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Genetic and phenotypic detection of beta-lactamase isolated from *Proteus mirabilis* bacteria in hospitals of Anbar Province

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ABSTRACT: The study aimed to isolated *Proteus mirabilis* and determined the genotype and phenotype of the beta-lactamase enzymes produced by these bacteria. 242 samples were collected from various pathogenic cases during the period of August 2023 to January 2024. A fifty-three isolates of *Proteus mirabilis* were isolates Distributed as follows: 47 isolates (88.7%) were obtained from urinary tract infection, 5 isolates (9.4%) from otitis media, while one isolate (1.9%) from vaginitis. The samples were diagnosed culturally using appropriate culture media, microscopically using the Gram stain and biochemically. Then the isolates were confirmed using the VITEK Compact 2 device. After confirming the isolates, a sensitivity test was conducted against some antibiotics that belong to different groups, and the results showed that the highest resistant was against the antibiotic cefotaxime with a rate of 56.6%, and Amikacin with a rate of 41.5% followed by Amoxicillin-Clavulanic acid with 35.9%, while ciprofloxacin, levofloxacin, cefepime, and azithromycin the resistant rates were moderate with percentages 30.2%, 28.3%, 26.4%, and 20.8% respectively. The lowest percentages of resistance shown by the isolates was against Azeteronam with 5.7% and imipenem with 3.8%, while for meropenem, all isolates were sensitive to this antibiotic. The results showed that 43.4% of the isolates were Multi-Drug Resistance, as the number of isolates that have resistance to more than three of the selected antibiotics is 23 isolates. Phenotypic results according to the PST test results showed that 32 isolates (60.38%) tested positive, indicating a high probability of beta-lactamase enzyme production according to the PCT test results. PCR and gel electrophorese revealed that, blaTEM gene were present in 32.1% of the isolates, while blaSHV gene were present in 5.7% only. Interestingly, blaCTX-M1 was present in all the isolates that produce beta-lactamases enzymes, 41.5%. Metalobeta-lactamases genes, blaIMP and blaGIM were present in 13.2% and 64.2%, respectively.

Keywords: *P. mirabilis*, Genotypic, MDR, beta-lactamases

1. INTRODUCTION

Gram-negative bacteria, motile and urease-producing it is member of the Enterobacteriaceae family. Urinary tract infections (UTIs) have long been linked to *Proteus* species, in individuals without risk factors as well as those with indwelling catheters or aberrant urinary architecture [1]. *Proteus* species are common in nature and contribute to the normal flora of the human digestive system. Only six species in this genus have been identified from human clinical specimens: *P. mirabilis, P. vulgaris, P. terrae, P. penneri, P. hauseri, and P. faecis* [2]. Microbes have evolved a number of defense mechanisms against the ß-lactam antibiotics' inhibitory effects. The main mechanism of resistance is caused by ß-lactamases, such as penicilinases and cephalosporinases, hydrolyzing the ß-lactam ring [3]. Numerous virulence factors, including fibrillae, fagellae, urease enzyme, hemolysin synthesis, protease enzyme synthesis, biofilm synthesis, and quorum sensing, are present in *P. mirabilis* [4]. *P. mirabilis* has the ability to generate biofilms in addition to its virulence components. These structures give the pathogenic bacteria more opportunities to multiply their pathogenicity and strengthen their antibiotic resistance [5]. *P. mirabilis* Vegetative swimmer cells differentiate into elongated, multinucleated, and strongly flagellated swarmer cells, which produce typical swarming movement [6]. Swarming is a surface movement characterized by flagellum dependence, as opposed to swimming through liquid or soft agar [7]. A more complex infection may sometimes result in bloodstream infection, infection spreading to the blood, and in extremely rare instances, infectious endocarditis (IE) [8]. Microbes may become resistant to multiple drugs (MDR), increasing the likelihood of recurrent infections and perhaps making antibiotic therapy ineffective [9]. Due to their numerous diseases-causing potential, these bacteria were selected and aim of the study detecting P. mirabilis's capacity to produce beta-lactamases using phenotypic and genetic techniques.

