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Morphological and Molecular Study of the Biofilm of *Proteus Mirabilis* Isolated from Urinary Tract Infections

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Abstract

Proteus mirabilis is one of the most common causes of urinary tract infections, especially in catheter injected patients. The aim of study was to investigate morphological and molecular study of the biofilm of Proteus mirabilis isolated from urinary tract infections. One hundred urine samples were collected from outpatients and inpatients at Al-Diwaniyah Teaching Hospital and the Maternity and Children's Hospital who were diagnosed with urinary tract infections. The results showed that molecular detection of the genes responsible for the bacteria under study was performed on all samples using PCR technique. After transferring the result of the multiplication in agarose gel, it was noted that 10 isolates (100%) of the isolates possessed the int1 genes under study. In general, the virulence factor (Biofilm) in *P. mirabilis* was investigated, as the results of the phenotypic detection showed the ability of the bacteria under study to produce biofilm in 10 isolates at a rate of 100%.

Keywords: *biofilm, Proteus mirabilis, urinary, rsbA, luxS, MrpA*

دراسة مورفولوجية وجزيئية للغشاء الحيوي لبكتيريا *Proteus mirabilis* المعزولة من التهابات المسالك البولية

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الخلاصة

تُعدّ بكتيريا *Proteus mirabilis* من أكثر مسببات التهابات المسالك البولية شيوعًا، لا سيما لدى المرضى الذين يستخدمون القسطرة .هدفت هذه الدراسة إلى فحص الخصائص المورفولوجية والجزيئية للغشاء الحيوي لبكتيريا *Proteus mirabilis* المعزولة من حالات التهابات المسالك البولية .جُمعت مئة عينة بول من مرضى العيادات الخارجية والداخلية في مستشفى الديوانية التعليمي ومستشفى الولادة والأطفال، والذين شُخّصت إصابتهم بالتهابات المسالك البولية .أظهرت النتائج أن الكشف الجزيئي عن الجينات المسؤولة عن البكتيريا المدروسة قد أُجري على جميع العينات باستخدام تقنية تفاعل البوليميراز المتسلسل .(PCR) بعد نقل نتائج التكاثر إلى هلام الأغاروز، لوحظ أن جميع العزلات العشر (100%) تحمل جينات int1 المدروسة .وبشكل عام، دُرست عوامل الضراوة (الغشاء الحيوي) في بكتيريا*P. mirabilis* ، حيث أظهرت نتائج الكشف الظاهري قدرة البكتيريا المدروسة على إنتاج الغشاء الحيوي في جميع العزلات العشر بنسبة 100%.

الكلمات المفتاحية: *الغشاء الحيوي، Proteus mirabilis, urinary, rsbA, luxS, MrpA*

1. Introduction

Proteus mirabilis is an opportunistic pathogen, meaning it causes infections in people with weakened immune systems. This bacterium possesses several virulence factors, or pathogenicity traits, which are essential for colonizing new areas, including host tissues and organs [1].

One of these pathogenicity factors is its ability to grow for extended periods [2]. Its morphology is constantly changing, contributing to the formation of what are called long cells, measuring 10–80 μm in length, with numerous cilia on their surface and undivided nucleotides. These long Proteus mirabilis cells migrate uniformly across the surface of a solid substrate. If the bacteria detach and find single cells a considerable distance apart, they stop growing and break down into shorter bacilli [3]. Both the pathogen and biofilm production of Proteus mirabilis may be affected by diffuse growth, according to reports indicating that this phenomenon promotes biofilm development and may be involved in urinary tract infections caused by urinary tract infections. Long cells have also been found to be characterized by increased production of substances such as urease, HpmA hemolysin or ZapA metalloprotease, which are pathogenic factors [4].

