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Molecular Identification of Extended- Spectrum β-lactamase Genes in Klebsiella pneumoniae Strains by Multiplex PCR in Arak, Iran

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Abstract
Introduction: One of the enzymes that is produced by Gram negative bacteria is Extended spectrum-β-lactamases (ESBLs) which was first described in the 1980s. This study aims at identifying three common genes (blaTEM, blaSHV and blaCTX-M) in Klebsiella pneumoniae isolated from patients in Arak, Iran.
Methods: A total number of 112 isolates were selected during 2012-2013. To identify phenotypic specifications, Combination Disk Method based on CLSI guidelines. The ESBL's genotype was analyzed by multiplex PCR and DNA sequencing.
Results: Among 10 examined antibiotics, the most resistant and susceptible antibiotics were ampicillin and imipenem, respectively. The phenotypic determination of ESBL showed that 46.42% of isolates produced ESBL. The molecular survey of genes showed that 35.89% had CTX-M, 5.12% had both CTX-M and TEM and 2.56% had TEM, SHV, CTX-M.
Conclusion: According to the findings, the high prevalence (73.07%) of ESBL producing K. pneumoniae was observed with a new pattern of blaCTX-M distribution differed from other countries.
Keywords: Klebsiella pneumoniae; ESBLs; Drug Resistance; Multiplex-PCR

Introduction
Klebsiella are gram negative bacteria usually separated from different contamination and infections and are opportunist pathogens (1) For the people and human one of the infection caused by Klebsiella spp. is multidrug resistant and also it can cause an expanding rate to produce extended-spectrum beta-lactamases (ESBLs). We know that resistance to penicillins (such as ampicillin or amoxicillin), newer cephalosporins like cefotaxime, ceftazidime, resistance to first generation cephalosporins, cefoxitin and ceftiofur, and also to aztreonam is created by these enzymes (2). For the first time and at the beginning extended-spectrum β lactamases (ESBLs) were accounted in Klebsiella pneumoniae in Germany in 1983 (3) and since that time ESBLs have been reported as worldwide. Gram negative bacteria produce Extended-spectrum-β-lactamases (ESBLs) enzymes were first described in the 1980s. They have been observed in Klebsiella species, and subsequently in Escherichiacoli (E. coli), Serratiamarcescens (S. marcescens), Pseudomonasaeruginosa (P. aeruginosa), and other gram negative bacilli which have been known as an important resistance practice and

manner against of oxyimino-cephalosporin antibiotics during the last 2 decades (4). The ability to resist to extended-spectrum cephalosporins and aztreonam is mostly mediated by β-lactamases through hydrolyzing the β-lactam ring (5) The ESBL's genes generally exist on plasmids, along with genes which are responsible for resistance totrimethoprim- sulfamethoxazole and aminoglycosides. Furthermore, resistances to quinolones in Klebsiella pneumoniae (K. pneumoniae) isolates that are ESBL+ are notably more frequent than strains that are non ESBL producing and it is considered as a significant cause of nosocomial infections. Today infants and immunocompromised hosts are the most important population that are at risk (6-7). Extended-spectrum beta-lactamases become manifest mainly because of mutations in b-lactamases encoded by the blaSHV, blaTEM, and bla CTX-M genes. Different ESBLs that have been detected up to now are more than 300 variants (8). That which was observed in 1989 concurrently in an Escherichiacoli strain isolated in Germany and in a Salmonella typhimurium isolated in Argentina was a new kind of ESBL that caused resistance in a high level to cefotaxime and a low level of activity against ceftazidime (9). To name one of the plasmid ESBLs of class A, the cefotaximaze enzyme was considered and named (CTX-M) family. Dissemination and spread of ESBLs is different all over the world. Generally, TEM-type ESBLs is predominating type in the USA, and in Western Europe the SHV-type of ESBLs is dominant type of the ESBLs (3). The most frequently isolated ESBLs in Japan, South America, Eastern Europe, Kenya and Spain is Cefotaximases (CTX-M) (10–12). During the recent decade, TEM and SHV types are the most usual ESBLs and strains with CTX-M ESBLs are going to distribute in many of countries and the most frequent not any TEM and not any SHV ESBLS type (13). By cefotaxime selective hydrolysis rather than ceftazidime, CTX-M b-lactamases are separated. Therefore, CTX-M-15 a type of CTX-M types, may really hydrolyses ceftazidime (14). Overall, by mutation occurrence in classical plasmid, most ESBLs mediated SHV and TEM genes. At first ESBLs were described in K. pneumoniae from Western Europe but now are mostly published all over the world (15). There are frequent reports for nosocomial distribution of infecting disease caused by bacteria producing ESBL, but the spread of ESBL mediated resistance is not established well enough for most hospitals and medical centers (16). ESBLs have a high epidemiological and clinical importance which is responsible for therapeutic shortage and increase hospital costs and government spending (17). Gram negative bacilli which produce ESBL enzymes and cause multidrug resistance infections have been reported with increasing distribution of antimicrobial resistance in hospitals and specially in intensive-care units and are associated with remarkable deaths and morbidity (18).

