

# Genotyping of New and Old *Proteus mirabilis* Isolates from Mansoura Hospitals in Egypt by *rpoB* Sequence Analysis

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## Abstract

Sequence analysis of the RNA polymerase B subunit encoding gene (*rpoB*) has been proposed as a useful tool for bacterial identification. This method has been implemented to differentiate five well-defined *Proteus* species: *P. mirabilis*, *P. hauseri*, *P. penneri*, *P. vulgaris*, and *P. myxofaciens*. In this study, we evaluated the usefulness of *rpoB* sequencing for intraspecies discrimination of *P. mirabilis*. The sequence of *rpoB* 909 bp region was analyzed in 15 newly isolated strains and 5 of 8 years old isolates from different clinical sources. Three respective groups were obtained. The first group included 13 of the new strains showing similarity with *Proteus mirabilis* (ATCC 29906) strain. The second group including 3 of the old strains differs from the first group with a divergence of 0.22%. Group 3 contains only a single new strain 33. The sequence of this strain shows differences in the *rpoB* 909 bp region analyzed from the members of group 1 and the second group by 1.65% and 1.87% divergence respectively. According to our results, genetic differences could be detected within the *P. mirabilis* species. Therefore much more effort should be made to re-evaluate *rpoB* method and validate its usefulness as a molecular diagnostic method.

## Keywords

**Proteus, Genetic Analysis, *rpoB* Sequencing, Ribotyping**

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## 1. Introduction

As a member of the *Enterobacteriaceae*, *Proteus* sp. constitutes a part of the normal flora of the intestinal tract of both humans and animals. Opportunistic infections, commonly found in the human intestinal tract, are usually associated with *Proteus*. Catheterization and surgery of the urinary tract are considered as predisposing factors

for urinary tract infections in hospital patients.

The genus *Proteus* consists of five species (*P. hauseri*, *Proteus myxofaciens*, *Proteus penneri*, *Proteus mirabilis*, and *Proteus vulgaris*) [1]. Three species namely *Proteus mirabilis*, *P. penneri* and *P. vulgaris* are associated with most common causes of complicated urinary tract infections [2] [3] and are notable for their swarming abilities, which are directly linked to their ability to cause diseases [2] [4]. *Proteus mirabilis* strains account for about 10% of uncomplicated urinary tract infections [5], and they are one of most common causes of nosocomial urinary tract infections and sepsis in patients [3] [6]. Furthermore, *P. mirabilis* has been implicated in cases of empyema [7], neonatal meningoencephalitis [8], osteomyelitis [9], and bacteremia [10].

In Egypt, *P. mirabilis* constitutes the third most commonly isolated pathogen of urinary tract infections after *Escherichia coli* and *Klebsiella pneumoniae* [11]. For both taxonomic and epidemiological studies of the genus *Proteus*, several phenotypic typing methods such as serology and phage typing have been used in the past for identification of *Proteus* species. In recent years, molecular typing methods were implemented for investigation of *Proteus* species. Sequencing of 16S rRNA gene [12] has been used. However, divergence was not suitable to detect some of *Proteus* species among clinical isolates. Sequence analysis of the RNA polymerase  $\beta$  subunit encoding gene (*rpoB*) has been proposed as a useful tool for bacterial identification [13]. In this study, *rpoB* sequencing is examined as a tool for intra-species discrimination of *Proteus mirabilis* clinical isolates.

## 2. Materials and Methods

### 2.1. Bacterial Strains

Bacterial strains: A total of 20 bacterial isolates were obtained from Mansoura Hospitals in Egypt including 15 new isolate: (5) wound, (1) oral swab, (1) sputum, (4) stool, (2) Endo-tracheal and (2) urine and (5) seven years old Urine samples (Table 1).

**Table 1.** Different *Proteus mirabilis* isolates, and their *rpoB* gene accession numbers, age and source of isolation.