2. MATERIALS AND METHODS

2.1. Collection of samples and isolation and identification:

In this study, 242 samples were taken from clinical cases the samples set comprised 159 cases of urinary tract infection, 60 cases of otitis media, 11 cases of wound infection, 7 cases of vaginitis, and 5 cases of burn infection at Ramadi Hospitals between August 2023 to January 2024. Diagnosis was based on the gram stain, cultural features, biochemical tests, and identification was confirmed with the Vitek2 compact system as required were used to identify of *P. mirabilis* [10,11]

2.2. Antimicrobial Susceptibility Test

The test was conducted to determine the sensitivity of bacteria to the chosen antibiotics, based on the Kirby-Bauer method, according to what was stated in CLSI [12], as follows:

The isolates were grown on MacConkey Agar medium, then one colony was transferred to the normal saline, and the solution was measured to be approximately 1.5 x 10^8 cells/ml. The bacterial solution was then spread on Mueller-Hinton Agar medium using sterile cotton swabs and then left to dry at room temperature for 5 minutes, then the antibiotic tablets were distributed on the plates and incubated for 24 hours at 37 C.

2.3. Phenotypic Detection

Four antimicrobial drugs were tested for resistance in isolates using a phenotypic screening test (PST) for b-lactamase production: cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), and aztreonam (30 µg). It was thought that resistance to at least one antibiotic indicated the possibility of b-lactamase synthesis. A phenotypic confirmatory test (PCT) using a combination disk approach was applied to isolates that tested positive for PST. For PCT test combination of amoxicillin (AMX; 25 µg) and AMX plus clavulanic acid (AMC; 20 plus 10 µg) where used [13].

2.4. Genotypic Detection

DNA was extracted based on the instructions of the processing company (Trans Company). After that, the purity and concentration of the DNA was confirmed using the Nano drop device.

Target gene	**Oligonucleotide primer sequence (5′→3′)**	**Size of product (bp)**	**Referance**
blaTEM	F:5′TCCTTGAGAGTTTTCGCCCC3′ R:5′TTGTTGCCGGGAAGCTAGAG3′	452 bp	(Ghaima,2021)
blaSHV	F:5′ATGCGTTATATTCGCCTGTG3′ R:5′ TGCTTTGTTCGGGCCAA3′	747 bp	(Ghaima,2021)
blaCTX-M1	F:5′ACGCTACCCCTGCTATTT 3′ R:5′ CCTTTCCGCCTTCTGCTC3′	850bp	(Ghaima,2021)
blaIMP	IMP-F/GGAATAGAGTGGCTTAAYTCTC IMP-R/ GGTTTAAYAAAACAACCACC	233bp	(Seyedi, 2022)
blaGIM	GIM-F/ TCGACACACCTTGGTCTGAA GIM-R/ AACTTCCAACTTTGCCATGC	477bp	(Seyedi, 2022)

Samples were prepared for placement in a thermocycler (Bio-Rad) by adding 12.5 microliters of Master Mix (Trans), 9.5 microliters of free nuclease water, 1 microliter of forward primer, 1 microliter of reverse primer, and 1 microliter of DNA. Samples were placed in the device according to the conditions of each gene.

Table 1. Program of PCR for blaTEM gene

PCR steps	Temperature	Time	No. of cycles
Initial denaturation	95 ºC	5 min	1
Denaturation	95 ºC	30 sec	30
Annealing	63 ºC	30 sec	30
Extension	72 ºC	30 sec	30
Final extension	72 ºC	7min	1

Table 2. Program of PCR for blaSHV gene

PCR steps	Temperature	Time	No. of cycles
Initial denaturation	94 ºC	2 min	1
Denaturation	94 ºC	1 min	30
Annealing	58.5 ºC	1 min	30
Extension	72 ºC	1 min	30
Final extension	72 ºC	5min	1

Table 3. Program of PCR for blaCTX-M1 gene

PCR steps	Temperature	Time	No. of cycles
Initial denaturation	94 ºC	2 min	1
Denaturation	94 ºC	1 min	30
Annealing	59.6 ºC	1 min	30
Extension	72 ºC	1 min	30
Final extension	72 ºC	5 min	1