Factors such as the portability of resistance factors mediated by transposons, gene cassettes in integrons, and plasmid along with the irregular and indiscriminate use of antibiotics, help to the rise in resistance of antibiotic in bacterial pathogens (19). The current therapy for ESBL producing K. pneumoniae infection is Imipenem as an example of broad-spectrum agent. Anyway, there are several reports of therapeutic failures of this drug due to multiple β -lactamases producer strains. Due to the limited therapeutic choice for some of these organisms, the extended-spectrum β -lactamases producers will challenge clinical microbiologists and clinicians (20). Because of the confined information about ICU-associated infections in referral hospitals and clinical centers in Arak, it is necessary to understand the prevalence rates and pattern of such nosocomial infection to obtain satisfactory results in managing these infections. Therefore, in this study, we arrange a bilateral study in order to determine the prevalence rate and genotypic and phenotypic

characteristics of K. pneumoniae infections. The molecular characterization of ESBL genes (TEM, SHV and CTX-M) from isolates of K. pneumoniae was completed and done by Multiplex PCR. By using this method, the TEM, SHV and CTX-M genes could be identified simultaneously and characterized and detected at molecular level (21). The aim of this perusal and study was to isolate and identify the types of extended spectrum β - lactamases (ESBL) produced by Klebsiella pneumoniae isolated from various specimens (urine, blood, sputum and wound) of patients hospitalized and outpatient in Arak, Iran. In this study, we used a combination of phenotypic tests and multiplex PCR to peruse the frequency of ESBL genes among isolates of Klebsiella pneumoniae.

Methods

Klebsiella isolates**.** The bacterial strains used in this study included 112 Klebsiella pneumoniae were gathered together from hospitals and clinical centers in Arak, Iran and were selected for the molecular study from the clinical samples including blood (n=10), urine (n=64), sputum (n=23), and wound (n=15) over a 6 month period from January 2013 up to June 2013. The biochemical tests for phonotypical identification were based on the colony morphology on Eosin Methylene Blue, Mac Conkey agar and Blood agar, and it was speciated by standard biochemical tests (22). The isolates were protected in TSB broth with 30% glycerol at -70 °C for further analysis.

The antibiotic vulnerability of bacteria was primarily detected by the combined disk method using Mueller Hinton agar plates according to the NCCLS guidelines (23-24). The antibiotics were tested ceftazidim, (30 µg), cefotaxime (30 µg), aztreonam (30 µg), co trimoxazole (25 µg), ampicillin (10 µ g), ciprofloxacin (5 µg), cefepime (30 µg), gentamicin (10 µ g), amikacin (30 µg), imipenem (10 µ g), (Himedia, India). According to our findings, minimum inhibitory concentration (MIC) was reported by the E-test (AB-Biodisk, Sweden) for cefotaxime antibiotic based on CLSI standard procedure (17).

In this study, extended spectrum beta lactamases were ascertained by the Combined Disc method. In brief, according to CLSI standard procedure we used discs in pairs including cefotaxime (30 µg) and ceftazidime (30 µg) with and without clavulanic acid (10 µg) (MAST Co. UK) that were placed on the same plate including Muller Hinton agar and facing sides (at a distance of 20-30 mm). It was explained for a positive test result as a≥5mm expansion in zone diameter compared to a disk without clavulanic acid (23).

