

## Prevalence of *blaSHV*, *blaNDM* beta-lactamase genes using multiplex PCR in clinical isolates of *Proteus mirabilis*

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### Abstract:

#### Background:

*Proteus mirabilis* is a Gram-negative bacterium belonging to the family Enterobacteriaceae that cause numerous diseases, including pyelonephritis, urinary stones, prostatitis, catheter-associated urinary tract infections, and diarrhea in humans. A total of 180 collected samples from different clinical sites (urine, discharge, otitis media, wounds, and vaginitis) during September 2024 to December 2024 from Baquba Teaching Hospital and Al-Batoul Maternity and Children's Hospital in Diyala Governorate/Iraq.

#### Aims:

This study aimed to detect *P. mirabilis* with multi drug resistance by morphological and biochemical methods and to determine some virulence factors by phenotype methods as well as detecting of genes encoding antibiotic resistance including (*blaNDM* and *blaSHV*) through multiplex PCR, in clinical samples.

#### Results:

Results revealed that 20 (18.18%) isolates of *P. mirabilis* were obtained. The incidence rate of isolation in females was higher than in males, 12 (60%) and 8 (40%) respectively. In this study, a higher rate of positive isolation was recorded in urine samples (65%). Also, 45% of isolates were extended spectrum beta-lactamase (ESβL) producers and 30% were Metallo beta-lactamase (MβL) producers. The most effective antimicrobial agents against all isolates are meropenem followed by Amikacin. Resistant of *P. mirabilis* for many antibiotics, especially Trimethoprim and ampicillin. Molecular detection of beta lactamase genes revealed that only 6 (30%) had *blaSHV* gene while *blaNDM* only in 7 (35%) of isolates.

## Conclusion:

*P. mirabilis* was resistance for many antibiotics, especially Trimethoprim and ampicillin and meropenem was the most effective followed by Amikacin. All isolates showed different rates of ESBL and MBL production and this reflect different rates of resistance genes expression such as *blaNDM* and *blaSHV*.

**Keywords:** *P. mirabilis*, ESBL, Metallo-βL, *blaSHV*, *blaNDM*, multiplex PCR



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## Introduction:

*Proteus mirabilis* is a major causative agent of several diseases such as pyelonephritis, urinary tract stones, prostatitis, catheter-associated urinary tract infections, and diarrhea in humans (Armbruster & Mobley, 2012). *P. mirabilis* is a medically significant organism that typically causes the majority of common nosocomial opportunistic infections, including infections of the urinary system, wounds, ears, and other area. It is listed as a medically significant nosocomial agent due to several virulence characteristics, including genes that resist antibiotics. Several nosocomial infection outbreaks and community-acquired diseases have been linked to this bacterium in various regions of the world (Alabi *et al.*, 2017).

In some situations, the ESBL enzyme may be responsible for a relatively high prevalence of multidrug-resistant (MDR) strains of *P. mirabilis*. MDR may also result from genetic alterations in resistance genes or horizontal migration of resistance genes (Sfaciotte *et al.*, 2021). Misuse or non-specific use of antibiotics has led to increased levels of drug resistance and widespread distribution of different resistance genes among clinical *P. mirabilis* isolates. In addition, *P. mirabilis* has intrinsic resistance to tetracycline, tigecycline, and polymyxin (Sanchez *et al.*, 2023). Because of the existence of multi-resistant genes, isolates of *P. mirabilis* that produce ESBL are frequently found to be MDR. These genes allow *P. mirabilis* to endure a variety of antibiotics, including beta-lactams (Firmo *et al.*, 2020).

The gene encoding New Delhi metallo-beta-lactamase-1 (*blaNDM-1*), which leads to resistance to most beta-lactam antibiotics, was identified as early as 2006, and its occurrence was initially confined to the Indian subcontinent (Yong *et al.*, 2009). *blaSHV-1* encoded enzyme (sulfhydryl variable reagent) has been shown to be active against penicillins and first-generation cephalosporins and was confirmed as part of the conjugative plasmid p453 (Liakopoulos *et al.*, 2016). Allelic

variants of this gene have recently been described, which have developed resistance to third-generation cephalosporins, monobactams and carbapenems (Poirel *et al.*, 2003).

This study aims to analyze and identify gene sequences encoding antibiotic resistance, specifically *blaNDM* and *blaSHV*, using multiplex PCR. These resistance genes are concomitant with mobile genetic elements and play a crucial role in the multidrug resistance of *P. mirabilis* in clinical specimens.

