Investigation of Introns in Proteus Mirabilis Bacteria and Extent of their Resistance to Antibiotics

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ABSTRACT
Proteus mirabilis is an opportunistic pathogen meaning that causes infection in people with reduced natural immunity. These bacteria possess many virulence factors or pathogens which is essential for colonizing new areas, including tissues and hosts. The study aimed to investigate phenotypically or genetically the introns (MIs) in P. mirabilis by estimate the extent of its resistance to antibiotics. The results showed that the appearance of genetic packages for all migrated isolates at one level. This is an indication of the binding of the primer to its complementary sequence in the DNA strand. The molecular weights of the resulting genetic packages were estimated based on known standard packages, the apparent molecular weights in the path (M) with a graduated size (100- 200 bp). The results also showed a clear similarity of the molecular weight of the resulting bands (1500 bp) with the molecular weight of the diagnostic gene rRNA 16s. In addition, 20 isolates were examined using PCR technology for the three classes of introns gene. All isolates were carried the chromosome intron gene. The appearance of the intron 1 gene on all isolates was (100%).

1. Introduction

Proteus mirabilis is a type of Gram-negative bacteria. These bacteria are usually straight or slightly curved rods, about 0.5-1.0 µm wide and 1.5-4 µm long. They are known for their ability to produce urease and show a unique pattern of movement on agar plates (Kamil and Jarjes., 2019). It has been recommended that this organism be given the new name *Cosenzaea myxofaciens*. Recent research suggests that *Proteus myxofaciens* may be a distinct genus unrelated to the tribe Proteaeae (Mohammed et al., 2016). These bacteria are present in the natural environment including soil, water, organic fertilizers. They are part of the physiological flora of the human digestive system. It means causes infection in people with reduced natural immunity. These bacteria possess numerous virulence factors, or pathogens, which are essential for colonizing new areas, including tissues.

Integrons are a genetic module that acquires exogenous medicine resistance genes and allows them to be expressed in bacteria. It is an important factor in the horizontal transmission of bacterial drug resistance. Under the influence of introns, bacteria take it up and express foreign medicine resistance genes through the intron gene cassette system, thus showing multidrug resistance (Halaji et al., 2020). Regarding to β-lactamases, integron-borne gene cassettes containing Ambler class A, B, and D-lactamase enzymes have been mostly detected in *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and several Enterobacteriaceae species, resulting in broad β-lactam resistance with few exceptions. Only eight separate families of β-lactamases of classes 1 and 3 integrin structures have been described originating from geographically distinct regions (Gmiter and Kaca, 2022). Moreover, the same gene can be sequenced in structure and arrangement between different strains, which also proves that introns have the ability to capture, carry, and reorganize gene and can also spread among different bacteria (Japoni et al, 2013).

Antrones are made up of two centers, the first made of the integron-integrase (intI) gene and its promoter (PintI), an integration site called the integron attI attachment site, and a constitutive promoter (Pc) for gene cassettes integrated into the attI site (John, 2016). The second is a cluster containing up to 200 gene cassettes, commonly transcribed in the opposite direction relative to the integron-integrase. Gene cassettes, the genetic cassette are not essentially part of the integrons. However, it becomes part of an integron when it is ligated. Integrase deletes and adds gene cassettes to and from the integron, but integrons themselves are not motile (Partridge et al., 2018). Integrons are classified into two groups depending on their location, integrons (MIs), closely related to plasmids and transposons, which are the main vehicles of multiple resistance in bacteria (Stankowska et al., 2012). Therefore, the study aimed to investigate phenotypically or genetically the introns in *Proteus mirabilis* by estimate the

extent of their resistance to antibiotics.

2. Methodology

Preparation of culture

These medium have prepared in the laboratory according to the manufacturers; the instructions for each medium and the pH were adjusted to 7.2±0.2 and then boiled in a water bath to ensure that all components were completely dissolved. The medium was sterilized by autoclaving at 121 °C P 15 psi for 15 minutes. Plates and tubes containing different media were incubated at 37°C to ensure no contamination and then stored at 4°C until use.

