






Molecular Characterizations and Antimicrobial Susceptibility of Extended-Spectrum β -lactamase (ESBL) Producing *Proteus* spp. Clinical Isolates in Babol, Northern Iran

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ABSTRACT

Introduction: *Proteus* spp. are opportunistic members of *Enterobacteriaceae*, accounting for 10% of urinary tract infections and other primary clinical infections. They produce extended-spectrum beta-lactamases (ESBL) that can confer resistance to beta-lactam antibiotics. This study aimed to investigate the prevalence, antimicrobial susceptibility, molecular characteristics, and genetic relationship of ESBL-producing *Proteus* spp. clinical isolates in Babol, Northern Iran. **Methods:** In this cross-sectional study, out of 112 clinical samples, 30 *Proteus* spp. isolates were identified via specific biochemical assays. According to the Clinical and Laboratory Standards Institute (CLSI) guidelines, antibiotic susceptibility was evaluated using disc diffusion and agar dilution methods, and polymerase chain reaction (PCR) was used to detect *bla*_{TEM} and *bla*_{SHV} genes. **Results:** The resistance rate to tetracycline and sulfamethoxazole was highest by disk diffusion and agar dilution. Multiple drug-resistant (MDR) isolates were 86% and 60% in disk diffusion and agar dilution assays. Seven (23.3%) isolates had the *bla*_{TEM} genes and 18 (60%) *bla*_{SHV}. **Conclusion:** ESBL-producing *Proteus* spp. was highly prevalent, and the *bla*_{SHV} was the most common resistance contributing gene. These findings and relatively high resistance to ampicillin demand more care in prescribing antibiotics. Also, the high prevalence of MDR isolates in patients infected with ESBL-producing *Proteus* spp. requires continuous surveillance.

INTRODUCTION

Proteus spp. are opportunistic members of *Enterobacteriaceae* responsible for 10% of urinary tract infections, cystitis, polio-nephritis, prostatitis, ulcer, eye, and intra-abdominal infections. *Proteus vulgaris*, *Proteus mirabilis*, and *Proteus penneri* are common pathogens affecting immunosuppressed individuals. Also, *Proteus* members cause ~15% of nephrolithiasis through alkalization. These bacteria were documented as extended-spectrum beta-lactamase (ESBL)-producers in 1987 [1]. Extended-spectrum β -lactamase (ESBL) producing *Enterobacteriaceae* is a public health concern worldwide [2, 3]. They produce enzymes responsible for the hydrolysis of oxyimino-beta-lactam antibiotics [4]. The spread of β -lactam antibiotic-resistant isolates occurs by a wide range of ESBL genes, e.g., *bla*_{TEM} and

*bla*_{SHV} [5, 6]. These narrow-spectrum β -lactamases are located on plasmid cassettes and contribute to resistance to β -lactam antibiotics. The rapid increase in cephalosporin-resistant *Enterobacteriaceae* containing *bla*_{TEM} and *bla*_{SHV} genes poses a major therapeutic challenge [6-8].

Misusing antibiotics has led to the spread of multi-drug resistant (MDR) strains, making it a significant challenge for the medical community. The ESBL resistance increases over time. In community-onset, there is a 0.91% yearly increase in ESBL, while in healthcare onsets, it reaches up to 2.31%. In some countries, the phenotypic ESBL production is estimated at 65% in *Enterobacteriaceae* isolates, and in Europe, ESBL antibiotics were the first-line therapy for the associated

infectious diseases. Therefore, the first reports of resistance to ESBL antibiotics came from Europe, and it did not take long for similar reports to be received from around the world [9-13]. The gradual evolution of antibiotic-resistant strains has led to the expression of resistance genes in antibiotic-sensitive bacterial strains through gene mutation and horizontal gene transfer, increasing the MDRs prevalence worldwide [14, 15]. The class I integron is a common factor in distributing and spreading antimicrobial resistance. This class carries more than 40 resistance genes related to aminoglycosides, beta-lactams, chloramphenicol, macrolides, sulfonamides, and disinfectants [16].

This study investigated the *bla*_{TEM} and *bla*_{SHV} resistance genes in *Proteus* spp. and their correlations with antibiotic resistance patterns in hospitalized patients.

MATERIAL AND METHODS

The setting, bacterial isolates, and study design.

From March 2018 to April 2019, we collected 112 blood samples from the inpatients at Ayatollah Rohani Hospital, Babol, Northern Iran. *Proteus* spp. were identified based on conventional biochemical and microbiological tests, i.e., biotyping assays. All isolates were stored in Luria Bertani broth (Merck, Germany) containing 20% glycerol at -80 °C for further use.