## Materials and Methods

### Collection of samples

Samples were collected under the supervision of a specialist physician and included (180) specimens from different clinical sources including (urine, stool, otitis media, wounds, and vaginitis) from both males and females in different ages during the period from September 2024 to December 2024 at Baquba Teaching Hospital and Al-Batoul Maternity and Children's Hospital in Diyala Governorate/Iraq.

### Identification of bacterial isolates

The collected specimens were streaked on McConkey media, blood agar media, and nutrient agar media, after ensuring that the patient had not taken antibiotics for at least three days for isolation and initial diagnosis. *P. mirabilis* isolates were diagnosed according to morphological features on culture media, microscopic examination (Gram's stain) and biochemical tests (Indole, Oxidase, Catalase, Urease). The diagnosis was confirmed by using VITEK2 System (Pincus, 2011).

### Antibiotic susceptibility test (AST)

Antibiotic susceptibility testing of each isolate was tested against 11 antibiotics (Amikacin, Azithromycin, Ampicillin, Ceftriaxone, Ciprofloxacin, Levofloxacin, Meropenem, Gentamicin, Cefixime, Augmentin, Trimethoprim) according to Clinical and Laboratory Standards Institute (CLSI 2023) recommendations, using Kirby-Bauer disc diffusion method on Mueller-Hinton agar (MHA) and the inhibitory zones' diameters were noted.

### Extended spectrum Beta lactamase production

ES $\beta$ L production in *P. mirabilis* was determined by double disc synergy test (DDST) according to Patel *et al.* (2017).

### Metallo beta lactamase production

This test was detected according to Anoar *et al.* (2014).

### Genotypic detection of beta-lactamase genes

Twenty *P. mirabilis* isolates were subjected for amplification of two of beta-lactamase genes (*blaNDM* and *blaSHV*). Genomic DNA was extracted according manufactured company directions using a bacterial genomic DNA extraction kit (Promega/USA). The presence of *blaNDM* and *blaSHV*

genes were screened for by PCR amplification. Primers was confirmed using the NCBI website. Primers, PCR components and program demonstrated in tables (1), (2) and (3)

Table 1: Primers used in the study

Genes	base pair sequences (5'–3')	Product size/ bp	References
<i>blaNDM</i>	F: TGGCAGCACACTTCCTATC R: AGATTGCCGAGCGACTTG	488	Nasri <i>et al.</i> , 2017
<i>blaSHV</i>	F-AGCCGCTTGAGCAAATTA AAC R-ATCCCGCAGATAAATCACCAC	713	Hammond <i>et al.</i> , 2005

Table 2: The size of the PCR mix components for amplifying genes in this study.

Name of target gene	Type of reaction	Master mix	F primer 10 pmol	R primer 10 pmol	Volume of template	Nuclease Free Water	Final volume
<i>blaNDM</i>	Multiplex PCR	12.5 µl	1 µl	1 µl	5 µl	5.5 µl	25 µl
<i>blaSHV</i>		12.5 µl	1 µl	1 µl	5 µl	5.5 µl	25 µl

Table 3: PCR program

Amplified gene		Primary denaturation	cycles	Denaturation	Annealing	Elongation	Final elongation
Multiplex	<i>SHV</i>	95°C/ 3min	35	95°C/ 60sec	58°C/60 sec	72°C/40 sec	72°C/2min
	<i>NDM</i>	95°C/ 3min	35	95°C/ 60 sec	58°C/60 sec	72°C/40 sec	72°C/2min

## Results and discussion

### Isolation and identification of *P. mirabilis*

Twenty isolates (18.18%) of *P. mirabilis* were identified, while other bacterial species accounted for 90 isolates (81.82%), as shown in Table (4). These findings align with several international studies, which have reported the prevalence of this bacterium to range between 5-18% among positive bacterial growth cases in clinical samples (Chakkour *et al.*, 2024). However, the results of the current study disagree with those of Al-Dulaimi (2019) in Diyala, Al-Obeidi (2021) in Diyala, and Hafiz *et al.* (2024) in Riyadh, where the isolation rates of *P. mirabilis* were 88.88%, 8.3%, and 74.14%, respectively.

Table (4): Isolation types and percentage

Positive growth	<i>P. mirabilis</i>	Other types of bacteria
110	20	90
100%	18.18%	81.82%

### *P. mirabilis* isolates distribution in clinical specimens

The percentage of *P. mirabilis* among clinical samples was variable. The results showed that the highest percentage was from urine samples 13 (65%), followed by stool samples 5 (25%), and one isolation for each of otitis media and vaginitis 1 (5%) respectively, while there was no isolation from wounds as shown in Table (5).

