Original Article

Molecular Analysis of Virulence Genes HpmB and rsbA among *proteus* Species Isolated from Different Infectious Cases in Iraq

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How to cite this article: **Mathlum Al-Khafaji K, Ayyal Mutar Alrashedi A, Al-Rubaeaee AA. Molecular Analysis of Virulence Genes HpmB and rsbA among *proteus Species* Isolated from Different Infectious Cases in Iraq. Archives of Razi Institute. 2023;78(4):1295-303.**

DOI: 10.32592/ARI.2023.78.4.1295

Article Info:
Received: 10 December 2022
Accepted: 20 December 2022
Published: 31 August 2023

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ABSTRACT

Proteus species (spp.) is considered one of the widely spread pathogens worldwide. *Proteus* spp. can be detected in contaminated water, soil, and manure, aiding the decomposition of organic substances from animals. *Proteus* is a gram-negative bacterium that causes a wide range of human illnesses. This study aimed to find some virulence genes in *Proteus* spp. from different sources, including the laboratories of government hospitals in Karbala, Al-Hussies, and Al-Muthanna, Iraq. Fifty swab samples were collected from patients' wounds, ears, and sputum. Clinicians collected swab samples for identification. In total, 17 sputum samples, 13 ear samples, and 20 wound samples were collected from 27 (54%) females and 23 (46%) males. The virulence genes *hpmB* and *rsbA* were identified after the genomic diagnosis of *Proteus* spp. Thirteen *Proteus* isolates were identified using the *hpmB* primer, and 16 isolates were identified using the *rsbA* primer. The DNA sequence analysis of *rsbA* and *hpmB* genes revealed that all samples shared 99.52% identity for the *rsbA* gene, whereas the *hpmB* gene differed from one sample to the next. The sequence results are available at the NCBI under the accession numbers (LC661938) and (LC661939), respectively.

Keywords: Antimicrobial resistance, PCR, *Proteus* species, Virulence gene

1. Introduction

Proteus is a considerable threat to animals and humans around the world. It may be found in various foods and animal sources (1). This bacterium has several resistant variants, indicating a major food hazard (2). Control strategies and preventive measures are essential to avoid and combat the outbreaks of forborne diseases from chicken, given the numerous outbreaks of infectious diseases linked to the *Proteus* set of bacteria and the growing likelihood of *Proteus*-related contaminated food (3). *Proteus*, a member of the *Enterobacteriaceae* family, was recognized for perhaps the first time by Hauser in 1885. It belongs to the family *Proteaceae*, which also includes the genera *Providencia* and *Morganella* (4). The gram-negative, motile, aerobic, rod-shaped bacteria seen in *Proteus* spp. range in size from 0.3 to 1.0 m in width and 0.6 to 6.0 m in length (5). *P. vulgaris*, *P. mirabilis*, *P. hauseri*, *P. myxofaciens*, and *P. penneri* are the five species that belong to the genus *Proteus* (6). One of the most critical factors of virulence possessed by this type of germs is the phenomenon of swarming, which is expressed as the movement of bacterial cells in the form of waves. This movement helps in spreading through diffusion in the urinary tract in cases of injury. Other urinary tracts are among the factors of virulence in these bacteria. The proteolytic enzyme UTIs works to break down the peptide bonds of proteases in the protein, which link the amino acids and have several classes and thiol proteases (7). Most of the metalloprotease isolates of these bacteria are also characterized by their ability to adhere (6). Hemolysin isolates, which break down red blood cells, are produced by this bacterium. Hemolysis does not stop at the lysis of red blood cells, but in some species (spp.), it works on the analysis of lymphocytes and can stop the process of phagocytosis, as well as the chemical attraction of neutrophil cells (8). The sensitivity of *Proteus* spp. is influenced by several factors, all of which have been controlled and expressed via virulence genes transcribed in operons (7). These pathogenic genes increase the virulence of *Proteus* spp., such as urease, the most important enzyme in the creation of kidney and bladder stones (4, 9), and allow it to thrive (10). The *luxS* gene is involved in making autoinducer 2 (8, 10, 11), a bacterial protein that is used to convey cell density and metabolic potential. *P. mirabilis* swarming behavior is governed by the *rsbA* gene, which has been connected to biofilm generation and extracellular polysaccharide creation (12). Regular monitoring of chicken farms is required to determine the likely region of microbiological infections that have spread into the food supply. Urease is so important in *P. mirabilis* virulence. The urease operon transcript is effectively stimulated by *ureR*, a dimer of unique 293 amino acid polypeptides that bind urease, causing the protein to bind strongly to *ureR* and *ureD* promoters. The *ureR* then activates RNA polymerase, causing transcription to begin. The goal of this study was to use Polymerase Chain Reaction (PCR) to determine the identification of *Proteus*-linked virulence genes (13).

