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Original Article

A cross-sectional study on the prevalence of multidrug-resistant clinical isolates of Proteus species in Northern Iran

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Abstract

Introduction: Multidrug-resistant (MDR) bacteria like *Proteus* species have led to more prolonged hospitalizations, fewer care choices, higher treatment costs, and even death. The present study aims to evaluate the prevalence of MDR *Proteus* species in clinical samples and to suggest the best therapeutic options for the MDR *Proteus* species.

Methodology: Clinical samples were collected randomly from five hospitals in Golestan Province, Iran, from February 2017 to July 2019. Disk diffusion on Mueller–Hinton agar plates were used to perform antibiotic susceptibility testing (ASTs). By using a double-disc synergy test (DDST), isolates resistant to one of the third-generation cephalosporins were examined for phenotypic extended-spectrum β-lactamase (ESBL) development. A combined double disk synergy test (CDDST) was used to identify MBL-producing isolates.

Results: 61 Proteus isolates, including *P. Mirabilis* 44/61 (77.04%), *P. vulgaris* 7/61 (11.47%), *P. hauseri* 5/61 (8.19%), and *P. penneri* 2/61 (3.27%) were collected. Most of the isolates were obtained from urine samples. *P. hauseri* isolates were more frequent in females. Resistance to tetracycline and nitrofurantoin antibiotics was observed in most *Proteus* isolates. *P. penneri* isolates were all resistant to antibiotics. ESBL production was observed in five ceftazidime-resistant isolates ($p < 0.05$).

Conclusions: Cefepime and imipenem were found to have the lowest occurrence of antibiotic resistance among *Proteus* species, confirming that cefepime and imipenem can be used to treat *Proteus* infections.

Key words: *Proteus*; extended-spectrum β-lactamase; multidrug resistance; antimicrobial resistance.

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Introduction

Antibiotics have saved many lives and alleviated the suffering of many people worldwide. Due to the rise and growing antibiotic-resistant bacteria, scientists hope for the treatment of microbial diseases has faded quickly [1-3]. Multidrug-resistant (MDR) bacterial infections have led to more prolonged hospitalizations, fewer care choices, higher treatment costs, and even death. Hence, continuous monitoring of microbial drug resistance is crucial [4]. MDR bacteria are resistant to at least three different antibiotic families [5]. It is crucial to choose the appropriate antibiotic and determine the pattern of bacterial susceptibility to improve the effectiveness of treatment against infections caused by MDR bacteria [6].

Another form of antibiotic resistance was found in Gram-negative bacteria, which were resistant to extended-spectrum beta-lactamase (ESBL) [7,8]. ESBLs are β-lactamase hydrolyzing extended-spectrum cephalosporins, penicillin, and monobactams but not cephamycin and carbapenems. ESBLs are inhibited by β-lactamase inhibitors, including clavulanic acid, sulbactam, and tazobactam [8-10]. The *Proteus* species belong to the order *Enterobacterales* and the *Morganellaceae* family, Gram-negative bacteria [11-13]. These organisms are found in the natural flora of humans' intestinal tracts and the soil and water, where their presence is thought to be due to fecal contamination.

Proteus mirabilis (*P. mirabilis*), *Proteus vulgaris* (*P. vulgaris*), *Proteus penneri* (*P. penneri*), *Proteus hauseri* (*P. hauseri*), and three other unidentified genomospecies 4, 5, and 6 are currently divided into five specific genera [14-16]. The urease-producing *P. mirabilis* is well-known for its ability to differentiate into elongated swarm cells and a distinctive bull's-eye pattern of motility on agar plates [14,17]. This bacterium causes wounds, gastrointestinal tract, and urinary tract infections, but it is best known for infections of the catheterized urinary tract, also known as catheter-associated urinary tract infections (CAUTI). *P. mirabilis* can induce respiratory tract infections,

bacteremia, meningitis, cystitis, acute pyelonephritis, and renal calculi [18,19]. Furthermore, proteus infection has been linked to autoimmune disorders, including rheumatoid arthritis [18]. The pathogenicity of *Proteus* species requires several virulence factors, including the mannose-resistant Proteus-like (MR/P) fimbria, mannose-resistant *Klebsiella*-like hemagglutinin (MR/K), *P. mirabilis* fimbriae (PMF) [18–20], and the urease enzyme [21].