The Ethical Committee of Babol University of Medical Sciences approved this study (Code No.: MUBABOL.REC.1394.162).

Disk diffusion (DD) assay. Susceptibility testing was performed with ten antibiotics by standard disk diffusion (DD) technique according to CLSI (Clinical and Laboratory Standards Institute) standard procedure [17]. The antibiotics included gentamicin (10 µg), cefepime (30 µg), amikacin (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), cefotaxime (30 µg), ampicillin (10µg),

piperacillin/tazobactam (30 µg), sulfamethoxazole (100 µg), and tetracycline (10 µg) (MAST Diagnostics, Merseyside, UK). *Escherichia coli* ATCC 25922 was used as positive quality control.

Agar dilution (AD) method. After preparing stock solution from antibiotics according to CLSI 2018 standard [17], 1.5×10⁸ CFU/ml of microbial suspensions were cultured on Mueller-Hinton Agar containing the desired antibiotics (MAST Diagnostics, Merseyside, UK) and incubated at 37 °C for 18 to 24 h. A plate containing a medium with no antibiotics was included in assays as the negative control, and results were evaluated according to the CLSI2018 standard table.

Detection of *bla*_{SHV} and *bla*_{TEM} genes. According to the manufacturer's instructions, DNA extraction from all isolates was performed using a high pure PCR template preparation kit (Roche, Germany). The extracted DNAs were stored at -20 °C for subsequent steps. The ESBL-encoding loci, *bla*_{TEM}, and *bla*_{SHV} were amplified by conventional PCR using the primers and conditions described by others (Table 1). The 60 µl PCR reactions contained 10 µl of extracted template DNA, 5 µl of 10x buffer, 1.5mM MgCl₂, 0.2mM dNTPs, 50 pMole of each primer (Copenhagen, Denmark), 1.5U of *Taq* DNA polymerase (Amplicon Co., Denmark) and ddH₂O to the final volume. Amplification was performed in a thermocycler (Corbet, Australia) (Table 1), and PCR products were electrophoresed in 1.5% agarose gel. The PCR products were sequenced in both directions using the same primers used in amplification in an automated DNA sequencer device (Forster, USA). The standard strain integron-positive *Proteus* spp. (ATCC1209) was used as a positive control and integron-negative *Proteus* spp. (ATCC1053) as a negative control. The generated sequences were analyzed at the National Center for Biotechnology Information (NCBI), available at the (<http://www.ncbi.nlm.nih.gov/BLAST/>) website.

Table 1. Primers and PCR programs for amplifying *bla*_{TEM} and *bla*_{SHV} genes

Target	Primer	Primer sequence	Product size (bp)	No. Cycles	PCR Condition				Reference
					Denaturation	Annealing	Extension	Final Extension	
<i>bla</i> _{TEM}	TEM-F	5'-ATGAGTATTCAACATTTCGG-3'	851	30	94 °C for 30 S	55 °C for 30 S	72 °C for 1 min	72 °C for 4 min	[32]
	TEM-R	5'-TTAATCAGTGAGGCACCTAT-3'							
<i>bla</i> _{SHV}	SHV-F	5'-ATGCGTTATATTTCGCCTGTG-3'	735	35	94 °C for 1 min	60 °C for 1 min	72°C for 1 min	72 °C for 10 min	[33]
	SHV-R	5'-TGCTTTGTTATTTCGGGCCAA-3'							

RESULTS

Bacterial Isolation. From March 2018 to April 2019, 30 clinical *Proteus* spp. isolates were collected from 30 patients admitted to Ayatollah Rohani Hospital (Babol,

Northern Iran). Other isolates were excluded from the study.

Antibiotic Resistance Profile. MDR was evaluated by DD assay and AD method. All strains were screened for resistance to 10 antimicrobials by DD. The resistance

rates to tetracycline, sulfamethoxazole, ampicillin, cefotaxime, imipenem, gentamicin, cefepime and ciprofloxacin were 90%, 83.3%, 51.7%, 48.2%, 17.2%, 13.7%, 10.3% and %3.4, respectively. In contrast, there was no resistance to amikacin and piperacillin/tazobactam. In the DD method, 86.6% of isolates were MDR phenotype. In the agar dilution

method, the resistance rate to sulfamethoxazole, ampicillin, tetracycline, cefotaxime, and cefepime were 80%, 50%, 13.7%, 13.7%, and 10.3%, respectively. No resistance to ciprofloxacin, gentamicin, amikacin, imipenem, and piperacillin/tazobactam was detected (Table 2). Also, in the AD method, 60% of isolates were MDR phenotype (Tables 3 and 4).

