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High Prevalence of Multiple Drug Resistance among ESBLs-Producing *Klebsiella pneumoniae* Isolated from Hospitalized Patients in Isfahan, Iran

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ABSTRACT

***Background*:** This study was to evaluate the prevalence of CTX-M and TEM type ESBLs-producing *K. pneumoniae* and determination of MDR, XDR, and PDR phenotypes of these isolates as well as find out the genetic relationship and molecular typing of these isolates using phenotypic and genotypic methods.

***Methods*:** Non-repetitive 96 *K. pneumonia* isolates were isolated from hospitalized patients in Al-Zahra hospital of Isfahan, Iran. The antibiotic susceptibility test was assessed for 20 antibiotics using Kirby-Bauer disk diffusion method. The frequency of ESBL-producing isolates was determined by phenotypic confirmatory test. All ESBLs-producing isolates were assessed for bla_{TEM} and bla_{CTX-M} genes using PCR method. Molecular typing was performed by enterobacterial repetitive intergenic consensus sequence-based PCR (ERIC-PCR).

***Results*:** Among 96 isolates, 58 isolates (60.4%) were ESBL-producers. In this study, 85.7% and 30.3% of ESBL-producing isolates showed MDR and XDR phenotypes, respectively. No PDR isolate was found. PCR amplification on ESBL-producing isolates showed that 47 (81%) isolates were carried bla_{TEM} gene, while bla_{CTX-M} was detected in all isolates (100%). ERIC-PCR typing was characterized the high genetic similarity among ESBL-producing *K. pneumonia* isolates and revealed 32 band pattern for the isolates.

***Conclusion*:** This study showed high prevalence of important ESBL genes (bla_{CTX-M} and bla_{TEM} genes) among the *K. pneumoniae* isolated from in-patients. Constant following of ESBLs, also identification of their types, in bacteria isolated from hospitalized patients has an important clinical impact. It can provide valuable information for the choice of appropriate antibacterial therapy and decrease of antibiotic resistance.

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Introduction

Klebsiella pneumoniae is an important opportunistic nosocomial pathogen causing a variety of infections including urinary tract infections, pneumonia, septicemia, wound infections and infections in the intensive care units (1). This bacterium is among leading causes of community-acquired and nosocomial infections that result to high rate of mortality (2). The prevalence of *K. pneumoniae* with antibiotic resistance which leads to systemic infections and death has been increasingly reported (3). The treatment options for these infections are complex and limited due to the emergence of multidrug resistance in *K. pneumoniae* strains (4). The beta-lactam antibiotics are the most common drugs of any application in the treatment of bacterial infections (5).

Extended-spectrum beta-lactamases (ESBLs) are hydrolyzing enzymes that produced by bacteria. These enzymes are causing to resistance to penicillin, the first, -second and -third generation cephalosporins as well as aztreonam, but are susceptible to beta-lactamase inhibitors such as clavulanic acid (6).

According to Ambler classification, there are four groups of beta-lactamases: class A, C and D enzymes which have serine at their active sites and class B are zinc-dependent metalloenzymes. The class A consists of the majority of ESBL and has three main groups including SHV, TEM and CTX-M (7). During the last decade, the CTX-M types were the predominant group of ESBL enzymes which are widespread among nosocomial and community bacterial isolates (8). Studies conducted to date have revealed that CTX-M type ESBLs was the most frequent of ESBL in many countries including Russia (9), Spain (10), Switzerland (11), Argentina (12) and Iran (6).

The most important causes of increase of antibiotic resistance, particularly between *Enterobacteriaceae* family, are high consumption of antibiotics and rapid dissemination of transmissible elements among bacterial isolates (13). Production of ESBLs by gram-negative bacteria has been emerging as important challenges in treatment of infections and is limiting the antibiotic choice (14). The most common of ESBL-producing *Enterobacteriaceae* are *Escherichia coli* and *K. pneumoniae*. These bacteria are the causative of various infectious diseases in outpatients and in-patients (15).

K. pneumoniae has been identified as an important opportunistic pathogen that commonly leads to multi drug resistance (MDR), extensive drug resistance (XDR), or even pan drug resistant (PDR) infections (16). Antibiotic resistance is becoming a great concern worldwide (17). MDR was explained as obtained insusceptibility to at least one agent in three or more antimicrobial classes XDR was defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (i.e. bacterial isolates remain susceptible to only one or two categories) and PDR was defined as non-susceptibility to all agents in all antimicrobial categories (18).

