

Phylogenetic tree analysis based on the 16S sequence alignment for *Proteus mirabilis* Isolated from UTI patients in Wasit province, Iraq

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Abstract

One of the most common bacterial illnesses worldwide is urinary tract infections (UTIs). Out of 228 urine specimens collected from a patient with suspected UTIs, 40 (17.5%) *Proteus mirabilis* isolates were identified, the isolates were first identified as related to culture, microscopic examination, and biochemical tests. DNA was extracted from these 40 isolates, the sample's nucleic acid purity ranged from (1.8-2) while its concentration was between (50 - 360 mg/ul).

Isolates were detected as *Proteus mirabilis* by molecular methods of 16S rRNA gene for confirmatory diagnosis and phylogenetic tree. In the current study the phylogenetic tree analysis showed the significant selection potential of 16S rRNA to identify isolated *Proteus mirabilis* and the close with Iraqi strain.

Keywords: *Proteus mirabilis*; Urinary tract infections; Phylogenetic tree; Vitek 2; PCR

1. Introduction

One of the most prevalent illnesses studied in various clinical settings is urinary tract infection (UTI), which is mostly caused by bacteria, notably Enterobacteriaceae [1]. One of the most common illnesses in people and a common bacterial infection are urinary tract infections (UTIs). The most common cause is a member of the family Enterobacteriaceae. The diagnosis is based on the growth of bacteria in a urine sample, and the therapy is determined in light of the current antimicrobial resistance situation. These days, antibiotic resistance has become a serious concern for the management of urinary tract infections (UTIs) [2]. UTI is linked to significant morbidity and death, particularly in high-risk populations including pregnant women, kids, and those with impaired immune systems [3]. The collection of midstream or catheter urine with a bacteriological culture is the gold standard for diagnosing a UTI [4]. The rate of multidrug resistance (MDR) combined with the synthesis of ESBLs is rising among UPEC strains, which may result in treatment failure and challenging infection management [2]. A public health issue is the spread of Gram-negative, multidrug-resistant (MDR) bacteria [5]. A frequent pathogen that causes severe UTIs is *Proteus mirabilis* [6]. The *Proteus* genus is a motile, rod-shaped, Gram-negative opportunistic pathogen that is a member of the Enterobacteriaceae family. It is present in the environment, manure, and sewage in addition to the digestive systems of both people and animals [7]. Determining the spread of *Proteus mirabilis* from UTI patients in Kut City, Wasit Province, Iraq and characterizing it at the molecular level through analyses of 16S rRNA genes.

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2. Material and methods

In a cross sectional study a total of two hundred and twenty eight urine specimens were collected from patient with different ages admitted to: Al-Zahraa teaching hospital, Al-Karama teaching hospital, Al-Kut hospital for Gynecology obstetrics and pediatrics and from private clinics in Wasit province, Iraq from both sex male and female during a period from 15th of September 2021 to 1st of February 2022 , isolated bacteria have been recognized base on morphological, biochemical tests, API 20E kit and Vitek2 for some isolates

2.1 Molecular Analysis

DNA extraction and assessment of the concentration and purity of extracted DNA: The concentration of the isolates' DNA was estimated using the Nanodrop by placing 1 μ l of the obtained DNA in an instrument for the detection of the concentration in ng/m, and the purity was discovered by observing the OD 260/280 ratio for determining whether the DNA isolates were contaminated with protein.

2.2 PCR Technique

2.2.1 Primer's preparation

Primers were supplied by the manufacturer (Integrated DNA Technologies, USA) as lyophilized powder in Eppendorf tubes (1.5 ml) [2]. Then optimization of the primer, several trials of thermal cycles were done using Mastermix 20x to optimize the PCR reactions to obtain annealing temperature for the primers. For those annealing temperatures were gradient (53,55,57,59,61,62) °C [8]. As illustrated in table (1)

Table 1 Primer's sequence of genes

Primer type	Sequence 5'- 3'	Size (bp)	Reference
16S rRNA	F: CTGCCCGATAGAGGGGATA	383	This study
	R: GGAGTTAGCCGGTGCTTCTT		