2. Materials and Methods

2.1. Sampling

From April to August 2021, 50 samples were collected from patients at Al-Hussies, Al-Muthanna, and Karbala hospitals. Wounds, ears, and sputum swab samples were collected from patients whose identities were confirmed by doctors. The samples were immediately streaked over blood agar and MacConkey agar and incubated at 37°C for 24 h.

2.2. First Diagnostic Step

The isolates were recognized visually and biochemically. They were first identified based on the shape of the colonies on the MacConkey medium and blood agar, as well as the observation of the swarming phenomenon. Since a Gram stain was applied, they were also diagnosed based on morphological traits under a microscope (9).

2.3. Molecular Detection

The extraction of DNA was conducted using an EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech, China), according to the manufacturer's instructions. The DNA was extracted from culture media

that contained suspended bacterial cells. The PCR was used to examine the virulence genes of *Proteus* spp. The primer sets (5' TTGAAGGACGCGATCAGACC3') and (3' ACTCTGCTGTCCTGTGGGTA5') were used to amplify a 467-bp sequence of the *rsbA* gene, and (5' CAGTGGATTAAGCGCAAATG-3') and (5' CCTTCAATACGTTCAACAAACC-3') were used to amplify a 422-bp sequence of the *hpmB* gene. The primers were prepared by dissolving the lyophilized primers in the TE (Tris-EDTA) buffer to create a stock solution containing 100 mmole/mL, as directed by the manufacturer. The working solution for each primer was created by diluting the stock solution with TE buffer to obtain the final working solution (10 mmole/mL) after spinning it down. It was reserved overnight at 4°C. The PCR master mix reaction was performed using a OneTaq® Quick-Load PCR kit (New England Biolabs, USA), according to the manufacturer's instructions, as shown in table 1.

Table 1. Showed AProtocol for OneTaq® Quick-Load 2× Master Mix with Standard Buffer (M0486)

Component	25µl reaction
10 µM Forward Primer	0. 5 µl
10 µM Reverse Primer	0. 5 µl
Template DNA	1. 5 µl
OneTaq Quick-Load 2X Master Mix with Standard Buffer	12. 5 µl
Nuclease-free water	10 µl
Total	25 µl

Following that, the previously mentioned PCR master mix component was inserted in the PCR tubes containing the multiplex PCR while the lyophilized materials contained all other PCR reaction components, such as Taq DNA polymerase, dNTPs, and 6 mM $MgCl_2$, with a pH of 8.7. The tube was centrifuged for 3 min in an ExiSpin vortex centrifuge (Bioneer, South Korea). It was then transferred to a multigene PCR thermocycler to be processed further. Table 2 displays the PCR thermocycler parameters for each gene when using a standard PCR thermocycler setup. Three samples were sequenced using PCR by the Macrogen Company in South Korea. Nucleotide substitutions were discovered by matching data from the GenBank publications available at the NCBI (https://www.ncbi.nlm.nih.gov). The results were registered in the NCBI under the registration number (LC661938), which is available on the website (https://www.ncbi.nlm).

