the several Iraqi capital Baghdad hospitals for the period from the first of September 2019 to January 2020 . Each specimen is immediately inoculated onto the MacConkey's agar plates where it is considered a selective and differentiation agar which develops only gram-negative bacterial organisms; it could also distinguish gram-negative species on the basis of the lactose fermentation , the selective and differentiating property of MacConkey agar that allows use in both scientific and clinical applications [10]. On the other hand, to see the phenomenon of swarming more clearly , samples were cultured on blood agar plates which was the enriched product often used for cultivating fastidious microbes and the differentiation of bacteria depending on their hemolytic effects [11].

Identification system for Gram negative bacteria GN24 KIT

GN 24 is a standard recognition method for Gram negative bacteria common species . The test is dependent on 24-29 miniaturized biochemical tests and on the Internet database. Kit GN 24 consists of 24 wells microtitration plate strip in the classic 96 well format containing dehydrated substrates Reconstitution of substrates is performed by bacterial suspension inoculation. During incubation, color changes occur in wells due to microorganisms ' metabolic activity. Test results may be measured either by automated readers or visually depending on the color scheme, or by the color definition displayed in the flyer. The results of recognition can be collected from the evaluation table or by using evaluation tools located at (www.diagnostics.sk/idmicro).

DNA Extraction from Gram Negative bacteria

According to the ABIOpure (USA) Extraction method , genomic DNA was extracted from bacterial growth .

Quantitation of DNA

Quantus Florometer (Promega, USA) has been used to measure the concentration of sample DNA collected for downstream applications.

Detection of 16S rRNA Genes by Polymerase Chain Reaction (PCR)

Bacterial *16S* rRNA had amplified via specific primer pair designed in Macrogen Company (Korea) .**(F: 5`- AGAGTTTGATCCTGGCTCAG-3`)** and **(R: 5-` TACGGTTACCTTGTTACGACTT-3`)** The PCR condition was: Initial denaturation at 95°C for 5 min . followed by 30 cycles of denaturation at 95 °C for 30s, annealing at 60 °C for 30s and extinction at 72 °C for 1min . Final extension at 72 °C for 7 min , finally hold at 10 °C for 10 min.

Standard Sequencing

The PCR amplicon samples was analyzed in Macrogen Corporation labortary–Korea for Sanger sequencing using ABI3730XL, an automated DNA sequences . the findings were obtained and analysed Using genious software .

Results and Discussion

Of the 150 clinical samples, 110 gives positive results, including 19 (12.7%) isolates belonging to *Proteus mirabilis*, whereas the remaining 91 (60.7%) isolates belonged to other bacteria species as seen in the table (1).

Table 1: Number and percentage of bacteria that isolated from clinical cases.

Samples No.	Positive culture		Negative culture
	P. mirabilis	Other bacteria	
150	19	91	40
100%	12.7%	60.7%	26.6%

The outcome of the present study matched Abbas *et al* 2015 that found the *P. mirabilis* rate (12.6%) of one hundred and thirty-five specimens from patients with various clinical cases[12]. EL-Baghdady *et al* . (2009) got the results comparable to the present study , which had obtain to isolate *P. mirabilis* at rate reaching to (10%) [13]. Mishra *et al* . (2001) isolated *P. mirabilis* from different sources of infections at a percentage of 78.3%[14]. Furthermore, AL-Jumaa (2011) isolated just (7%) of *P. mirabilis* from the total of 100 samples obtained from various clinical samples of urine , blood, otitis media, burn, wound and perianus [15].

Proteus mirabilis Identification

Proteus mirabilis identification was carried out according to [16] based on the Colonial morphology (Shape, swarming, odor on MacConkey) and Microscopic analysis involving bacterial cell morphology was examined by Gram-Stain to determine the shape , cell arrangement, and form of Gram-Stain reaction. After staining, different biochemical tests are carried out for each isolate includes Catalase (+ ve) and Oxidase (-ve) In addition to use Identification system for Gram negative bacteria (GN24 KIT) which used as previously mentioned . Its results are shown in Figure (1) below

