Molecular detection of some virulence genes for Proteus mirabilis bacteria isolated from diabetic foot ulcers

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ABSTRACT

Molecular analysis was used in this investigation to identify the bacterium Proteus mirabilis, which was collected from individuals with diabetic foot ulcers. Two hundred fifty wound swab samples were obtained from diabetic patients receiving treatment at Al-Diwaniyah Teaching Hospital and patients receiving treatment at private clinics between October 2021 and February 2022. According to the findings, eighty (80) positive isolates were found by the bacteriological test, biochemical tests, and VITEK2 system. PCR was performed using a specific primer (16SrRNA) to identify the genus Proteus and distinguish it from the other Enterobacteria and bacteria. PCR methods were utilized in order to investigate the genes ureR, which is accountable for the production of the enzyme urease; hpmA, which is accountable for the production of hemolycin; zapA, which is accountable for the production of the protease enzyme; and flaA, which is accountable for the production of flagella. These genes showed up with relative frequencies of 68.75 percent, fifty percent, one hundred percent, and one hundred percent. The PCR technology does the sequence analysis of the Nitrogen bases on the samples used in this investigation demonstrates a level of consistency in strains of P. mirabilis of the WHO that reached up to 98 percent. This study demonstrated a high level of sensitivity and specificity of P. mirabilis diagnosis with the PCR technology, which is less expensive and more efficient than the conventional approaches currently utilized in medical facilities and research laboratories.

Keywords: Proteus mirabilis, diabetic foot ulcers, PCR, virulence Factors

Introduction:

DM causes elevated blood glucose levels due to insulin insufficiency, -cell dysfunction, and insulin resistance. Persistent hyperglycemia damages blood vessels, nerves, and organs. Global diabetes and its consequences are rising (Mostafa and Abdel-Hamid, 2021). Numerous genetic investigations have proven a definite hereditary component to diabetes and its consequences. Clinical risk factors and glycemic management alone cannot predict the development of vascular problems (Cole and Florez, 2020).

Diabetic foot infections are a significant source of fatality in people with diabetes. It's still unclear if the presence of anaerobes causes more severe symptoms (Toniolo *et al*., 2019). *S. aureus*, *E. spp*., Group B Streptococci, *Proteus spp*., *P. aeroginosa*, *K. pneumoniae*, and *E. coli* cause diabetic foot ulcers (Zubair *et al*., 2015).

The Proteus genus is an opportunistic Gram-negative bacillus, and its members are

widely distributed in the digestive system (animals and humans) and the environment (Armstrong *et al*., 2018). *Proteus* is nosopathogen community-acquired infection. The common Plasmodium fascicles have been reported to cause wound infections, respiratory tract infections, community-acquired urinary tract infections, and catheters (Yu *et al*., 2019). Common bacterial characteristics have the ability to adhere to epithelial surfaces, invasion of host cells, multiplication, colonization, enzymes, and toxins production. The cell surface of Proteus bacilli is very important for its strength, particularly its ability to adhere, colonize the urinary tract, and form stones (Arunagiri *et al*., 2017).

P. mirabilis has pathogenicity factors and morphological characteristics, such as capsules, flagellum, fimbria, and can produce enzymes, and toxins, which explain the difficulty in achieving clinical therapy success (Alamuri and Mobley, 2008). Evidence reveals a link between P. mirabilis' hemolytic activity and its invasive and cytotoxic capacity in Vero cells (Swihart and Welch, 1990). Our study aims to investigate the virulence factors that Proteus mirabilis has that are isolated from diabetic foot ulcers wounds in Al-Diwaniyia province.

Material and Methods

Collection of Samples:

A total of 250 Swabs were collected from patients with Diabetic Foot Ulcers. Samples were collected from patients during the period from October 2021 to February 2022 from patients at Al-Diwaniyah Teaching Hospital and private clinics.