Table 2. Showed pcr thermo cycler conditions in this study

Steps	Temperatures	Time
Initial Denaturation	94°C	30 sec
30 Cycles	94°C variable (as mentioned below every photo) 68°C	15-30 sec 15-60 sec 1 minute per kb
Final Extension	68°C	5 min
Hold	4-10°C	

3. Results

3.1. Identification of *Proteus* spp.

Fifty samples were collected from patients' wounds, ears, and sputum. Clinicians collected swab samples for identification. In total, 27 (54%) females and 23 (46%) males provided 17 sputum samples, 13 ear samples, and 20 wound samples. Table 3 shows that 16 isolates were obtained using the *rsbA* primer and 13 using the *hpmB* primer.

The DNA was extracted from culture media containing suspended bacterial cells using an EasyPure® Bacteria Genomic DNA Kit (TransGen Biotech, China). It was then identified using agarose gel electrophoresis, as shown in figure 1A. The results were identified using 1% agarose electrophoresis and UV light exposure, indicating that all isolates produce the same result for biochemical diagnosis, regardless of the primer type. The PCR products of *rsbA* and *hpmB* primers showed 467 bp at 55°C and 422 bp at 55°C, respectively, after being optimized by gel electrophoresis with different temperatures and primers (Figure1B). The virulence genes *hpmB* and *rsbA* were identified through the molecular investigation of *Proteus* spp., as shown in figure 2A and 2B).

Table 3. Showed samples positive with *proteus* spp.

NO	Primer name	PCR product	TM	Total samples	Positive Samples	Negative Samples
	rsbA	467bp	59c	25	16	34
	HpmB	422bp	55c	25	13	37

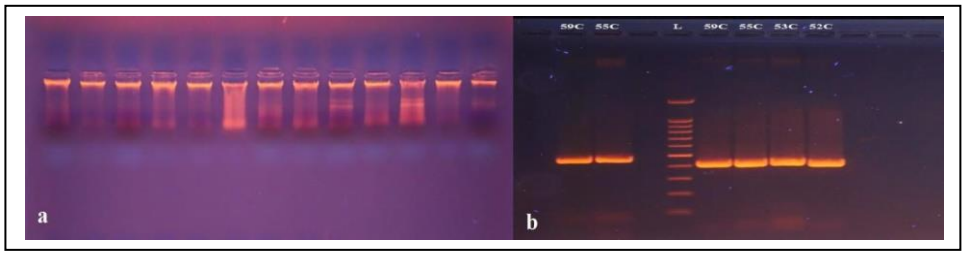

Figure 1. (a) Depicts an ethidium bromide-stained agarose gel electrophoresis appearance displaying DNA extracted from bacteria. **(b)** Gel electrophoresis for optimization process with different temperatures and different Pimers for PCR product of rsbA and HpmB primer which shows (467bp at 55°C and 422bp at 55°C) respectively (Agarose 1%, 10 min at 100 voltage and then lowered to 70 volts, 60 min). Lane L: 100-1500 bp DNA ladder

