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Preliminary survey of extended-spectrum β-lactamases (ESBLs) in nosocomial uropathogen *Klebsiella pneumoniae* in north-central Iran

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ABSTRACT

Infections caused by extended-spectrum β-lactamases (ESBLs) producing bacteria, including *Klebsiella pneumoniae* have increasingly subjected to therapeutic limitations and patients with these infections are at high risk for treatment failure, long hospital stays, high health care costs, and high mortality. The aim of this study was to screen the prevalence of the bla_{TEM},bla_{CTX-M} and bla_{SHV} ESBL genes in *K. pneumoniae* strains isolated from nosocomial urinary tract infections (UTIs). During the March 2016 to December 2017, one hundred isolates of *K. pneumoniae* were collected from urine specimens of patients suffering from nosocomial UTI referred to Khatam Al-Anbia hospital in Shahrud, north-central Iran. All isolates were identified by standard bacteriological tests. The pattern of antibiotic susceptibility was determined according to the CLSI guidelines. The presence of the ESBLs was investigated using the double-disc synergy test (DDST). Polymerase chain reaction technique was used to detect the bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes in DDST positive isolates. Most isolates showed remarkable resistance to tested antibiotics with highest rate against nitrofurantoin (75%) and trimethoprim/sulfamethoxazole (65%). The imipenem was the most effective antibiotic against *K. pneumoniae* isolates. ESBL phenotype was detected in 50 (50%) of isolates. The prevalence of bla_{TEM}, bla_{CTX-M} and bla_{SHV} genes among 50 ESBLs-positive isolates was 25 (50%), 15 (30%) and 35 (70%) respectively. The bla_{TEM} and bla_{SHV} genes were seen in 25 isolates (50%) simultaneously. The findings of this study indicated the 50% frequency rate of ESBL-producing *K. pneumoniae* in our geographic region. Since the treatment of infections caused by this bacterium is associated with many limitations, this high prevalence is a warning sign to adopt new control policies to prevent further spread of this microorganism.

1. Introduction

Urinary tract infection (UTI) is one of the most commonly diagnosed infections in both genders and across all age groups in the world [1]. Uropathogenic *Escherichia coli* (UPEC) and *Klebsiella pneumoniae* are classified among the most important microorganisms contributing UTI [2]. As a Gram-negative bacterium, *K. pneumoniae* is responsible for hospital-acquired UTI, septicemia, pneumonia, and soft tissue infections [3]. In recent decades, due to the excessive and improper use of antibiotics and the spread of organisms that produce extended-spectrum β-lactamases (ESBLs), the emergence of multidrug-resistant uropathogens such as *K. pneumoniae* has increasingly been raised [4].

Since first described in the 1980s, the ESBL-producing bacteria have been spreading throughout the world, and nowadays, they are frequently

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isolated from nosocomial and from community-acquired infections [5, 6]. ESBLs are bacterial enzymes have ability to cause resistance against various types of β-lactam antibiotics, including third-generation cephalosporins and monobactams, though inhibited by clavulanic acid. Meanwhile, they do not confer resistance against cephamycins and carbapenems [7]. So far, more than 400 ESBL enzymes have been identified, and most of them have evolved due to mutations in the active center of the classic plasmid β-lactamases, including TEM-1, TEM-2, and SHV-1, with over 150 members. The CTX-M enzymes are the second largest group of ESBLs [8, 9]. Until the late 1990s, the majority of ESBLs (mainly TEM and SHV type) were isolated from *K. pneumoniae* strains involved in nosocomial outbreaks [10].

Infections caused by ESBL-producing bacteria have increasingly subjected to therapeutic limitations, and patients with these infections are at high risk for treatment failure, long hospital stays, high health care costs, and high mortality [11, 12]. The clinical bacterial isolates all over the world vary in the presence of ESBL enzymes and their pattern is changing rapidly over time [13]. Currently, ESBL-producing *Klebsiella* species have been categorized as one of the six drug-resistant bacteria that need urgent new therapeutic compounds [14].

Owing to the serious problems in the treatment of ESBL-producing *K. pneumoniae* infections, identifying the most prevalent ESBL enzymes locally is of great importance for countries, to monitor the changing of antibiotic resistance patterns. These observations afford valuable information on ESBL epidemiology and could aid medical personnel in choosing the right and most effective treatment [14].