Bacterial diagnosis:

P. mirabilis bacteria were isolated using culture on both blood and MacConkey agar, which was cultured aerobically for 24 hours at 37°C. Biochemical tests such as oxidase, indole, citrate utilization, catalase, urease production, H2S formation, lactose fermented, Voges-proskauer reaction, Methyl red, Trible sugar iron test (TSI), and Simmon citrate were used to identify bacteria (Hawkey, 2006; Forbes *et al*., 2016; Jacobsen *et al*., 2008) as well as Vitek2 and PCR technique used to diagnosis the bacteria (Bunyan & Albakery, 2021).

Molecular Detection:

A. Urease detection: It was carried out in accordance with (Zhang *et al*., 2012).

B. Heamolysin Detection: It was carried out in accordance with (Senior and Hughes, 1988).

C. Extracellular Producing protease Detection: It was carried out in accordance with (McCartney *et al*., 1989).

D. Flagellin Production Detection: It was carried out in accordance with (Nielubowicz, 2010).

E. DNA Extraction: *P. mirabilis* DNA was isolated using (gDNA Extraction mini Kit) processed by a Favorgen firm and pursuant to the directions of the providing company.

F. DNA electrophoresis was carried out as described in (Reid, 1991).

G. DNA Primers: Table 1 lists the primer sequences used in the investigation.

H- Thermal cycles program to amplify the DNA: The thermal cycler PCR was used to carry out the enzyme polymerization process.

I. Contents of Reaction Mixture: Table 1: DNA amplification reaction mixture components (3). Using an agarose gel at (1-1.5 %) (Sambrook et al., 1989) created an agarose gel by dissolving agarose (1.5 gm) in TBE buffer (100 ml) in a microwave; after that, cooling to 50 C, and then adding 0.5 mg/ml ethidium bromide.

The comb formed wells for DNA sample on one end of the tray (5-50 contraction). Pour the agarose into the tray and let it firm for 30 min at 25 C. The comb was then carefully removed. 8 l of each PCR product, along with Gel loading dye and a 100 bp DNA ladder, were added into the wells, and the apparatus was turned on. Seventy-two minutes of 75-volt electrophoresis were performed. A UV device was used to see the DNA bands

Table (1): The primers, sequences

Primer	Sequence 5'-3'	Product Size (bp)	Reference
16S rRNA	F-CCTGGACAAAGACTGACGCT R-CGCTTCTCTTTGTATGCGCC	523	(Alatrash and Al-Yasseen, 2017)
UreR	F-GGTGAGATTTGTATTAATGG R- ATAATCTGGAAGATGACGAG	225	(Adnan, 2014)
zapA	F-ACCGCAGGAAAACATATAGCCC R-GCGACTATCTTCCGCATAATCA	540	(Ali and Yousif,2015)
hpmA	F-GTTGAGGGGCGTTATCAAGAGTC R-GATAACTGTTTTGCCCTTTTGTGC	717	(Ali and Yousif,2015)
flaA	F-AGGATAAATGGCCACATTG R- CGGCATTGTTAATCGCTTTT	417	(Ali and Yousif,2015)

F*: Forword R:Reverse**

Table (2): Thermal cycle's program for the interaction of single PCR

Target gene	Cycling Conditions					
	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	No. of cycles
ureR	95°C/2 min	95°C/ 30 Sec	58 °C/ 30 Sec	72°C / 30 Sec	72°C / 5 min	32 Cycles
zapA	95°C/2 min	95°C/ 30 Sec	59 °C/ 30 Sec	72°C / 60 Sec	72°C / 5 min	306 Cycles
hpmA	95°C/2 min	95°C/ 30 Sec	56.2 °C/ 30 Sec	72°C / 20 Sec	72°C / 5 min	34 Cycles
flaA	95°C/3 min	95°C/ 30 Sec	54.2 °C/ 30 Sec	72°C / 30 Sec	72°C / 5 min	35 Cycles