Table (5): Distribution of isolates in clinical samples

Samples type	No. of specimens	No. of isolates
Urine	90 (50%)	13 (65%)
Stool	50 (27.78%)	5 (25%)
Otitis	15 (8.33%)	1 (5%)
Vaginal	10 (5.65%)	1 (5%)
Wounds	15 (8.33%)	0 (0%)
Total	180 (100%)	20 (100%)

In this study, a higher rate of positive isolates was recorded in urine samples, this may due to the high number of urine specimens collected than other sources. Similar finding was reported by Hafiz *et al.* (2024), who demonstrated that urine had the highest incidence of *P. mirabilis* among other clinical sources.

This is usually associated with the possession of several virulence factors that are important for causing UTIs, and these virulence factors include adhesion ability, urease production, and flagella (Filipiak *et al.*, 2020).

Studies have confirmed the high percentage of proteobacteria in urine due to their natural presence in the intestinal tract, and when they move to the urethra, they lead to urinary tract infections. The presence of a urinary catheter is also an important factor in the ascent of microorganisms to the bladder due to the contact of the outer surface of the catheter tube with the urethral wall or the skin surrounding the urinary opening (Al-Dulaimi, 2019).

The current results showed that the percentage of *P. mirabilis* bacteria isolation from females 12 (60%) is higher compared to males 8 (40%) Table (6). The current study recorded the highest infection with *P. mirabilis* bacteria in females compared to males. This finding is agreed with AL-Ezzy *et al.* (2023) result, as the infection in females was higher than males by 58.3% and 41.7%,

respectively, while it disagree with Hassan (2008) study on *P. mirabilis* bacteria in the city of Nasiriyah, as he showed that the infection in males was higher than in females by 59% and 41% respectively.

Females are more infected than males with urinary tract infections due to physiological, anatomical, hormonal and immunological differences (Mestrovic *et al.*, 2021).

**Table (6): Relations between isolation and gender**

Genders	No. of isolates	Percentage %
Females	12	60%
Males	8	40%
Total	20	100%

### Antibiotic susceptibility test (AST)

Kirby-Bauer disk method was used to test the sensitivity of all 20 isolates under study against 11 antibiotics from different groups, most of which are used to treat multiple infections. The diameter of the inhibitory zone surrounding the disks was measured to record the results, which were then compared using AL-Ezzy *et al.* (2024). Table (7) shows the numbers and percentages of sensitive and resistant isolates toward antibiotics.

**Table (7): Numbers and percentages of sensitive and resistant isolates**

Antibiotic	No. of isolates (%)		
	Sensitive	Intermediate	Resistant
Cefixime	8(%20)	0 (%0)	12 (60%)
Ciprofloxacin	13(%65)	0 (%0)	7(%35)
Trimethoprim	1 (%5)	0 (%0)	19(%95)
Ceftriaxone	6(%30)	0 (%0)	14(%70)
Gentamicin	13(%65)	0 (%0)	7(%35)
Azithromycin	7(%35)	0 (%0)	13(%65)
Amoxicillin-Clavulanic acid	4(%20)	0 (%0)	16(%80)
Levofloxacin	19(%95)	0 (%0)	1 (%5)
Meropenem	20(%100)	0 (%0)	0 (%0)
Amikacin	19(%95)	0 (%0)	1 (%5)
Ampicillin	3(%15)	0 (%0)	17(%85)



The results showed resistance to cephalosporin antibiotics, as the resistance rate of the isolates to Cefixime reached 60% and Ceftriaxone 70%. This result was disagreed from Shabeeb and Alghanimi (2018) in Karbala, as their isolates were resistant to the antibiotic at a rate of 47.3%.

The isolates under study showed low resistance to aminoglycosides antibiotics, which include Amikacin and Gentamicin, as the resistance rate reached 5% and 35%, respectively. This result is reliable with Singla et al. (2015) finding, as the isolates showed low resistance to Amikacin antibiotic 11.4%. As for Gentamicin, this result was nearly to AL-Oqaili et al. (2017), as the isolates resistance was 54.68%.

By altering the ribosome's target site (S30), where a mutation takes place, gram-negative bacteria become resistant to aminoglycosides like amikacin and gentamicin. This results in a reduction in the antibiotic molecules' ability to pass through the membrane (Liu et al., 2023).