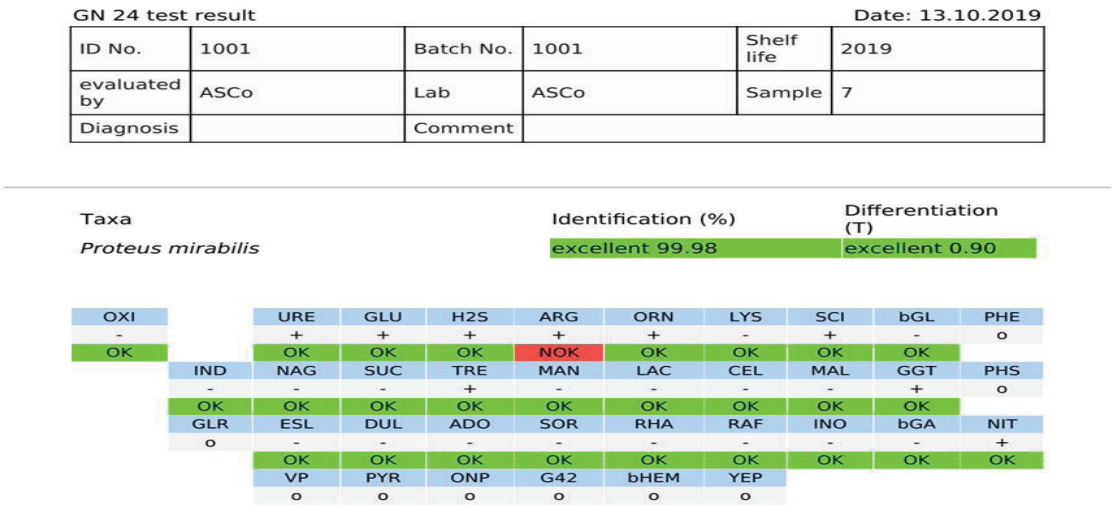


Figure 1. Diagnostic Results to one of *Proteus mirabilis* isolates Using GN24 kit Software.

genotyping assay

PCR identification

The results of PCR identification of (15 Isolates) *P. mirabilis* using *16S* rRNA showed all isolates were *P. mirabilis* and gave a good confirmative identification as shown in the figure (2). All *P. mirabilis* isolates yielded the same band size (1500 bp) which was the product size of primers used for identification.