In recent decades, resistance to antibiotics has increased significantly in Iran. This alarming increase has caused various researchers to conduct studies to investigate and identify antibiotic resistance [22]. According to the review of three studies conducted in the last few years in Iran, the most resistance in *Proteus* isolates was against the antibiotic cotrimoxazole. Also, the results of these studies showed high resistance to nitrofurantoin and ampicillin [23–27].

Due to the lack of knowledge of the antibiotic resistance and ESBL patterns of *Proteus* species and increased evidence of drug resistance to bacterial infection, it was aimed to evaluate the abundance distribution of MDR *Proteus* species from clinical samples. In addition, the best possible therapeutic regimen was suggested for the MDR *Proteus* species.

Methodology

Sample size and identification of Proteus species

This cross-sectional study was performed in the Department of Microbiology, Golestan University of Medical Science, Gorgan, Iran. The study was approved by the Ethics committee of Golestan University of Medical Sciences, Iran (ethical code: IR.GOUMS.REC.1397.194). All clinical samples were collected randomly with no-repeat from five hospitals, namely A, B, C, D, and F in Golestan Province, Iran, from February 2017 to July 2019.

The isolates were belonged to various sources, including urogenital, abscess, and wound swabs. All specimens were cultured on nonselective blood agar media (Merck, Darmstadt, Germany). *Proteus* species were identified by standard microbiological and biochemical methods, such as colony morphology and Gram staining, oxidase, catalase, swarming motility, indole, citrate, maltose, saccharose fermentation, urease, bile esculin, and ornithine decarboxylase. Positive *Proteus* isolates were stored in tryptic soy broth stocks (Merck, Darmstadt, Germany) with 15% glycerol at −70 °C. The isolates were further confirmed by 16 sRNA gene amplification as described before [28].

Antimicrobial susceptibility test

Antibiotic susceptibility tests (ASTs) of all isolates were performed by disk diffusion method on Mueller–Hinton agar plates (Merck, Darmstadt, Germany), according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [29]. The antibiotics, including amikacin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ciprofloxacin (5 µg), co-trimoxazole (25 µg), gentamicin (10 µg), imipenem (10 µg), nitrofurantoin (30 µg), nalidixic acid (30 µg) cefepime (30 µg), ceftriaxone (30 µg) and norfloxacin (10 µg) (Mast Co, Merseyside, UK) were used in disc diffusion test. Inhibition zone (IZ) diameters (mm) of each antibiotic disc on the plates were measured after 24 hours of incubation. The isolates were classified into three categories: resistant, intermediate, and susceptible. *E. coli* strain ATCC 35218 was used as a control.

Phenotypic detection of ESBL production

Each isolate resistant to one of the third-generation cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) was tested for phenotypic ESBL production. A double-disc synergy test (DDST) was used for phenotypic detection of ESBL production. In brief, the isolates with 0.5 McFarland standard turbidity were cultured on Müller-Hinton agar plates. According to CLSI guidelines, ceftazidime (30 µg), cefotaxime (30 µg), and ceftriaxone (30 µg) discs were placed at a distance of 15 mm away from the amoxicillin/clavulanic acid (30 µg) that was located in the center of the plate at 37 °C for 24 hours. After the incubation period, the plates with an IZ diameter > 5 mm around the amoxiclav disk compared to cephalosporins alone were considered ESBL producing isolates [4,24,29,30].

Phenotypic detection of Metallo-β-lactamase producing isolate(s)

Initial screening for the production of MBL isolates was based on carbapenem (imipenem, meropenem) resistance. In summary, imipenem-resistant isolates were evaluated by *a* combined double-disk synergy test (CDDST*)* according to the CLSI guideline. To determine MBL-producing isolates, a 0.5 McFarland turbidity suspension of imipenem-resistant isolates was cultured on Müller-Hinton agar medium. In addition, two discs of both imipenem and ceftazidime antibiotics were placed on the surface of the inoculated plate. Discs were incubated at 37 °C for 24 hours by adding 8 µL of EDTA solution. Increasing the IZ around the discs (Merck, Darmstadt, Germany) compared to the

Table 1. The information of Proteus isolates from wards, sources, and patients.