By virtue of the scarce data on the frequency rate of the most prevalent ESBL genes (bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{SHV}) in UTIs-isolated *K. pneumoniae* strains in our region at this time, our study was conducted to screen the presence of these genes among nosocomial uropathogen isolates of *K. pneumoniae* in Shahrud City, north-central Iran. Furthermore, the multidrug-resistant (MDR) (resistant to three or more antimicrobial categories) *K. pneumoniae* isolates were determined according to the most frequently used definition [15].

2. Materials and methods

2.1. Ethical consideration

This cross-sectional study was conducted in accordance with the Declaration of Helsinki and written informed consent was obtained from all patients.

2.2. Bacterial isolates

The isolates that were used in this study belonged to different wards (internal, surgery, intensive care unit, emergency, etc.) of Khatam Al-Anbia hospital in Shahrud, north-central Iran, that were collected from urine samples of patients with nosocomial UTI during the March 2016 to December 2017. Totally, 100 isolates of *K. pneumoniae* were collected from nosocomial UTI. The patients with primary negative urine culture, whose urine culture test showed a positive result after 48–72 hours of hospitalization, were considered as a nosocomial UTI. The 100 *K. pneumoniae* strains were isolated from 58 females and 42 males, respectively. The age of the patients was between 5-79 years (average 39 years).

2.3. Sample collection

For isolation of the *K. pneumoniae*, a 10 ml of clean-catch midstream urine specimen was collected in a sterile container. The specimens were cultured on blood agar and MacConkey agar (Quelab, Canada) media. A volume of urine measured using the calibrated loop method was inoculated into blood agar medium for colony counting. Densities of growth of *K. pneumoniae* greater than or equal to 10^4 CFU/ml (colony forming units per milliliter) were interpreted as positive for urinary tract infection [16].

2.4. Identification of K. pneumoniae

All isolates were confirmed by standard bacteriological tests, which included Gram staining, lysine iron agar (LIA), triple sugar iron agar (TSI), SIM (sulfide-indole- motility), Simon citrate, MR-VP (Methyl Red-Voges Proskauer), and urea broth [17]. All media were purchased from Merck Co, Germany. The verified *K. pneumoniae* isolates were suspended in trypticase soy broth (TSB, Merck, Germany) with 20% (v/v) glycerol and frozen at -80 °C for further investigations.

2.5. Antimicrobial susceptibility testing (AST)

Antibiotic susceptibility testing of the isolates was carried out on Mueller-Hinton (MH) agar (Merck, Germany) by the Kirby-Bauer method, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [18]. The antibiotics were as follows: amikacin (30μg), cefotaxime (30μg), ceftazidime (30 μg), ciprofloxacin (5μg), co-trimoxazole (25μg), gentamicin (10μg), imipenem (10μg), nitrofurantoin (30μg), nalidixic acid (30μg) cefepime (30μg), ceftriaxone (30μg) and norfloxacin (10μg) (Mast Co, Merseyside, UK). The MDR isolates were determined according to previous definition [15]. The *Escherichia coli* ATCC 25922 was used as a quality control strain.

2.6. Phenotypic detection of ESBL production

All isolates that were resistant to one of the third generation cephalosporins (ceftazidime, cefotaxime, and ceftriaxone) by AST were analyzed for ESBL production [18]. ESBL phenotypic detection was performed using a double-disc synergy test (DDST) standard on MH agar. Briefly, the bacteria with turbidity equivalent to 0.5 McFarland standards were swabbed on to MH agar plates, an amoxicillin/clavulanic acid (20/10μg) was placed in the center of the plate and ceftazidime (30μg), cefotaxime (30μg) and ceftriaxone (30μg) discs were placed 15 mm away from the central disc. The plates were incubated at 37 °C for up to 24 h. An increase of >5 mm inhibition zone for antibiotics around the amoxyclav disc compared to the cephalosporin discs alone was considered ESBL production. ESBL production was further confirmed by the phenotypic confirmatory test (PCT) by using both ceftotaxime/cefotaxime-clavulanic acid (30μg/10μg) and ceftazidime/ceftazidime-clavulanic acid (30μg/10μg) as described previously [9, 19]. *K. pneumoniae* ATCC 700603 was used as an ESBL-positive control.

