



**Original Article**

**Prevalence of *bla*TEM, *bla*SHV, and *bla*CTX-M Genes among ESBL-Producing *Klebsiella pneumoniae* and *Escherichia coli* Isolated from Thalassemia Patients in Erbil, Iraq**

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**Competing interests:** The authors have declared that no competing interests exist.

**Abstract. Background:** Due to the recent appearance of organisms that are resistant to several drugs (multidrug-resistant) like Enterobacteriaceae that produce extended-spectrum  $\beta$ -lactamase (ESBL), concerns have remarkably increased regarding the suitable treatment of infections. The present study was an investigation into ESBL molecular characteristics among clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* resulting in urinary tract infections (UTIs) and their pattern of antimicrobial resistance in order to come up with helpful information on the epidemiology of these infections and risk factors accompanied with them.

**Methods:** In order to conduct the study, 20 *K. pneumoniae* and 48 *E. coli* were isolated and retrieved from thalassemia center in Erbil, Iraq during July 2016 and September 2016. The collected strains were analyzed and the profile of their antimicrobial susceptibility was specified. In order to spot  $\beta$ -lactamase genes (i.e. *bla*TEM, *bla*SHV, and *bla*CTX-M), polymerase chain reaction was conducted.

**Results:** The findings obtained from multiplex PCR assay showed that out of the collected strains of ESBL-producing *E. coli*, had 81% *bla*TEM, 16.2% *bla*SHV, and 32.4% *bla*CTX-M genes. Similarly, 64.7% *bla*TEM, 35.2% *bla*SHV, and 41.1% *bla*CTX-M genes existed in the isolates of *K. pneumoniae*. It was found that antibiotic resistance pattern of *E. coli* and *K. pneumoniae* isolates to 20 antibiotics varied widely. It was also concluded that the majority of the *K. pneumoniae* and *E. coli* isolates were multi-drug resistant (MDR). Moreover, 75% and 87.5% of respectively *K. pneumoniae* and *E. coli* isolates showed the MDR phenotypes.

**Conclusion:** TEM prevalence was high among other types of ESBLs. Over all, the most active antimicrobial agents *in vitro* remained to be the carbapenems.

**Keywords:** *Escherichia coli*, *Klebsiella pneumoniae*, ESBL, *bla*TEM, *bla*SHV and *bla*CTX-M.

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**Introduction.** It has been reported that bacteria that belong to the Enterobacteriaceae family are etiologic factors of numerous nosocomial infections all over the world.<sup>1</sup> It is difficult to control diseases induced by bacilli Enterobacteriaceae given the limitation of therapeutic possibilities caused by constantly rising

resistance of such organisms to antibiotics. In fact, Ojdana *et al.* (2014) introduced ESBLs as one of the most well-known resistance mechanisms in Gram-negative bacilli.<sup>2</sup> ESBLs are a group of enzymes that lead to resistance increase in Aztreonam, Ceftazidime, Cefotaxime, related Oxyimino- $\beta$ -lactams,

cephalosporins, and penicillins, but Clavulanic acid inhibits them. TEM, SHV, and CTX-M are the 3 main types of ESBLs. CTX-M, which has become more prevalent than SHV and TEM, includes a rapidly expanding family which has spread among a wide range of clinically important bacteria and over wide geographic areas.<sup>3</sup> Furthermore, strains that produce ESBL often demonstrate resistance to antibiotics belonging to other classes (i.e. aminoglycosides, quinolones, and sulfonamides), which makes strategies of treatment more complex.<sup>4</sup>

In addition, Enterobacteriaceae family members such as *Klebsiella pneumoniae* and *Escherichia coli* often produce ESBLs; however, other genera of the *Enterobacteriaceae* family have recently been reported to contain some other enzymes. A higher level of resistance in such organisms was first observed in patients with prolonged hospital stays in intensive care units in Europe. However, isolates were identified in Africa, Asia, the Middle East, and South and North Americas, and ESBL GNB soon became a global problem and concern.<sup>5</sup>

Common ESBL genes coding for isolates of *K. pneumoniae* and *E. coli* were determined as CTX-M (cefotaximase that preferentially hydrolyzes cefotaxime), TEM (found and isolated in the early 80s from Teminora who was a Greek patient), and SHV (for variable of sulphhydryl which was first observed in a single *Klebsiella ozaenae* strain retrieved in Germany). These genes which are mediated by transposons, plasmids, or chromosomes are all sporadically described all over the world.<sup>6</sup>