The resistance of the isolates to quinolones, namely ciprofloxacin and levofloxacin, was 5% and 35%, respectively. The results disagree with a previous local study by Ramatla et al. (2024), that the resistance rate to ciprofloxacin was (61.5%). The resistance rate of the isolates to Azithromycin was 65%, which is close to what Al-Obeidi (2021) found, as the resistance rate was 76%.

Meropenem was the most effective and sensitive drug against *P. mirabilis* isolates in the present study, the resistance rate of isolates to Meropenem was (0%) and the sensitivity was (100%). This result was close to Mirzaei et al. (2019) who documented that *P. mirabilis* resistance was 4.5%. The results showed that the resistance of bacteria to Ampicillin was 85%. In other study, it was recorded at 75% (Attalah et al., 2020).

As for the Augmentin antibiotic, the resistance rate of the isolates under study reached 80%, Perween et al. (2016) recorded a resistance rate of 94.3% that differ from this study. The origin of resistance is due to the ability of bacteria to produce beta-lactamase enzymes such as ES $\beta$ Ls, which are plasmid or chromosome encoded genes, as they degrade penicillins and cephalosporins, resulting in antibiotics multi-resistance, or due to the target site modification and outer wall waste reduction (Guilfoile, 2007).

Trimethoprim showed 95% resistance; this result differed from Al-Dulaimi (2019) result which the resistance ratio for the same antibiotic was 75.7%. The reason for this resistance is due to reducing the concentration of the antibiotic inside the bacterial cell or producing a type of enzyme known as Dihydrofolate reductase, which is encoded by genes carried on the plasmid (Eliopoulos and Huovinen, 2001)

It is clear from the above that *P. mirabilis* isolates are resistant to most of the widely used antibiotics. This may be due to the bacteria adapting to the hospital environment and the continuous use of antibiotics, which allows mutations to occur in the genetic material. It has been shown that treatment with normal concentrations of antibiotics kills the bacteria present in the environment, while the bacteria inside the polysaccharide matrix remain vital and resist antibiotics, as they act as a focus for repeated infections (Ehrlich et al., 2002).

## Virulence factors of *P. mirabilis*

### ESBL and Metallo $\beta$ -lactamase production

Double disk synergy (DDC) technique was used to test the synthesis of extended-spectrum beta-lactam enzymes, which is one of the simplest and most precise techniques. The results showed that 9 (45%) of *P. mirabilis* isolates produced these enzymes, Figure (1). Similar results were reported by Shabaan et al. (2022) that *P. mirabilis* isolates produced ESBL (51.7%). While results showed that 6 (30%) of the isolates under study were producers of the metallo-beta-lactamase enzyme.

Infections due to ESBL-producing isolates represent a critical global issue, leading to substantial rises in morbidity and death rates among hospitalized individuals. Moreover, ESBL producers exhibit cross-resistance to many other classes of antibiotics such as quinolones, aminoglycosides, and sulfa drugs (Malaki *et al.*, 2022).

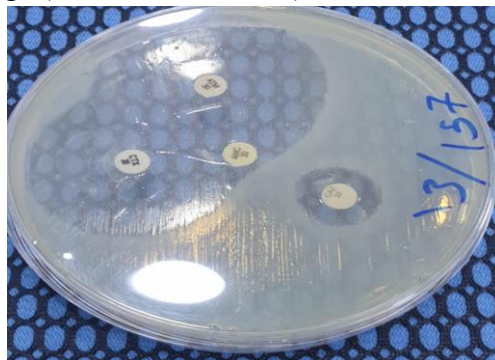


Figure 1:  $\beta$ -lactamase production on Mueller-Hinton medium.