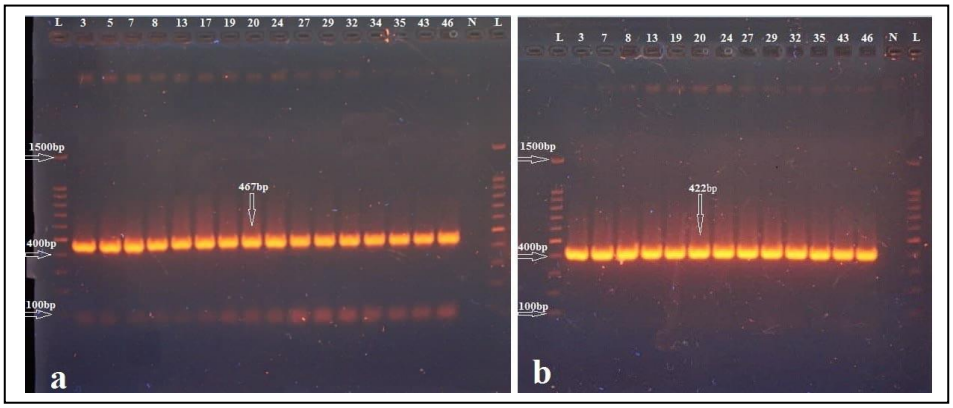

Figure 2. (a) Gel electrophoresis for the PCR product of the *rsbA* primer, which shows 467 bp at 55 °C (agarose 1%, 10 min at 100 volts, and then lowered to 70 volts, 60 min.) visualized under UV light after staining with ethidium bromide. Lane L: DNA ladder (1500-100) bp; Lanes 3, 5, 7, 8, 13, 17, 19, 20, 24, 27, 29, 32, 34, 35, 43, and 46: positive results; Lane N: negative control. **(b)** Gel lectrophoresis for PCR product of (*HpmB* primer) which shows 422bp Primer TM at (55C), (Agarose 1%, 10min.at 100 voltage and then lowered to 70 volts, 60 min). Visualized under U. V light after staining with ethidium bromide Lane L: DNA ladder (1500-100)bp , Lanes (3, 7, 8,13,19, 20, 24, 27, 29, 35, 43 and 46) represented positive results, Lane (N) represented Negative control

3.2. DNA Sequence Analysis of *rsbA* Gene

Three samples were sequenced using PCR sequences by the Macrogen Company in South Korea. The findings were submitted to the NCBI under the accession number LC661938 and can be found at the following link: https://www.ncbi.nlm.nih.gov/nocore/LC661938.1 Sample 1 had 99.52% identity when *rsbA* was matched to the genes with Sequence ID CP053684.1. Compared to the same genes in the CP053718.1, sample 2 had 99.52% identity for *rsbA*, while sample 3 had 99.52% identity for *rsbA* when matched to the same genes in the CP045257.1. Based on our results, the *rsbA* gene sequence analysis revealed three polymorphisms in the *rsbA* gene in sample 1. There was a nucleotide deletion at loci 2944607, 2944613, and 2944640. The *rsbA* gene sequence analysis also revealed two polymorphisms in the *rsbA* gene in sample 2. A nucleotide deletion was discovered at loci 1592842 and 1592869. The *rsbA* gene sequence analysis in sample 3 revealed two changes in the gene. A nucleotide deletion was discovered at locus 2461454, while a nucleotide insertion was discovered at locus 2461481. However, as shown in table 4, impact variation altered the sequence of amino acids in the protein, changing its function or causing the protein produced by the gene not to function normally.

Table 4. Type polymorphism sequence of in the rsbA gene in *Proteus* mirabilis

No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
1	Deletion	2944607	A>Gab(-)	GTA>GT-	Aspartate> No functional protein	The protein made by the gene may not function properly	2944601 to 2945021	CP053684. 1	760 bits (411)	419/422 (99%)	Proteus mirabilis rsbA
	Deletion	294461	A>Gab(-)	CGA>CG-	Arginine > No functional protein	The protein made by the gene may not function properly	2944601 to 2945021	CP053684. 1	760 bits (411)	419/422 (99%)	
	Insertion	2944640	Gab(-)>A	GT- >GTA	No functional protein > Aspartate	The protein made by the gene may not function properly	2944601 to 2945021	CP053684. 1	760 bits (411)	419/422 (99%)	
2	Deletion	1592842	A>Gab(-)	CGA>CG-	Arginine > No functional protein	The protein made by the gene may not function properly	1592427 to 1592838	CP053718. 1	750 bits (406)	411/413 (99%)	Proteus mirabilis rsbA
	Insertion	1592859	Gab(-)>A	GT- >GTA	No functional protein > Aspartate	The protein made by the gene may not function properly	1592427 to 1592838	CP053718. 1	750 bits (406)	411/413 (99%)	
3	Deletion	2461454	A>Gab(-)	CGA>CG-	Arginine > No functional protein	The protein made by the gene may not function properly	2461450 to 2461862	CP045257. 1	752 bits (407)	412/414 (99%)	Proteus mirabilis rsbA
	Insertion	2461481	Gab(-)>A	GT- >GTA	No functional protein > Aspartate	The protein made by the gene may not function properly	2461450 to 2461862	CP045257. 1	752 bits (407)	412/414 (99%)	