Preparing different culture:

Blood agar medium

The blood agar base was prepared according to the manufacturer's instructions. Instructions are prepared by dissolving 40 g of blood agar medium in 1000 ml of dry water. It is then sterilized at 121°C for 15 mins and cool it to 50 °C. Then, add 5% of fresh human blood, mix well until combined, then pour into sterile Petri dishes. Finally cool to 37°C and leave to freeze at room temperature. This medium was used to culture bacterial isolates and determine the ability of bacteria to lyse blood cells (Madigan et al., 2005).

Urea Agar medium

Filter-sterilized urea solution (5 ml) was prepared with a 0.20 µm µ filter, by dissolving 2 g of urea in (5 ml) of D.W after sterilization. It was added to 95 ml of urea agar base, and placed in sterile tubes. Finally, it is cooled to 37°C and left at room temperature to harden into a bent shape for use.

Peptone water medium

This medium was prepared by dissolving 20 g of peptone and 5 g of sodium chloride in the amount of D.W. The pH was adjusted to (7.2), and then using D.W the volume was completed to 1000 ml. They were then sterilized by autoclaving, transferred to sterile 5 ml tubes and stored at 4°C. It was used to demonstrate the ability of bacteria to hydrolyze the amino acid tryptophan to indole (Collee et al., 1996).

Motility test

It was prepared by dissolving 0.8 g of nutrient broth and 10 g of agar into 90 ml of D.W. The pH was adjusted to 7.0, the volume was completed to 100 ml, and it was sterilized by autoclaving at 121°C for 15 mins. Leave it to harden as a vertical slant. It has been used to identify motile and non-motile bacteria (Goldman and Lorrence et al., 2009).

Isolation identification

The isolated bacteria were identified based on morphological and biochemical tests and the Vitek2 system for some isolates.

Gram stain method

Preparation of a slide smear

The inoculation loop is used to transfer a small amount of bacteria from the colony which is distributed with a drop of normal saline on a clean slide to facilitate minimal colony transfer to the examination slide. The slide can be either air dried or dried with the help of heat over a gentle flame. Heat helps cell adhesion to the glass slide and prevents significant culture loss during washing (Tripathi and Sapra, 2020).

Gram staining

Crystal violet is added over the still farm. After 10 to 60 seconds, an iodine solution is used to cover

the swab for 10 to 60 seconds. The slide is washed with running water. A few drops of color remover are added to the slide. Color removers are often a mixed solvent of ethanol and acetone. This step is known as (solvent treatment). The slide is washed with water within 5 seconds (O'Toole 2016). The swab is counterstained with basic fuchsin solution for 40 to 60 seconds. The fuchsin solution is washed with water, lubricated and air dried after removing excess water.

Microscopic examination of a slide

The slide should be examined under a microscope and in oil immersion. The initial slide examination is with strength of 40X and emphasis on distributing the swab. Then it should be checked using 100X force after immersing it in oil. (Tripathi and Sapra, 2020).

Patients

25 samples were collected for different medical cases, including (urine from urinary tract infection patients, middle ear infection wound, skin) from different hospitals and of different ages during their visits to hospitals during the period from August 15, 2023, to November 15, 2023. The name, age and gender of each patient were recorded. The basic clinical status and date of sample collection were collected.

Diagnosis the Vitek-2 Bacterial Identification system

Recently, this system has been used to identify microorganisms (Winn et al., 2005). It has been provided with the identification database required for all routine identification tests which has provided improved efficiency in microbial diagnosis resulting in reduced time and need to perform any additional tests, which will be safe for the user of the system.This system was performed according to the manufacturer's instructions (Biomerieux France). This system consisted of:

1- A personal computer.

2- Reader/Incubator which consists of multiple internal components including: card cassette, card stuffing mechanism, cassette loading processing mechanism, card sealer, barcode reader, carousel, and incubator.

3-The system also contains: transmittance optics, waste handling, and instrument and stationary control electronics. This system was performed according to the manufacturer's instructions (BiomerieuxFrance) as follows:

A- Three ml of normal saline solution was placed in a test tube and inoculated with a complete ring of one colony in the culture overnight.

B- The test tube was inserted into the adens screening machine to standardize the colony to McFarland standard solution = (1.5×108 cells/ml).

C- Standardized vaccines were placed in the cassette.