Table 2. Frequency of Antibiotic susceptibility pattern of *Proteus* spp. evaluated by disk diffusion (DD) and Agar dilution (AD) methods

Antibiotics	Method	Resistant	Intermediate	susceptible	Total number	P-value
Ciprofloxacin	Disk diffusion	n = 1 (3.4%)	n = 2 (6.8%)	n = 27 (90%)	n = 30	NS*
	Agar dilution	n = 0 (0.0%)	n = 1 (3.4%)	n = 29 (96.6%)	n = 30	
Amikacin	Disk diffusion	n = 0 (0.0%)	n = 2 (6.8%)	n = 28 (93.3%)	n = 30	<0.001
	Agar dilution	n = 0 (0.0%)	n = 0 (0.0%)	n = 30 (100%)	n = 30	
Tetracycline	Disk diffusion	n = 27 (90%)	n = 0 (0.0%)	n = 3 (10.3%)	n = 30	<0.001
	Agar dilution	n = 4 (13.7%)	n = 23 (76.6%)	n = 3 (10.3%)	n = 30	
Gentamicin	Disk diffusion	n = 4 (13.7%)	n = 0 (0.0%)	n = 26 (86.6%)	n = 30	NS
	Agar dilution	n = 0 (0.0%)	n = 3 (10.3%)	n = 27 (90%)	n = 30	
Cefotaxime	Disk diffusion	n = 14 (48.2%)	n = 9 (31%)	n = 7 (23.3%)	n = 30	NS
	Agar dilution	n = 4 (13.7%)	n = 10 (34.4%)	n = 16 (53.3%)	n = 30	
Ampicillin	Disk diffusion	n = 15 (51.7%)	n = 3 (10.3%)	n = 12 (40%)	n = 30	NS
	Agar dilution	n = 15 (50%)	n = 0 (0.0%)	n = 15 (50%)	n = 30	
Cefepime	Disk diffusion	n = 3 (10.3%)	n = 21 (72.4%)	n = 6 (20%)	n = 30	<0.05
	Agar dilution	n = 3 (10.3%)	n = 0 (0.0%)	n = 27 (90%)	n = 30	
Imipenem	Disk diffusion	n = 5 (17.2%)	n = 1 (3.4%)	n = 24 (80%)	n = 30	NS
	Agar dilution	n = 0 (0.0%)	n = 5 (17.2%)	n = 25 (83.3%)	n = 30	
Piperacillin tazobactam	Disk diffusion	n = 0 (0.0%)	n = 0 (0.0%)	n = 30 (100%)	n = 30	<0.001
	Agar dilution	n = 0 (0.0%)	n = 0 (0.0%)	n = 30 (100%)	n = 30	
sulfamethoxazole	Disk diffusion	n = 24 (80%)	n = 0 (0.0%)	n = 6 (20%)	n = 30	<0.001
	Agar dilution	n = 24 (80%)	n = 0 (0.0%)	n = 6 (20%)	n = 30	

Table 3. Multi-drug resistant (MDR) pattern in isolated antibiotic-resistant *Proteus* spp.

Method	MDR	Antibiotics	Resistant sample count (%)	Total number (%)
Disk diffusion	Double-resistant	T+ SXT	n = 5 (16.6%)	n = 7 (23.3%)
		T + CTX + SXT	n = 1 (3.3%)	
		T + CTX	n = 1 (3.3%)	
	Triple-resistant	T + GM + STX	n = 4 (13.3%)	n = 9 (30%)
		T + CTX + SXT	n = 1 (3.3%)	
		T + AP + CTX	n = 3 (10%)	
		T + CTX + CPM	n = 1 (3.3%)	
	Quadruple-resistant	T + CTX + AP + SXT	n = 4 (13.3%)	n = 6 (20%)
		T + CTX + CPM + SXT	n = 2 (6.6%)	
	Quintuplet-resistant	T + CTX + AP + CP + SXT	n = 3 (10%)	n = 3 (10%)
Sextuplet-resistant	T + GM + CTX + AP + CP + SXT	n = 1 (3.3%)	n = 1 (3.3%)	
Agar dilution	Double-resistant	AP + SXT	n = 10 (33.3%)	n = 12 (40%)
		CTX + CPM	n = 2 (6.6%)	
	Triple-resistant	T + AP + SXT	n = 3 (10%)	n = 5 (16.6%)
		CTX + AP + SXT	n = 2 (6.6%)	
	Quadruple-resistant	T + CTX + CPM + SXT	n = 1 (3.3%)	n = 1 (3.3%)