Because there is an increase in the rates of bacterial resistance every year, leading to rising global concern, it is highly significant to understand susceptibility patterns as hospital stays may prolong and mortality rates increase due to inappropriate empirical antimicrobial therapy, which can be controlled given appropriate therapy.<sup>7</sup> Acquiring additional PBPs insensitive to  $\beta$ -lactam or changing the normal PBPs are known as the commonest cause of resistance in cocci such as MRSA and pneumococci which are gram positive. However, a mixture of endogenous acquired  $\beta$ -lactamases with natural efflux and up-regulated impermeability is the main reason for resistance in the gram-negative bugs.<sup>8</sup> It should be noted that there are well-prepared documents on the fact that routine disc-diffusion tests fail to detect ESBL production. Moreover, the significance and detection method of ESBLs are not fully recognized by many clinical laboratories; therefore, there may be a lack of enough resources in laboratories to reduce the spread of these mechanisms of resistance.<sup>9</sup>

A wide variety of ESBLs including SHV, TEM, OXA, CTX, AmpC, and so forth exist; however, most of them are derivatives of SHV, TEM, and CTX-M enzymes which are most often found in *K. pneumoniae*

and *E. coli*. In this regard, the current study was aimed at determining the prevalence of the ESBL phenotype and examines the existence of *bla*SHV, *bla*CTX-M, and *bla*TEM genes in isolates.

## Materials and Methods

**Isolates of bacteria.** In total, 68 consecutive non-duplicate of *K. pneumoniae* and *E. coli* isolates (n = 20 and 48, respectively) were retrieved from specimens of urine at a Thalassemia center in Erbil, Iraq. The samples were obtained from both outpatients and inpatients between July 2016 and September 2016. Standard microbiological techniques were used for isolation.<sup>10</sup> Conventional microbiological procedures were employed to identify the isolates. Besides, the VITEK 2 compact system was utilized to re-identify them (BioMerieux, France).

**Antimicrobial susceptibility testing.** According to the guidelines of the Clinical and Laboratory Standards Institute (CLSI), the isolates were screened by the disc diffusion method (Kirby-Bauer disc diffusion method) on Mueller-Hinton agar (MHA) plates in order to test their antimicrobial susceptibility.<sup>11</sup> The utilized antimicrobials included Amoxicillin+Clavulanic acid (20+10 $\mu$ g), Amikacin (10  $\mu$ g), Azithromycin (15 $\mu$ g), Cefixime (5 $\mu$ g), Cefotaxime (30 $\mu$ g), Chloramphenicol (30 $\mu$ g), Ceftazidime (30 $\mu$ g), Ciprofloxacin (10 $\mu$ g), Doxycycline (30 $\mu$ g), Imipenem (10 $\mu$ g), Gentamicin (10 $\mu$ g), Kanamycin (30 $\mu$ g), Nalidixic acid (30 $\mu$ g), Meropenem (10 $\mu$ g), Nitrofurantoin (100 $\mu$ g), Norfloxacin(10 $\mu$ g), Ofloxacin (5 $\mu$ g), Streptomycin (25 $\mu$ g), Piperacillin (100 $\mu$ g), and Tobramycin (10 $\mu$ g).

**Testing for production of ESBL (MDDST).** Using a disc of Amoxicillin-Clavulanate (20/10  $\mu$ g) with four cephalosporins of Ceftriaxone, 3GC-Cefotaxime, 4GC-Cefepime, and Cefpodoxime, the Modified Double Disc Synergy Test (MDDST) was employed to test all strains in terms of their production of Extended Spectrum Beta-Lactamase (ESBL). A lawn culture belonging to the organisms was created on a Mueller-Hinton agar plate following the recommendations by CLSI.<sup>11</sup> A disc that contained Amoxicillin-Clavulanate (20/10  $\mu$ g) was put in the middle of the plate. The 3GC and 4GC discs were placed respectively 15mm and 20mm center-to-center apart from the center of the amoxicillin-clavulanate disc.<sup>12</sup> Any increase or distortion in the zone toward the Amoxicillin-Clavulanate disc was regarded positive for the production of ESBL. According to CLSI guidelines, the combined disc test was used to confirm ESBL production.