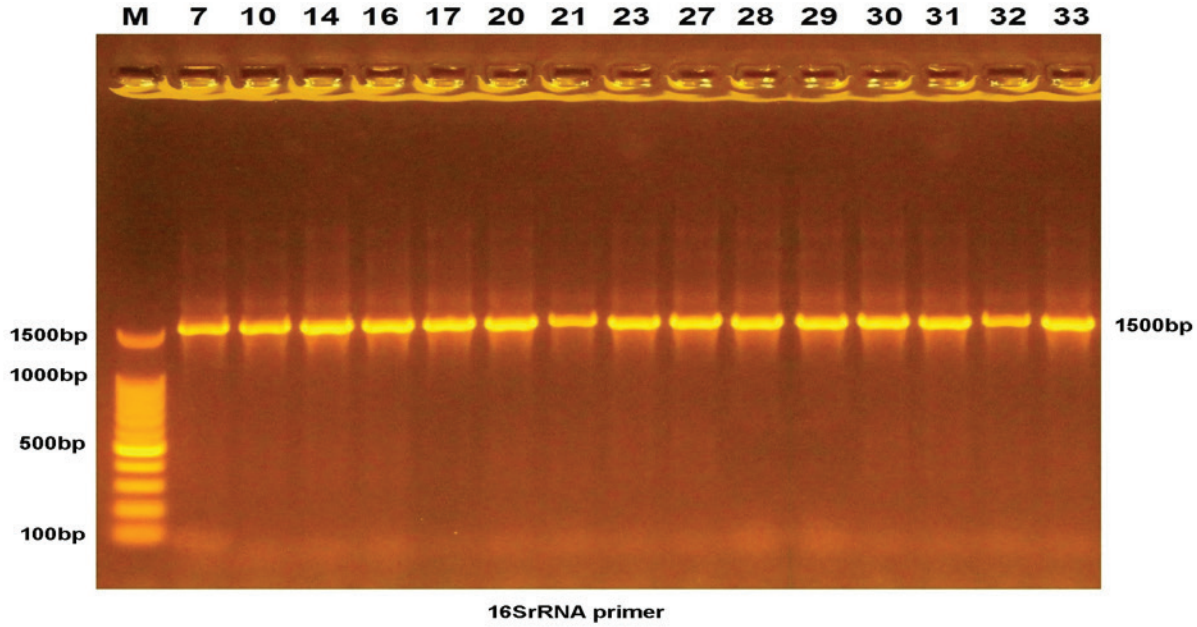


Figure 2. Results of the amplification of *16S* rRNA gene of *Proteus mirabilis* samples were fractionated on 1% agarose gel electrophoresis stained with Eth.Br. Lane1:100bp DNA marker.

The present research showed that *16S* rRNA was a strong selective power to determine isolated *P. mirabilis*, which was compatible with the Saleh *et al*.(2019) *16S* rRNA test to recognize *Proteus* spp. isolated from urinary tract infection patients and the Mukhtar *et al*. (2018) finding the *16s* rRNA gene used to identify *Proteus mirabilis* on banknotes ,the two above research have been reported to be a strongly selective force for *Proteus* spp recognition by *16S* rRNA [9;17].

The 16S rRNA gene, which has a length of 1.5 kilobase (kb), has proved to be a valuable molecular target because it is found in all bacteria, whether as a single copy or in multiple copies, and is strongly conserved in a species over time. [18] [19] .

Sequencing and Phylogenetic of 16S rRNA gene

The results of the analysis of gene sequence of the present study showed a match in the sequence of the nitrogenous bases of the 16S rRNA gene of *P.mirabilis* local isolates with *P.mirabilis* global isolates which saved in (NCBI-Genebank), Where the proportion of identical of fourteen isolates were (100%) and the identical ratio of last isolate was (99.63%) , as the result of the current study was close to what Jian-ke *et al*.,(2015) reached where he was found that the *16S* rRNA gene for the diagnosis of *P.mirabilis* bacteria is more than (99%) identical when analyzed at (NCBI-BLAST) site . The same researcher also found that the Phylogenetic tree analysis for *16S* rRNA gene of 20 global *P.mirabilis* strains had very high identical ratio in the gene-bank and the match rate was (98.9-99.7%) this ratio was Close to the result of the phylogenetic tree analysis for the current study for the *16S* rRNA gene as shown in figure 3 below . Where the results showed in the current study that there was a group of strains that were 100% identical with each other, and these are the following samples (10-14-16-27-29-30-33) these strains differ with some strains in the gene bank in the rate of ($6*10^{-5}$) , The other group includes a group of strains that are 100% identical with each other, as well as other strains in the genebank at 100%. an example of this was

the numbered strain (AOUC-001) which matching with samples (20-21-23-28-32) , While the sample No. (17) was identical to the gene bank sample (TL14(1)) at the rate of (100%) and differs from the other local and global strains above in ratio about ($1.84*10^{-4}10^{-4}$) also, the sample (31) was 100% identical to the sample (ALK042) on the other hand, it differs from the first group strains above by ($6*10^{-5}10^{-5}$) . Finally, with regard to sample No. (7), it did not match with any other strain (100%), whether they were local or global strains, and the percentage of difference with all strains mentioned previously was (0.002). It will be discussed more broadly in the next topic .