Table 4. Program of PCR for blaIMP gene

PCR steps	Temperature	Time	No. of cycles
Initial denaturation	94 ºC	2 min	1
Denaturation	94 ºC	1 min	30
Annealing	55 ºC	1 min	30
Extension	72 ºC	1 min	30
Final extension	72 ºC	5 min	1

Table 5. Program of PCR for blaGIM gene

PCR steps	Temperature	Time	No. of cycles
Initial denaturation	95 ºC	3 min	1
Denaturation	95 ºC	30 sec	34
Annealing	60 ºC	30 sec	34
Extension	72 ºC	1 min	34
Final extension	72 ºC	5 min	1

3. RESULTSAND DISCUSSION

3.1. Isolation and identification

Fifty-three *Proteus mirabilis* isolates were is from isolated 242 samples collected, including 47 isolates from urinary tract infection, 5 isolates from otitis media, and one isolate from vaginitis. Microscopic test results showed Gram-negative rods and biochemical tests results as shown in the table (6). The isolates were strongly positive for the urease test and positive for the TSI test, as some of the isolates were A/A and some were K/A and all isolates were H2S producers and non-gas producers. The isolates were also positive for catalase, methyl red and the citrate utilization test, while they were negative for the oxidase, indole, and Voges Proskauer tests.

Table 6. Biochemical Test

Biochemical test	Result
1-Indole	-
2-Methyl red	+
3-Voges Proskauer	-
4- Citrate utilization test	+
5-TSI	A/A , K/A , gas _ , H2S +
6-Catalase	+
7-Urease	+
8- Oxidase	-

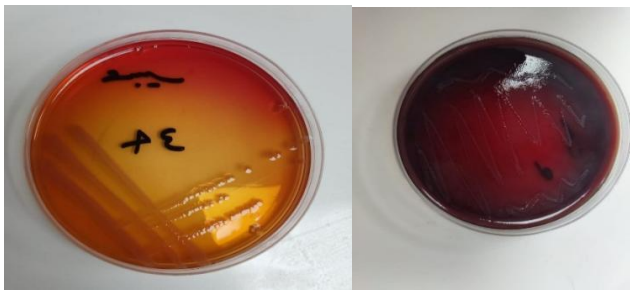

Figure 1. Growth *Proteus mirabilis* on MacConkey agar and Blood agar

3.2. Sensitivity test

In this study, a sensitivity test was conducted against 10 antibiotics using the disc diffusion method. The results showed that the highest percentage of resistance was against Cefotaxime at a rate of 56.6%, followed by Amikacin and Amoxicillin-Clavulanic acid with 41.5% and 35.9%, respectively. The isolates showed moderate levels of resistance to the antibiotics Ciprofloxacin, Levofloxacin, Cefepime, and Azithromycin with 30.2%, 28.3% ,26.4%, and 20.8% respectively. The isolates were showed low level of resistance against Azeteronam with 5.7%, and to a lesser extent, against Imipenem with 3.8%. Surprisingly, the resistance rate of the isolates to meropenem was 0%, As the isolates did not show resistance to this antibiotic. A study was conducted in Al-Diwaniyah Governorate by [13], it showed that the highest rate of resistance was against Cefotaxime with 53.62%. The study also showed that the lowest rate of resistance was towards Meropenem at 5.6%, and this is consistent with the results of this study, but the study that was conducted in Diwaniyah Governorate showed that the resistance rate of isolates to ciprofloxacin is 4.34%, and this percentage is far from the percentage that appeared in this study, as the resistance rate of isolates to the ciprofloxacin was 30.2%.

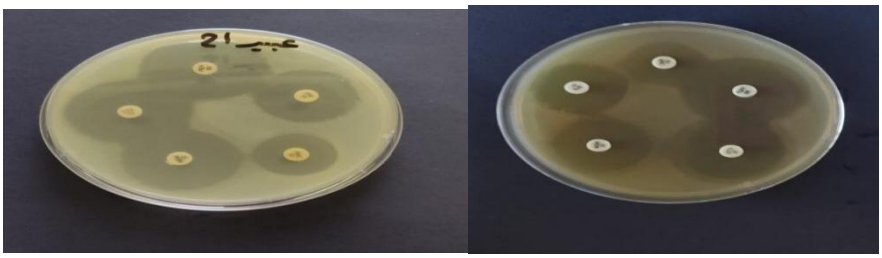

Figure 2. Sensitivity test of *Proteus mirabilis*

3.3. Phenotypic result

- According to the PST test results, 32 isolates (60.38%) tested positive, indicating a high probability of beta-lactamase enzyme production. 22 isolates (41.51%) were positive for beta-lactamase enzyme production, according to the PCT test results.