Table (3): Polymerase Chain Reaction Mixture

No.	Mixture Contents	Volume (µl)
1.	Master Mix	12.5
2.	Forward Primer	2.5
3.	Reverse Primer	2.5
4.	Template DNA	5
5.	Nuclease -Free Water	2.5
Total		25

Results and Discussion:

1. Isolation and Identification of *Proteus mirabilis:*

This study revealed the prevalence of *P. mirabilis* 80(32%) of 250 swabs in diabetic foot ulcers patients, detection of this bacteria by the culture and biochemical tests. The culture on MacConkey agar showed *P. mirabilis* looked pale, yellow, and lactose nonfermenters, similar to (Forbes *et al*., 2016). On blood agar, *P. mirabilis* culture exhibits the swarming phenomenon and the fish odor, mucoid and non-hemolytic colonies which agree with (Al-Aabideen, 2005).

Biochemical assays were utilized to *prove that P. mirabilis showed positive for Catalase,*

citrate, Urease, H2S production, and Methyl Red, all of which were positive. In contrast, Oxidase, Indole, Lactose Fermentation, and Voges-Proskauer (VP) were all negative; the present research's biochemical tests accord with those of a previous study (Hawkey, 2006; Forbes *et al*., 2016; Jacobsen *et al*., 2008). The findings of this investigation match those of (Jabur *et al*., 2013), who identified *P. mirabilis* from urinary tract infections. Also, *P. mirabilis* was isolated from patients with UTI by (El-Baghdady *et al*.,2009; Wang *et al*.,2014).

Furthermore, the results of *P. mirabilis* identification using the Vitek2 system revealed that all isolates were *P. mirabilis*, with a percentage of identification ranging from (95 to 99%). This percentage was consistent with (Sung *et al*., 2000), who reported that the identification of Proteus mirabilis by the VITEK 2 system was (97%). Manual biochemical assays are commonly employed for bacterial identification. The advantages of traditional procedures were that they were inexpensive. However, the downsides were that they took time and were prone to contamination present compared to automated biochemical testing like the VITEK 2 system. In several prior experiments, the VITEK 2 technology discovered bacteria faster, more efficiently, and without contamination. The present study's biochemical testing confirmed that all isolates were P. mirabilis, as in the prior study (Drzewiecka et al., 2016).

Eighty bacterial isolates were selected to perform the PCR technique to diagnose bacteria *P. mirabilis* using gene 16S rRNA; the current study results showed that it contained 80 isolation of bacteria *P. mirabilis* on the 16S rRNA gene, which represents the diagnostic gene for this bacteria. As DNA using the kit used for this purpose and migrating bacteria Figure(1) extracted the Electrolytic (5.1%) and detected by using ethidium bromide dye and examined in agarose gel Ultraviolet (UV).

As indicated in Figure (1), PCR identification of *P. mirabilis* using 16S rRNA revealed that all isolates were *P. mirabilis* and provided an excellent confirmative identification. The product size of the primers utilized for identification was (523 bp), which was the same for all *P. mirabilis* isolates (Al-Dawah *et al*., 2015), This finding matched (Schabereiter-Gurtner et al., 2001) and (Lu et al., 2000), who was using 16S rRNA for detection Proteus spp. in monomicrobial and polymicrobial ocular diseases (CSF).

According to the studies mentioned above, 16S rRNA has a strong discriminating ability for identifying *Proteus spp*. Furthermore, it enables the identification of dead cells and living but non-cultivable cells, which is acceptable (Postollec *et al*., 2011). (El-Bakkali *et al*., 2013) The Sequencing of the 16S rRNA gene is now used and based on it in identifying Bacterial isolates isolated from hospitals and different environments. The identification rate was using Sequencing of the 16s rRNA gene (93.1%). Compared to other methods that identify the bacterial isolate, the ratio (60.34%) was higher.