Strain number	Source	GenBank number	Age
2	Wound	(KR083867)	New
7	Wound	(KR083868)	New
8	Wound	(KR083869)	New
9	Wound	(KR083870)	New
12	Oral swab	(KR083871)	New
14	Sputum	(KR083872)	New
15	Endo-tracheal tube	(KR083873)	New
48	Stool	(KR083874)	New
54	Stool	(KR083875)	New
58	Stool	(KR083876)	New
78	Urine	(KR083877)	New
55	Stool	(KR083883)	New
11	Endo-tracheal aspirate	(KR083884)	New
33	Urine	(KR083886)	New
19	Wound	(KR083885)	New
303	Urine	(KR083878)	Old
313	Urine	(KR083879)	Old
302	Urine	(KR083880)	Old
305	Urine	(KR083881)	Old
346	Urine	(KR083882)	Old

## 2.2. Molecular Identification of *Proteus* Isolates

### 2.2.1. Amplification of *rpoB* Gene

Verification of the genus and species was confirmed by the molecular identification which was carried out by polymerase chain reaction (PCR) amplification of *rpoB* gene. *Proteus* isolates were grown on nutrient agar at 37°C for overnight then the colonies were suspended in 300 µl of distilled water and boiled for 10 min. The supernatant was transferred into a new tube and used as DNA template.

A portion of the coding region of the *rpoB* gene was amplified with primers CM7 (59-AACCAGTTCCGC-GTTGGCCTGG-39) and CM31b (59-CCTGAACAACACGCTCGGA-39), according to the technique described by Mollet *et al.* (1997). The PCR amplicons generated were sequenced with primers CM81 (59-CAG-TTCCGCGTTGGCCTG-39) and CM31b (seq) (59-TGAACAACACGCTCGG-39) (Mollet *et al.*, 1997) to obtain partial *rpoB* gene sequences. A reaction mixture containing 0.5 µM of each primer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 1 U Taq polymerase (Thermoscientific Dream Taq Green DNA polymerase), 5 µl of template DNA and nuclease free water was added for a total volume of 25 µl per reaction. The PCR was performed under the following conditions: 2 min. initial denaturation at 94°C, 35 cycles of denaturation (45 s at 94°C), annealing (60 s at 55°C), and extension (60 s at 72°C); a final extension at 72°C for 10 min. Finally, PCR product was analyzed by electrophoresis through 1% agarose gel and then visualized under UV light by staining with ethidium bromide.

### 2.2.2. Determination of the Gene Sequence of *rpoB* Gene

Amplified *rpoB* gene fragments were purified from 20 strains using the PCR Purification Kit (MEGA quick-spin fragment DNA purification INTRON biotechnology, Sangdaewon-dong, Korea) for subsequent Sequencing. Purified PCR products were used as a template in sequencing reactions carried out with the ABI PRISM® BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Bio-systems, Foster City, USA). The reaction mixtures were analysed on an ABI 3730 DNA analyzer (Applied Bio-systems, Foster City, USA). Amplicons were sequenced on both strands and predicted peptide sequences analyzed by the online BLAST of the NCBI website software (<http://www.ncbi.nlm.nih.gov/BLAST/>).