3.3. DNA Sequence Analysis of *hpmB* Gene

Macrogen Company in South Korea sequenced the three samples using PCR sequences. Nucleotide variations were measured by analyzing data from the GenBank publications, which are accessible at the NCBI (https://www.ncbi.nlm.nih.gov). The results were registered in the NCBI under the accession number (LC661938), which can be found on the website (https://www.ncbi.nlm.nih.gov/nuccore/LC661939.1). Sample 1 had 99.20% identity for *hpmB* when matched to the genes with Sequence ID CP053683.1. Sample 2 had 99.95% identity for *hpmB*, compared to the same genes in CP053719.1, and sample 3 had 99.21% identity for *hpmB*, compared to the same genes in CP042857. In sample 1, the findings of the *hpmB* gene sequence analysis revealed three polymorphisms, as shown in table 2. Nucleotide deletion was discovered at loci 322785, 322793, and 322811. There were four polymorphisms in the *hpmB* gene in sample 2. Nucleotide deletion was discovered at loci 248428, 248436, and 248454. Nucleotide deletion and insertion were discovered at locus 248739. There were also three polymorphisms in the *hpmB* gene in sample 3. Nucleotide deletions were discovered at loci 281114 and 281112. A nucleotide deletion was also discovered at locus 2811140. However, as shown in table 5, effect variation caused a change in the sequence of amino acids in the protein, which alters the protein's function or causes the protein produced by the gene to malfunction.

3.4. DNA Sequence Analysis of *rsbA* and *hpmB* Genes

A phylogenetic tree is a subset of phylogeny that examines hereditary molecular distinctions, primarily in DNA sequences, to determine an organism's evolutionary relationships based on the *rsbA* gene. A specialized genotyping technology can identify any microorganism's genetic profile, which is as unique as a fingerprint (9). Phylogenies derived from a single gene, on the other hand, should be treated with caution (7). Figure 3 shows a phylogenetic tree built from *rsbA* gene sequences from three clinical strains of *P. mirabilis* samples with varying sequences available at the NCBI.

P. mirabilis (LC661938) is closely related to *P. mirabilis* (CP045257.1, CP053684.1, and

CP053718.1). Figure 4 shows a phylogenetic tree built from *hpmB* gene sequences of three clinical strains of *P. mirabilis* samples and other NCBI sequences, demonstrating that *P. mirabilis* (LC661939) is related to *P. mirabilis* (CP053683.1, CP053719.1, and CP042857. 1). Because they contain a high conservation region that differs between spp., 1.5 kb 16S rRNA sequences have been recognized and widely used in bacterial taxonomy. The *rsbA* gene was discovered to be an effective and acceptable molecular marker for *P. mirabilis* differentiation, which has the potential to be used as an effective molecular tool for determining *P. mirabilis* phylogenetic relationships, as well as a powerful tool for investigating numerous microorganisms. Understanding the regulatory and metabolic structure that connects chromosomal genes requires a thorough understanding of the genome sequence (1). The *rsbA* gene sequence was used as a molecular clock in our study to assess bacterial relationships (phylogeny). The combination of molecular phylogeny with traditional methods, such as morphology, physiology, and biochemistry, could improve bacterial identification (14).