**Detection of ESBL genotypes by multiplex PCR amplification.** Using the method utilized by Monstein *et al.* (2007) with slight modifications, multiplex PCR

was employed to examine the positive isolates in the initial screening test for ESBL production for the existence of *bla*SHV, *bla*CTX-M, and *bla*TEM genes.<sup>13</sup> Freshly cultured isolates bacteria were used to prepare template deoxyribonucleic acid (DNA) was prepared using Presto™ Mini gDNA bacterial kit. All reactions of PCR were conducted by utilizing 2 µl DNA template (density of 10 ng/µl), the Master Mix consisting of 3 mM MgCl<sub>2</sub>, 0.2% Tween® 20, 20 mM Tris-HCl pH 8.5, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.4 mM of each dNTP, 0.4 µM of each primer, and 0.2 units/µl Ampliqon Taq DNA polymerase. The conditions of polymerase chain reaction amplification were set up as follow: primary denaturation step for 10 minutes at 95°C; 30 denaturation cycles for 30 seconds at 94°C, annealing 30 seconds at 60°C for, extension for 2 minutes at 72°C, and a final extension step for 10 minutes at 72°C. Using agarose gel electrophoresis, size separation PCR amplicons were utilized to detect respective genes (**Table 1**).

## Results.

**Antimicrobial susceptibility profile.** In total, 68 consecutive non-duplicate of *K. pneumoniae* and *E. coli* isolates (n = 20 and 48, respectively) were retrieved, and their antimicrobial resistance profile against 20 different antimicrobial agents was tested. The current results revealed that *K. pneumoniae* and *E. coli* isolates vary widely to different antimicrobials.

The resistance rates of isolates of *K. pneumoniae* and *E. coli* against the selected 20 antimicrobial agents obtained from urine samples. It was found that a majority of the *K. pneumoniae* and *E. coli* isolates were resistant to several drugs (multi-drug resistant: MDR) where a total of 87.5% and 75% of respectively *E. coli* and *K. pneumoniae* isolates indicated MDR phenotypes.

Furthermore, the results of the antimicrobial susceptibility test against *E. coli* revealed that *E. coli* showed 81.25% resistance to Amoxicillin+Clavulanic acid (**Table 2**), whereas susceptibility to doxycycline decreased to 39.5%. Similar patterns were observed for Piperacillin. Substantial decrease of 16.6–37.5% was observed in the susceptibility for all Cephalosporins. Imipenem, Meropenem, Amikacin, Gentamicin, Ciprofloxacin, Tobramycin and Ofloxacin with susceptibility rates of respectively 100%, 100%, 95.8%, 95.8%, 95.8%, 95.2%, and 93.75% were the most active agents against *E. coli*. Resistance to Azithromycin Norfloxacin and Streptomycin was comparatively less (10.5%, for them). On the other hand, *E. coli* showed a different sensitive rate to Chloramphenicol, Kanamycin, Nalidixic acid and Nitrofurantoin with 85.4%, 62.5%, 50% and 75%, respectively. Meanwhile, similar results were observed for *K. pneumoniae* which revealed that *K. pneumoniae* showed 65% resistant to Amoxicillin+Clavulanic acid, whereas susceptibility to Piperacillin dropped to 50% (**Table 2**).

**Table 1.** List of primers used for Multiplex PCR amplification.

Target gene	Primer	Sequence (5'-3')	Amplicon size	References
<i>bla</i> TEM	Forward	TCG CCG CAT ACA CTA TTC TCA GAA TGA	445-bp	[30]
	Reverse	ACG CTC ACC GGC TCC AGA TTT AT		
<i>bla</i> SHV	Forward	ATG CGT TATATT CGC CTG TG	747-bp	[12]
	Reverse	TGC TTT GTT ATT CGG GCC AA		
<i>bla</i> CTX-M	Forward	ATG TGC AGY ACC AGT AAR GTK ATG GC	593-bp	[30]
	Reverse	TGG GTR AAR TAR GTS ACC AGA AYC AGC GG		