T = Tetracycline; CTX = Cefotaxime; SXT = sulfamethoxazole; GM = Gentamycin; AP = Ampicillin; CPM = Cefepime; CP = Ciprofloxacin

PCR amplification of Gene Cassettes. Of 30 *Proteus* spp. isolates, 7 (23.3%) and 18 (60%) were positive for *bla*_{TEM} and *bla*_{SHV} genes, respectively (Fig. 1).

BLAST and Nucleotide Sequence Accession Number. The positive *bla*_{TEM} and *bla*_{SHV} strains were

sequenced and blasted against similar sequences in the Genbank database. After alignment, the homologous sequences were excluded, and the novel ones were deposited in the GenBank database under the accession numbers MH724198, MH724199, MH724200, and MH724201.

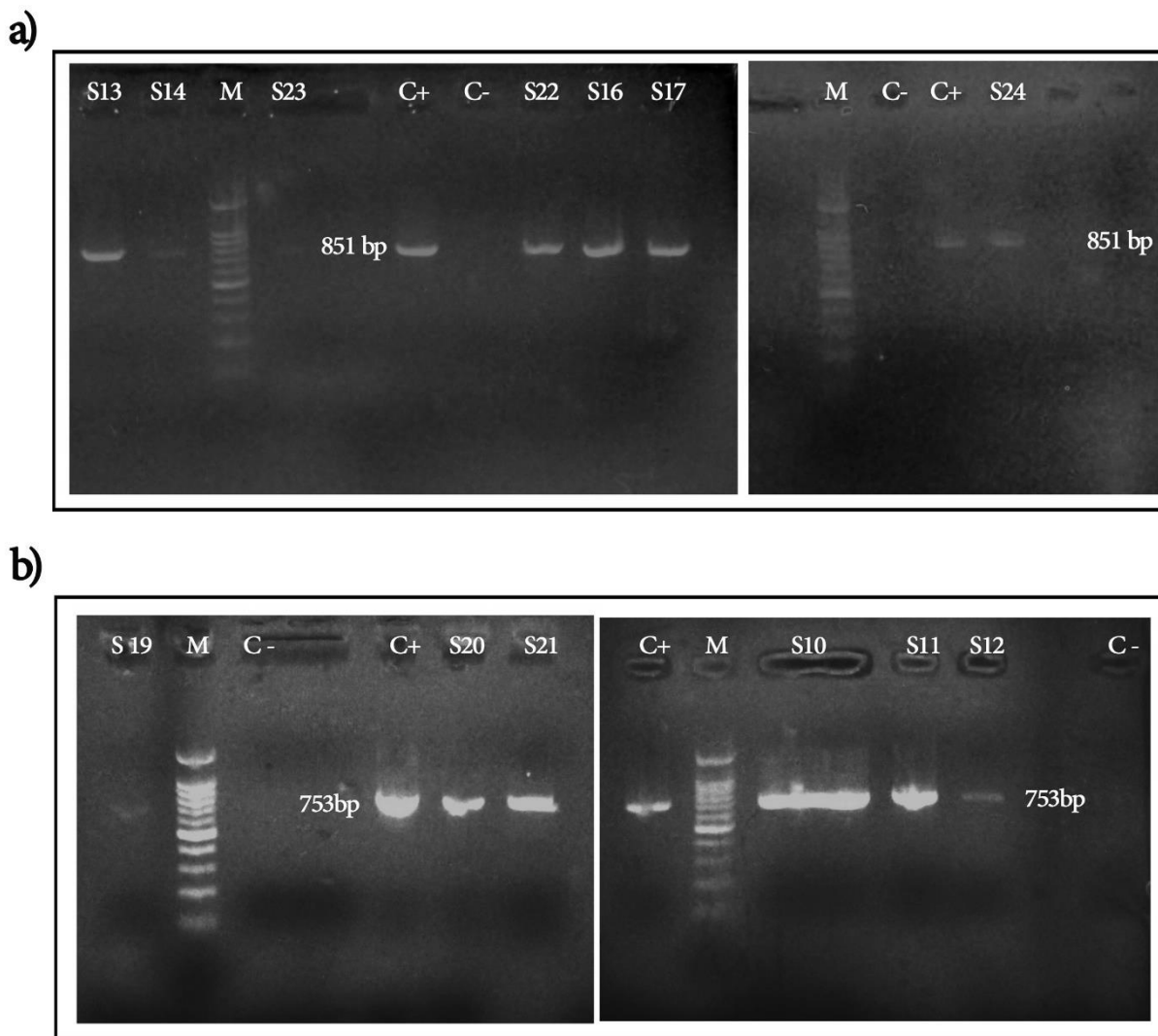


Fig. 1. PCR amplification of *bla*_{TEM} (a) and *bla*_{SHV} (b) genes. Lane M: DNA size marker (100bp); C-: negative control (ATCC 1053); C+: positive control (ATCC 1209).

Table 4. Correlation between *bla*_{TEM} and *bla*_{SHV} genes and antibiotic resistance

Antibiotics	Antibiotic evolution method	Presence/Absence of Genes		Antibiotic resistance pattern			P-value
				Resistant	Sensitive	Total samples	
Ciprofloxacin	Agar Dilution	TEM	TEM+	0 (0%)	7 (100%)	n = 7	NS*
			TEM-	1 (4.3%)	22 (95.7%)	n = 23	
	Disk Diffusion	SHV	SHV+	0 (0%)	18 (100%)	n = 18	NS
			SHV-	1 (8.3%)	11 (91.7%)	n = 12	
		TEM	TEM+	1 (14.3%)	6 (85.7%)	n = 7	NS
			TEM-	2 (8.7%)	21 (91.3%)	n = 23	
	SHV	SHV+	1 (5.6%)	17 (94.4%)	n = 18	NS	
		SHV-	2 (16.7%)	10 (83.3%)	n = 12		
	TEM	TEM+	0 (0%)	7 (100%)	n = 7	< 0.001	

Tetracycline	Agar Dilution	SHV	TEM-	0 (0%)	23 (100%)	<i>n</i> = 23	< 0.001	
			SHV+	0 (0%)	18 (100%)	<i>n</i> = 18		
	Disk Diffusion	TEM	SHV-	0 (0%)	12 (100%)	<i>n</i> = 12	NS	
			TEM+	0 (0%)	7 (100%)	<i>n</i> = 7		
		SHV	TEM-	2 (8.7%)	21 (91.3%)	<i>n</i> = 23		
			SHV+	1 (5.6%)	17 (94.4%)	<i>n</i> = 18		