Species	Ward				Mean age	Gender		Source					
	Urology	Outpatient	ICU	Burn		Male	Female	Urine	Wound	Abscess	Tracheal	Vaginal	Total
P. mirabilis	0	38	8	1	31.5 ± 16.72	17	30	38	3	3	2	1	47
P. vulgaris	3	3	0	1	35.5 ± 16	3	4	6	1	0	0	0	7
P. hauseri	0	5	0	0	49 ± 14.94	1	4	5	0	0	0	0	5
P. penneri	0	2	0	0	45 ± 6	1	1	2	0	0	0	0	2
Total	**3**	**48**	**8**	**2**	**34 ± 17.1**	**22**	**39**	**51**	**4**	**3**	**2**	**1**	**61**

initial state. The discs with IZ ≥ 8 mm were considered MBL producing isolates [29–31].

Statistical analysis

The data analysis was performed by SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). Categorical and continuous quantitative variables were analyzed by Pearson's Chi-squared and independent samples *t*-tests. A $p \leq 0.05$ was considered significant with a 95% confidence interval (CI).

Results

Characterization of Proteus isolates

A total of 61 *Proteus* isolates were collected. The most prevalent species have belonged to *P. Mirabilis* 44/61 (77.04%), *P. vulgaris* 7/61 (11.47%), *P. hauseri* 5/61 (8.19%), and *P. penneri* 2/61 (3.27%). 36/61 (59%) isolates were recovered from females, and the remaining 25/61 (41%) were from males. The mean patient was 34 years (1 to 74 years old).

P. hauseri was more common in older patients (mean 49 years) (Table 1). *P. penneri*, *P. hauseri* isolates, *P. vulgaris* (86%), and *P. mirabilis* (80.85%) species were isolated from urine. *P. hauseri* isolates were more frequent in females (4/5). Interestingly, *P. hauseri* and *P. penneri* isolate was commonly detected from outpatients, while 57% of P.vulgaris isolated were inpatients (Table 1).

Antibiotic susceptibility test

As shown in Table 2, resistance to tetracycline and nitrofurantoin antibiotics was observed in the most *Proteus* isolates. All *P. mirabilis* isolates were resistant to nitrofurantoin. Only one isolate of *P. mirabilis* was sensitive to tetracycline. Surprisingly, none of the *P. penneri* isolates were resistant to the four families of antibiotics. Accordingly, P. penneri species were more sensitive to antibiotics among the *Proteus* species. Unlike *P. penneri* isolates, antibiotic resistance was more common among *P. vulgaris* isolates, but in general, no significant relationship was observed between antibiotic resistance and type of *Proteus* species ($p > 0.05$). Further information is provided in Table 2.

ESBL and Metallo-β-lactamase

From 16 isolates resistant to ceftazidime, ceftriaxone, or both, 5/16 (31.25%) produced ESBL, of which four isolates were *P. mirabilis,* and one isolate was *P. hauseri* (Figure 1). Three isolates producing ESBL were isolated from urine, and the other two isolates were isolated from wounds and trachea. No metallo-β-lactamase producing isolates were observed in imipenem-resistant cases. However, the frequency of resistance to imipenem, gentamicin, ceftazidime, ciprofloxacin, chloramphenicol, cefepime, cefotaxime, and ampicillin was higher in ESBL-producing isolates. A significant correlation was found between ESBL production and resistance to ciprofloxacin, ampicillin, cefepime, and cefotaxime ($p < 0.001$).

Discussion

Proteus spp infects humans due to its numerous virulence factors, such as the enzyme urease, flagella, fimbriae, biofilm formation, and produce toxins such as hemolysin and mirabilysin [19]. Therefore, assessments

Table 2. Antibiotic susceptibility of clinical isolates of Proteus species.