2.7. Preparation of DNA template for polymerase chain reaction

Bacterial DNA templates were extracted by suspending some colonies of an overnight growth on Luria–Bertani agar (Merck, Germany) in 500 ml DNase- and RNase-free water. The suspension was boiled at 95 °C for 10 min in a dry bath incubator (Fisher Scientific, USA), then centrifuged at 14000 rpm for 10 min at 4 °C. Finally, 0.5 ml of the supernatant was used as DNA template for PCR [9]. The yielded DNA was stored at -20 °C for molecular screening.

2.8. Molecular characterization of ESBL genes

The phenotypic ESBLs-positive *K. pneumoniae* isolates were investigated for presence of bla_{SHV}, $bla_{CTX\text{-}M,}$ and bla_{TEM} ESBL genes by polemerase chain reaction (PCR). The primers used in this study are listed in Table 1. The PCR reactions were carried out in an Eppendorf thermal cycler (Germany) in a final volume of 25 μL containing 2.5 μL of 10X PCR buffer, 0.6 mg/μL MgCl2, 200 μM of deoxynucleotide triphosphates (dNTPs), 0.5 units of Taq polymerase, 10 pmol of each primer, and 5 μL of sample DNA. The PCR carried out in the following conditions: initial denaturation at 94 °C (5 min), followed by 35 cycles of denaturation at 95 °C (30 sec), different annealing temperatures (Table 1) (30 sec), and extension at 72 °C (30 sec), with final extension

period of 72 °C (5 min). To determination of expected products, the PCR bands were separated by electrophoresis on 1% agarose gel stained with ethidium bromide and evaluated using an ultraviolet transilluminator (ENDURO™ UV, Labnet International, CA, USA). Positive gene controls prepared from the Pasteur Institute of Iran were used in each PCR run.

2.9. Statistical analysis

The data analysis was done by using SPSS™ software, version 22.0 (IBM Corporation, Armonk, NY, USA). The results are presented as descriptive statistics in terms of relative frequency. Values were expressed as the percentages of the variables.

3. Results

3.1. Antibiotic resistance patterns

The results of AST of the 100 *K. pneumoniae* isolates against 12 antibiotics are shown in Table 2. Overall, from 100 *K. pneumoniae* isolates, 75 (75%) of them were resistant to nitrofurantoin, followed by 65 (65%) to trimethoprim/sulfamethoxazole and norfloxacin, 60 (60%) to ciprofloxacin, 55 (55%) to nalidixic acid and ceftazidime, 50 (50%) to cefepime and amikacin, 40 (40%) to ceftriaxone and cefotaxime, and 30 (30%) to gentamicin. The results revealed that imipenem was the most effective antibiotic against *K. pneumoniae* isolates with 80% susceptibility. Among the *K. pneumoniae* isolates studied, 45 (45%) were resistant to at least one of third-generation cephalosporins. In total, 60 (60%) isolates were resistant to third-generation cephalosporins. The antibiotic resistance rate in ESBL-positive isolates was as follows: nitrofurantoin (76%), nalidixic acid (50%), cefepime (58%), gentamicin (22%), ceftazidime (74%), cefotaxime (66%), ciprofloxacin (70%), cefteriaxon (64%), trimethoprim/sulfamethoxaz (78%), norfloxacin (62%), imipenem (16%), and amikacin (58%) (Table 2).

3.2. MDR patterns

Regarding the result of AST, all 100 *K. pneumoniae* isolates were resistant to at least three antibiotics and all of the isolates (n = 100, 100%) were MDR with 19 different patterns (Table 3). The profile II (nitrofurantoin- nalidixic acid- cefepime- norfloxacin- ciprofloxacin) was the most predominant resistance pattern with 10% frequency.

3.3. Phenotypic results for ESBLs

Among the 60 isolates that were resistant to third-generation cephalosporins, ESBL production was detected in 45 and 50 isolates using DDST and PCT, respectively.

3.4. PCR investigation of ESBL genes

Based on the results of this study, among the phenotypic ESBLs-positive isolates, the PCR assay showed that 25 (50%), 15 (30%) and 35 (70%) of *K. pneumoniae* isolates were positive for bla_{TEM}, $bla_{CTX\text{-}M}$ and bla_{SHV} gene, respectively and 25 (50%) of isolates contained both bla_{TEM} and bla_{SHV} genes (Fig. 1 A,B,C). In the ESBLs-producing *K. pneumoniae*, 25 isolates (50%) harbored one type of ESBL genes, and 25 isolates (50%) carried two types of ESBL genes.