Biofilms are communities of one or more types of microorganisms (bacteria and fungi) that can grow on a variety of surfaces. Bacteria within biofilms produce extracellular polymer (EPS), which is typically a polymeric aggregate of extracellular sugars, proteins, lipids, and nucleic acid [5]. The production of P. mirabilis biofilms is an important resistance mechanism because it promotes the transfer of resistance genes, making bacterial colonies resistant to antibiotics [4]. Peptide flow, a component of the polymeric matrix produced by biofilms, may play a role in biofilm formation. Additionally, peptides may be involved in the quorum sensing (QS) system of P. mirabilis, suggesting that their flow can influence signaling during biofilm development [5].

P. mirabilis is a catheter biofilm-forming agent that rapidly contaminates the surface of newly inserted urinary catheters. The adhesion, enzymes, and toxins produced by this bacterium play a role in the development of disease, which can invade bladder epithelial cells, damage the bladder epithelium, and cause significant histological pathology [6]. P. mirabilis contains a urease-catalyzed enzyme that degrades urea in urine to carbon dioxide and ammonia, providing the bacteria with a rich source of nitrogen. This enables the formation of polyvalent ions, reduces urine pH, and produces struvite and apatite crystals [7]. Therefore, the study aim to study morphological and molecular study of the biofilm of Proteus mirabilis isolated from urinary tract infections.

2. Materials and Methods

2.1 Sample Collection

One hundred midstream urine samples were collected from outpatients and inpatients at Al-Diwaniyah Teaching Hospital and the Maternity and Children's Hospital who were diagnosed with urinary tract infections, as well as from the Kidney and Urinary Stone Center, across various age groups and both sexes, during the period from September 20, 2025, to January 10, 2026. After in vitro culture, 80 (80%) of the samples showed bacterial growth, while 20 (20%) did not show any bacterial growth.

Table 1- Number of samples and bacterial isolates obtained from patients with urinary tract infections

Bacterial growth status	Isolates number	Percentage (%)
Bacterial growth	80	%80
Non-Bacterial growth	20	%20
Total number	100	%100

2.2. Bacterial DNA Extraction

Genomic DNA was extracted from Proteus mirabilis isolates using the Geneaid Genomic DNA Purification Kit (Turkey) according to the manufacturer's instructions. The bacterial culture was inoculated in 10 mL of nutrient broth medium and incubated overnight at 37°C. Bacterial cells (up to 1 × 10^9) were transferred to a 1.5 mL microcentrifuge tube and centrifuged for 1 minute at 15,000 × g. The supernatant was then discarded.

1. A total of 180 µL of GT Buffer was added, and the cell pellets were resuspended using a vortex or pipette. 20 µL of Proteinase K was added (ensure the addition of ddH2Was). The mixture was then incubated at 60°C for at least 10 minutes, with the tube being inverted every 3 minutes during incubation.
2. Two hundred µL of GB Buffer was added, then the mixture was vortexed for 1 second and incubated at 70°C for at least 10 minutes to ensure the sample was thoroughly rinsed. During incubation, the tube was inverted every 3 minutes. At this time, the required elution buffer (200 µL per sample) was heated to 7°C for use in the next step (DNA elution).
3. Two hundred µL of absolute ethanol was added and immediately mixed by vigorous shaking. If a precipitate appeared, the shaking was stopped as much as possible using a pipette. The GD column was then placed in a 2 mL collection tube, and the mixture (including any insoluble precipitate) was transferred to the GD column and centrifuged at 15,000 × g for 2 minutes. The 2 mL collection tube containing the flux was discarded, and the GD column was placed in a new 2 mL collection tube.
4. Four hundred µL of W1 Buffer was added to the GD column and centrifuged at 15,000 × g for 30 seconds. The flux was discarded by placing the GD column back into a 2 mL collection tube.
5. Six hundred µL of wash solution (with ethanol) was added to the GD column, and it was centrifuged at 15,000 × g for 30 seconds. The flux was discarded, and the GD column was placed back into a 2 mL collection tube and centrifuged again at 15,000 × g for 3 minutes to dry the column matrix.
6. The dried GD column was transferred to a clean 1.5 mL centrifuge tube, and 1 µL of preheated rinse solution was added to the center of the column matrix. It was left to stand for at least 3 minutes and then centrifuged at 15,000 × g for 30 seconds to remove the purified DNA. The DNA was then stored at 2–8°C. 3-9-2