Antibiotics	*P. mirabilis,* n (%)	*P. vulgaris,* n (%)	*P. hauseri,* n (%)	*P. penneri,* n (%)	Total, n (%)	*Sig.*
Ampicillin	11 (23.40)	0 (0.00)	0 (0.00)	2 (40.00)	13 (21.31)	0.20
Cefoxitin	10 (21.27)	0 (0.00)	0 (0.00)	0 (0.00)	10 (16.40)	0.20
Cefazolin	12 (25.53)	4 (57.10)	0 (0.00)	2 (40.00)	18 (29.50)	0.20
Ceftazidime	8 (17.02)	2 (28.60)	0 (0.00)	2 (40.00)	12 (19.67)	0.20
Cefotaxime	7 (14.89)	2 (28.60)	0 (0.00)	3 (60.00)	12 (19.67)	0.20
Cefepime	5 (10.63)	2 (28.60)	0 (0.00)	1 (20.00)	8 (13.11)	0.50
Imipenem	7 (14.89)	2 (28.60)	0 (0.00)	0 (0.00)	9 (14.75)	0.40
Gentamicin	8 (17.02)	1 (14.30)	0 (0.00)	0 (0.00)	9 (14.75)	0.60
Ciprofloxacin	6 (12.76)	2 (28.60)	0 (0.00)	1 (20.00)	9 (14.75)	0.60
Cotrimoxazole	15 (31.93)	2 (28.60)	0 (0.00)	1 (20.00)	18 (29.50)	0.60
Chloramphenicol	18 (38.29)	3 (42.90)	0 (0.00)	2 (40.00)	23 (37.70)	0.50
Nitrofurantoin	47 (100)	6 (85.80)	1 (50.00)	3 (60.00)	57 (93.44)	0.001
Tetracycline	46 (97.87)	7 (100.00)	2 (100.00)	5 (100.00)	60 (98.36)	0.90

of antibiotic resistance and ESBL patterns of *Proteus* species is crucial for suggesting correct antibiotic regimen. Proteus is a known bacterial genus that causes urinary tract infections and is more likely to be isolated from urine than other sources [18,19]. Most of the *Proteus* species were isolated from urinary tract samples in the present study. This finding was consistent with the previous reports [30,32,33].

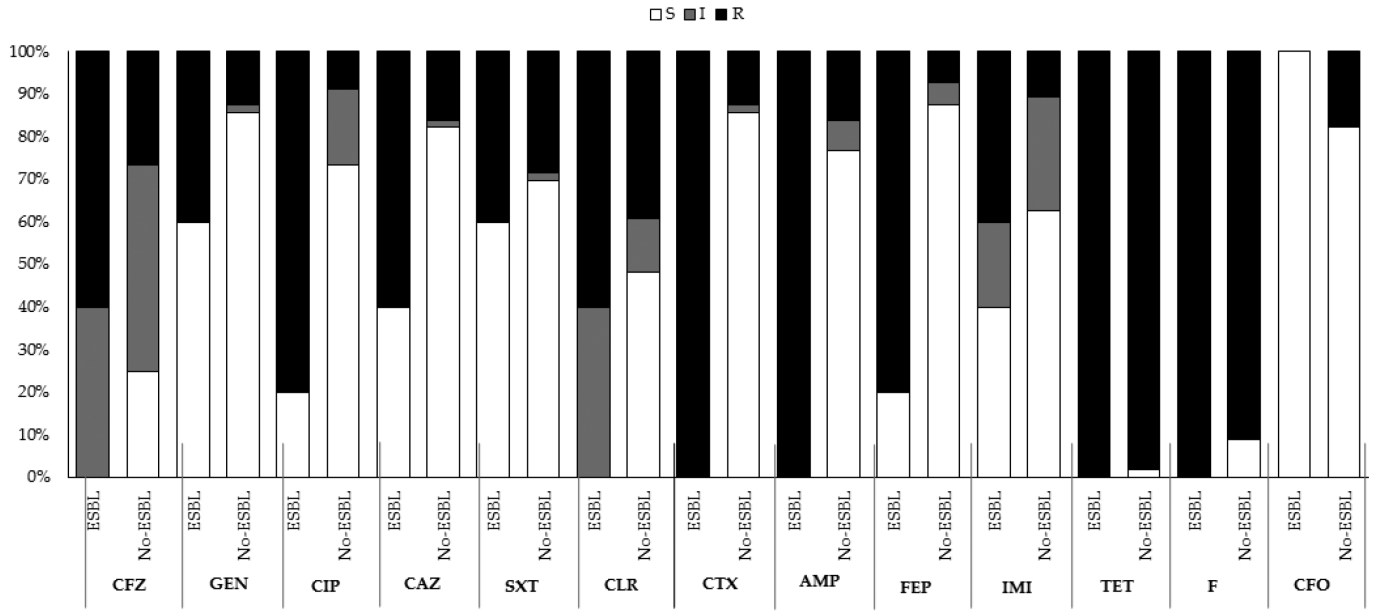
As a result, *P. mirabilis* was the most prevalent isolate, followed by *P. vulgaris*, *P. Hauseri*, and *P. penneri*. Similar results are also reported from Iran [30]. One of the exciting findings was the identification and isolation of *P. hauser* by biochemical diagnosis. The bacterium was differentiated from *P. vulgaris* in 2000 by doctor Hauser. *P. hauseri* is indole positive but negative for salicin and esculin tests. These tests are the distinguishing feature of *P. hauseri* from *P. vulgaris*. Due to the similarity to *P. vulgaris*, accurate statistics of *P. hauseri* frequency in the world have not been reported.

