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Phenotypic Detection of Beta-lactamases among *Proteus mirabilis*, *Enterobacter cloacae*, and *Citrobacter freundii* Isolates from Urinary Samples in Gorgan, Northeast Iran

Ameneh Fendereski[1], Ania Ahani Azari[2*], Teena Dadgar[2]

[1]MSc, Department of Microbiology, Gorgan Branch, Islamic Azad University, Gorgan, Iran; [2]PhD, Department of Microbiology, Gorgan Branch, Islamic Azad University, Gorgan, Iran

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***Correspondence**
Email: ania_783@yahoo.com
Tel: +981732547074
Fax: +981732547074

ABSTRACT

Introduction: The data on members of the genera *Proteus*, *Pseudomonas*, *Enterobacter*, *Citrobacter*, and *Staphylococcus* as the etiologic agents of urinary tract infections (UTIs) is not much. This study investigated the frequency of various beta-lactamases in urine isolates of *Proteus mirabilis*, *Enterobacter cloacae*, and *Citrobacter freundii* in Gorgan, Golestan province. **Methods:** A total of 632 urine samples were collected from hospitalized patients in a teaching hospital. The samples were cultured on blood agar and Eosin Methylene blue agar and incubated overnight at 37°C. The cultures with a ≥105 CFU/mL bacterial count were defined as positive for UTI. Bacteria identification was performed using standard biochemical methods and the API20E enteric identification system. The antibiotic resistance pattern was determined by the Kirby-Bauer disk diffusion method, and a phenotypic confirmatory test was used for detecting ESBL, MBL, and AmpC beta-lactamases producers. **Results:** Out of 632 samples, 317 (50.1%) were positive for UTIs, and 27 (8.5%), 21 (6.6%), and 12 (3.7%) were positive for *Enterobacter cloacae*, *Citrobacter freundii*, and *Proteus mirabilis* isolates, respectively. All the isolates were sensitive to piperacillin-tazobactam and colistin. The prevalence of ESBL and AmpC beta-lactamases in *P. mirabilis* isolates was higher than the other isolates, but No MBL producers were detected. **Conclusions:** In this study, the high frequency of ESBL and AmpC beta-lactamases in *P. mirabilis* isolates may suggest an increasing trend in resistance to cephalosporins and monobactams, which could have a significant impact on the management and treatment of UTI caused by this organism. Therefore, continuous monitoring is required to control the spread of β-lactamase-producing isolates in different geographical areas.

INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial infections and are estimated to affect 150 million people annually worldwide [1]. They are the second most common infections, with approximately 8.1 million visits to medical centers annually that sometimes require hospitalization [2, 3]. The most common cause of UTI is *Escherichia coli*, followed by *Klebsiella pneumonia*, but members of the genera *Proteus*, *Pseudomonas*, *Enterobacter*, *Citrobacter*, and *Staphylococcus* are also known to be etiologic agents of UTIs [4]. UTIs are usually treated with common antibiotics, but the prevalence of antibiotic resistance among urinary pathogens has increased globally due to overuse and misuse of these antimicrobial agents [5].

One of the most common resistance mechanisms for β-lactam resistance is the production of various beta-lactamases that hydrolyze the beta-lactam ring of β-lactams [6]. This mechanism is known as the primary mechanism for β-lactams resistance among members of the Enterobacteriaceae family. Resistance to different β-lactams such as penicillins, cephalosporins, monobactams, and carbapenems is frequently attributed to the production of several types of beta-lactamases including AmpC β-lactamases, extended-spectrum β-lactamases (ESBLs), and metallo β-lactamases (MBLs) [7].

ESBLs can hydrolyze a wide range of β-lactams except for cephamycins and carbapenems. Among

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Enterobacteriaceae, ESBLs are mainly found in *Klebsiella* spp. and *E. coli*, but they have also been described in other genera, such as *Enterobacter*, *Citrobacter*, *Proteus*, *Morganella*, *Providencia*, *Salmonella*, and *Serratia* [8]. AmpC β‑lactamases are a type of cephalosporins, rendering Enterobacteriaceae resistant to most β-lactams but are not active against cefepime and carbapenems [7, 9]. MBLs or carbapenemases are responsible for carbapenem resistance and are increasingly detected among Enterobacteriaceae bacteria [7].

Most studies have focused on the prevalence of beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae* as a common cause of UTIs. However, few studies are available regarding the prevalence of beta-lactamases among *Proteus mirabilis* (responsible for 1-10% of all UTIs), *Enterobacter cloacae* (responsible for 1-10% of UTIs), and *Citrobacter freundii* (responsible for 5 -12% of UTIs in adults) [4, 10, 11], so this study aimed to evaluate the frequency of various beta-lactamases in urine isolates of these organisms in a teaching hospital in Gorgan, Golestan province.