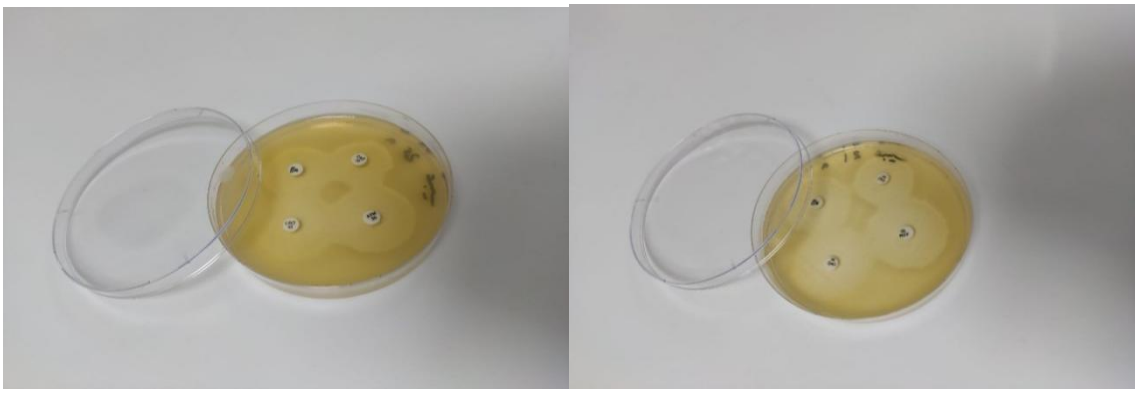

Figure 3. Phenotypic test, phenotypic screening test (PST) test, positive result.

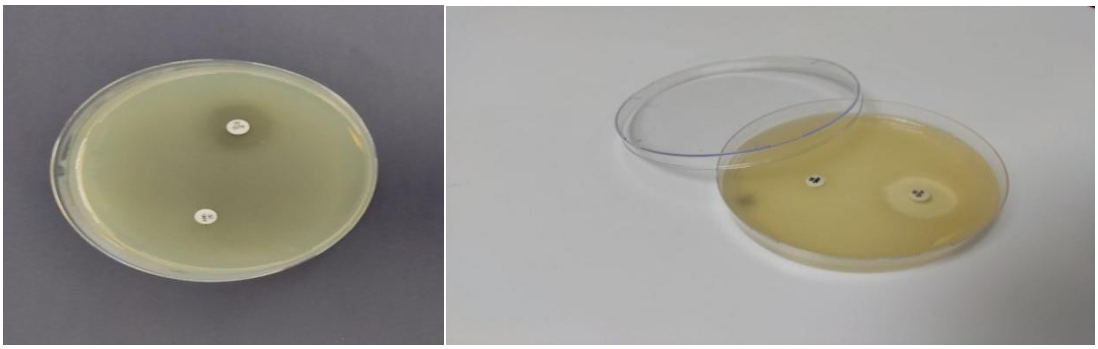

Figure 4. Phenotypic test , phenotypic confirmatory test (PCT) test , positive result.

3.4. Genotypic results

The results of PCR and gel electrophoresis showed that seventeen isolates out of a total of 53 isolates producing beta-lactamase enzymes contained the blaTEM gene (32.1%), while the results showed that all isolates producing beta-lactamase enzymes contained blaCTX_M1 (41.5%). and in contrast, with regard to the blaSHV gene, only 3 isolates contain this gene (5.7%). the blaIMP gene is present in 7 isolates (13.2%), and as for the blaGIM gene, it was found that 34 isolates contain this gene (64.2%).