4. Discussion

This preliminary hospital-based study aimed to identify the ESBL-producing UTI-causing *K. pneumoniae* isolates and their antibiotic susceptibility pattern. Therefore, our study did not focus on concerns such as incidence and prevalence of other ESBL-UTI causing organisms in north-central Iran. Globally, UTIs afflict 150 million people every year [20]. Available data regarding the UTI-causing *K. pneumoniae* and its antimicrobial resistance profiles in specific geographical regions may assist physicians in selecting the best empirical antimicrobial therapy. Although *K. pneumoniae* is the second or third most etiologic agent of UTIs after *E. coli*, the former creates a problem for clinicians because of the multidrug resistance expressed by this pathogen [21]. The current investigation explored the resistance patterns of *K. pneumoniae* strains in patients suffered from UTIs in Shahrud, north-central Iran. Moreover, we showed a high resistance rate to antibiotics in patients with nosocomial urinary infections, except for imipenem with the susceptibility rate of 80%. Unlike our study, Latifpour *et al.* [22] in Sharekord, Iran, reported lower antibiotic resistance rate against norfloxacin (59%), trimethoprim/sulfamethoxazole (61%), and nitrofurantoin (55%). However, the resistance rate

Table 1
PCR primers used for detection of ESBL genes in *K. pneumoniae* isolates.

Target Genes	Primer Sequence (5′ to 3′)	Amplicon Size, bp	Annealing Temperature, °C
$bla_{CTX\text{-}M}$	F: CGCTTTGCGATGTGCAG R: ACCGCGATATCGTTGGT	550	60
bla_{SHV}	F:CGCCTGTGTATTATCTCCCTGTTAGCC R:TTGCCAGTGCTCGATCAGCG	843	62
bla_{TEM}	F: CATTTCCGTGTCGCCCTTATTC R: CGTTCATCCATAGTTGCCTGAC	800	60

Table 2
The antibiotic susceptibility testing results of 100 *K. pneumoniae* isolates based on CLSI 2015.

Antibiotic	Total *K. pneumoniae* No. (%)			ESBL-producing *K. pneumoniae* No. (%)		
	Resistant	Intermediate	Sensitive	Resistant	Intermediate	Sensitive
Nitrofurantoin	75 (75%)	5 (5%)	20 (20%)	38 (76%)	2 (4%)	10 (20%)
Nalidixic acid	55 (55%)	5 (5%)	40 (40%)	25 (50%)	2 (4%)	23 (46%)
Cefepime	50 (50%)	5 (5%)	45 (45%)	29 (58%)	1 (2%)	20 (40%)
Gentamicin	30 (30%)	10 (10%)	60 (60%)	11 (22%)	3 (6%)	36 (72%)
Ceftazidime	55 (55%)	5 (5%)	40 (40%)	37 (74%)	3 (6%)	10 (20%)
Cefotaxime	40 (40%)	15 (15%)	45 (45%)	33 (66%)	8 (16%)	9 (18%)
Ciprofloxacin	60 (60%)	10 (10%)	30 (30%)	35 (70%)	4 (8%)	11 (22%)
Cefteriaxon	40 (40%)	15 (15%)	45 (45%)	32 (64%)	8 (16%)	10 (20%)
Trimethoprim/Sulfamethoxazole	65 (65%)	5 (5%)	30 (30%)	39 (78%)	0 (0%)	11 (22%)
Norfloxacin	65 (65%)	10 (10%)	25 (25%)	31 (62%)	4 (8%)	15 (30%)
Imipenem	15 (15%)	5 (5%)	80 (80%)	8 (16%)	0 (0%)	42 (84%)
Amikacin	50 (50%)	10 (10%)	40 (40%)	29 (58%)	6 (12%)	15 (30%)

Table 3
Multidrug-resistance patterns of *K. pneumoniae* isolates.