MATERIALS AND METHODS

Sample collection and identification of isolates. This cross-sectional study was conducted on urine samples of hospitalized patients in different wards of a teaching hospital in Gorgan, Golestan province, from Apr. to Sep. 2020. Written informed consent was obtained from all patients, and their data remained anonymous. Midstream urine samples were collected and assessed for the presence of leucocytes and bacteriuria. The urine samples were cultured with a 0.001-mL loop on blood agar and Eosin Methylene blue agar (Merck, Germany). After overnight incubation at 37°C, all cultures with a $\geq 10^5$ CFU/mL bacterial count were considered positive for UTI and included in the study. Bacterial identification was performed using standard biochemical methods and the API20E enteric identification system (bioMe'rieux, Marcy l' Etoile, France) [7].

Antibiotic susceptibility test. The Kirby-Bauer disk diffusion method was deployed using various antibiotic disks, including imipenem (10µg), meropenem (10 µg), aztreonam (30 µg), ciprofloxacin (5µg), gentamicin (10µg), cefotaxime (30µg) , ceftazidime (30µg), cefoxitin (30 µg) , cefepime (30 µg), doxycycline (30 µg), co-trimoxazole (1.25 µg), nitrofurantoin (30µg), amikacin (30µg), carbenicillin (100µg), ampicillin (30µg), amoxiclav (30µg), piperacillin-tazobactam (110 µg) and colistin (10 µg) (PattanTeb, Iran) according to the Clinical Laboratory Standards Institute (CLSI) [12]. The standard strain *E. coli* ATCC 25922 was included in the assay as quality control. After overnight incubation at 37°C, the results were interpreted by measuring the inhibition zone diameter. The susceptibility of the isolates to each antibiotic was interpreted as sensitive (S), intermediate (I), or resistant (R).

Detection of Beta-lactamases

ESBL. To determine ESBL-producing isolates, ceftazidime (CAZ) or cefotaxime (CTX) resistant isolates were selected for ESBL production by double disk-diffusion test. In this test, Mueller-Hinton agar plates with the disks containing 30µg of CAZ and CTX, with and without 10 µg of clavulanic acid, were used. After incubation, the inhibition zones diameter of each isolate was measured. If the inhibition zone surrounding at least one combination disk was 5 mm larger than that produced around the corresponding antimicrobial disk without clavulanic acid, the isolate was considered as an ESBL producer [11, 13].

AmpC. Cefoxitin-resistant isolates were tested for AmpC β-lactamases production by boronic acid double disk diffusion test. In this test, two disks containing cefoxitin (30µg) and cefoxitin+boronic acid (30/400µg) were placed on the inoculated Muller-Hinton agar plates. After overnight incubation at 37°C, if the inhibition zone diameter surrounding the cefoxitin + boronic acid disk was 5mm greater than the inhibition zone diameter around the cefoxitin disk alone, AmpC production was considered as positive [12, 14].

MBL. Imipenem (10µg) resistant isolates were screened for the presence of MBL by the IMP-EDTA double disk-diffusion test. Two disks containing imipenem (10µg) and imipenem +EDTA (10µg/750µg) were placed on the inoculated Muller-Hinton agar plates and incubated overnight at 37°C. If the inhibition zone diameter around the imipenem+ EDTA disk was 5mm greater than the inhibition zone diameter surrounding the imipenem disk alone, MBL production was considered as positive [15].

RESULTS

The Results of Sampling and Identification of Isolates. From Apr. to Sep. 2020, 632 urine samples were collected. Out of all samples, 317 (50.1%) with colony count $\geq 10^5$ CFU/ml with a high white blood cell count were considered positive for UTIs. One hundred eighty-eight (59.3%) samples belonged to females and 129 (40.7%) to males. Of 317 samples, 27 (8.5%), 21 (6.6%), and 12 (3.7%) samples were positive for *Enterobacter cloacae*, *Citrobacter freundii*, and *Proteus mirabilis*, respectively. Most of the isolates were recovered from the surgery ward (23 out of 60), followed by the intensive care unit (ICU) (18 out of 60).