In present study DNA was extracted from all isolates which were phenotypically strains that produce ESBLs and some non-producing strains, using CinnaPure DNA extraction kit (Cinnagen Co, Tehran, Iran). To ensure the quality of the extracts, amplification of 16srRNA gene was evaluated. To this end, GenoMab 16s Univer PCR detection kit (GenoMab, Tehran, Iran) was used according to manufacturer's instruction. Screening the presence of SHV, TEM, and CTX-M genes, the extracts were analyzed by using GenoMab Multi ESBLs PCR detection kit (GenoMab, Tehran, Iran).The Multiplex PCR reactions were completed and done in a final 25 µL volume containing 12.5 µl of reaction mix, 1 µl primer mix, 7.5µl DW, 1 µl of Taq polymerase and 3 µl extracted DNA. Two tubes containing positive and negative controls were used in each PCR run. Description conditions were as follows: initial denaturation step at 95 °C for 15 min, 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 2 min followed by a final extension step at 72 °C for 10 min (25). PCR amplicons were detached electrophoretically on a 1.5% (w/w) agarose gel and stained with ethidium bromide.

Results

In this study a total number of 112 isolates of K. pneumoniae were isolated from a range of

clinical samples of patients hospitalized and outpatient from hospitals and clinics, Arak, Iran. Out of 112 isolates of K. pneumoniae that WERE collected and confirmed by biochemical methods, a total of 64 (57.14%) were isolated from urine, 15 (13.39%) wound, 10 (8.92%) blood, and 23 (20.53%) sputum.

Primary antimicrobial susceptibility test of 112 species of K. pneumoniae showed the most resistance of these isolates was to ampiciline (98.2%) while the most susceptibility of these isolates was to imipenem. The results of phenotypic confirmatory test (Fig. 1) showed that out of 112 isolates of K. pneumonia, 52 (46.42%) were ESBLs-positive isolates (Table 1).

16S rRNA amplification analysis showed a distinct 465 bp band on gel agarose in all extracted DNA so confirmed the samples to be used in the subsequent experiment. A commercial multiplex PCR reaction was applied to discover blaSHV, blaTEM, and blaCTX-M genes in the K. pneumoniae isolates. Gel agarose electrophoresis of the PCR-products showed specific bands related to blaTEM (445 bp), blaCTX_M (600 bp) and blaSHV (750 bp) genes (Fig. 2). These results demonstrated that 75% of the phenotypic ESBLs producing isolates were genotypically containing the ESBLs coding genes. The blaTEM gene alone was present in 25.64% (n=10) of the isolates, the blaCTX-M gene alone was found in 35.89% (n=14) and the blaSHV gene alone was found in 30.76% (n=12) of the isolates (Table 2). The blaTEM and the blaCTX-Mgenes were present in 5.12% (n=2) of the isolates. The blaTEM and the blaSHV genes were present in 5.12% (n=2) of the isolates. The blaCTX-M and the blaSHV genes were present in 2.56% (n=1) of the isolates. Finally, the three TEM, SHV, CTX-M genes were found in 2.56% (n=1) of isolates. No PCR products were obtained in 25% (n=13) of 52 phenotypic ESBLs producing isolates.

Table 1. Comparison of resistance rates to antibiotics in all isolated strains of KlebsiellaPneumonia

Antibiotics	Resistant	Intermediate	Sensitive
Ceftazidime	48 (42.8%)	14 (12.5%)	50 (44.6%)
Cefotaxime	52 (46.4%)	27 (24.1%)	33 (29.4%)
Cefepime	50 (44.6%)	17 (15.1%)	45 (40.1%)
Aztreonam	37 (33%)	27 (24.1%)	48 (42.8%)
Ampicilin	110 (98.2%)	2 (1.7%)	0 (0.0%)
Amikacin	15 (13.3%)	12 (10.7%)	85 (75.8%)
Gentamicin	20 (17.8%)	14 (12.5%)	78 (69.6%)
Co-trimoxazole	46 (41%)	16 (14.2%)	50 (44.6%)
Ciprofloxacin	24 (21.4%)	10 (8.9%)	78 (69.6%)
Imipenem	10 (8.9%)	0 (0.0%)	102 (91%)