2.2.2 Conventional PCR reaction mixture

Twenty microliters of mixture solution of PCR were used as the total volume in the current study as follows

Table 2 The content of mixture conventional PCR working for current study genes

Component	Reaction volume (μ l)
DNA Template	5
Forward primer	1 μ l for each primer
Reverse primer	1 μ l for each primer
Free water DD. W	13
Master mix(20x)	20

2.2.3 Thermocycling Conditions of PCR

Table 3 Thermal cycling program for 16S rRNA gene

Genes	PCR cycling profile	Products size
16S rRNA	<p>30 Cycles</p> <p>25°C 94°C 94°C 59°C 72°C 72°C 4°C</p> <p>5 1 30 5 min. 30 sec.</p>	383bp

2.3 Gel Electrophoresis and Documentation

PCR products and the DNA ladder were loaded in well of (1.5-2) % agarose gel with 1 ul Ethidium Bromide and the electrophoresis runs at (70 or 100) volts. Agarose was removed from tank visualized under U.V light to measure the DNA bands with DNA ladder according to [9].

2.4 Sequencing of PCR amplicons

The resolved PCR amplicons were sequenced by MacroGen Corporation - Korea (MacroGen Inc. Geumchen, Seoul, South Korea) using the identical forward and reverse PCR primers and an automated DNA sequencer (ABI3730XL). BioEdit and MEGA 11 software were used to analyze the obtained ABI sequence files by clearing any unwanted nucleotides sequences. In order to identify the obtained nucleotide sequences, BLAST tool of the NCBI website was employed.

2.5 Interpretation of sequencing data

Using BioEdit Sequence Alignment Editor Software Version 7.1, the sequencing results of the PCR products were edited, aligned, and assessed in accordance with the corresponding sequences in the reference database (DNASTAR, Madison, WI, USA). The detected nucleic acids were assigned numbers in the PCR amplicons and at the appropriate locations in the reference genome. The exact target locations were retrieved using the NCBI – BLAST tool and sequence matches were obtained for each sample.

2.6 Comprehensive phylogenetic tree construction

In this work, a specific comprehensive tree was built using the cladogram building method explained by [10]. Using the NCBI-BLASTn service, the detected variation was compared to its neighbor homologous reference sequences [11]. Based on the Clustal omega suit [12], multiple sequence alignments were made for the retrieved nucleic acid sequences. Subsequently, an inclusive tree was built by the neighbor-joining method and visualized using BioEdit and MEGA 11 software. The observed variants as well as its corresponding reference sequences were incorporated in the constructed comprehensive cladogram. The obtained phylogenetic tree was optimized to be properly presented.

3. Results and discussion

The results of identification revealed the detection of 40 (17.5%) isolates belong to *Proteus mirabilis* out of 228 urine samples collected from patient suspected UTIs with different ages admitted to: Al-Zahraa teaching hospital, Al-Karama teaching hospital, Al-Kut hospital for gynecology obstetrics and Pediatrics, and from private clinics in Wasit province. The isolates were first determined to be members of the Proteus genus by the swarming phenomenon on blood agar, the cultures, distinctive smell, and the pale appearance of bacteria (non-lactose fermenting) on MacConkey agar and convex, round, and smooth colonies with a distinct fishy smell [13]. Additionally, through microscopic analysis of the bacteria, which were gram-negative and looked as straight rods when stained with gram stain. Several common biochemical assays were run on the presumed *Proteus mirabilis* isolates to learn more about them. All of the isolates tested positive for catalase, urease, citrate, and motility, but they all tested negative for indole and the oxidase test. These results coincide with mentioned by [14–17]. Also, the findings of this investigation match those of [18], who identified *Proteus mirabilis* from urinary tract infections. Under the right circumstances, *Proteus mirabilis* can produce a range of opportunistic nosocomial infections [19]. This disease can infect numerous anatomical areas of the body due to its varied modes of transmission [19], including those of the urinary tract causes complicated UTIs with a higher

frequency compared to other uropathogens and formation of urinary stones [20]. Further confirmation done using API 20E system depending on 20 biochemical tests related with *Proteus mirabilis* metabolism activities. Further confirmation done using Vitek2. Additionally, *Proteus mirabilis* identification using the Vitek2 system confirmed that all isolates were *Proteus mirabilis*. The VITEK 2 technology found bacteria more quickly, more effectively, and without contamination. [21].