The Results of Antibiotic Susceptibility Test. The highest resistance in *E. cloacae* isolates exhibited against gentamycin (40.7%) and cefoxitin (40.7%) followed by ceftazidime (37%). Among the *C. freundii* isolates, the highest resistance was observed to ciprofloxacin (61.9%), followed by ampicillin (38%). For *P. mirabilis* isolates, the highest resistance was to gentamycin (75%),

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followed by cefoxitin (58.3%) and ceftazidime (41.6%), the same as *E. cloacae* isolates. All the isolates were sensitive to piperacillin-tazobactam and colistin. About 39.2%, 42.8%, and 52.5% of the *E. cloacae*, *C. freundii*, and *P. mirabilis* isolates exhibited multidrug resistance (MDR) phenotype, respectively.

Table 1. Frequency of antimicrobial-resistant among the isolated organisms

	Resistance No (%)		
Antimicrobial agents	*Enterobacter cloacae*	*Citrobacter freundii*	*Proteus mirabilis*
Imipenem (10µg)	8 (29.6)	5 (23.8)	2 (16.6)
Meropenem (10 µg)	7 (25.9)	6 (28.5)	3 (25)
Aztreonam (30 µg)	5 (18.5)	2 (9.5)	3 (25)
Ciprofloxacin (5µg)	8 (29.6)	13 (61.9)	0 (0)
Gentamicin (10µg)	11 (40.7)	5 (23.8)	9 (75)
Cefotaxime (30µg)	5 (29.4)	6 (28.5)	2 (16.6)
Ceftazidime (30µg)	10 (37)	6 (28.5)	5 (41.6)
Cefoxitin (30 µg)	11 (40.7)	6 (28.5)	7 (58.3)
Cefepime (30 µg)	0 (0)	2 (9.5)	2 (16.6)
Doxycycline (30 µg)	0 (0)	2 (9.5)	0 (0)
Co-trimoxazole (1.25 µg)	7 (25.9)	5 (23.8)	2 (16.6)
Nitrofurantoin (30µg)	5 (18.5)	0 (0)	2 (16.6)
Amikacin (30µg)	8 (29.6)	5 (23.8)	3 (25)
Carbenicillin (100µg)	0 (0)	2 (9.5)	0 (0)
Ampicillin (30µg)	8 (29.6)	8 (38)	3 (25)
Amoxiclav (30µg)	5 (18.5)	2 (9.5)	0 (0)
Piperacillin-tazobactam (110 µg)	0 (0)	0 (0)	0 (0)
Colistin (10 µg)	0 (0)	0 (0)	0 (0)

Detection of ESBLs: All the isolates resistant to ceftazidime or cefotaxime were examined for the possibility of positive ESBLs by combined disk assay. Among the screened *E. cloacae*, *C. freundii*, and *P. mirabilis* isolates, 53.3% (8/15), 58/3% (7/12), and 100% (7/7) were ESBLs producers.

Detection of AmpC β-lactamases: The isolates that were resistant to cefoxitin were selected for AmpC production. Among the isolates, 27.2% (3/11), 57.1% (4/7), and 83.3% (5/6) of the *E. cloacae*, *P. mirabilis*, and *C. freundii* isolates were found to be AmpC producers. None of the isolates showed the coexistence of ESBL and AmpC in the same isolate.

Detection of Metallo β-lactamases: The imipenem resistant isolates were screened for the detection of MBL production. Among the isolates, 40% (2/5) and 50% (4/8) of the *C. freundii* and *E. cloacae* isolates were detected as MBL producers. No MBL producers were detected for *P. mirabilis* isolates. None of the isolates showed the coexistence of ESBL and MBL. Table 2 shows the Frequency of ESBLs, AmpC, and MBL production within the total isolates.

DISCUSSION

Today, resistance to the β-lactam antibiotics by the production of ESBL, AmpC, and MBL enzymes has become a worldwide health problem [16]. Most urinary tract isolates are resistant to these antibiotics, which have long been used for treating UTIs [17]. Most studies have focused on the prevalence of beta-lactamases in *E. coli* and *K. pneumoniae* as a common cause of UTIs. However, few studies are available regarding other Enterobacteriaceae family members, including *P. mirabilis, E. cloacae,* and *C. freundii* as common urinary pathogens in hospitalized patients. Hence, this study aimed to evaluate the frequency of the ESBL, AmpC, and MBL enzymes in urine isolates of these organisms in a teaching hospital in Gorgan, Golestan province.