D-The sample identification number was then entered into the computer programs via barcode. The Vitek-2 card was then connected to the sample ID number.

E- The cassette was placed in the filler unit, when the cards were filled, the cassette was moved to the reader/incubator unit.

Preservation and maintenance of bacterial isolates

Bacterial isolates were maintained on nutrient agar at 4°C. Isolates were maintained monthly by reculturing on fresh medium. Nutrient broth supplemented with 15% glycerol was used for long-term preservation and the isolates were kept frozen at −20°C (freezing) for several months (long-term maintenance) (Forbes et al., 2007).

Preparation of bacterial inoculum

At least 3-5 well-isolated colonies were resuspended in 4-5 ml brain heart infusion. The broth culture

was incubated at 37°C for 8 h. The turbidity of the active-growth broth culture was adjusted using sterile broth to obtain a turbidity visually comparable to a 0.5 McFarland tube of standards (growth equivalent to 1.5×108 cells/ml).

Preparing the reaction mixture of PCR

The reaction mixture was prepared using the Applied Biomaterials Manufacturing (ABM) kit according to the company's instructions as follows: The PCR mixture was prepared in tubes equipped with a kit containing the reaction components, and the other components were added to the reaction mixture according to the company's instructions (Table 1). After completing the preparation of the reaction mixture, the tubes were closed and mixed carefully with a Vortex device for 10 seconds. The tubes were transferred to a PCR Thermocycler to complete amplification of the target gene.

Table (1): Contents of the PCR reaction mixture used in this study

NO	Contents of reaction mixture	Volume
1-	PCR master mix	25Ml
2.	DNA template	5Ml
3.	Forward Primer 10pmol	3Ml
4.	Reverse Primer 10pmol	3µL
5.	Nuclease free water	14µL

Statistical analysis

The statistical investigation was performed using the statistical software (IBM, SPSS V.23). Data were statistically defined in the order of frequencies (number of cases) and relative frequencies (percentages). The least significant Pearson correlation was calculated at $p < 0.01$ and $p < 0.05$.

3. Results and discussion

Molecular Characterization of *Proteus mirabilis*

The 20 isolates of *P. mirabilis* isolated from clinical cases under study were selected for molecular diagnosis using polymerase chain reaction technology, using a specialized primer that targets the sequence of the 16s rRNA diagnostic gene. The programming for this gene was entered into the polymerase chain reaction device, the reaction took place, and then the reaction products were migrated on an agarose gel at a concentration of (1.5%) for (60) mins. It was observed that genetic bands appeared for all the migrated isolates, and at one level, and this gives us an indication of the binding of the primer to its complementary sequence in the DNA strand. Then, the molecular weights of the resulting genetic packages were estimated based on standard packages with known apparent molecular weights in the path (M) in a graduated size (100-200 bp).

The results showed a clear similarity of the molecular weight of the resulting packages, which was (1500 bp), with the molecular weight of the diagnostic gene rRNA. 16s. The results of the molecular diagnosis were identical to the results of the Vitek-2 device, as the diagnostic results showed that 20% of the isolates belonged to *P. mirabilis*, with each isolate possessing genetic bands of 1500+. Despite the fact that isolates for which the diagnostic gene did not appear upon migration were given a positive result for the phenotypic diagnosis on the culture media and for biochemical and microscopic tests. It indicates that these isolates belong to the *P.mirabilis*. These results were agreed with. The percentage of bacterial isolates that possess s rRNA16 is 100%. It also agrees with Zahra (2022) that the percentage of isolates that possess s rRNA16 reached 100%. It is similar to Fanar (2023), where the results for the diagnostic gene s rRNA16 showed a percentage of (100%).