Table 2. Details of the Results of Molecular Identification of Isolates

Isolate number	Species	Origin	Multiplex PCR		
			CTX-M	TEM	SHV
1	K. pneumoniae	Urine	+		
2	K. pneumoniae	Wound			
3	K. pneumoniae	Wound			
4	K. pneumoniae	Urine			+
5	K. pneumoniae	Urine			+
6	K. pneumoniae	Urine	+	+	
7	K. pneumoniae	Urine			+
8	K. pneumoniae	Urine			
9	K. pneumoniae	Urine		+	+
10	K. pneumoniae	Urine	+		
11	K. pneumoniae	Sputum	+		
12	K. pneumoniae	Urine		+	
13	K. pneumoniae	Urine			+
14	K. pneumoniae	Urine			+
15	K. pneumoniae	Urine			+
16	K. pneumoniae	Urine			
17	K. pneumoniae	Urine			
18	K. pneumoniae	Urine	+		
19	K. pneumoniae	Urine			+
20	K. pneumoniae	Urine	+	+	+
21	K. pneumoniae	Sputum	+		
22	K. pneumoniae	Urine		+	
23	K. pneumoniae	Urine		+	
24	K. pneumoniae	Urine			
25	K. pneumoniae	Urine	+		
26	K. pneumoniae	Urine		+	
27	K. pneumoniae	Urine			+
28	K. pneumoniae	Urine		+	+
29	K. pneumoniae	Urine			
30	K. pneumoniae	Wound		+	
31	K. pneumoniae	Urine			
31	K. pneumoniae	Urine	+		
32	K. pneumoniae	Blood		+	
33	K. pneumoniae	Urine			
34	K. pneumoniae	Urine	+	+	
35	K. pneumoniae	Urine	+		
36	K. pneumoniae	Urine			+
37	K. pneumoniae	Urine		+	
38	K. pneumoniae	Urine	+		+
39	K. pneumoniae	Wound	+		
40	K. pneumoniae	Urine	+		

41	K. pneumoniae	Urine				
42	K. pneumoniae	Urine				
43	K. pneumoniae	Urine				
44	K. pneumoniae	Urine				+
45	K. pneumoniae	Urine				+
46	K. pneumoniae	Urine			+	
47	K. pneumoniae	Urine	+			
47	K. pneumoniae	Blood	+			
48	K. pneumoniae	Urine			+	
49	K. pneumoniae	Urine				+
50	K. pneumoniae	Sputum			+	
51	K. pneumoniae	Urine	+			
52	K. pneumoniae	Urine				
53	K. pneumoniae	Urine	-		-	-
54	K. pneumoniae	Urine	-		-	-
55	K. pneumoniae	Urine	-		-	-
56	K. pneumoniae	Urine	-		-	-
57	K. pneumoniae	Urine	-		-	-

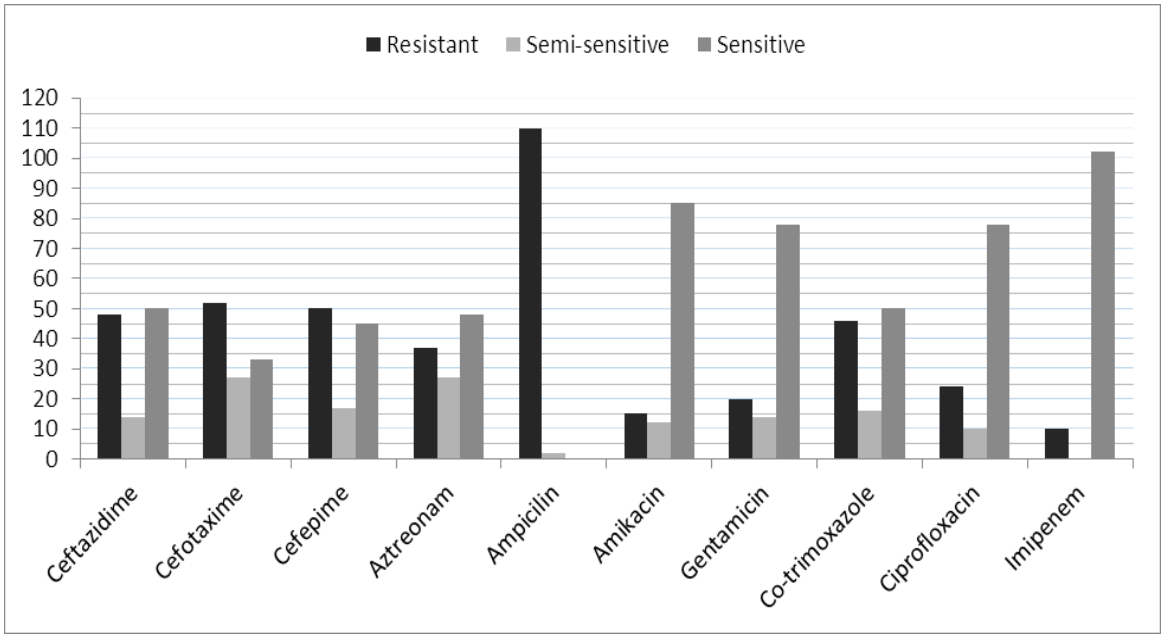

Figure 1. Comparison of resistance rates to antibiotics in all isolated strains of Klebsiella Pneumonia