Table 4 Morphological and biochemical tests for identification of *Proteus mirabilis*

Biochemical test	Result
Gram-stain	G-ve bacilli
Oxidase	Negative
Catalase	Positive
Indole	Negative
Vogas-Proskauer	Negative
Citrate Utilization	Positive
Triple-Sugar Iron Agar	K/A++
Methyl Red	Positive
H2S production	Positive
Motility	Positive swarming
Urease	Positive

(+) Positive test, (-) Negative test, (K) Alkaline, (A) Acid.

3.1 Molecular analysis

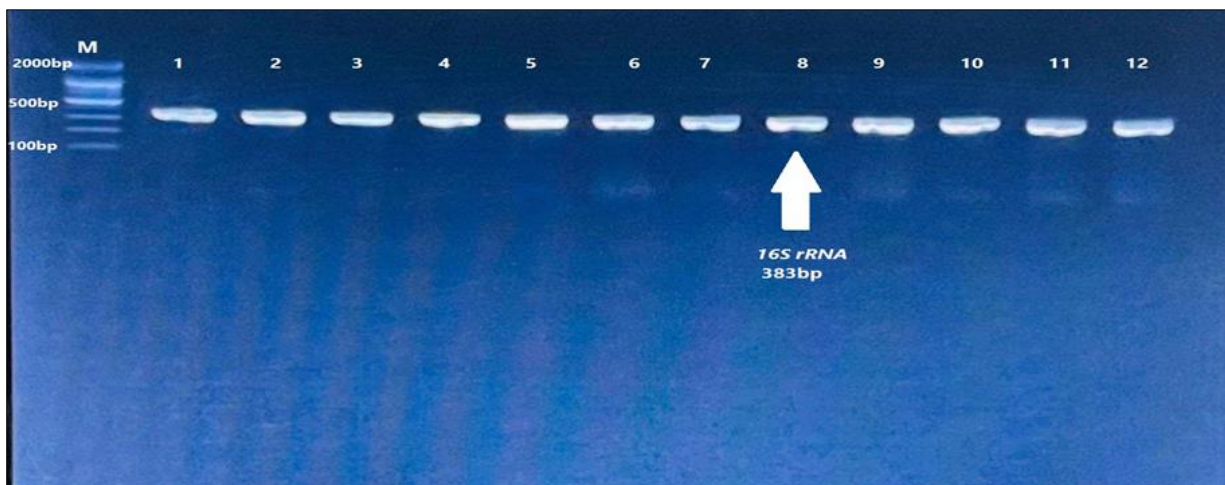


Figure 1 Using standard PCR, the 16S rRNA gene from *Proteus mirabilis* was amplified and then electrophoresed. Agarose 1.6%, 100 V/cm for 25 min, ethidium bromide dye staining, and UV transilluminator visualization. Lane (M): 2000-bp DNA ladder Lane (1–12): 16S rRNA gene amplicons

Forty isolates' DNA was extracted, and Nanodrop was used to check the purity and concentration. The sample included nucleic acid with a purity of (1.8-2) and a concentration of (50-360 mg/ul). A useful indication of the extraction process is purity DNA, which also verifies the absence of any contaminants that can obstruct the procedure [22].

Results revealed that all isolates were *Proteus mirabilis*, 16S rRNA gene sequencing has become prevailing in medical microbiology as a quick and inexpensive alternative to traditional methods of bacterial identification and more confident [23]. The 16S rRNA gene can be utilized as a substitute method for identifying bacteria down to the species level since it is highly conserved both within and across species of the same genus [24,25]. according to Figure 1.