**Table 2.** Antibiotic resistance pattern of *K. pneumoniae* and *E. coli* isolates.

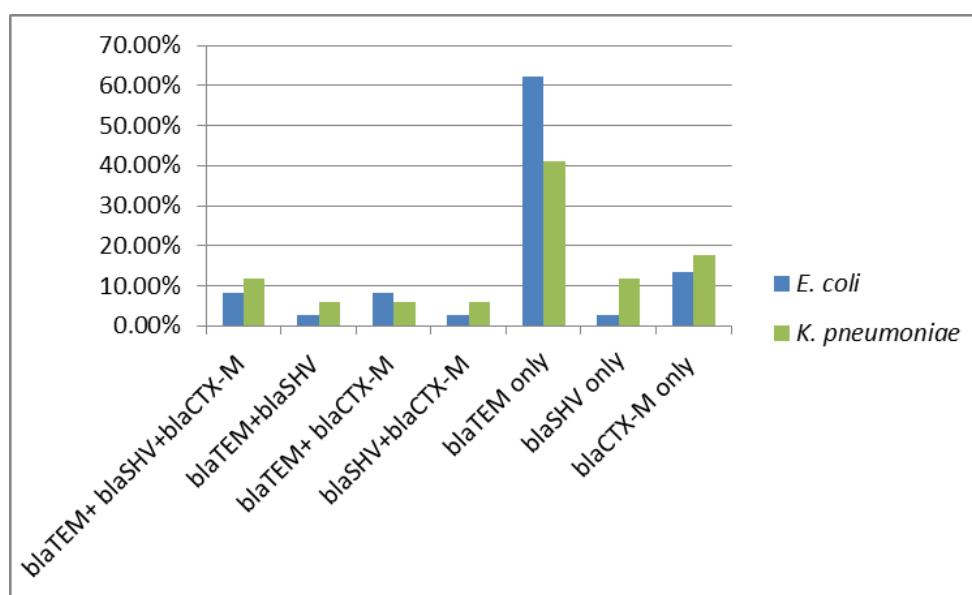
Name of Antibiotic	Symbol	<i>E. coli</i>		<i>K. pneumoniae</i>	
		Susceptible (%)	Resistance (%)	Susceptible (%)	Resistance (%)
Amikacin,	AK	95.8	4.2	100	00
Amoxicillin-Clavulanic acid	AMC	18.75	81.25	35	65
Azithromycin	AZM	89.5	10.5	90	10
Cefixime	CFM	16.6	83.4	35	65
Cefotaxime	CTX	16.6	83.4	40	60
Ceftazidime	CAZ	37.5	62.5	30	70
Chloramphenicol	C	85.4	14.6	95	05
Ciprofloxacin	CIP	95.8	4.2	100	00
Doxycycline	DOX	39.5	60.5	95	05
Gentamicin	CN	95.8	4.2	100	00
Imipenem	IPM	100	00	100	00
Kanamycin	KAN	62.5	37.5	90	10
Meropenem	MEM	100	00	100	00
Nalidixic acid	NA	50	50	50	50
Nitrofurantoin	F	75	25	70	30
Norfloxacin	NOR	89.5	10.5	100	00
Ofloxacin	OFL	93.75	6.25	100	00
Piperacillin	PIPER	33.3	66.7	50	50
Streptomycin	S	89.5	10.5	95	05
Tobramycin	TOB	95.2	4.8	100	00

Similar patterns were noticed for Nalidixic acid. In addition, a substantial drop of 30–40% was observed in the susceptibility for all Cephalosporins. Nevertheless, *K. pneumoniae* showed a different sensitive rate to Chloramphenicol, Doxycycline, Streptomycin, Azithromycin, Kanamycin and Nitrofurantoin with 95%, 95%, 95%, 90%, 90% and 70% respectively.

**ESBL screening of *E. coli* and *K. pneumoniae*.** Out of the 48 *E. coli* isolates, a total of 37 isolates (77%) showed positive results in initial screening test of ESBL production by MDDST and phenotypic confirmatory test of ESBL production. Meanwhile, out of the 20 *K. pneumoniae* isolates, a total of 17 isolates (85%) showed positive results in initial screening test of ESBL production and phenotypic confirmatory test

of ESBL production.

In PCR detection of ESBL genotypes, it was found that all of the ESBL screening positive *K. pneumoniae* and *E. coli* isolates had one or more ESBL genes that were tested in the present study. Overall, 85% (17/20) of *K. pneumoniae* and 77% (37/48) of *E. coli* isolates were positive for one or more ESBL genes. The multiplex PCR assay results indicated that 32.4% *bla*CTX-M genes, 16.2% *bla*SHV genes, and 81% *bla*TEM genes were detected in the *E. coli* isolates. Similarly, the isolates of *K. pneumoniae* contained 64.7% *bla*TEM, 35.2% *bla*SHV, and 41.1% *bla*CTX-M genes. The overall incidence of ESBL genotypes in *K. pneumoniae* and *E. coli* isolates is illustrated in **Figure 1**.