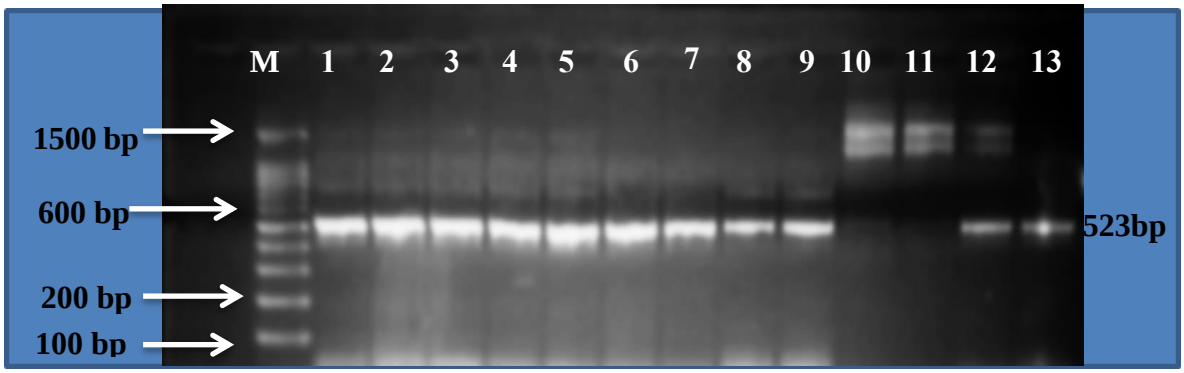

Figure (1): agarose gel electrophoresis showing the results of 16S rRNA (523 bp) polymerization for bacteria *P. mirabilis*, the Pits (1-13) Bacterial isolation, M = marker (100-1500 bp).

Virulence factors of *P. mirabilis:*

In order to determine virulence factors, phenotypic and genotypic features were evaluated for all *P. mirabilis* isolates in this investigation. The existence of (UreR, hpmA, ZapA, and flaA) genes was tested using particular primers.

The PCR findings for the *P. mirabilis* isolates revealed that 55 of 80 isolates tested positive for the UreR gene (68.75%), as shown in Figure (2).

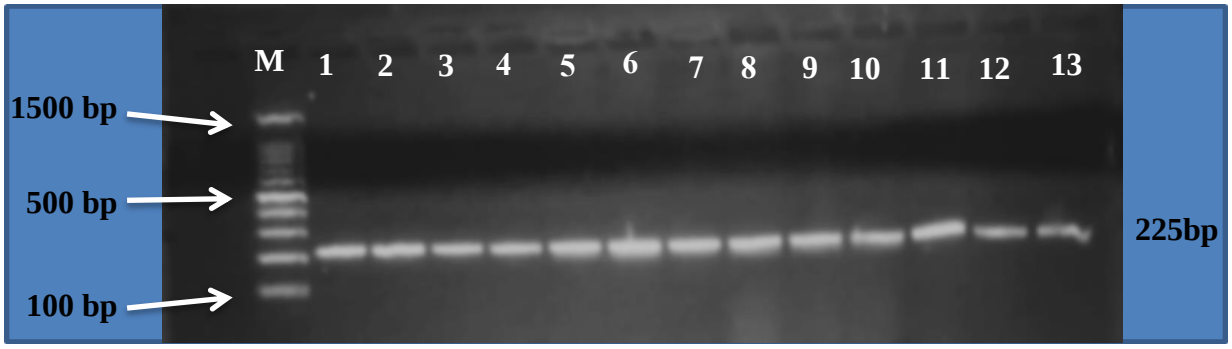

Figure (2):Agarose gel electrophoresis showing the results of UreR gene (225 bp) polymerization for bacteria *P. mirabilis*, the Pits (1-13) Bacterial isolation, M = marker (100-1500 bp).