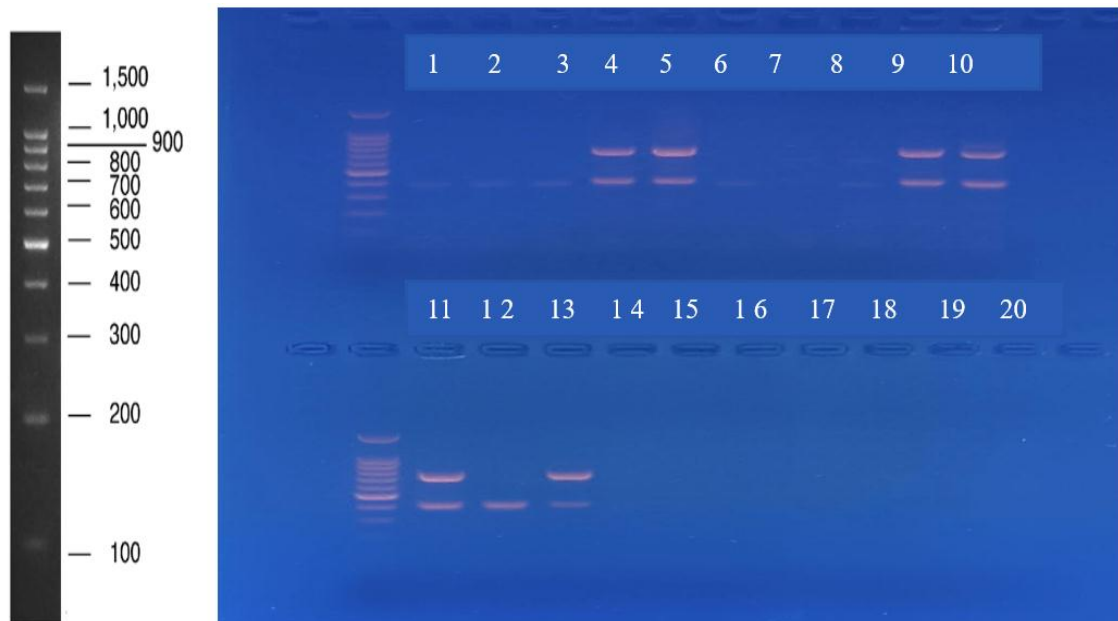
### DNA extraction and purification

Twenty *P.mirabilis* isolates were subjected to amplification of virulence genes (*blaNDM* and *blaSHV* gene) based on their possession of the most potent virulence factors. Accordingly, genomic DNA was extracted using an extraction kit (Alpha DNA, USA). The concentrations and purity of DNA were measured by Nano Spectrometry. All samples had DNA concentrations ranging from (62.1-12.5) ng/ $\mu$ l, while the purity ranged from (1.8-1.98). DNA from six isolates was exposed to 1.5% agarose gel electrophoresis to approve the integrity of DNA. The results revealed a unique discrete band indicating successful isolation of pure DNA.

### Detection of *blaNDM* and *blaSHV* gene

The results of the current study showed that 6 (30%) of the isolates had *blaSHV*, while showed that 7 (35%) of the isolates contained *blaNDM*, Figure 2.





**Figure (2):** Agarose gel electrophoresis (1.5% agarose, 7 V/cm<sup>2</sup> for 60 min) for A: SHV gene (amplicon 713 bp), B: NDM gene (amplicon 488 bp); size ladder 100 bp.

*P. mirabilis* is infamous for its capacity to aggressively spread genes of antibiotics resistance across horizontally gene transferring, and the multi-resistant isolates, comprising some carrying *bla*NDM, have caused nosocomial infections in several counties (Yang *et al.*, 2022).

Over the past several years, a number of excellent publications have been published that describe the biology, genetics, and evolution of SHV  $\beta$ -lactamases. In addition to describing the prevalence of plasmid-associated SHV enzymes in Enterobacteriaceae and their epidemiological implications.

However, SHV enzymes did not experience the rapid spread seen in CTX-M-type variations (Canton *et al.*, 2012), With increasing allele variety, they have lately been discovered in several environmental niches and in a number of Enterobacteriaceae other than the usual clinical hosts, *Klebsiella pneumoniae* and *Escherichia coli* (<http://www.lahey.org/studies>).

Multiplex PCR allows the simultaneous detection of several bacterial genes in a single tube by combining several primer pairs for various target sequences. Reagents and time are both saved by this method. This is a very sensitive and unique detection technique for identifying and detecting many bacterial diseases, including Enterobacteriaceae species, is multiplex PCR (Zhang *et al.*, 2019).

## Conclusions

Study revealed *P. mirabilis* resistance for many antibiotics, especially Trimethoprim and ampicillin. The most effective antibiotic against all isolates was meropenem followed by Amikacin. All isolates showed different rates of ES $\beta$ L and M $\beta$ L production. *P. mirabilis* isolates express different rates of resistance genes such as *bla*NDM and *bla*SHV.

*P. mirabilis* pathogenicity has evolved through several virulence factors that have been genetically investigated. It was found that detecting *P. mirabilis* using modern molecular methods

represented by multiplex polymerase chain reaction (PCR) technique is much better than traditional methods due to its high sensitivity, speed, and low cost, and its result does not depend on the opinion of the person working, and it can also deal with a large number of samples at one time.

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### Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article. The research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

### Authors contribution

All authors reviewed and approved the final version of the manuscript and agree to be accountable for its contents.

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