Production of ESBLs along or with multi drug resistance in bacteria is a serious threat and endangers patient's survival. There are a number of studies done on determination of antibiotic resistance pattern in ESBL-producing *K. pneumoniae* isolates from Iran; nevertheless, the rate of MDR, XDR, and PDR phenotype of these isolates are vastly unknown. This study aims to evaluate the prevalence of CTX-M and TEM type ESBL-producing *K. pneumoniae* and determination of MDR, XDR, and PDR phenotypes of these isolates as well as to find out the genetic relation and source of these isolates by using phenotypic and genotypic methods in a subspecialty hospital of Isfahan, Iran.

Materials and methods

Isolation of bacteria

K. pneumoniae isolates were obtained from 96 non-repetitive clinical samples including urine, trachea, wound, blood, sputum, catheter,

bronchoalveolar lavage, abscess and cerebrospinal fluid (CSF). These samples were collected from inpatients in different wards of a subspecialty hospital of Isfahan (Al-Zahra hospital), during January to August 2015. Collected samples were cultured on microbiological media and *K. pneumoniae* strains were identified based on standard microbiology methods (19, 20).

Antimicrobial susceptibility testing

Resistance pattern and antibiotic susceptibility of isolates were down using disk diffusion method (Kirby-Bauer) and based on clinical and laboratory standards institute (CLSI) guidelines (21). The used antibiotic discs (MAST, Merseyside, UK) were included imipenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5µg), ceftazidime (30 µg), cefotaxime (30 µg), aztreonam (30 µg), cefepime (30 µg), nitrofurantoin (300µg), tetracycline (30 µg), chloramphenicol (30 µg), colistin sulphate (25 µg), cefoxitin (30µg), meropenem (10µg), cefazoline (30 µg), tigecycline (15µg), ceftaroline (30 µg), ampicillin/sulbactam (10/10µg), piperacillin/ tazobactam (PTZ,100/10µg), and trimethoprim/ sulfamethoxazole (1.25/23.75µg). *E. coli* ATCC 25922 was applied for quality control of antibiotic discs (22). The data entry and analysis were done via the WHONET 5.6 software.

ESBL screening

The *K. pneumoniae* isolates were screened for ESBL-production by combined disc method accordance to CLSI guideline (22). The phenotypic confirmatory test was performed by ceftazidime (30 µg) alone, combined with clavulanic acid (10 µg) as well as cefotaxime (30 µg) alone, combined with clavulanic acid (10 µg) discs. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were applied as negative and positive control, respectively.

DNA extraction and PCR amplification of bla_{BLATEM} and $bla_{CTX\text{-}M}$ genes

After primary screening, the ESBLs-producing isolates in phenotypic confirmatory test were selected to evaluate of bla_{TEM} and $bla_{CTX\text{-}M}$ genes using PCR assay. Bacterial genomic DNA of these isolates were extracted by boiling method (8, 23). PCR amplification of $bla_{CTX\text{-}M}$ and bla_{TEM} genes was performed via using specific primers for $bla_{CTX\text{-}M}$ (forward: 5'CGT GCT GAT GAG CGC TTT GC3' and reverse: 5'AGA TCA CCG CGA TAT CGT TG3') with product size of 568 bp and bla_{TEM} (forward: 5'AGT ATT CAA CAT TTC CGT GTC G3' and reverse: 5'GCT TAA TCA GTG AGG CAC CTA TC3') with product size of 850bp. *K. pneumoniae* K.P 7881 containing $bla_{CTX\text{-}M}$ and bla_{TEM} genes was used as positive control for PCR reactions.

PCR was performed using commercially available PCR master mix (Ampliqon, Denmark) according to the manufacturer's instructions. The PCR reactions were prepared in 20µl volume. Briefly, 1 µl template DNA (~100 ng/mL), 1 µl of each primer (10 pmol/mL), and 7 µl DNase-free water were added to 10 µl of master mix.

Amplification for $bla_{CTX\text{-}M}$ gene was performed with an initial denaturation step at 95 °C for 5 min; 27 cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 40 s, with a final extension of 7 min at 72 °C in the last cycle.