2.3. Estimation of DNA Concentration

The extracted genomic DNA was analyzed using a Nano-Drop spectrophotometer, which measures DNA concentration (nanograms/µL). DNA purity was assessed by measuring absorbance at 260/280 nm. The instrument was operated as follows:

1. After switching on the Nano-Drop spectrophotometer, the DNA was measured using the selected program.
2. The cortical substrate was filtered twice by applying 2 µL of ionic distilled water to the surface of the spectrophotometer using a sterile micropipe.

3. One µL of each extracted DNA sample was placed on the spectrophotometer substrate, and the OK button was pressed to begin the DNA concentration measurement. The spectrophotometer was then cleaned again to measure the other sample. 4- The purity of the extracted DNA samples was determined by reading the absorbance using a nanodroplet spectrophotometer at two wavelengths (280/260 nm). The extracted DNA was considered pure when the absorbance ratio was 1.8.

2.4. Agarose Gel Electrophoresis of Extracted DNA

Agarose sheets were prepared by dissolving agarose powder in 1X TBE buffer. The amount of agarose to be dissolved depended on the intended use of the agarose sheet. A 0.7% agarose sheet was used for visualizing DNA after extraction, while a 1.5%–2% agarose sheet was used for visualizing the PCR product. The ethidium bromide concentration for staining was 10 mg/ml. Only 0.5 µL of ethidium bromide dye was added to 100 ml of the dissolved agarose gel [8].

2.5. Primer Pair Preparation

The primers used in the current study were prepared from IDT (Canada) according to the manufacturer's instructions. Deionized distilled water (ddH2O) was added to the freeze-dried primers, based on the volumetric ratio, and the mixture was thoroughly mixed using a vortex mixer to obtain 100X (picomoles/microliter) stock solutions. These were then stored at -20°C. Ten microliters of the stock solution were transferred to an RNase-DNase-free tube, and 90 microliters of ddH2O were added. This was then thoroughly mixed using a vortex mixer to obtain a 10X primer concentration and stored at -20°C.

2.6. Preparation of the PCR Reaction Mixture

The reaction mixture was prepared using the Biomaterials Manufacturing (BMM) kit according to the manufacturer's instructions as follows:

The PCR reaction mixture was prepared in tubes equipped with a kit containing the reaction components. Other components were added to the reaction mixture according to the manufacturer's instructions, as shown in the following table:

Table 2- Contents of the reaction mixture of PCR used in this study

NO	Contents of reaction mixture	Volume
1-	PCR master mix	25Ml
2.	DNA template	5Ml

2.7. Gel Electrophoresis of Acarose

The polymerase chain reaction (PCR) products for all virulence genes were analyzed by loading 1.5% acarose as follows (Sambrook et al., 2001):

1. The acarose gel was prepared by dissolving 1.5 g of acarose in 100 mL of 1X TBE and then allowing it to cool to 45–50°C.

2. 0.5 µL of ethidium bromide dye was then added to the acarose gel solution.

3. The acarose gel solution was placed in a container, the comb was positioned appropriately, and then allowed to solidify for 30 minutes at room temperature. The comb was then carefully removed from the container. 4. The gel tray was placed in the electrophoresis chamber and filled with 1 x TBE solution.

5. 10 µL of PCR product was added to each tray, and 10 µL of (100 bp ladder) was added to one well.

6. An 80 V current was then applied for 40 minutes.
7. The PCR products were imaged using the gel electrophoresis system.