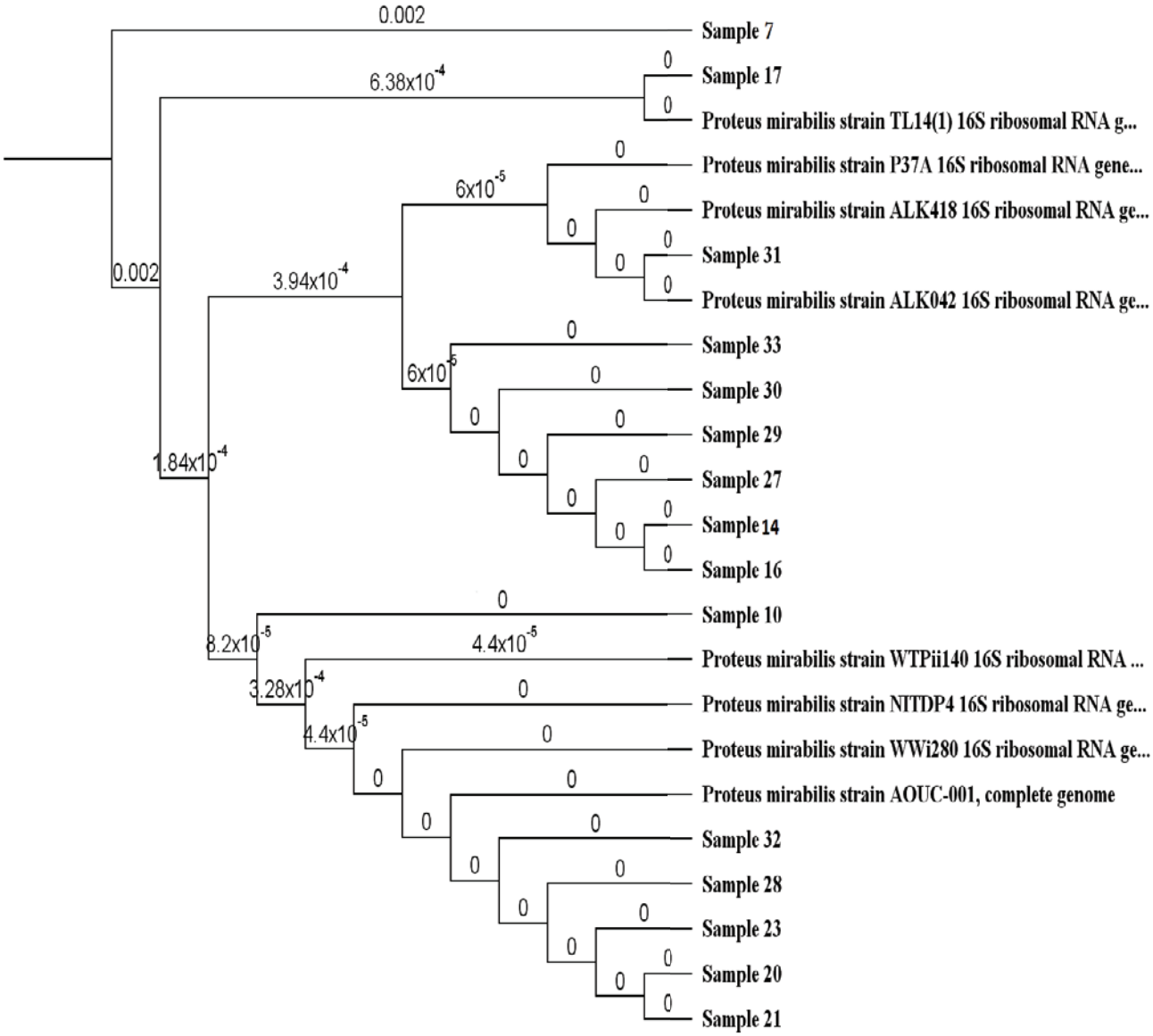


Figure 3 . The Phylogenetic tree of *16S* rRNA gene for fifteen strains of *P.mirabilis* bacteria included in the current study in addition to a number of strains of the same species of bacteria from the strains stored in the gene-bank database of NCBI .

Detect novel strain

After the *16S* rRNA nucleotide sequences were scanned for similarity sequences using online NCBI-BLAST analysis one of the isolates,was discovered which did not match any strain, at 100% of the strains stored in a NCBI-Genebank . The above-mentioned isolation registration was approved by the (NCBI-Genebank) as a new global strain and it was given an accession number (MN 700085). The 16S rRNA nucleotide sequences of new strain were scanned for similarity sequences using

online BLAST to create phylogenetic tree as appear in figure (4). All isolates retrieved from the NCBI GenBank subjected to the analysis of multiple sequence alignment using genius software .