	Gentamicin	Agar Dilution	SHV	SHV-	1 (8.3%)	11 (91.7%)	<i>n</i> = 12	NS
				TEM+	6 (85.7%)	1 (14.3%)	<i>n</i> = 7	
				TEM-	21 (91.3%)	2 (8.7%)	<i>n</i> = 23	
		Disk Diffusion	TEM	SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18	
				SHV-	12 (100%)	0 (0%)	<i>n</i> = 12	
				TEM+	6 (85.7%)	1 (14.3%)	<i>n</i> = 7	
Cefotaxime	Agar Dilution	SHV	TEM-	21 (91.3%)	2 (8.7%)	<i>n</i> = 23	NS	
			SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
	Disk Diffusion	TEM	SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
			TEM+	6 (85.7%)	1 (14.3%)	<i>n</i> = 7		
Cefepime	Agar Dilution	SHV	TEM-	21 (91.3%)	2 (8.7%)	<i>n</i> = 23	NS	
			SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
	Disk Diffusion	TEM	SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
			TEM+	6 (85.7%)	1 (14.3%)	<i>n</i> = 7		
Ampicillin	Agar Dilution	SHV	TEM-	21 (91.3%)	2 (8.7%)	<i>n</i> = 23	NS	
			SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
	Disk Diffusion	TEM	SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
			TEM+	6 (85.7%)	1 (14.3%)	<i>n</i> = 7		
Imipenem	Agar Dilution	SHV	TEM-	21 (91.3%)	2 (8.7%)	<i>n</i> = 23	NS	
			SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
	Disk Diffusion	TEM	SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
			TEM+	6 (85.7%)	1 (14.3%)	<i>n</i> = 7		
Piperacillin tazobactam	Agar Dilution	SHV	TEM-	21 (91.3%)	2 (8.7%)	<i>n</i> = 23	< 0.001	
			SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
	Disk Diffusion	TEM	SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
			TEM+	6 (85.7%)	1 (14.3%)	<i>n</i> = 7		
Piperacillin tazobactam	Agar Dilution	SHV	TEM-	21 (91.3%)	2 (8.7%)	<i>n</i> = 23	< 0.001	
			SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
	Disk Diffusion	TEM	SHV+	15 (83.3%)	3 (16.7%)	<i>n</i> = 18		
			SHV-	12 (100%)	0 (0%)	<i>n</i> = 12		
			TEM+	6 (85.7%)	1 (14.3%)	<i>n</i> = 7		