Table 5. Type polymorphism of in the HpmB gene sequence in Proteus mirabilis

No of sample	Type of substitution	Location	Nucleotide	Nucleotide change	Amino acid change	Predicted effect	Range of nucleotide	Sequence ID	Score	Identities	Source
1	Deletion	322785	A>Gab(-)	TCA > TC-	Threonine >No functional protein	The protein made by the gene may not function properly	322780 to 323156	CP053683. 1	676 bits (366)	374/377 (99%)	Proteus mirabilis *rsbA*
	Deletion	322793	A>Gab(-)	TGA>TG-	Arginine > No functional protein	The protein made by the gene may not function properly	322780 to 323156	CP053683. 1	676 bits (366)	374/377 (99%)	
	Deletion	322811	A>Gab(-)	GCA>GC-	Alanine > No functional protein	The protein made by the gene may not function properly	322780 to 323156	CP053683. 1	676 bits (366)	374/377 (99%)	
2	Deletion	248428	A>Gab(-)	TCA>CG-	Threonine > No functional protein	The protein made by the gene may not function properly	248420 to 248799	CP053719. 1	750 bits (406)	376/380 (99%)	Proteus mirabilis *rsbA*
	Deletion	248436	A>Gab(-)	TGA>TG-	Arginine > No functional protein	The protein made by the gene may not function properly	248420 to 248799	CP053719. 1	750 bits (406)	376/380 (99%)	
	Deletion	248454	A>Gab(-)	CGA>CG-	Arginine > No functional protein	The protein made by the gene may not function properly	248420 to 248799	CP053719. 1	750 bits (406)	376/380 (99%)	
	Deletion	248739	A>Gab(-)	CCA>CC-	Proline> No functional protein	The protein made by the gene may not function properly	248420 to 248799	CP053719. 1	750 bits (406)	376/380 (99%)	
3	Deletion	2811114	A>Gab(-)	TCA>TC-	Threonine > No functional protein	The protein made by the gene may not function properly	2811108 to 2811485	CP042857. 1	678 bits (367)	375/378 (99%)	Proteus mirabilis *rsbA*
	Deletion	2811122	A>Gab(-)	TGA>TG-	Arginine > No functional protein	The protein made by the gene may not function properly	2811108 to 2811485	CP042857. 1	678 bits (367)	375/378 (99%)	
	Deletion	2811140	A>Gab(-)	GCA>GC-	Alanine > No functional protein		2811108 to 2811485	CP042857. 1	678 bits (367)	375/378 (99%)	

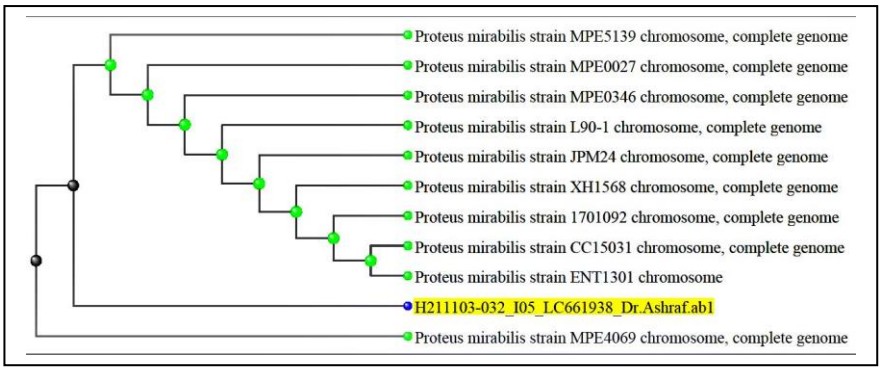

Figure 3. Phylogenetic tree of *Proteus* species based on *rsbA* gene sequence analysis**.**

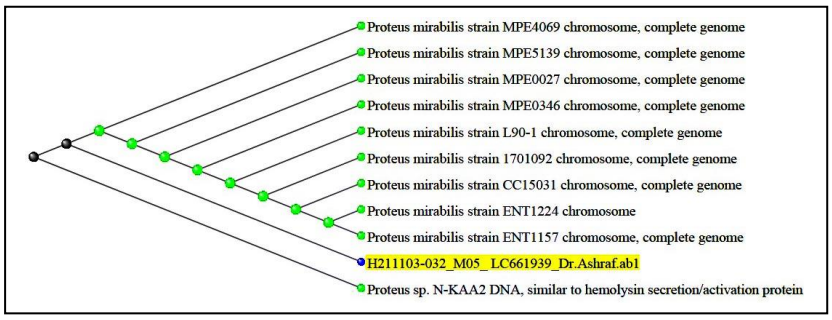

Figure 4. Phylogenetic tree of *Proteus* species based on *HpmA* gene sequence analysis