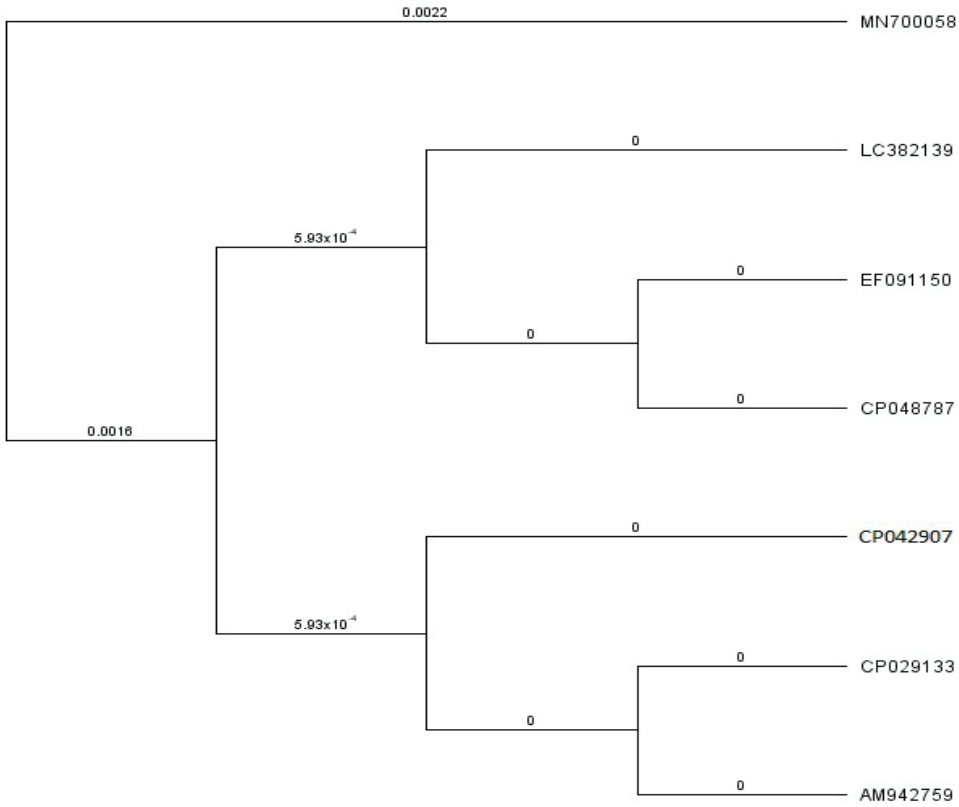
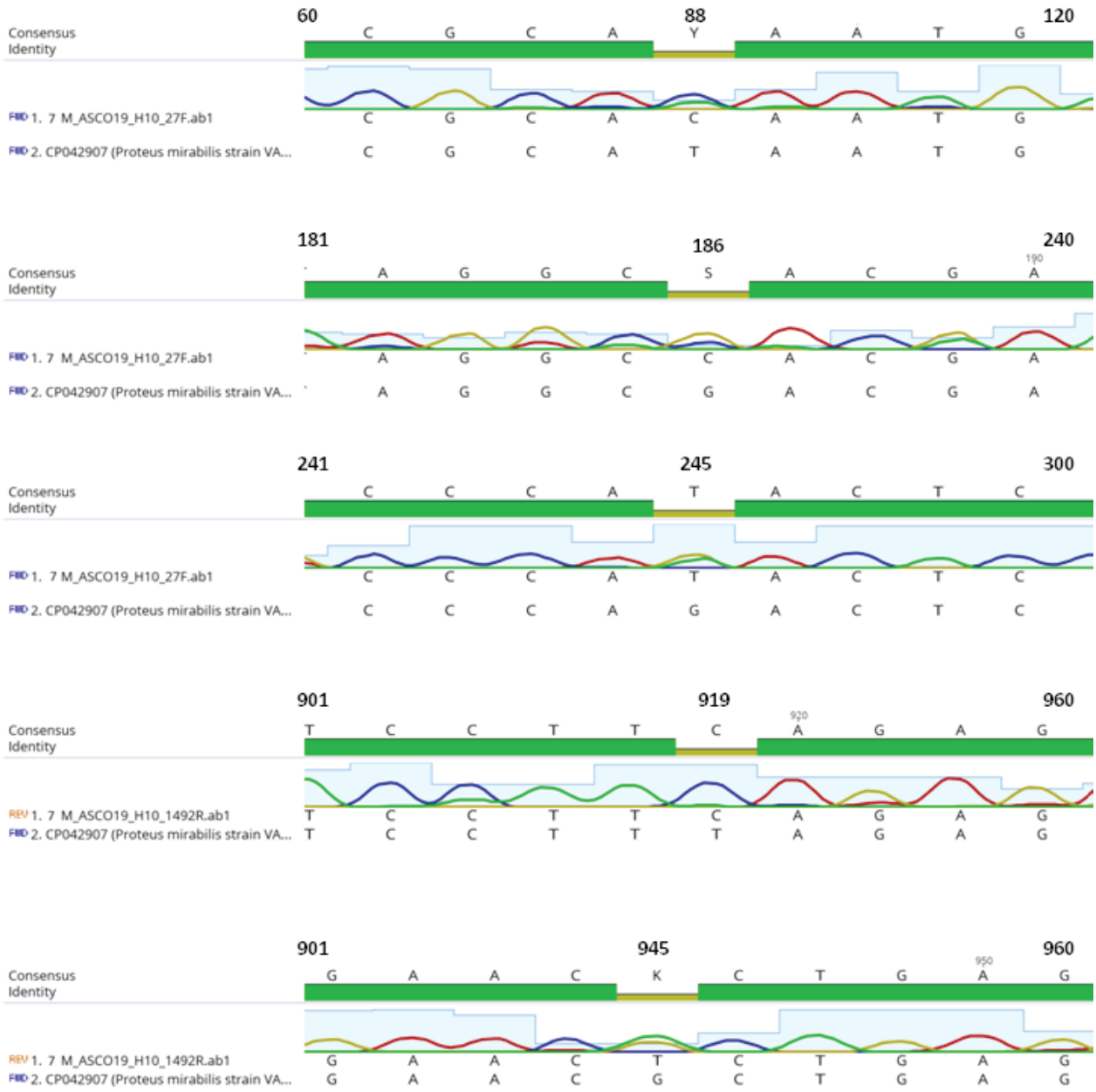