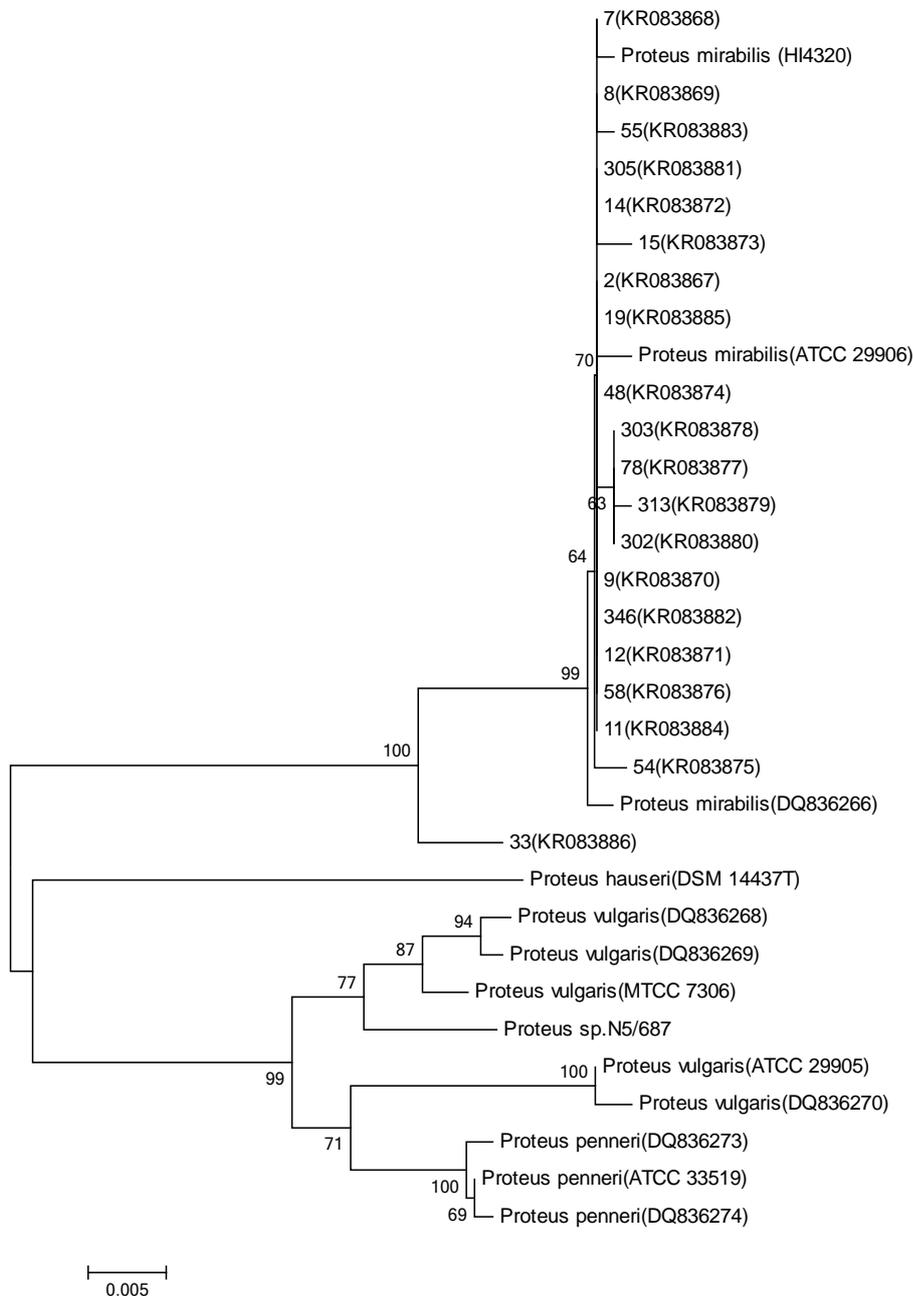
Sequence alignment and phylogenetic analysis was carried out on 909 bp of *rpoB* gene fragments using the CLUSTAL W algorithm (Thompson *et al.*, 1994). To ensure the stability and reliability of phylogenetic relationships among strains used in this study, phylogenetic trees were reconstructed by using the Neighbour-joining and Maximum-parsimony methods in MEGA 4.1 package.

Sequences of this study were deposited under the following accession numbers (**Table 1**): KR083867, KR083868, KR083869, KR083870, KR083871, KR083872, KR083873, KR083874, KR083875, KR083876, KR083877, KR083878, KR083879, KR083880, KR083881, KR083882, KR083883, KR083884, KR083885, and KR083886.

## 3. Results

### *rpoB* Sequence Analysis for *Proteus* Species Identification

The phylogenetic tree is derived from partial (909 bp) *rpoB* gene sequences of clinical strains of *Proteus mirabilis* (**Figure 1**). By analyzing partial *rpoB* DNA sequences, a phylogenetic tree was derived from *rpoB* sequencing results with >1000 bootstrap values. As a result, six *rpoB* groups could be described. All strains of our study were almost grouped in three respective branches more closely related to *Proteus mirabilis* reference strains installed from the GenBank. One branch (group 1) included the following 13 new strains: 14, 2, 12, 8, 55, 9, 15, 5, 7, 48, 11, 19 and 54 in addition to 346 and 305 showing similarity to *Proteus mirabilis* (ATCC 29906) strain. The other branch (group 2) differs from the first branch with a divergence of 0.22% including three old strains: 313, 302 and 303 in addition to the new isolate 78. Last branch (group 3) contains only a single newly isolated strain 33. The sequence of this strain shows differences in the *rpoB* 909 bp region analysed from the members of branch one and the second branch by 1.65% and 1.87% respectively. Strains of group 1 differs from *P. penneri*, *P. vulgaris* (MTCC 7306), *P. vulgaris* (DQ836268), *P. vulgaris* (ATCC 29906) and *P. hauseri* with a divergence 6.4%, 6.38%, 6.6%, 7% and 6.7% respectively. Strains of group 2 differs from *P. penneri*, *P. vulgaris* (ATCC 29906), *P. vulgaris* (DQ836268), *P. vulgaris* (MTCC 7306) and *P. hauseri* with a divergence 6.6%, 7.2%, 6.7%, 6.5% and 6.7% respectively. *P. mirabilis* strains of group 3 showed 5.72% and 6.27% nucleotide