Women are more prone to urinary tract infections because they have a shorter bladder outlet than men and a shorter distance to the anus. As expected, most isolates were obtained from women, which has been the subject of many studies in other parts of the world and Iran [34–36]. Moreover, most *Proteus* isolates were found in hospitalized patients, while *P. penneri* and *P. hauseri* isolates and a large portion of *P. mirabilis* isolates were obtained from outpatients. *Proteus* is an opportunistic bacterium, and one of the most critical risk factors for infections is an extended hospital stay and the use of a catheter [19]. Due to these reasons, the prevalence of this bacterium was expected to be higher in hospitalized patients than in outpatients. Accordingly, therapeutic approaches in these patients are crucial.

Since *Proteus* species are inherently resistant to nitrofurantoin and tetracycline [37,38], the results showed a high degree of resistance to these antibiotics (91% and 98% to nitrofurantoin and tetracycline, respectively). Unlike other *Proteus* species, *P. penneri* isolates were more sensitive to nitrofurantoin. Antibiotic resistance has been seen in various forms among Gram-negative bacteria. Resistance to broad-spectrum β-lactams is one type of resistance that has become a global problem among Gram-negative bacilli [39]. As a result, ESBL production was detected in only 8.2% of isolates, falling within the reported range of 0 to 11.8% for ESBL-producing *Proteus* isolates [24,30,40]. Furthermore, the finding was lower than that reported for other parts of the world, such as Japan (45.6%), India (48.86%), and Taiwan [32,41,42].

Antibiotic resistance was detected among ESBL isolates to imipenem, gentamicin, ciprofloxacin, cefepime, cefotaxime, chloramphenicol, and ampicillin. There was also concurrent resistance between ciprofloxacin, ampicillin, cefotaxime, and cefepime. Some investigations have found a link between ESBL production and ciprofloxacin resistance [24]. The ESBL-producing gene could be on a chromosome or a plasmid. Many genes conferring resistance to aminoglycosides, trimethoprim-sulfamethoxazole, and fluoroquinolones have been found on the plasmid harboring the ESBL gene. The

Figure 1. ESBL production by Proteus isolates. The figure shows the sensitivity of Proteus isolates to different types of antibiotics.



association between the simultaneous existence of these genes and being ESBL in *Klebsiella* was explored in one study, and it was found to be statistically significant. Another reason for this synchrony could be changing the bacterium's outer membrane proteins. Mutations at the nan locus in Gram-negative bacteria are linked to ciprofloxacin resistance and the presence of ESBL enzymes [43-44].

Conclusions

In imipenem-sensitive bacteria, metallo β-lactamase production was not found. This shows that imipenem could treat *Proteus* species that produce metallo β-lactamases. Furthermore, cefepime and imipenem were found to have the lowest occurrence of antibiotic resistance among *Proteus* species, confirming that cefepime and imipenem can be used to treat *Proteus* infections.

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