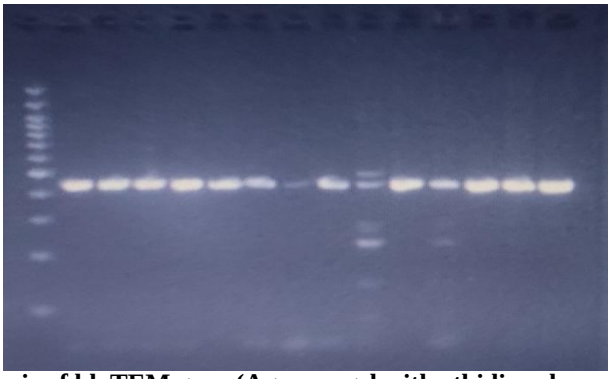

Figure 5. Gel electrophoresis of blaTEM gene (Agarose gel with ethidium bromide stained of mono-plex PCR amplified product from extract DNA of *P. mirabilis* isolates with blaTEM gene primers

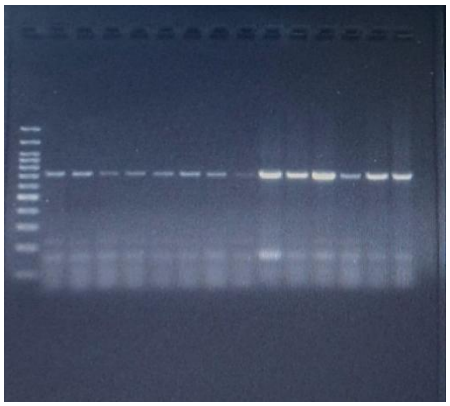

Figure 6. Gel electrophoresis of blaCTX-M1 gene (Agarose gel with ethidium bromide stained of mono-plex PCR amplified product from extract DNA of *P. mirabilis* isolates with blaCTX-M1 gene primers

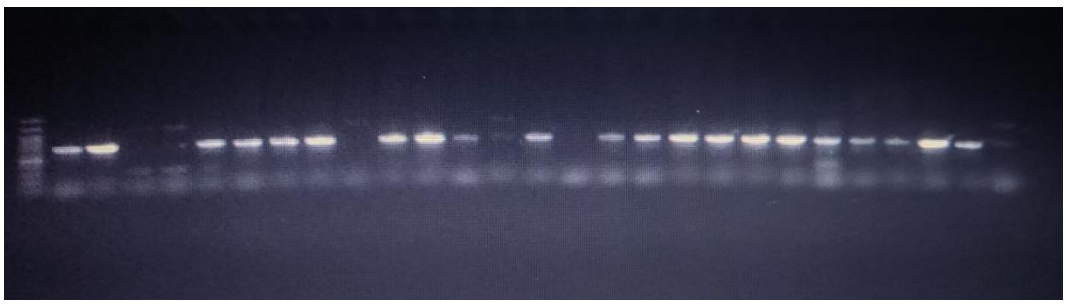

Figure 7. Gel electrophoresis of blaGIM gene (Agarose gel with ethidium bromide stained of mono-plex PCR amplified product from extract DNA of *P. mirabilis* isolates with blaGIM gene primers).

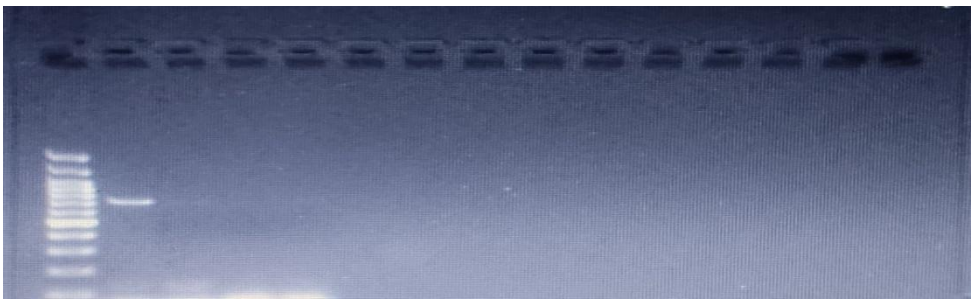

Figure 8. Gel electrophoressis of blaSHV gene (Agarose gel with ethidium bromide stained of mono-plex PCR amplified product from extract DNA of *P. mirabilis* isolates with blaSHV gene primers).

