Molecular characterization of *Proteus mirabilis* virulence factors isolated from patients with otitis media in Diwaniyah, Iraq

**ABSTRACT**

The current study's goal was to identify virulence factors (genes) in *P. mirabilis*. A total of 25/100 (25%) *Proteus mirabilis* were obtained from patients with otitis media and identified using culture and biochemical characteristics, and Polymerase chain reaction (PCR). The PCR technique was used to detect the 16S rRNA gene and all virulence factors, including the *ureC* gene, which encodes urease synthesis, the *flaA* gene, which encodes flagella and fimbriae production, the *hpmA* gene, which encodes hemolysin production, and the *zapA* gene, which encodes protease. The results revealed that *Proteus mirabilis* isolates have 100% of the 16S rRNA gene, 100% of the *ureC* gene, 95% of the *flaA* gene, 95% of the *hpmA* gene, and 100% of the *zapA* gene.

The *ureC*, *flaA*, *hpmA*, and *zapA* genes were sequenced using the DNA sequencer technique and BLAST software and compared to the gene sequence of a standard isolate. The results revealed that the genes in local *Proteus mirabilis* isolates were similar to *Proteus mirabilis* isolates globally registered on the NCBI-Genbank website at a percentage of 98.91-99.78%.

In conclusion, this study demonstrated the high prevalence of the *ureC*, *flaA*, *hpmA*, and *zapA* genes in *P. mirabilis* isolated from otitis media patients.

### 1. Introduction

Otitis media is an infection of the middle ear membrane. One of the most common types of bacteria that cause otitis media is *Proteus mirabilis* which belongs to the family of *Enterobacteriaceae* [1]. It is a Gram-negative rod that is non-spore-forming, an opportunistic pathogen of human causes (nosocomial infections) and is multi-resistant to antibiotics [2,3]. *Proteus mirabilis* colonies on MacConkey agar are colorless or pale yellow because they do not ferment lactose but do ferment glucose, sucrose, and galactose, but they are brown on chromogenic agar [4]. *P. mirabilis* forms a swarming phenomenon with
dense growth rings on blood agar [5]. Virulence factors involved in the pathogenesis of *P. mirabilis* include the *ureC* gene, which produces urease, the *flaA* gene, which produces flagella and fimbriae, the *hpmA* gene, which produces hemolysin, and the *zapA* gene, which produces protease [6,7,8]. One of the most crucial virulence mechanisms is swarming, which helps bacteria invade the infection site and increases the pathogenicity of *P. mirabilis* [9].

The current study used PCR and DNA sequencing to detect virulence factors (*ureC, flaA, hpmA,* and *zapA* genes) in *P. mirabilis* isolates.

2. Materials and Methods

2.1. Collection of the specimens and data

A total of 100 samples were collected during the period from December 2021 to July 2022 from patients of both sexes suffering from otitis media. The specimens were labeled and transported to the Microbiology Laboratory.

2.2. Identification of isolated bacteria

The shape, size, and color of the colonies on MacConkey agar, as well as the swarming phenomenon on blood agar, were used to diagnose *P. mirabilis* colonies. PCR method, and DNA sequencing were used to confirm the results [10].

2.3. PCR technique

PCR was used to confirm the diagnosis of *P. mirabilis* based on 16S ribosomal RNA gene and some of their virulence factor genes (*ureC, flaA, zapA,* and *hpmA*) using specific primers, as shown in the table (1). The Presto™ Mini gDNA Bacteria Kit was used to extract the DNA from *P. mirabilis*. A nanodrop spectrophotometer was used to calculate the DNA concentration. According to the GoTaq ®Green PCR master kit instructions, a total volume of 20 l of the PCR master mix was applied. The reaction was carried out in a thermo-cycler as shown in table (2). Electrophoresis with a (2%) agarose gel and UV light was used to obtain the PCR products [11].
Table 1. The PCR primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')</th>
<th>Product Size</th>
<th>Genbank</th>
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<tbody>
<tr>
<td>16S rRNA gene</td>
<td>F: CCTGGACAAAGACTGACGCT</td>
<td>344bp</td>
<td>LT745977.1</td>
</tr>
<tr>
<td></td>
<td>R: TTCACAACACGAGCTGACGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UreC gene</td>
<td>F: AAGTTGTGCTGGAGAAGGT</td>
<td>462bp</td>
<td>MF975538.1</td>
</tr>
<tr>
<td></td>
<td>R: ACACATGGATCAGTCAGGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>zapA gene</td>
<td>F: AGCCCGCTCTTCTTCATGATA</td>
<td>512bp</td>
<td>HM217133.1</td>
</tr>
<tr>
<td></td>
<td>R: CCATTATTATCTGCGGCTGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FlaA gene</td>
<td>F: GAGCGTGCTGTCTTCTGGTCT</td>
<td>528bp</td>
<td>AF221596.1</td>
</tr>
<tr>
<td></td>
<td>R: CAGTTGCGCACTGCATTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HpmA gene</td>
<td>F: CGACTTGCTGCTCTTTTACG</td>
<td>593bp</td>
<td>NC_010554.1</td>
</tr>
<tr>
<td></td>
<td>R: GCGGCAGTTGATTTCTCTCT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F: Forword                    R: Reverse