The ureR gene is an important gene in Bacteria's diagnostic process (Mobley *et al*., 1995). Consider researcher (Zhang *et al*., 2012) the ureR gene is a highly specialized gene in diagnosing type *P. mirabilis*. Who is responsible about the production of the enzyme urease, which is present in the species *P. mirabilis* ureR. Genes have been investigated It is responsible for the secretion of urease from bacterial isolates And isolated cases of diabetic foot ulcers, and used qualitative primers Customized, after several trials, the best concentration was determined It was around 25 ng/µl. And used Scores (50-56-58-60 centigrade) were selected conditions based on the best results that have appeared; the best glow was under the ultraviolet rays. The best specialized single packages for reaction product at The temperature is 58°C, and it has been relied upon to give A positive result for the detection of ureR genes, By comparing the results with the standard DNA size by an electrical relay as it is close to (225 bp).

The authors (Limanskiĭ *et al*. 2005) indicated that it could be assumed that the ureR gene method should be more specific for characterizing *P. mirabilis* samples. The PCR technique was adopted as it diagnosed these bacteria specifically by the 16sRNA gene and the ureR gene (Postollec *et al*., 2011).

Figure 2 shows that the ureR gene was found in 55 of these isolates. The ureR gene is responsible for making urease at a 68.75% rate. The urease enzyme that P. mirabilis makes is more powerful than the urease enzyme that other bacteria make. Stankowska et al. (2008), all of the isolates made urease enzyme, do not agree with this study. At the same time, the Abbas et al. (2015) study showed that 18% of bacterial isolates made the urease enzyme.

The results of this study also showed that all the isolates have zapA gene, which is responsible for proteins making at a 100% rate. This was checked using PCR, Figure (3), and that agrees with Bunyan and Albakery (2021) found about p. mirabillis, which was that it had the zapA gene at a ratio of 1:1. (100 percent). The rotease is thought to be one of the most important enzymes because it can break down IgA and IgG antibodies, which decreases the immune response and makes the bacteria that make them more dangerous. The P. mirabilis could make case protein of the type (Metaloprotease), which had been found in swabs taken from people with infected diabetic foot ulcers. On the other hand, strains of the genus Proteus that do not cause disease are not as good at making these enzymes (Belas et al., 2004)

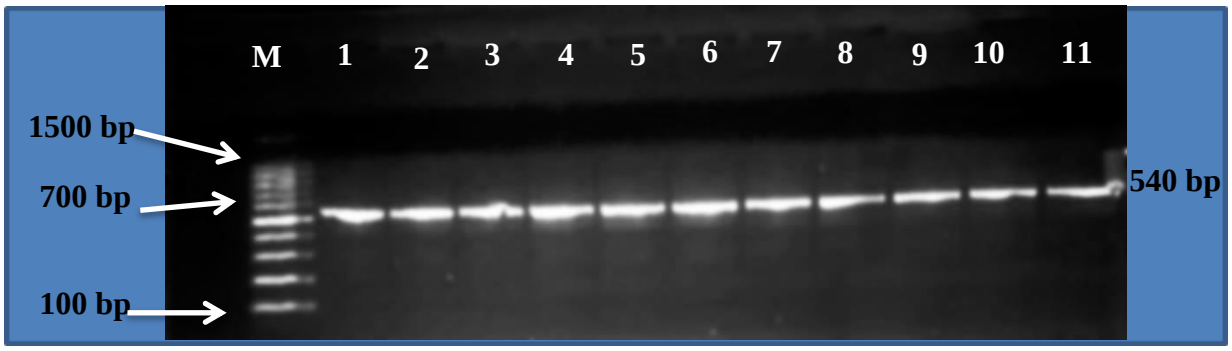

Figure (3): the amplified zapA gene for *P. mirabilis* by Single PCR, wherever, M is ladder (from 100 to1500).