**Figure 1.** Phylogenetic analysis of partial nucleotide sequences of the *rpoB* gene of reference and clinical strains of the genera *Proteus*. The tree was generated using the Neighbour-joining method.

differences compared to *P. penneri* and *P. hauseri* strains. This group differs by 5.61%, 6.05%, 6.49% from *P. vulgaris* (MTCC), *P. vulgaris* (DQ836268) and *P. vulgaris* (ATCC 29906).

#### 4. Discussion

The conventional typing methods are based on the presence of specific bacterial surface structures, which may change during the course of chronic infection [14]. Conventional typing methods, if applied alone, may sometimes lead to miss-classification of *Proteus* strains from different clinical source.

Identifying different types of organisms within a species is called typing. Traditional typing systems based on

phenotype, such as anti-biogram, biotype, serotype and phage-type have been used for many years. Genotyping is mainly used to link the epidemiologically related isolates collected during an outbreak of nosocomial disease to one another. In other cases, genetic events (mutation, plasmid acquisition, etc.) may occur during the outbreak, so it may not be enough to know if strains are identical or not but rather may be necessary to know how related (or not) are the isolates [15]. In addition, 16S rRNA gene sequencing followed by phylogenetic analysis of members of the family Enterobacteriaceae is associated usually with taxonomic problems which cannot be easily resolved because of the high degree of conservation in closely related species [16]. As an example, *P. penneri* and *P. vulgaris* could not be easily differentiated by this method [17].

For this reason, the *rpoB* gene, encoding the RNA polymerase  $\beta$ -subunit, a highly conserved housekeeping gene is now used as a tool for molecular identification. Partial *rpoB* gene sequencing and analysis was implemented to provide more sensitivity [13]. In addition, it was previously reported that upon comparison of *rpoB* and 16S rRNA sequences more than 2.5% nucleotide divergence sequencing data of genera *Proteus*, *Morganella* and *Providencia* could be produced allowing the definition of *rpoB* clades [18]. This method was used to test the divergence of 14 clinical strains of species of the family Enterobacteriaceae, including type strains of *P. mirabilis* [13]. In the same manner, *rpoB* gene sequence was used to test the intra-species discrimination of *rpoB* sequences of the genus *Proteus* [19].

Our 20 *Proteus mirabilis* strains isolated in this study were almost grouped in three respective branches. One branch (group 1) included the following new strains: 14, 2, 12, 8, 55, 9, 15, 5, 7, 48, 11, 19 and 54 in addition to 346. The other branch (group 2) differs from the first branch with a divergence of 0.22% including three old strains: 313, 302 and 303 in addition to 78. Last branch (group 3) contains only a single strain 33. The sequence of this strain shows differences in the *rpoB* 909 bp region analysed from the members of branch one and the second branch by 1.65% and 1.87% respectively. This is in contrast to another study [19], as *Proteus mirabilis* strains were grouped into one designated group. However, in the same study [19], two groups of *P. vulgaris* sequences could be described, one of them including the new strain type, differing from each other by 3.3% - 3.6% of their nucleotides and for both of these there was a nucleotide difference of 6.5% - 6.8% from the standalone former *P. vulgaris* strain type. These results provide further evidence for the existence of genetic differences within *P. vulgaris rpoB* sequences.

Moreover, in a previous study [20], strain types of four species of the genus *Proteus* showed remarkably different rRNA gene restriction patterns after digestion with *EcoRV* and *HincII* 17. As a result, different ribo-groups could be defined depending on the number and size of the bands observed among clinical strains. Strains of *P. mirabilis* had identical profiles to those of their respective type strains. Neither old nor new strains have their own discriminatory group. However, in strains belonging to other species such as *P. vulgaris*, distinct ribo-groups could be defined, as these strains showed a considerable pattern did not sufficiently perform variability.

## 5. Conclusions

In conclusion, *rpoB* sequencing proved able to characterize the different species of the genus *Proteus* on a molecular basis. However, our results provide further evidence for the existence of genetic differences within *P. mirabilis*. These findings indicate that *rpoB* sequencing method is not quite sufficient for fine typing and epidemiological purposes.

It appears that other new typing methods using another housekeeping gene must be further tried.

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