2.8. Biofilm Formation

Microtitre-plate test was used to detect the biofilm formation for all bacterial isolates as follow

1. A 20 µl of bacterial isolates overnight cultures were used to Inoculate 96-microtitier wells plate containing 180 µl of TSB. Negative control wells contained the broth only.
2. Cultures were removed and the wells were rinsed with PBS (pH 7.2).
3. After drying at room temperature for 15 min., 200 µl per well of crystal violet (1%) was added to the wells for 20 min.
4. The stained biofilms were rinsed three times with PBS (pH 7.2), and allowed to dry at room temperature for 15 min., and then. extracted twice with 200 µl per well of 95% ethanol.
5. The OD of each well was estimated using automatic microtiter plates reader. All assays were performed in duplicate.
6. The average OD values were calculated for all tested isolates and negative controls.
7. The cut-off value (ODC) was established, it was defining as a three standard deviations (SD) above the OD mean of the negative control.
8. The OD values of a tested isolates was expressed as average OD value of the isolate reduced by ODc value. OD average OD of isolate-ODc).
9. Finally, the biofilm results of isolates were divided into the following categories:

Non-biofilm producer (OD ODC)
Weak biofilm producer (ODCOD ≤ 2 x ODC)
Moderate biofilm producer (2 x ODC OD ≤ 4 x ODC)
Strong biofilm producer (4 x ODcOD)

2.9. Statistical Analysis

The statistical analysis was performed using the statistical software package (IBM, SPSS v.23). The data were statistically defined as frequency order (number of cases) and relative frequency (percentages). Pearson's least significant correlation was calculated at $p < 0.01$ and $p < 0.05$.

3. Results and Discussion

3.1. Distribution of Proteus spp. Isolates from Urinary Tract Samples

Fifteen *Proteus spp.* isolates were obtained from urine samples collected from various hospitals between September 2025 and January 2026. All samples were cultured in nutrient broth at 37°C for 18–24 hours and then cultured on blood agar and MacConkey agar using the line-panel method to observe colony morphology (color, shape, surface texture, rim, size, height, etc.). *Proteus mirabilis* was identified by biochemical tests and the integrated VITEK 2 system. The percentage of isolates per sample from the total number of bacterial isolates (Table 3).

Table 3- Distribution of *Proteus spp*. Isolates from Urinary Tract Samples

Proteus spp.	Number	Percentage (%)
Proteus mirablilis	10	66.7%
Proteus vulgaris	5	33.3%
Proteus penneri	0	0%
Total	15	100%
X^2 value	15	
probability value	<0.001*	

The results showed that infected with *P. mirabilis* (66.7%) were more commonly than urine samples. These differences in percentage between urine and other infections may be due to the high susceptibility of the exposed wound area to microbial invasion. It became more susceptible to infection due to inadequate hygiene practices and a lack of attention to sterilization protocols by hospital staff, as well as other factors related to the personal hygiene of both the patient and medical personnel. *P. mirabilis* is an opportunistic pathogen, meaning it can cause infection when there is a breach in the body's natural defenses[8] (
These bacteria are a common site for such breaches, as they create an opening for bacteria to enter the body [9]. This finding is consistent with that of [10], who reported that 15% of wound samples were isolated with *P. mirabilis*. Local studies conducted by [11] in Erbil found higher rates of *P. mirabilis* isolation from wound samples, at 36% and 24%, respectively. Regarding urine samples, the percentage of *P. mirabilis* isolates detected. This finding is consistent with those of [12], who reported 6% isolation from urine samples. The urinary tract is designed to eliminate waste and foreign materials through urine production and excretion. This continuous cleansing process helps reduce the risk of infection by flushing out bacteria. The role of the innate immune system in otitis media may be a contributing factor, specifically the role of middle ear epithelial cells, neutrophils, phagocytes, fibroblasts, and mast cells. Natural killer cells also protect the middle ear from pathogens [13].