**Figure 1.** The overall incidence of ESBL genotypes in screening positive *K. pneumoniae* and *E. coli* isolates.

**Discussion.** As a global challenge, antimicrobial resistance in pathogenic bacteria is accompanied with high rates of mortality and morbidity. In addition, because of multidrug resistant patterns, infections have been reported to be difficult or even impossible to treat with conventional antimicrobials. Because many healthcare centers fail to diagnose causative microorganisms and their patterns of antimicrobial susceptibility timely in patients with bacteremia and other serious infections, antibiotics are broadly, liberally and mostly unnecessarily used.<sup>14</sup>

In the current study, high prevalence of MDR isolates of *K. pneumoniae* and *E. coli* was noticed in the clinical samples. The overall prevalence of MDR phenotypes in *K. pneumoniae* and *E. coli* isolates was respectively 75% and 87.5%. Among the MDR isolates of *E. coli* and *K. pneumoniae*, a majority of them were producers of ESBL. Similar to the results of the present study, also in a research by Bora *et al.* (2014) reported the same ratios.<sup>15</sup>

In the current study, the antimicrobial susceptibility patterns were determined in all isolates, and the results obtained from the test of antimicrobial susceptibility against *E. coli* and *K. pneumoniae* revealed that isolated bacteria were different in their susceptibility to the tested antimicrobials. Liao *et al.* (2017)<sup>4</sup> and Tabar *et al.* (2016) reported similar results.<sup>16</sup> Carbapenems are often the final influential therapy that exists for infections resulting from MDR Enterobacteriaceae.<sup>17</sup> According to other studies, 100% sensitivity was seen with Imipenem and Meropenem, which has been reported to be the most effective antibiotic including the isolates that produce ESBLs. This is an important result of the present study because many infections can be treated with Carbapenemes. This result can be relevant to the fact that these antibiotics are more expensive and thus used less in this region.

Paterson *et al.* (2001) stated that even if ESBL producers show an *in vitro* susceptibility, they are intrinsically resistant to all cephalosporins.<sup>18</sup> In the

present study, 9% and 13% of the producers of ESBL were found to have false susceptibilities respectively to cefotaxime and Ceftazidime. This can be attributed to the fact that different ESBL enzymes possess various optimal substrate profiles.<sup>19</sup>

In fact, ESBLs are reported to be a challenge among hospitalized patients all over the world. It has also been reported that ESBLs have different prevalence rates among clinical isolates in different parts of the world, and there is a rapid continuous change in their prevalence rate over time.<sup>20</sup> Given the increased prevalence of ESBLs-producing Enterobacteriaceae, it is highly crucial to develop laboratory testing methods in order to accurately diagnose the existence of such enzymes in clinical isolates.<sup>21</sup> Among all ESBL detection methods, modified double disc synergy tests were the most sensitive ones.<sup>22</sup> A study carried out by<sup>23</sup> presented similar findings and indicated positive MDDST in 40/40 isolates, while it was positive in 25/40 and 39/40 isolates respectively in double disk synergy test (DDST) and phenotypic confirmatory disc diffusion test (PCDDT).

By following the MDDST screening criteria for ESBL production, respectively 85% and 77% of *K. pneumoniae* and *E. coli* isolates were screened for detecting production of ESBL. Existence of one or more ESBL genes in all screened positive isolates revealed that *K. pneumoniae* and *E. coli* isolates that produce ESBL are highly frequent in the geographical region under investigation. In India, Kaur *et al.* (2013) observed that 63.4% *E. coli* and 60.3% *K. pneumoniae* isolates produced ESBL.<sup>24</sup> Phenotypic tests for detection of ESBL can only confirm ESBL production but fail to recognize the subtypes of ESBL. As reported by Nüesch-Inderbinen *et al.* (1996), molecular methods have been proved to be sensitive, but they costly and conducting them requires a long time, expertise, and specialized equipment.<sup>25</sup> Ultimate identification is only probable through methods of molecular detection. The results of a study conducted by (Navon-Venezia *et al.*, 2003) revealed that it is necessary to periodically evaluate these phenotypic tests because introduction of new enzyme can change their performance.<sup>26</sup> In their study of phenotypic and genotypic methods of ESBL detection, (Grover *et al.*, 2006) stated that PCR is a reliable method for detecting ESBL.<sup>27</sup> In the present study, multiplex PCR amplification assay was utilized to detect *bla*CTX-M, *bla*SHV, and *bla*TEM genes in the retrieved clinical isolates of *K. pneumoniae* and *E. coli* because one of the advantages of this assay rapid screening of large numbers of clinical isolates, moreover, if it is required, further molecular epidemiological studies can take advantage of the DNA that is isolated via this assay.<sup>13</sup>