Table 2. Frequency of ESBLs, AmpC, and MBL production among total isolates

Isolates	ESBLs No (%)	AmpC No (%)	MBL No (%)
E. cloacae	8/27 (29.6)	3/27 (11.1)	4/27 (14.8)
P. mirabilis	7/12 (58.3)	4/12 (33.3)	0 (0)
C. freundii	7/21 (33.3)	5/21 (23.8)	2/21 (9.5)

In the current study, of 60 isolates identified as *E. cloacae* (27/60), *C. freundii* (21/60), and *P. mirabilis* (12/60), most (23 out of 60) were from the surgery ward. All the isolates were sensitive to piperacillin-tazobactam and colistin. *E. cloacae* isolates showed a higher resistance rate to gentamycin and cefoxitin. Among the *C. freundii* and *P. mirabilis* isolates, the highest resistance was observed against ciprofloxacin and

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gentamycin, respectively. Besides, 39.2%, 42.8%, and 52.5% of the *E. cloacae*, *C. freundii*, and *P. mirabilis* isolates exhibited multidrug resistance (MDR) phenotype.

Among the isolates, 29.6%, 33/3%, and 58.3% of the *E. cloacae*, *C. freundii*, and *P. mirabilis* isolates were detected as ESBLs producers. Meanwhile, the AmpC rates were 11.1%, 23.8%, and 33.3% in the *E. cloacae*, *C. freundii*, and *P. mirabilis* isolates. The frequency of MBL in the *E. cloacae* and *C. freundii* isolates were 14.8% and 9.5%, respectively. No MBL producer was detected for *P. mirabilis* isolates.

Similar to the current study results, in a study in Korea, *Citrobacter* spp. contributed to 9.4% of all UTIs in hospitalized patients, and the resistance rate to ciprofloxacin (61%) was almost the same as our findings study [17]. In contrast to the present study, a study from India reported 82.30% (107/130) carbapenem resistance among *C. freundii* recovered from clinical specimens, and 65% isolates were considered MDR [18].

In Italy, the occurrence of ESBL among the *E. cloacae*, *C. freundii*, and *P. mirabilis* isolates was remarkably lower than in our study [8]. In India, the prevalence of MBL in *Citrobacter* spp. isolates in a tertiary care hospital was significantly higher than the current study (26.9%) [19]. In Iraq, out of 120 *E. cloacae* isolates, 53.3% were MBL producers, and none of the isolates showed the ESBL [20]. In Azerbaijan, northwest Iran, 35.7% (5/14) and 100% (14/14) of the *E. cloacae* isolates were ESBL and AmpC producers, and 80% isolates of *P. mirabilis* and the only *C. freundii* isolate were AmpC producers. In this study, the *C. freundii* isolate was also tested positive for ESBL production (7). In India, among the *C. freundii* isolates recovered from clinical specimens, 35.4% and 26.9% were positive for ESBL and MBL [18].

In China, out of 84 carbapenem-resistant *E. cloacae* isolates from three tertiary hospitals, 50 (59.5%) were MBL producers [19]. In agreement with our results, a study in North West Bank-Palestine reported 14.6% MBL and 11.1%AmpC β-lactamases among the *E. cloacae* isolates [20], but in Babol, north Iran, higher rates were reported for *E. cloacae* isolates from clinical specimens [21]. In India, out of 251 Gram-negative bacteria isolates from clinical specimens, 7 (2.7%) and 25 (9.9%) were *Proteus* spp. and *Citrobacter* spp., respectively. The incidence of ESBL and AmpC production among the isolates was dissimilar to our finding results, but a similar rate was for MBL [22]. In a similar study in India, out of 807 Gram-negative bacteria isolated from clinical specimens, 20 (2.4%) *Proteus* spp. and 15 (1.8%) *Citrobacter* spp. isolates were identified. The occurrence of MBL production in the *Proteus* isolates and AmpC production in *Citrobacter* isolates was similar to our findings [23]. In Egypt, 25% of *P. mirabilis* strains isolated from UTIs in Egyptian Hospitals were positive for AmpC production [24]. In agreement with the present study in Nigeria, 22.2% of *Citrobacter* species recovered from clinical specimens were AmpC producers [25].

According to our findings and other studies in Iran and other countries, the prevalence of beta-lactamases among the tested bacteria varies in different geographical regions. Therefore, continuous monitoring for controlling the spread of β-lactamase-producing strains in different geographical areas is required to adopt appropriate strategies for controlling these bacteria. Restricting the use of carbapenem and third-generation cephalosporins and applying appropriate infection control measures should be taken to control the spread of ESBLs, AmpC, and MBL-producing isolates.

In the present study, the high frequency of ESBL and AmpC beta-lactamases in *P. mirabilis* isolates may suggest an increased resistance to cephalosporins and monobactams that could have a significant impact on the management and treatment of UTI caused by this organism.

The present study limitation was that we performed it at a single-center for six months in a teaching hospital in Gorgan. To reveal the increasing trend of drug-resistant bacteria, performing a multicenter study involving all types of medical systems in the region for at least one year is recommended.

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CONFLICT OF INTEREST

None of the authors declared any competing interest associated with this article.

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