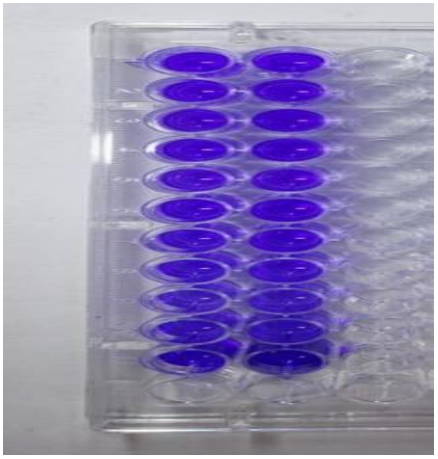

Figure -1 Biofilm production morphologically of Proteus isolates

3.2. Detection of Proteus mirabilis Virulence Genes by PCR

Ten isolates of *P. mirabilis*, isolated from clinical cases under study, were selected for molecular diagnosis using polymerase chain reaction (PCR). A specialized primer targeting the rsbA gene was used. The gene's programming was entered into the PCR, the reaction was initiated, and the products were centrifuged on a 1.5% agarose gel for 60 minutes. Genetic bands appeared in all centrifuged isolates at a single level, indicating the binding of the primer to its complementary DNA sequence. The molecular weights of the resulting genetic bands were then estimated using standard bands with known molecular weights, specifically in the M-track at a gradient of 467 bp. The results showed a clear correlation between the molecular weight of the resulting bands (1500 bp) and the molecular weight of the rsbA gene. The results of molecular diagnosis were consistent with those obtained using the VITEK-2 system, indicating that 20% of the isolates belonged to *P. mirabilis*, based on each isolate possessing genetic bands larger than 1500. Even isolates that did not show the diagnostic gene during migration were positive for phenotypic diagnosis on culture media and for

biochemical and microscopic tests, confirming that these isolates belonged to *P. mirabilis*. These results are consistent with those of [14], where 100% of the bacterial isolates possessed rsbA gene. They also agree with [15], where 100% of the isolates possessed srRNA16, and with [16], where rsbA gene was found in 100% of the isolates.

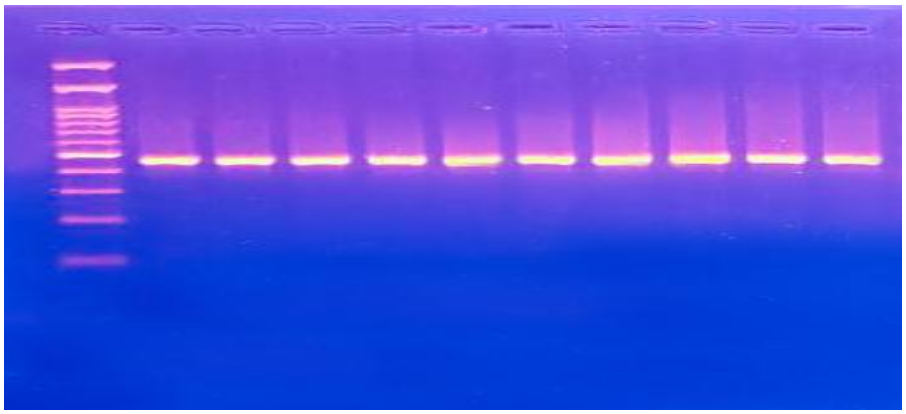

Figure -2 Electrophoresis of agarose gels obtained from a sequential PCR reaction using a gene-specific primer (rsb) gene (467)bp, It regulates the creeping movement in bacteria.

The genes were genetically investigated by detecting the luxS gene, which is responsible for diagnostic characteristics in *P. mirabilis*. Eight out of ten isolates produced the luxS gene using a primer specific to the antinuclear gene. Polymerase chain reaction (PCR) was applied to all isolates, followed by electrophoresis. This resulted in the appearance of genetic bands with a single molecular size (464 bp).