Figure 4 . phylogenetic tree of *16S* rRNA nucleotide sequences of new strain *Proteus mirabilis* strain M.K.84 accession number (MN 700085) .

Through the phylogenetic tree shown in the figure above, we divide the strains into two groups for the purpose of comparison among them in the first group the results show that the numbered strain (EF091150) was matching with strain (CP048787) . The two strains differ with the strain that had accession number (LC382139) in a ratio ($5.93*10^{-4}$) . In the second group the the strain which had accession number (CP029133) was matching with strain (AM942759) . The two strains also differ with the strain that had accession number (CP042907) in a ratio ($5.93*10^{-4}$) . The rate of difference between the strains of the first group and the second group was (0.0016) . The end result of the new strain (MN 700085) was It differs with the strain of both groups in ratio (0.0022) . The nucleotides substitutions that appeared in the new strain (MN 700085) was shown in Figure. 5.



Figures 5 . Consensus identity of new strain (MN 700085) sequence after analyzing it in genius software .

During the analysis of the sequence of the new strain in the genius program, the result was a match with the strain of accession number (CP042907) at (99.63%) and by five nucleotides in the form of substitutios at the sequences (88-186-245-919-945) (C-T : C-G: T-G: C-T: T-G).

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Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both MOH and MOHSER in Iraq