Resistance pattern	Phenotypic resistance	Resistant isolates N (%)
I	FM-NA-SXT	5 (5%)
II	FM-NA-FEP-NOR-CIP	10 (10%)
III	FM-NA-CAZ-NOR-CIP	5 (5%)
IV	FM-NA-FEP-IPM-NOR-CIP	5 (5%)
V	FM-NA-SXT-CAZ-FEP-CRO-NOR-CIP	5 (5%)
VI	FM-NA-CAZ-FEP-CRO-GM-CTX-NOR-CIP	5 (5%)
VII	FM-NA-SXT-CRO-GM-CIP-CTX-NOR -AN	5 (5%)
VIII	FM-SXT-CTX-AN	5 (5%)
IX	FM-SXT-CAZ-NOR-CIP-AN	5 (5%)
X	FM-SXT- CAZ-CRO-GM-CIP-CTX	5 (5%)
XI	FM-SXT-CAZ-CRO-GM-CTX-AN	5 (5%)
XII	FM-SXT-CAZ-CRO-GM-CTX-AN-NOR-CIP	5 (5%)
XIII	FM-SXT-CAZ- CRO-CTX-AN -FEP- CIP	5 (5%)
XIV	FM-IMP-NOR	5 (5%)
XV	NA-CAZ-FEP-NOR	5 (5%)
XVI	NA-SXT-IPM-NOR	5 (5%)
XVII	NA-SXT-FEP-NOR-AN	5 (5%)
XVIII	SXT-CAZ-FEP-NOR-CIP-AN	5 (5%)
XIX	SXT-CAZ-FEP-CRO-GM-CIP-CTX-AN	5 (5%)

Abbreviations: FM, Nitrofurantoin; NA, Nalidixic acid; CTX, Cefotaxime; CRO, Ceftriaxone; CAZ, Ceftazidime; IPM, Imipenem; CIP, Ciprofloxacin; NOR, Norfloxacin; SXT, Trimethoprim/Sulfamethoxazole; FEP, Cefepime; GEN, Gentamicin; AN, Amikacin.

to nalidixic acid (72%) was higher, and the ciprofloxacin resistance was the same as the present survey. On the other hand, Najar Peerayeh *et al.* [23] displayed 30.7% and 51.1% resistance to gentamicin and ceftazidime, respectively, which was in line with our results. Furthermore, consistent with our investigation, they introduced the imipenem as the most effective antibiotic against *K. pneumoniae*. A previous study by Dallal *et al.* [24], suggested higher resistance for nalidixic acid and ceftazidime antibiotics [24]. The resistance rate of our isolates against third-generation cephalosporin groups, ceftriaxone and cefotaxime, were 40%. However, some other studies demonstrated a higher resistance rate for cephalosporin antibiotics [24, 25, 26]. The occurrence of MDR *K. pneumoniae* is a worldwide concern, and many types of research with diverse results have been performed in the recent decade [27].

The current study showed the occurrence of 100% for MDR *K. pneumoniae* strains, but in other studies conducted in Iran, the frequency of these strains was reported to be 74% and 46.6%, which is less than our results [28, 29]. The possible explanation for this higher rate of MDR characteristic, mainly in developing countries, could be irregular antibiotic prescription, sampling biases, genetic variations, geographic differences, social behaviors, and dissimilar patients' characteristics [28].

More than 75% of the studies have reported ESBL-producing infections with *K. pneumoniae* [30]. Our findings revealed that 50% of the *K. pneumoniae* isolates were ESBL producers. ESBL producing rate in this study was higher than those reported in the United States (12%) and Europe (33%) [31]. Conversely, higher prevalence rates of ESBL-producing *K. pneumoniae* isolates were identified in Tehran (69.7%), and Zahedan (66.7%), cities of Iran [29]. In the present study the PCT method detected more ESBL-positive isolates than DDST. DDST found 45% of ESBL producers *K. pneumoniae*, whereas PCT identified 50%. The results of the PCR assay in this study demonstrated the bla_{SHV} as the most prevalent ESBL gene, a finding which was similar to Khosravi *et al.* [32] study in Ahvaz, Iran. The prevalence of bla_{SHV} gene in clinical isolates of *K. pneumoniae* has differently been reported in some previous studies from Iran, which ranged from 43.1% to 67.4% [32, 33, 34, 35].