These results are consistent with those of [17]study, which found that the antinuclear gene was present in 77.2% of urine samples in various clinical cases of Gram-negative *E. coli*. This finding is consistent with the results of [18], where luxS gene were successfully detected in 21% of *P. mirabilis* isolates. This is the first report from Egypt to reveal clinical P. mirabilis isolates carrying luxS gene, suggesting possible horizontal transmission from other bacterial species. However, it contradicts the findings of [19], where the luxS gene was found in 0% of 150 *P. mirabilis* isolates, and it also contradicts the findings of [20], who reported no presence of the luxS gene in their study.

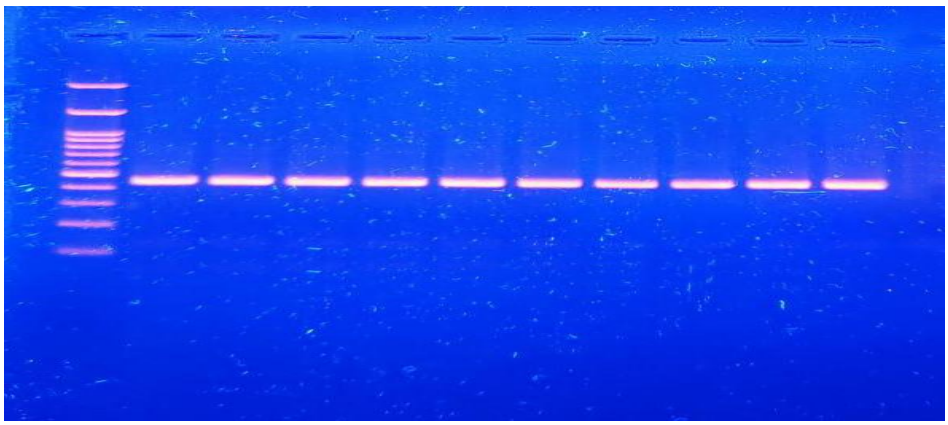

Figure -3 Electrophoresis of agarose gels obtained from a sequential PCR reaction using a gene-specific primer (luxS) gene (464)bp.

3.3. Quorum sensing (bacterial communication) and regulation of biofilm formation and involvement in the system

MrpA gene was genetically investigated by detecting the MrpA gene responsible for diagnostic traits in *P. mirabilis* bacteria. Cilia genes (Mrp)gene (565)bp helps in the adhesion and initial formation of the biofilm. Twenty isolates produced the MrpA gene out of a total of 20 isolates using a primer specific to the MrpA gene production. After applying polymerase chain reaction (PCR) to all isolates and electrophoresis, the genetic bands appeared at a single level and with a single molecular size of (565 bp) at a rate of (100%) (Figure 3). The results of this study are consistent with those of [21], whose study revealed the presence of the MrpA gene in 16.6% and the anti-cinetrione 2 gene in 83.25% of Gram-negative bacteria. They also align with the study by [22], which showed the presence of anti- MrpA gene in urine samples at rates of 52.2% and 38.6%, respectively. Furthermore, the MrpA gene appeared in 80% and the anti-cinetrione 2 gene in 66.6%. However, they do not agree with the findings of [23], who found that only 26% and 15% of their P. mirabilis isolates, respectively, harbored both MrpA gene.