Conflict of Interest: None

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References

1. Hamilton A L, Kamm M A, Ng S C, Morrison M. Proteus spp. as putative gastrointestinal pathogens. *Clinical microbiology reviews* 2018; *31*(3):e00085-17.
2. Armbruster C E, Mobley H L, Pearson M M. Pathogenesis of *Proteus mirabilis* infection. *EcoSal Plus* 2018; *8*(1): 10.1128/ecosalplus.ESP-0009-2017.
3. Adeolu M, Alnajar S, Naushad S, Gupta R S. Genome-based phylogeny and taxonomy of the '*Enterobacteriales'*: proposal for *Enterobacterales* ord. nov. divided into the families *Enterobacteriaceae*, *Erwiniaceae* fam. nov., *Pectobacteriaceae* fam. nov., *Yersiniaceae* fam. nov., *Hafniaceae* fam. nov., *Morganellaceae* fam. nov., and *Budviciaceae* fam. nov. *International journal of systematic and evolutionary microbiology* 2016; *66*(12): 5575-5599.
4. Alabi O S, Mendonça N, Adeleke O E, da Silva G J. Molecular screening of antibiotic-resistant determinants among multidrug-resistant clinical isolates of *Proteus mirabilis* from SouthWest Nigeria. *African health sciences* 2017; *17*(2): 356-365.
5. Chen C Y, Chen Y H, Lu P L, Lin W R, Chen T C, Lin C Y. *Proteus mirabilis* urinary tract infection and bacteremia: risk factors, clinical presentation, and outcomes. *Journal of Microbiology, Immunology and Infection* 2012; *45*(3): 228-236.
6. Pelling H, Nzakizwanayo J, Milo S, Denham E L, MacFarlane W M, Bock L J, Jones B V. Bacterial biofilm formation on indwelling urethral catheters. Letters in applied microbiology 2019; 68(4): 277-293.
7. Tshikhudo P, Nnzeru R, Ntushelo K, Mudau F. Bacterial species identification getting easier. African Journal of Biotechnology 2013; 12(41): 5975-5982.
8. Janda J M, Abbott S L. *16S* rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *Journal of clinical microbiology* 2007; *45*(9): 2761-2764.
9. Mukhtar A A, Alfadil N A A, Mohamed M S, Altayb H N, Elzak S G, Hassan M S. Identification of *Proteus mirabilis* on banknotes using *16s* rRNA gene in Khartoum State. *Sudan Journal of Medical Sciences* 2018; *13*(3): 175-186.
10. Jung B, Hoilat G J. MacConkey Medium. In StatPearls [Internet]. StatPearls Publishing 2020.
11. Buxton R. Blood agar plates and hemolysis protocols. American Society for Microbiology 2005.
12. Abbas K F, Jawad K, Khafaji A L, Maysaa S, Shukri A L. Molecular detection of some virulence genes in *Proteus mirabilis* isolated from Hillaprovince. *Int. J. Res. Stud. in Biosci.(IJRSB)* 2015; *3*(10): 85-89.
13. El-Baghdady K Z, Abooulwafa M, Ghobashy M O, Gebreel H M. Plasmid mediated virulence factors of some *Proteus* isolates. *Egypt. Acad. J. biology. Sci 2009;*1(1): 7-22.
14. Mishara M, Thakar YS, Pathak A A. Haemagglutination, haemolysin production and serum resistance of *proteus* and related species isolated from clinical sources. *Indian J Med Microbiol* 2001; 19 (2):5-11.
15. AL-Jumaa M H J. Bacteriological and Molecular Study of Some Isolates of *Proteus mirabilis* and *Proteus vulgaris* in Hilla Province. M.Sc. thesis. University of Babylon 2011.
16. Forbes B A, Daniel F S, Alice S W. Baily and Scott's Diagnostic microbiology. 12thed. Mosby, USA 2007.
17. Saleh T H, Hashim S. T, Malik S N, AL-Rubaii, B A L. Down-Regulation of flil Gene Expression by Ag Nanoparticles and TiO2 Nanoparticles in Pragmatic Clinical Isolates of *Proteus mirabilis* and *Proteus vulgaris* from Urinary Tract Infection. *Nano Biomed. Eng* 2019; *11*(4): 321-332.
18. Reller L B, Weinstein M P, Petti C A. Detection and identification of microorganisms by gene amplification and sequencing. Clinical infectious diseases 2007; 44(8): 1108-1114.
19. Sabat A J, van Zanten E, Akkerboom V, Wisselink G, van Slochteren K, de Boer R F , Kooistra-Smid A M M. Targeted next-generation sequencing of the 16S-23S rRNA region for culture-independent bacterial identification-increased discrimination of closely related species. *Scientific reports* 2017; *7*(1): 1-12.