Nowadays, there are reports presenting the prevalence of other ESBL enzymes in different countries [9, 19, 31]. The co-existence of varied ESBL genes within the same isolate, as detected in this study, has also been reported in other regions of Iran and other countries [9, 29, 32, 36]. Furthermore, most of the bla_{SHV} ESBL positive strains in our study were detected to be bla_{TEM} positive. This investigation has some limitations such as short period time of sample collection, small sample size, single hospital evaluation, and focusing only on bla_{CTX-M}, bla_{TEM}, and bla_{SHV} ESBL genes. However, they could be the subject of ongoing studies.

5. Conclusions

Even with the above-mentioned limitations, this report offers an insight into the current prevalence and molecular types of ESBL-producing *K. pneumoniae* in Shahrud, north-central Iran, contributing to a better understanding of the epidemiology of these enzymes at local and national levels. In addition, to the best of our knowledge, we have

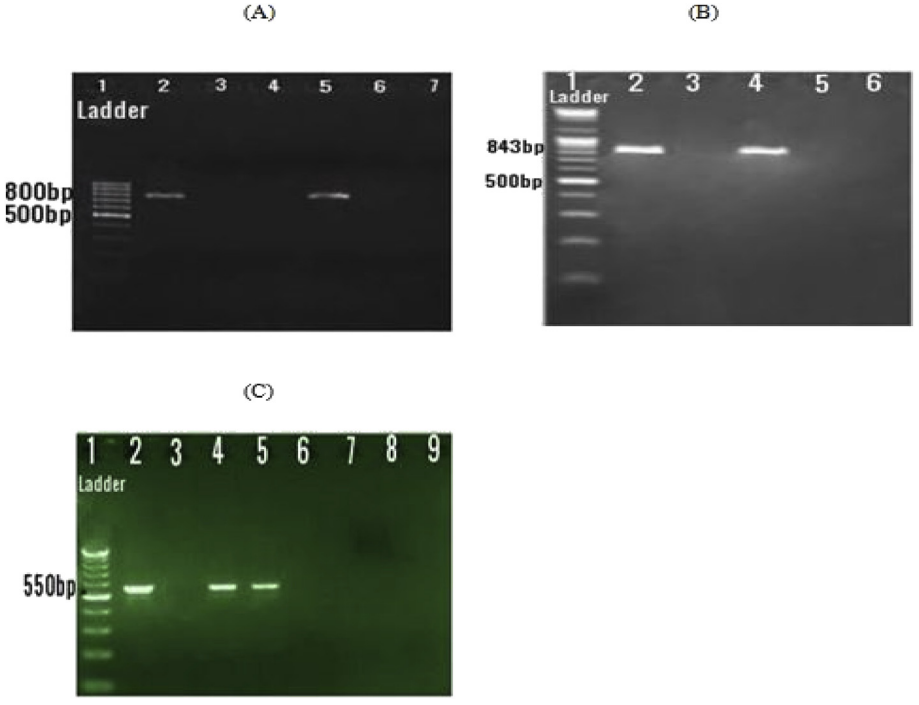

Fig. 1. Gel electrophoresis of PCR assays of different ESBL genes. (A) Lane 1: 100 bp DNA marker; lane 2: positive control (bla_{TEM} - 800bp); lane 3: negative control; lane 4,6, and 7: negative isolate; lane 5: positive isolate. (B) Lane 1: 100 bp DNA marker; lane 2: positive control (bla_{SHV} - 843bp); lane 3: negative control; lane 5, and 6: negative isolate; lane 4: positive isolate. (C) Lane 1: 100 bp DNA marker; lane 2: positive control (bla_{CTX-M} - 550bp); lane 3: negative control; lane 4, and 5: positive isolate; lane 6,7,8, and 9: negative isolate.

presented the first report regarding the ESBL-producing *K. pneumoniae* in north-central Iran. The findings of this study indicated the 50% frequency rate of ESBL-producing *K. pneumoniae* in our geographic region. Since the treatment of infections caused by this bacterium is associated with many limitations, this high prevalence is a warning sign to adopt new control policies to prevent further spread of this microorganism. Our results emphasize the necessity for the adequate screening of ESBL-producing strains in our region.

Declarations

Author contribution statement

Sajjad Yazdansetad, Miaad K. Alkhudhairy: Conceived and designed the experiments; Performed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Reza Najafpour, Elika Farajtabrizi, Reham M. Al-Mosawi: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Morteza Saki, Elham Jafarzadeh, Farokh Izadpour, Atefeh Ameri: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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