3.2 Results of sequencing reactions

Within this locus, one sample was included, which showed approximate length of the ribosomal fragment. By using NCBI BLASTN, the sequencing reactions revealed the amplified product's verified identification. The sequenced sample and *Proteus mirabilis* sequences shown a significant degree of sequence similarity in the NCBI BLASTN engine. The NCBI BLAST engine found that these predicted targets covered the defined regions of the rRNA gene sequences with roughly 99% identity.

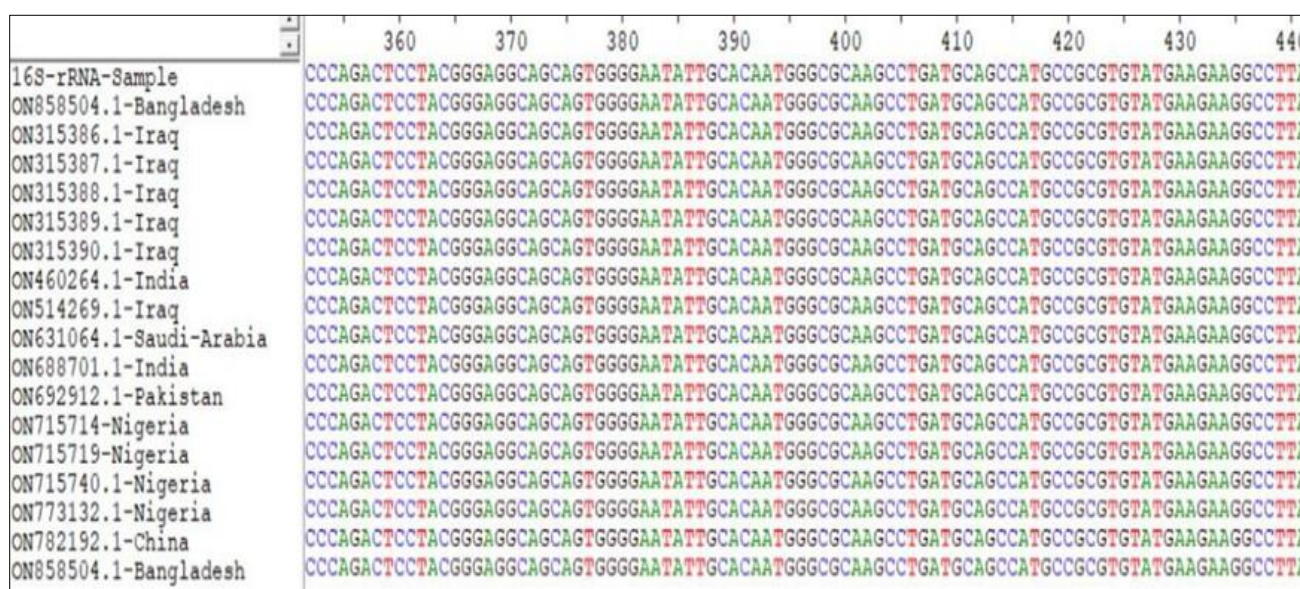


Figure 2 Nucleic acid sequence alignment of isolated bacterial sample with the most relevant deposited genomic sequence

Based on the examined 16S rRNA sequences in the examined bacterial samples, a thorough phylogenetic tree was produced. This phylogenetic tree included our screened bacterial sample together with the other deposited DNA sequences, linked with their closely related sequences in a neighbour-joining manner. This complete tree required the presence of a single distinct species, which corresponds to the tree's only included nucleic acid sequence. *Proteus mirabilis* was this species. The BLAST program placed this sample close to the Gene Bank accession numbers from several sources of *Proteus mirabilis* that had been deposited from various international sources. This sample has similar match with two Gene Bank accession numbers of (ON315387.1), which were belonged to Iraq strain of the same species. Additionally, the sample also resided beside two GenBank accession numbers of (ON315388.1 and ON315390.1) respectively, that belong to the same bacterial species deposited from Iraq. It is important to mention that our isolate alignment gave high similarity (around 94%) with bacterial isolates in NCBI database including mirabilis and rest belong another *Proteus spp.* When doing dendrogram for the isolate result showed that it has unique clone as compared to all other similar sequences Figure (3).