Table 2. PCR Thermocycler

<table>
<thead>
<tr>
<th>PCR Step</th>
<th>Repeat cycle</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>1</td>
<td>95°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>30</td>
<td>95°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td></td>
<td>58°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td></td>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>1</td>
<td>72°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Hold</td>
<td>-</td>
<td>4°C</td>
<td>Forever</td>
</tr>
</tbody>
</table>

2.4. DNA sequencing method

The study of the DNA sequence was performed using Mega 6.0 (Molecular Evolutionary Genetics Analysis version 6.0) and Multiple Sequence Alignment Analysis of the Partial Genes based on ClustalW Alignment Analysis. Phylogenetic tree UPGMA method was used to calculate the evolutionary distances using the Maximum Composite Likelihood approach. The homology sequence identity and mutation analysis were carried out by using NCBI BLAST analysis [12].

3. Results and Discussion

3.1. Identification of Proteus mirabilis

From otitis media patients, a total of 100 specimens were obtained. The results revealed that 25/100 (25%) of the isolates were classified as P. mirabilis using culture and microscopic characteristics, biochemical tests, and Molecular investigation, as follows.
3.2. Culture and Biochemical tests

The isolates were diagnosed based on the phenotypic characteristics of the colonies. The colonies of *P. mirabilis* appear at MacConkey agar as pale-colored colonies, non-lactose ferment, as well as the smell of bacterial growth similar to the smell of rotting fish, at Chromogenic agar appear as brown colonies, while swarming phenomenon formed on the blood agar, as shown in Figure (1). The results of *P. mirabilis* isolates' biochemical testing were shown in Table (3).

3.3. Molecular diagnosis using 16SrRNA gene

Isolates of *P. mirabilis* were diagnosed using Monoplex PCR to detect the 16SrRNA gene. The 16SrRNA gene's specific primers were employed to accurately identify *P. mirabilis*. The PCR product was electrophoresed on Agarose gel, documented on a gel document, and observed under ultraviolet rays (UV). The result of the PCR reaction revealed that the amplified DNA is 344 bp for the 16SrRNA gene. All isolates of *P. mirabilis* have the 16SrRNA gene at a percentage of 25/25 (100%) as in Figure (2).

3.4. Molecular investigation of virulence factors

Using particular primers, the following virulence genes were tested.

3.4.1. DNA amplification of *ureC* gene

The result of the mono-plex PCR reaction revealed that the amplified DNA is 462 bp for the *ureC* gene as in Figure (3). It is responsible for the urease enzyme's synthesis. The result of the current study revealed that 25/25 (100%) isolates gave positive results for the *ureC* gene. The importance of the urease enzyme in the pathogenesis of bacteria comes through the decomposition of urea to ammonia and then protects bacteria from toxic and high concentrations of urea [13]. The urease enzyme helps *P. mirabilis* to consume urea as a source of nitrogen for the synthesis of DNA and protein [6].

3.4.2. DNA amplification of *flaA* gene:

The mono-plex PCR reaction result showed that the amplified DNA is 528 bp for the *flaA* gene as in Figure (4), which is responsible for producing the flagella and fimbriae. A large number of genes on the Proteus chromosome are involved in the formation of flagella, which is essential for motility and swarming. The genes *flaA* and *flaB* are responsible for producing flagellin in *P. mirabilis* [14]. The *flaA* gene encodes the flagellin protein, which makes up the majority of the filamentous part of the *P. mirabilis* flagellum [15].

3.4.3. DNA amplification of *hpmA* gene

The result of the monoplex PCR reaction revealed that the amplified DNA is 593 bp for the *hpmA* gene as in Figure (5), which is responsible for the production of hemolysin. The result of the current study revealed that 21/25 (95%) isolates gave positive results for the *hpmA* gene.
Hemolysin damages red blood cells by making tiny holes in cell membranes and epithelial cells. Due to its cytotoxicity, host tissues are destroyed, and its presence is a key factor in the supply of iron to bacteria, thus increasing the pathogenicity of bacteria [16].

3.4.4. DNA amplification of zapA gene

The result of the mono-plex PCR reaction revealed that the amplified DNA is 512 bp for the zapA gene as in Figure (6), which is responsible for the production of protease. The result of the PCR revealed that 25/25 (100%) isolates gave positive results for the zapA gene. The protease enzyme is crucial because it can destroy IgA and IgG antibodies, reducing the immune response and making the bacterium more virulent [17].

3.5. DNA sequencer technique of some virulence genes

DNA sequencer technique was done to determine the sequence of nucleotides in ureC, flaA, hpmA, and zapA genes. The blast program was used to analyze the nucleotide sequence of (ureC, flaA, hpmA, and zapA genes) and compare it with the wild type sequences of standard isolates. The results showed that the sequences of the ureC, flaA, hpmA, and zapA genes were 98.91–99.78% similar to the sequence of the standard isolate.

There was one substitution mutation in the ureC genes. As shown in figure (7). The results of the ureC gene analysis showed that adenine was converted to guanine in site (296) at Subjct (241) and, and the percentage of the matching with the ureC gene of the standard isolate in the NCBI website was 99.78%.