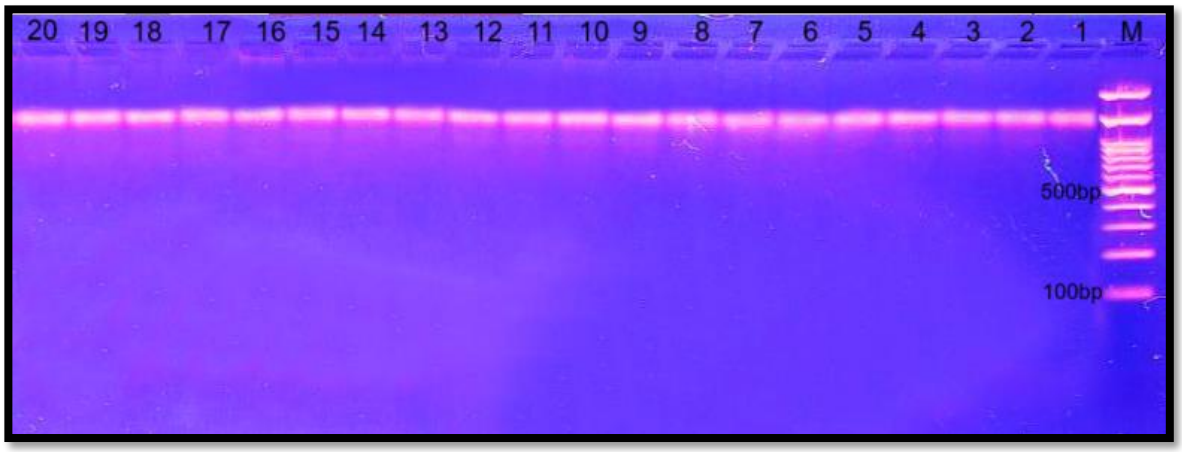

Figure (1) Electrophoresis results of the diagnostic gene 16SrRNA (1500bp) of *P.mirabilis* isolates on an agarose gel at a voltage of (80 volts); (M) represents the size index (100-1500)

Classes of Integrons isolated from urine, otolitis and skin

All DNA results revealed adequate quality. 20 isolates were examined using PCR technology for the three categories of the intron gene 20 (100%). All isolates carried the chromosome intron gene. The appearance of the inticron1 gene in all isolates was (100%), while the appearance of the inticron2 gene was (95%), while the appearance of the inticron3 gene was (80%). This is due to Isolates 4, 18, 19, and 20 did not produce intron gene (Table 2). Compared to the study (Jaber and Almiyah, 2022) on Gram-negative bacteria, specifically *E.coli*, these results are consistent with the results of their study, which showed that the percentage of presence of the inticron 1, 2, and 3 genes was (100%), (90%), and (65%), respectively. It is also consistent with the study of (Mahasin, 2021) on Gram-negative bacteria. According to the results of this study, intron 1 and 3 were the most common among the isolates.

Table (2) chromosomal introns in *p. mirabilis*

***Proteus mirabilis* isolate**	**sample**	**IL1**	**IL2**	**IL3**
IQD-No.1	Urine	+	+	+
IQD-No.2	Urine	+	+	+
IQD-No.3	Urine	+	+	+
IQD-No.4	Urine	+	+	-
IQD-No.5	Urine	+	+	+
IQD-No.6	Urine	+	+	+
IQD-No.7	Urine	+	+	+
IQD-No.8	Urine	+	+	+
IQD-No.9	Urine	+	+	+
IQD-No.10	Urine	+	+	+
IQD-No.11	Urine	+	+	+
IQD-No.12	Urine	+	+	+
IQD-No.13	Skin	+	+	+
IQD-No.14	Skin	+	+	+
IQD-No.15	Skin	+	+	+
IQD-No.16	Skin	+	+	+
IQD-No.17	Middle ear	+	-	+
IQD-No.18	Middle ear	+	+	-
IQD-No.19	Middle ear	+	+	-
IQD-No.20	Middle ear	+	+	-

Integron 1 on chromosomal

The integron was genetically estimated through the emergence of the integron gene responsible for the diagnostic characteristics in *P.mirabilis*. 20 isolates produced the integron gene out of a total of 20 isolates through the use of a primer specific for the integron *p*roduction gene after applying the polymerase chain reaction technique to all the isolates and electrophoresing them, which led to the emergence of genetic packages at one level and with a single molecular size of (254bp) for integron1 and a ratio of (100 %) (Figure 2).