flaA is a virulence factor that helps this bacteria move quickly and causes infection; the main component is a protein called flagellin (Umpiérrez *et al*., 2013). *P. mirabillis* has two types of genes responsible for the generation of flagellin: flaA و flaB (Hatt and Rather, 2008). Flagella production is a crucial step in motility and swarming, including many genes on the *proteus* chromosome. The flagellin protein FlaA, encoded by flaA, makes up most of the filamentous part of the *P. mirabilis* flagellum (O'May *et al*., 2008). As it was observed through the current study of the genetic aspect of flagella by using a molecular technique that 80 swabs of *p. mirabillis* bacteria possessed the flaA gene at ratio (100%), as shown in Figure (4). the results of the current study came close to the finding of (Bunyan and Albakery, 2021), where the ratio (100%). At the same time, the results of (Ali and Yousif, 2015) found that *p. mirabillis* possess flaA at a ratio (86.66%).

Figure (4): the amplified flaA gene for *P. mirabilis* by Single PCR wherever, M is the ladder (from 100 to 1500)

The hmpA gene is responsible for the production of the virulence factor Hemolysin, which is important for *P. mirabilis*. Hemolysin damages the membrane of red blood cells by creating tiny holes in the cell membranes and epithelial cells. Its presence is an important factor in the supply of iron to bacteria, and because of its cytotoxicity (cytotoxic), it leads to the destruction of host tissues (Liaw *et al*.,2000) and thus increases the pathogenicity of bacteria when present in the tissues of the host (Abed-Wahed *et al*., 2001). HpmA was analyzed using the PCR technique to confirm their ability to produce extracellular hemolysin. The results of the current study showed that these bacteria possess hpmA gene in 40 isolates(50%) of the total 80 bacterial isolates, as shown in Figure(5), and this result was close to what was reached (Uphoff and Welch, 1990), The percentage of *P. mirabilis* that possesses the gene (hpmA) was (46.7%), while the study (Filimon and Iacob, 2007; Cestari *et al*., 2013) as they reported that the gene rate in P. mirabilis was (97.15%), and (Sosa *et al*.,2006; AL-Jumaa *et al*.,2011; Jabur *et al*.,2013 and Mohammed,2014; Ali and

Yousif,2015; Bunyan and Albakery,2021) indicated that the presence of this gene in these bacteria was (100%) as already mentioned. This enzyme is considered one of the virulence factors that contribute to the pathogenicity of these bacteria, as it works to provide nutrients resulting from the degradation processes carried out by this enzyme

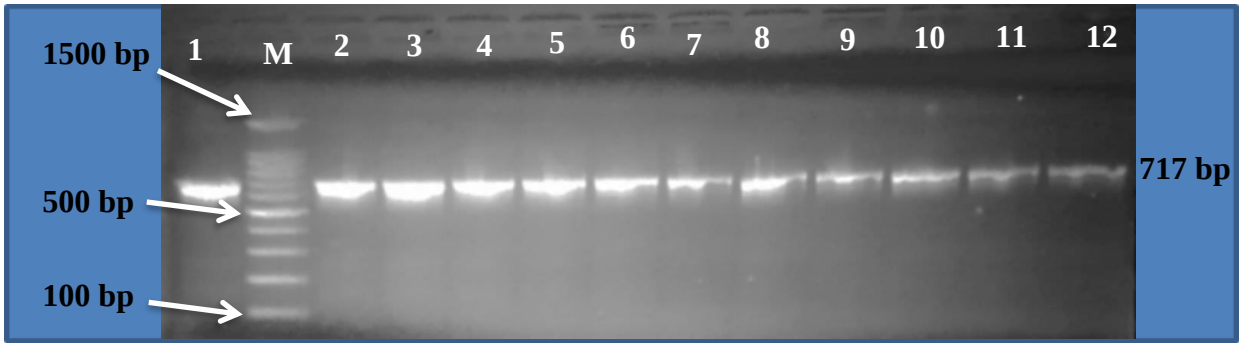

Figure (5): Results of amplified gene hmpA (717 bp) for *P. mirabilis* using Single PCR .M (DNA Ladder 100-1500), (1-12) bacterial isolates.

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