Co-trimoxazole	Agar Dilution	TEM	TEM+	5 (71.4%)	2 (28.6%)	<i>n</i> = 7	NS
			TEM-	19 (82.6%)	4 (17.4%)	<i>n</i> = 23	
		SHV	SHV+	14 (77.8%)	4 (22.2%)	<i>n</i> = 18	NS
		SHV-	10 (83.3%)	2 (16.7%)	<i>n</i> = 12		
	Disk Diffusion	TEM	TEM+	5 (71.4%)	2 (28.6%)	<i>n</i> = 7	NS
			TEM-	19 (82.6%)	4 (17.4%)	<i>n</i> = 23	
SHV		SHV+	14 (77.8%)	4 (22.2%)	<i>n</i> = 18	NS	
		SHV-	10 (83.3%)	2 (16.7%)	<i>n</i> = 12		

DISCUSSION

The prevalence of ESBL-producing *Proteus* spp. is increasing worldwide, including in the United States, Asia, and Europe [18]. In the present study, the prevalence of *bla*_{TEM} and *bla*_{SHV} were 23.3% and 60%. These differences in the distribution of *bla*_{TEM} and *bla*_{SHV} may be due to geographical distribution, type of organisms, and source of infections. In Iraq, the prevalence of *bla*_{TEM} was 60% among *P. mirabilis* isolates [19], while in China, it was around 52% in the same bacteria [20]. In Argentina, investigating the resistance to β -lactam/ β -lactamase inhibitors in enterobacteria revealed that all *Proteus* spp. isolates harbored the *bla*_{TEM} gene [21]. In India, the *bla*_{TEM} rate among *P. mirabilis* isolates was 81.9% [22], while in Egypt, it was 35% [23]. In Tehran, Iran, Malekjamshidi *et al.* (2010) estimated *bla*_{TEM} prevalence at 83% among ESBL-positive *P. mirabilis* specimens [24]. Other studies indicated variable rates ranging from 8.3% to 91% [25-29].

In a study by Hamid *et al.*, no *P. mirabilis* isolates in Iraq had *bla*_{SHV} [19]. In India, the *bla*_{SHV} prevalence among *P. mirabilis* isolates was 7% [22]. In Tehran, Iran, the prevalence of *bla*_{SHV} prevalence was 8% in ESBL-positive *P. mirabilis* isolates [24]

The correlation between *bla*_{TEM} and *bla*_{SHV} gene and resistance to some antibiotics showed a significant correlation. The *bla*_{TEM} and *bla*_{SHV} genes significantly correlated with the resistance to piperacillin/tazobactam obtained by the disk diffusion method. Also, there was a significant correlation between *bla*_{TEM} and *bla*_{SHV} genes and resistance to piperacillin/tazobactam and amikacin in the agar dilution method. According to the disk diffusion and agar dilution assays, piperacillin/tazobactam, amikacin, gentamicin, and imipenem are proper choices for treating *Proteus* spp. Given that most ESBL-positive strains showed increased resistance to tetracycline, sulfamethoxazole, cefotaxime, and ampicillin, *bla*_{TEM} and *bla*_{SHV} genes might help confer resistance to these antibiotics. Conza *et al.* (2014) showed a significant association between the *bla*_{TEM} gene and resistance to amoxicillin-clavulanic acid [21]. Also, Li *et al.* (2022) showed a substantial correlation between *bla*_{TEM} and resistance to chloramphenicol, ciprofloxacin, and trimethoprim-sulfamethoxazole in *P. mirabilis* isolates [20].

In our study, the highest antibiotic resistance rates were against sulfamethoxazole, tetracycline, and ampicillin. The results of MDR strains in both disk diffusion and agar

dilution methods were 86% and 60%, almost similar to other studies in different countries [17, 26, 27, 30, 31]. Due to the high prevalence of MDR strains, which indicates misuse of antibiotics, studying the physiological properties of β -lactamase genes has received much attention. *Proteus* spp., an opportunistic bacterium, accounts for 10% of urinary tract infections. Therefore, identifying resistance genes is essential for implementing infection control programs and preventing the spread of resistant strains [10-13].

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

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