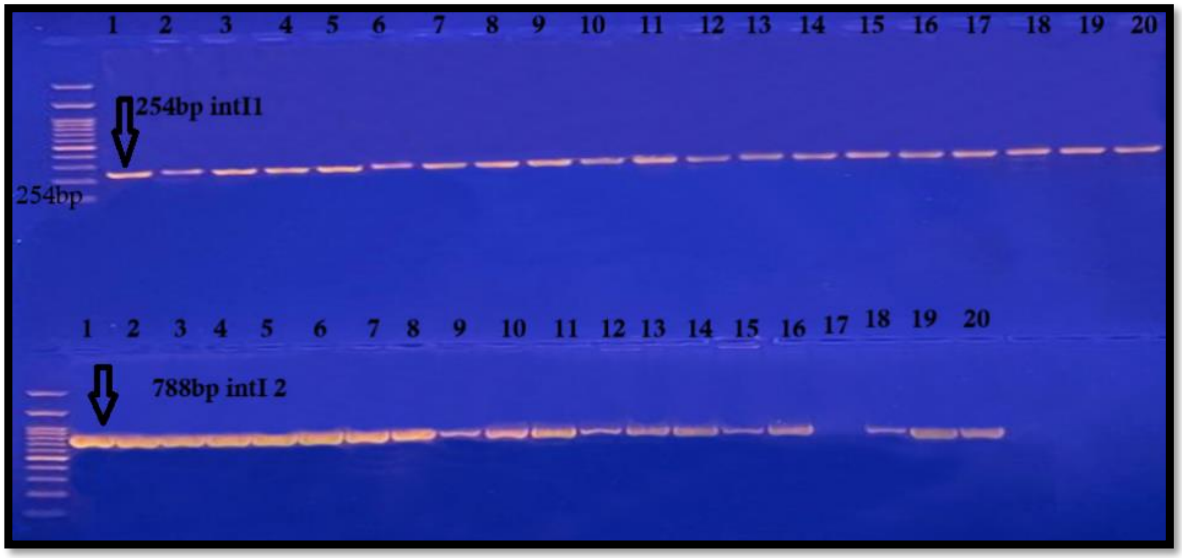

Figure (2) Agarose gel electrophoresis resulting from PCR obtained using a primer specific for the intI1 gene. Lanes 1-20 represent the intI1 gene and the product is bp in size (254 intI1), and M represents the 100 bp DNA line.

The results of this study were agreed with Mustafa (2023), as the results of his study revealed the presence of the anticron1 gene in Gram-negative bacteria Proteus.ssp at a rate of (16.6%) and the presence of the anticron2 gene at a rate of (83.25). They also converge with the study of Mahasin (2021), which showed the presence of a gene. Antrons1 and 3 were found in urine samples at a rate of (52.2%) and (38.6%), respectively. It also converges with Amel et al., (2023) in Egypt, where the intchron1 gene appeared at a rate of (80%), while the intchron 2 gene appeared at a rate of (66.6%). It does not agree with Li et al., (2020) who found that only 26% and 15% of their *P. mirabilis* isolates respectively harbored both integron1 and 2 together.

Combination of Integrons from chromosomal DNA

The result of this isolated study shows the distribution of introns as triplicate, combined and single. Of the 44 *E. coli* isolates, 9 (20.4%) had three integron classes, 14 (31.8%) had co-integration class I and II, I and III, and II and III and 11 (25.0%) had one type. The results of this study indicate that the prevalence of intron (81.8%). However, 8 (18%) of the MDR strains did not carry an intron on their chromosome DNA. Two isolates did not have an integrons gene on their chromosomal DNA, possibly due to an alternative mechanism of drug resistance. Therefore, thirty-six of the *E. coli* isolates had Integrase I, II, and III genes on the chromosomal DNA, indicating that these virulence factors are integrated into the genetic information of the bacteria.

4. Conclusion and future scope

The study concludes that the molecular diagnosis were identical to the results of the Vitec-2 device. The diagnostic results showed that 20% of the isolates belonged to Proteus mirabilis, with each isolate possessing genetic bands of 1500+, despite the fact that isolates that did not show the diagnostic gene upon migration were given a positive result for the phenotypic diagnosis on culture media and for biochemical and microscopic tests. As for examination using PCR technology for the three categories

of integrons gene, all isolates carried the chromosomal integrons gene. The appearance of the integrons1 gene on all isolates was 100.

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