4. Discussion

Proteus spp. was found in numerous human sources, with no clinical indications (15). Because *Proteus* spp. is an opportunistic infection, healthy turtles can carry the pathogen without becoming ill (16). In this study, 50 samples were collected from patients' wounds, ears, and sputum, including 17 from sputum, 13 from ears, and 20 from wounds. They were collected from 27 (54%) females and 23 (46%) males. When grown on blood agar plates, all typical *Proteus* isolates exhibited swarming motility 100% of the time. *Proteus* spp. has several distinguishing characteristics within the *Enterobacteriaceae* family, such as swarming motility, a coordinated multicellular activity involving flagella, occurring when cells begin on solid, rich media or sticky surfaces (15, 17, 18). Antimicrobials, such as aminoglycosides, amikacin, and gentamicin, were the most effective against *Proteus* spp., with a 95.9% success rate, but tetracycline exhibited the highest resistance (25.7%). According to Omoruyi and Evangelista (14), *Proteus* has moderate resistance to amikacin and gentamicin, as well as overall resistance to tetracycline. *Proteus* spp. resistance to ciprofloxacin was modest (4.1%) in this study, compared to the 36.8% resistance reported by Pearson, Rasko (18). The isolates' lower resistance to ciprofloxacin may be attributed to the drug's small size, which makes it more soluble in diluents and increases its penetration power through the cell into the cytoplasm of the target organism where it acts (19). There were 25 *Proteus* isolates discovered. The *rsbA* gene could not be amplified in *P. vulgaris* or *P. hauseri*. However, the majority of positive strains were *P. mirabilis*, which is consistent with previous research findings (20). All of the studied isolates had phenotypic characteristics of swarming in wounds and possibly the septum. Due to the large number of genes and operons involved in the process, swarming-regulated genes are not required for swarming (8). The findings are consistent with previously published studies (21). It was discovered that all of the isolates produced the urease enzyme. The *hpmB* gene of *P. mirabilis*, which produces hemolysin, is thought to be a major virulence factor. A phylogenetic tree is a subset of phylogeny, based on the *rsbA* gene, which investigates hereditary molecular characteristics, mostly in DNA sequences, to discover an organism's evolutionary relationships. Specialized genotyping tools can detect the genetic profile of a microorganism, which is as unique as a fingerprint (9). On the other hand, phylogenies generated from a single gene should be treated with caution (7). In Harada, Niina (22), a phylogenetic tree was constructed using *rsbA* gene sequences from three *P. mirabilis* clinical strains and other NCBI sequences, demonstrating that *P. mirabilis* (LC661938) is related to *P. mirabilis* (LC661938). In Ghebremedhin, Layer (8), a phylogenetic tree was constructed using *HpmB* gene sequences from three *P. mirabilis* clinical strains with different sequences available at the NCBI, demonstrating that *P. mirabilis* (LC661939) is related to *P. mirabilis* (LC661939). Because they contain a high conservation section with a variable portion in

different species, 16S rRNA sequences with a size of 1.5 kb are assessed and extensively used in taxonomy. The *rsbA* gene was discovered to be an effective molecular marker for *P. mirabilis* specialization, which might be utilized as an alternative molecular tool for assessing *P. mirabilis* genetic relationships, as well as a powerful tool for microorganism research. The genome sequence reveals a great deal about the regulatory and metabolic network that links chromosomal genes (1). In our study, the sequence of the *rsbA* gene was used as a molecular clock to assess the relationships between bacteria (phylogeny). The combination of molecular phylogeny with more traditional techniques, such as morphological, physiological, and biochemical characteristics, could lead to better bacterial diagnosis (14).

Authors' Contribution

Study concept and design: K. M. A.
Acquisition of data: K. M. A.
Analysis and interpretation of data: A. A. M. A.
Drafting of the manuscript: A. A. M. A.
Critical revision of the manuscript for important intellectual content: A. A. M. A.
Statistical analysis: A. A. A.
Administrative, technical, and material support: A. A. A.

Ethics

The study protocol was approved by the ethics committee of the 1. AL Safwa University College, Karbala City – 56001, Iraq.

Conflict of Interest

The authors declare that they have no conflict of interest.

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