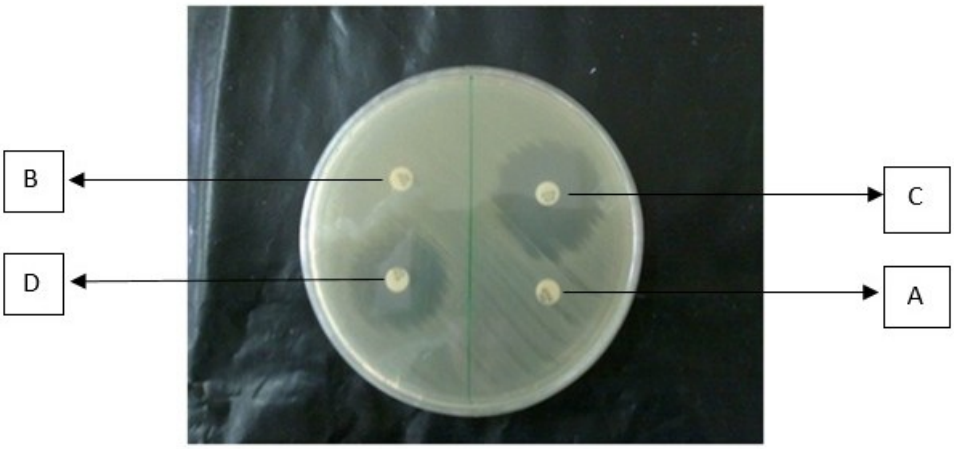

Figure 2. ESBLs phenotypic confirmation by Combination Disk Method. Disks of containing (A) Cefotaxime (CTX=30µg) and (B) Ceftazidime (CAZ = 30µg) without inhibition zone and their combination (C and D) with Clavulanic acid (CA = 10 µg) with inhibition zone around of them, have been compared

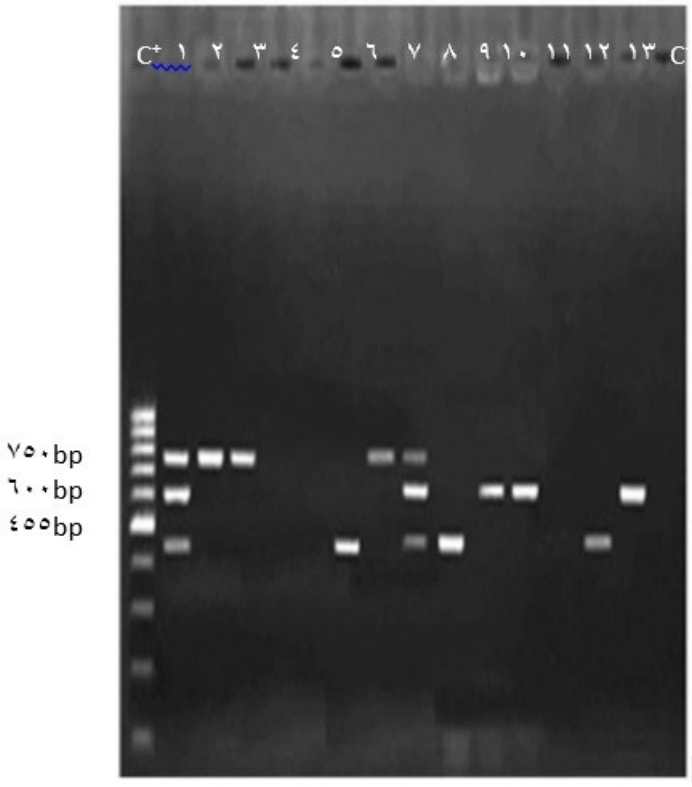

Figure 3. Multiplex PCR for TEM, SHV and CTX-M from 13 clinical isolates K. Pneumonia with blaTEM (445 bp), blaSHV (750 bp) and bla CTX-M (600 bp)

Discussion

In the last decade, extended–spectrum beta-lactamases (ESBLs) producing bacteria have been causing a lot of resistance problems to beta-lactam antibiotics and consequently it has been known as a healthy problem in the remedy of patients with infection due to these bacteria (26). In the present study, primary antimicrobial susceptibility test of K. pneumoniae isolates showed the most resistance to ampiciline, cefotaxime, cefepime and ceftazidime. Some reports also show resistance to β-lactam antibiotics and are increasing worldwide. However, all clinical isolated strains in our study were susceptible to imipenem. It can be expressed that most ESBLs have developed gradually through mutation from natural β-lactamases, especially TEM, CTX-M and SHV. TEM, CTX-M and SHV enzymes are usually found in gram-negative bacteria, especially Enterobacteriaceae.