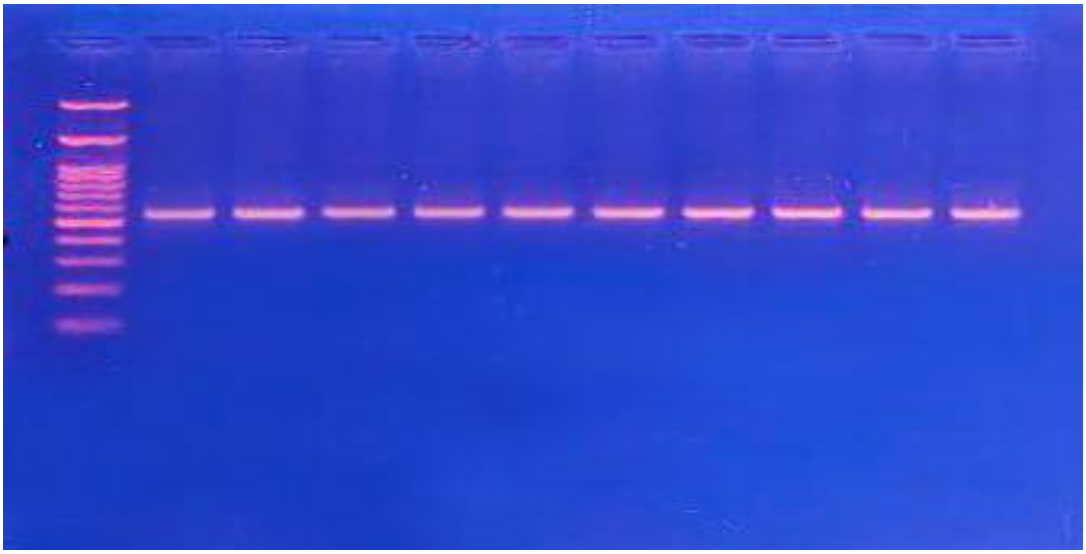

Figure -4 cilia genes (Mrp)gene (565)bp helps in the adhesion and initial formation of the biofilm

The results showed that *P. mirabilis* utilizes a variety of virulence factors to induce catheter-associated urinary tract infections. Ureases, fimbriae, flow pumps, and the polysaccharide capsule are all virulence features that have been linked to its ability to form biofilms. The first step in biofilm formation on catheter surfaces is the adhesion of fimbriae to the protein layer on the catheter surface, either from physiological fluids or directly to the catheter material.

The formation of bacterial biofilms is an adaptation that grants the organism resistance to environmental conditions, provides protection against antibiotics, and evades the host's immune defenses. Consequently, bacterial biofilms are particularly prevalent in chronic and hospital-acquired infections [24]. The initial step in developing biofilms on catheter surfaces involves the binding of fimbriae adhesins either to a protein layer derived from body fluids on the catheter surface or directly to the catheter material itself. In the genome of the P. mirabilis isolate, at least 17 fimbriae operons were identified through whole-genome sequencing. This number is significantly higher than other sequenced bacterial genomes and

is associated with P. mirabilis's exceptional ability to adhere to catheter surfaces among Gram-negative bacteria[25] .

Biofilms are collections of one or more types of microorganisms (bacteria and fungi) that can grow on many different surfaces. Bacteria within biofilms produce extracellular polymers (EPS), which are typically polymeric aggregates of extracellular sugars, proteins, lipids, and nucleic acid. The production of P. mirabilis biofilms is an important resistance mechanism. Because it promotes the transfer of resistance genes, and makes bacterial colonies resistant to antibiotics [26].

Table 4- virulence genes from different isolate samples

Isolate samples	Gene type		
	rsbA	Mrp	lux
Isolate 1	+	+	+
Isolate 2	+	+	+
Isolate 3	+	+	+
Isolate 4	+	+	+
Isolate 5	+	+	+
Isolate 6	+	+	+
Isolate 7	+	+	+
Isolate 8	+	+	+
Isolate 9	+	+	+
Isolate 10	+	+	+
Positive No.	10	10	10
Percentage	100%	100%	100%
X2 value		19.51	
probability value		0.001*	

4. Conclusions

The study concludes that molecular detection of the genes responsible for the bacteria under study was performed on all samples using PCR technique. After transferring the result of the multiplication in agarose gel, it was noted that 10 isolates (100%) of the isolates possessed the int1 genes under study. In general, the virulence factor (biofilm) in *P. mirabilis* was investigated, as the results of the phenotypic detection showed the ability of the bacteria under study to produce biofilm in 10 isolates at a rate of 100%.

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