Furthermore, it is essential to identify beta-lactamase in order to conduct a reliable epidemiological investigation into antimicrobial

resistance. The current study was conducted to survey antimicrobial drug resistance, ESBL phenotypes, and *bla*SHV, *bla*TEM and *bla*CTX-M genes detection in *K. pneumoniae* and *E. coli* isolates retrieved from urinary tract infections in Erbil, Iraq.

The most globally common type of ESBL appeared to be CTX-M-type ESBLs with their higher incidence in most locations compared to SHV and TEM ESBLs.<sup>28</sup> Among the three ESBL genotypes included in this study, the most prevalent one was found to be *bla*TEM (81%) and *bla*TEM (64.7%) respectively in ESBL-producing isolates of *E. coli* and *K. pneumoniae*. The less prevalent ESBL genotype was *bla*SHV, and the prevalence rate of *bla*SHV in ESBL-producing *K. pneumoniae* isolates (35.2%) was higher than *E. coli* isolates (16.2%). Also, the prevalence rate of *bla*CTX-M in ESBL-producing *K. pneumoniae* isolates (41.1%) was higher than *E. coli* isolates (32.4%). It was found that all of the ESBL-producing isolates of both organisms were positive for one or more ESBL genotypes. It was observed that *bla*TEM alone was more prevalent in *E. coli* (62.16%, 23/37), and in *K. pneumoniae* (41.17%, 7/17), while *bla*CTX-M and *bla*TEM together predominated in *E. coli* (8.1%), while *bla*SHV, *bla*TEM, and *bla*CTX-M together predominated in isolates of *K. pneumoniae* (11.76%). A study conducted by Manoharan *et al.* (2011) reported similar findings.<sup>29</sup> In the present study; however, TEM ESBL was the prevalent genotype and CTX-M-type ESBL was not prevalent. The discrepancy is assumed to be because of regional variations, since the strains collected and evaluated in the current study were only from Erbil, Iraq.

Furthermore, in another study, Moghnieh *et al.* (2018) have reported that *E. coli* and *Klebsiella spp* resistance to third-generation cephalosporins is usual in whole countries, with outbreak reaching over 50% in Egypt and Syria<sup>30</sup> and in our study, 30–40% was observed in the susceptibility for all Cephalosporins, which this prevalence is close to other Arabia countries, as well as in Moghnieh study, they reported that carbapenem resistance is emerging, albeit with a prevalence of less than 10%.<sup>30</sup> In parallel, we have found that the most active antimicrobial agents *in vitro* remained to be the carbapenems. Khalaf and Al-Ouqaili *et al.* (2018) in Baghdad, during a period one year demonstrated that SHV gene was detected only in 12.5% *E. coli*, and 56.25% in *K. pneumoniae*.<sup>31</sup> Approximately, we found close to findings above that 16.2% SHV genes in *E. coli* and 35.2% SHV genes existed in the isolates of *K. pneumoniae*. Of course, according the above findings several studies by Teawtrakul *et al.* (2015) Girmenia *et al.* (2016), Ricciardi *et al.* (2016) and Devrim *et al.* (2018) have shown that the rates and types of *Klebsiella* and *Escherichia* strains isolated are differed in other countries.<sup>32-35</sup> These outcomes highlight require for

structured national plans in the zone to target infection prevention and antimicrobial supervision.

**Conclusions.** Knowledge of the antimicrobial resistance patterns and resistance genes of bacterial pathogens in a geographical area is important for control and surveillance of antibiotic resistance. The results of the present study revealed that MDR was highly prevalent. In addition, the Carbapenems,

Amikacin, and Ciprofloxacin were found to be the most to the least active antimicrobial agents *in vitro*. Based on the results obtained in the present study, TEM was highly prevalent among other types of ESBLs.

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