In the flaA genes, there were three substitution mutations. The flaA gene analysis revealed that adenine was converted to thymine in site (204) at Subjct (181), adenine was converted to guanine in site (296) at Subjct (241) and guanine was converted to cytosine in site (341) at Sbjct (301), and the percentage of matching with the flaA gene of the standard isolate in the NCBI website was 99.34%, as shown in figure (8).

The hpmA gene included two substitution mutations. The results of the hpmA gene analysis showed that guanine was converted to adenine in site (132) at Subjct (121) and adenine was converted to guanine in site (296) at Subjct (241), and the percentage of the matching with the hpmA gene of the standard isolate in the NCBI website was 99.56%, as shown in figure (9).

In the zapA gene, there were five substitution mutations. The results of the zapA gene analysis showed that guanine was converted to adenine in site (132) at Subjct (121), adenine was converted to thymine in site (202) at Subjct (181), cytosine was converted to thymine in site (250) at Subjct (241) adenine was converted to guanine in site (296) at Subjct (241), and cytosine was converted to adenine in site (383) at Subjct (361), and the percentage of the matching with the zapA gene of the standard isolate in the NCBI website was 98.91% as shown in the figure (10).

The PCR and DNA sequencer technique is essential for biological research and is also used in biotechnology, virology, and medical diagnostics [18]. Substitution mutations are 20 times more common
on the Lagging strand than on the Leading strand, according to previous research on *P. mirabilis*. Substitution mutations can occur spontaneously as the result of a DNA polymerase III replication error or they can be caused by physical or chemical factors. These genetic changes frequently increase the rate of mutations in bacteria, which promotes the accumulation of harmful alleles [19].

The structure and function of a gene are altered as a result of mutations in that gene, and these changes affect gene expression, which results in the substitution of one amino acid for another. Because the genetic code consists of three nitrogen bases and the change occurred in one of them, replacing one of them does not always affect the structure and function of the protein. The genetic variation between local and standard isolates may be due to point mutations or substitution mutations within the sequence of the nucleotides of the genes, causing alteration of a single nitrogen base in the DNA sequence. During replication, these changes were replicated, thus causing a permanent change in the genome [20]. In point mutations, if purines were replaced with purines, it is called transition mutation, if Purines were replaced with pyrimidines or vice versa, it is called transversion mutation [21].

![Chromogenic agar](image1.png) ![Blood agar](image2.png)

**Figure 1: Culture results of *P. mirabilis***

<table>
<thead>
<tr>
<th>Test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catalase production</td>
<td>+</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+</td>
</tr>
<tr>
<td>Indole production</td>
<td>-</td>
</tr>
<tr>
<td>Methyl red</td>
<td>+</td>
</tr>
<tr>
<td>Motility</td>
<td>+</td>
</tr>
<tr>
<td>Oxidase production</td>
<td>-</td>
</tr>
<tr>
<td>Urease production</td>
<td>+</td>
</tr>
<tr>
<td>Voges-proskauer</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 3: Results of biochemical tests for *P. mirabilis* isolates**
<table>
<thead>
<tr>
<th>Lactose ferment</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSI</td>
<td>Acid/Alkaline, H2S production</td>
</tr>
</tbody>
</table>

(+): positive result  (-): Negative result

Figure 2: PCR product analysis of 16SrRNA gene in P. mirabilis. M (Marker ladder 100-2000bp). Lane (1-20) showed a positive 16SrRNA gene at 344bp product size.

Figure 3: PCR product analysis of ureC gene in P. mirabilis. M (Marker ladder 100-2000bp). Lane (1-20) showed a positive ureC gene at 462bp product size.

Figure 4: PCR product analysis of flaA gene in P. mirabilis. M (Marker ladder 100-2000bp). Lane (1-20) showed some a positive flaA gene at 528bp product size.

Figure 5: PCR product analysis of hpmA gene in P. mirabilis. M (Marker ladder 100-2000bp). Lane (1-
20) showed some a positive $hpmA$ gene at 593bp product size.

**Figure 6:** PCR product analysis of $zapA$ gene in *P. mirabilis*. M (Marker ladder 100-2000bp). Lane (1-20) showed a positive $zapA$ gene at 512bp product size.

**Figure 7.** Comparing *P. mirabilis' ureC* gene's multiple sequence alignment using NCBI-BLAST.
Figure 8. A comparison between NCBI-BLAST *P. mirabilis* and multiple sequence alignment of the *flaA* gene in that species.

Figure 9. Comparing multiple sequence alignment results for the *hpmA* gene in *P. mirabilis* to *P. mirabilis* NCBI-BLAST.
Figure 10. Multiple sequence alignment results of zapA gene in \textit{P. mirabilis} compared to NCBI-BLAST \textit{P. mirabilis}.

Conclusions

The present study was demonstrated the prevalence of virulence factors in \textit{P. mirabilis} isolated from otitis media patients, and local isolate sequences were 98.91–99.78% identical to those of the standard isolates.

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Conflict of interest

The authors declare that there is no conflict of interest.

References