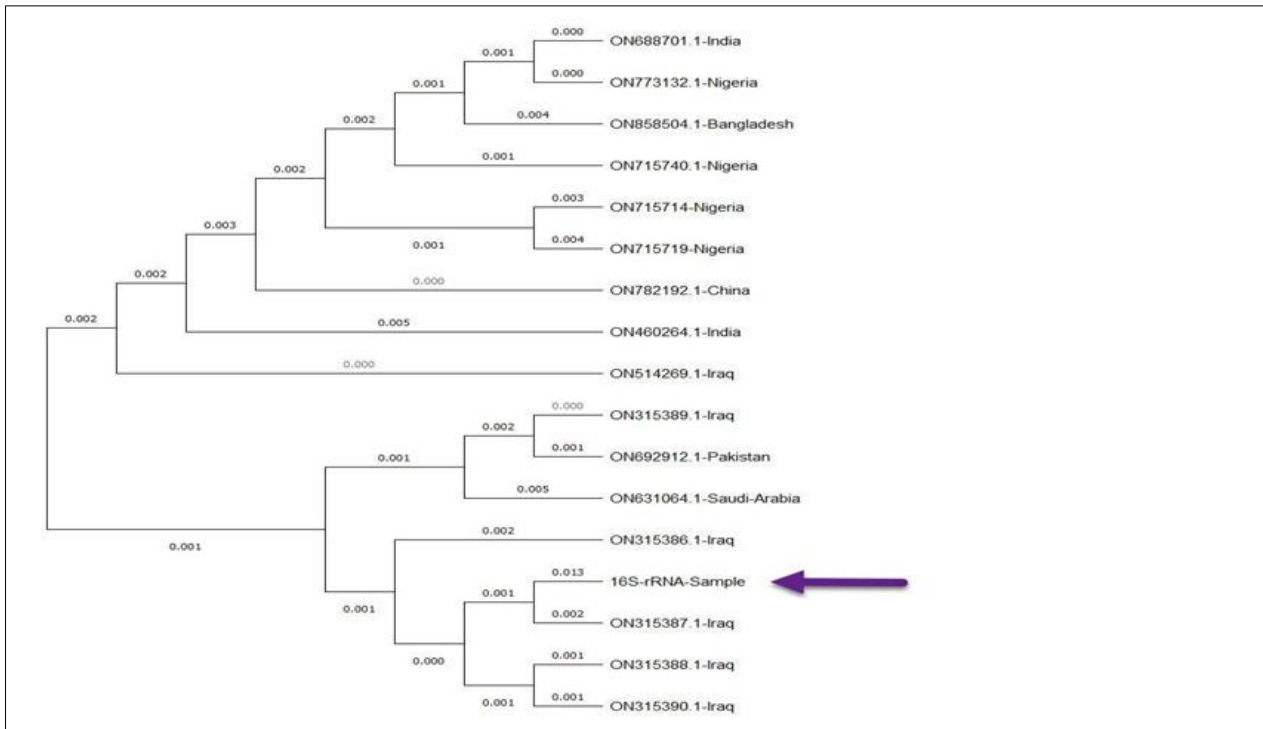


Figure 3 The comprehensive phylogenetic tree of the *16S rRNA* sequences from NCBI database and sequences from the tested sample

4. Conclusion

Proteus mirabilis was a significant pathogen responsible for a number of infections, particularly urinary tract infections (UTI), for which it accounted for 17.5% of all bacterial isolates. Phylogenetic tree analysis revealed that the *16S rRNA* gene had a good selection capacity to identify isolated *Proteus mirabilis* and the strain that was closest to the Iraqi strain.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The author hereby declares that there are no conflicts of interest concerning this paper.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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