The results of the current study are consistent with Abdul-hussein and Hassan [15], every *P. mirabilis* isolate (100%) in their study was multidrug resistant (MDR), meaning it showed resistance to at least three different antibiotic classes.
Recently, there was observed association between susceptibility to certain antibiotic drugs, ability to form biofilms, and several virulence linked genes [16].
However, in order to create efficient treatment plans and stop the spread of isolates that are resistant to many drugs, it is imperative that the status of antibiotic resistance be continuously, frequently, and regularly monitored [16]. And choosing the right empirical antibiotic to treat a *Proteus mirabilis* infection will be made easier by having an understanding of the antibiotic sensitivity in the patient's locality [17]. Levofoxacin, sulfamethoxazole, and cefuroxime should no longer be the main choices for empirical antibiotic therapy due to the significant proportion of female patients with urolithiasis brought on by *Proteus mirabilis* infection. Rather, third-generation cephalosporins such ceftazidime and second-generation cefoxitin should be used [17].
A phenotypic screening test (PST) test was conducted to assess the isolates' productivity for beta-lactamase enzymes. The findings revealed that 32 isolates met the apparent test criteria, which is resistance to at least one of the antibiotics cefotaxime, cefetraxone, azethronam, and ceftazidime. The proportion of isolates that met the test criteria was 60.38 %. After that, the isolates were put through a phenotypic confirmatory test (PCT) test, in which amoxicillin, a beta-lactam antibiotic, was combined with amoxicillin-clavulanic acid, a beta-lactam inhibitor. According to the test results, 22 isolates (41.51%) produced beta-lactamase enzymes, indicating that they passed the test. These isolates changed from being resistant to sensitive due to their inability to produce the enzymes necessary to inhibit beta-lactam antibiotics. Specifically, these isolates were resistant to amoxicillin but sensitive to amoxicillin plus clavulanic acid because the latter inhibited beta-lactamase. The results of the phenotypic test of a study conducted in Najaf Province confirmed that, 26.8% of the isolates produced beta-lactamases enzymes [19].

The results of PCR and gel electrophoresis showed that 17 isolates (32.1%) contained the blaTEM gene. This result is a little far from what Ghazi and Ghaima [18] confirmed in their study, where he confirmed that 84.6% of the isolates contained the blaTEM gene. While the number of isolates containing the blaSHV gene was only 3 (5.7%), and this result is close to the result that was mentioned in a study conducted in Najaf Province [19], as only one isolate (5.3%) contained this gene. The results of this study showed that all isolates producing the beta-lactamase enzyme, 22 isolates (41.5%), contained the blaCTX_M1 genes and this result is not consistent with what was confirmed by Ghazi and Ghaima [18], as they mentioned in there study that (12.8%) of the isolates contain the blaCTX_M1 gene and (7.7%) contain the blaCTX_M2 gene, and this is a little far from the results of the current study. On the contrary, with regard to the blaIMP gene, only 7 isolates (13.2%) contain this gene, and this result is close to what was mentioned in a study conducted in Iran about *Pseudomonas*, where the study confirmed that only (10.6%) of the isolates contain this gene [20]. As for the blaGIM gene, it was found that, 34 isolates have this gene (64.2%), and this result does not agree with what [20], mentioned in their study. The results of their study confirmed that the blaGIM gene is not present in any of the isolates of *Pseudomonas* that also produce beta-lactamases. A (5.7%) of the isolates contained a combination of Extended-spectrum beta-lactamase genes (TEM, SHV, blaCTX_M1 and blaCTX_M2), and (32.1%) contained only blaTEM and blaCTX_M Extended-spectrum beta-lactamase genes, while the percentage of isolates containing The blaIMP and blaGIM genes together are (9.4%).

4. CONCLUSIONS

The antibiotics imipenem and meropenem are considered the best option for treating infections caused by Proteus mirabilis, Proteus mirabilis widely produces beta-lactamase enzymes include the blaSHV gene, which belongs to Extended Spectrum Beta_Lactamases, is rare in Proteus mirabilis, as is the blaIMP gene, which belongs to Metalobeta_lactamases and blaTEM, blaCTX_M1, is the most abundant genes in beta-lactamase-producing isolates.

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