In this study, we described strains of K. pneumoniae harboring CTX-M, TEM and SHV ESBL isolated from patients in Arak, Iran and in this city there is any register of inspection of molecular epidemiology of ESBL K. pneumoniae. High level of morbidity and mortality. K. pneumoniae caused a Nosocomial bacterial infection that is known as one of the main organisms that make nosocomial infections in the hospital and has been known in every association acquired ESBL. The perception of ESBL producing K. pneumoniae strains in this study can be considered as an alarm and this could be imputed to the clutter and common and irregular use of antibiotics, especially betalactamase antibiotics to treat patients. The percentage of ESBLs belonging to hospitals (in-patient) is more than outpatient in this study and it was high (73.07%). It seems that care treatment and control practices are not acceptable and treatment policies in both in-patient and outpatient are disputable. We also detected 43 (82.6%) isolates with ESBL+ phenotype from urine. It appears that ESBLs producing strains create major therapeutic problems in diseases affiliated with urinary extent infections.

The distribution of blaCTX-M, blaSHV and blaTEM genes in this study was 35.89%, 30.76% and 25.64% separately in the order mentioned which are diverse from the results of the multi-national study group [7]. In Iran, Feizabadi et al. in 2009 showed that 69.7% of K. pneumoniae isolated from Tehran was ESBL positive and the distribution of blaTEM, blaSHV, blaCTX-M-I and blaCTX-M-III of these isolates was 54%, 67.4%, 46.51% and 29%, respectively (27). The count of CTX-M type ESBLs is quickly increasing. They have been discovered in some geographic areas where similar studies have been carried out and are today the most dominant ESBL type worldwide (8).

What was observed in this study was the presence of strains with positive phenotype of ESBL but negative in genotype survey. This indicates that ESBLs other than SHV, TEM and CTX-M enzymes may also emerge in Klebsiella in the near future. Demonstrating that the blaTEM, blaCTX-M and blaSHV and because of variation occurrence by single nucleotide sequence, 3 genes may not be present or the PCR primer sequences may not produce any PCR products under the conditions used. The presence of ESBL producing K. pneumoniae particularly in the NICU of hospitals could lead to main treating and epidemiological results if care is not taken of multiple occurrence and K. pneumoniae has been shown to be the most dominant contained organism. The results of this perusal determined that the common rate of ESBLs, beta-lactamase genes and resistance to multiple antibiotics were considerable among K. pneumoniae isolates. We all should pay attention to the fact that using many ineffective and improper antibiotics and possibility in spreading of ESBL genes between the species of Enterobacteriaceae by transferable genes will help to spread ESBL-producing isolates that can be like a bacterial

bomb. Therefore, we propose that associated therapeutic regimens such as beta-lactam antibiotics and beta-lactamase impediment or carbapenems can be used only for patients with important infections be limited only to patients with serious infections not all patients without any examination of antibiotic resistance and be planned according to the antibacterial sensitivity test. The appearance and extent of ESBL-producing K. pneumoniae strains in all over the world is alarming and usage of antibiotics such as cephalosporins opposite of these isolates is ineffectual and helps to greater resistance. According to our study and our findings as imipenem is the alternative antibiotic for serious infection disease nowadays, spread usage of this drug in the remedy of infection caused by resistant isolates will increase although we should be careful in using this antibiotic because if its consumption continues like other antibiotics in the past, it may be a serious threat of the cure. What is important is that laboratory services should be accessible to lead and provide each infection control schedule for every infection, especially infections related to drug resistance. Regretfully, there is shortcoming in co-operation among clinical settings, doctors and laboratories in Iran. It is critical that we always remind competent remedy of serious infections, and prevention of development of drug resistance will only occur according to the collaboration among clinical and laboratory employees, so we can save on the high costs of treatment and drug preparation.

Conclusion

According to the findings, the high prevalence (73.07%) of ESBL producing K. pneumoniae was observed with a new pattern of blaCTX-M distribution differed from other countries.

Ethical issues

No applicable.

Authors' contributions

All authors equally contributed to the writing and revision of this paper

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