For bla_{TEM} gene, PCR was carried out using the following conditions: an initial denaturation at 95 °C for 5 min, followed by 25 cycles of amplification at 95 °C for 30 s, annealing at 61 °C for 25 s, extension for 45 s at 72 °C, with a final extension of 7 min at 72 °C in the last cycle. The PCR products were analyzed after electrophoresis on 1.7% agarose gel.

Performing of ERIC-PCR on ESBL-producing isolates

To determine any genetic relationship among the isolates, all ESBLs-producing *K. pneumoniae* isolates were typed by ERIC-PCR method. The

primers and PCR conditions described by Norouzi et al. (24) were used. Finally, the results were analyzed by PyElph 1.4 software.

Statistics analysis

The results were evaluated using SPSS Software (version 18) as well the descriptive-statistic methods (percentage and frequency (regarding qualitative variables.

Results

Table 1 represents the pattern of antibiotic resistance of all isolates (96 isolates) for 20 different antimicrobial agents. Results of phenotypic confirmatory test showed that among 96 isolates, 58 isolates (60.4%) were ESBL-producers. Table 2 is demonstrating the antimicrobial resistance pattern of ESBLs-producing isolates (58 isolates) for applied antibiotics. Table 3 and 4 are representing the distribution of prevalence of *K. pneumoniae* isolates divided to hospital wards and type of clinical specimens, respectively.

Among all isolates, 39 isolates (40.6%) showed MDR phenotype. The highest antimicrobial resistance was observed to aztreonam (98%), followed ampicillin/ sulbactam (96%), tetracycline (92%), meropenem (89%), and nitrofurantoin (85%). The lowest antimicrobial resistance was observed to colistin sulfate (2%), followed amikacin (31.5%), and tigecycline (37%). Moreover, 33.3% of isolates (32 isolates) were showed XDR phenotype. All XDR isolates were susceptible to colistin sulfate and more of them were not resistance to amikacin and tigecycline.

PCR amplification on ESBL-producing isolates showed that 47(81%) isolates had *bla*$_{\text{TEM}}$ gene, while 58 (100%) isolates were positive for *bla*CTX-M.

ERIC-PCR typing was characterized the high genetic similarity among ESBLs-producing *K. pneumoniae* isolates and revealed 32 band patterns for these isolates. With considered a maximum of 96.5% difference in fingerprinting patterns, these isolates grouped in two main clusters. This subject was showed that these isolates were from same source. Figure 1 is showing genetic relatedness in ESBL-producing *K. pneumoniae* isolates in the form of a dendrogram.

Discussion

Antibiotic resistance in bacteria, especially against beta-lactam antibiotics, usually is higher in nosocomial isolates than community isolates, maybe due to rapid transmission of antibiotic resistance genes between hospitalized isolates. Thus, control of prevalence of ESBL-producing organisms in hospital is critical (25). There is very variability in outbreaks of some beta-lactamase types like the CTX-M and TEM types geographically, which mostly depend on variable usage of beta-lactam antibiotics in different regions (26). According to a study conducted in 2007, the frequency of ESBL-production was highest among *K. pneumoniae* isolated in Latin America (44.0%), followed by Asia/Pacific Rim (22%), Europe (13%), and North America (7.5%) (27). In this study, 60% of isolates considered as ESBL-positive. Among these isolates (ESBL-positive isolates), the prevalence of *bla*$_{\text{TEM}}$ was 81% and *bla*$_{CTX\text{-}M}$ gene was detected in all of them. Taherpour et al. in their study in Tehran (capital of Iran) in 2013, showed 57% of *K. pneumoniae* isolates were ESBL-positive and the frequency of *bla*$_{\text{CTX-M}}$ and *bla*$_{TEM}$ genes were 50% and 58.3%, respectively (28). In another study that was performed by Feizabadi et al. in Labbafinejad hospital of Tehran, 69.7% of *K. pneumoniae* isolates were ESBL-producers. The prevalence of *bla*$_{TEM}$ and *bla*$_{\text{CTX-M}}$ were 54% and 46.5%, respectively (13). Shahraki-Zahedani et al. (6) in another study in Zahedan (Southeast Iran) in 2015 reported the

Table 1. Antimicrobial susceptibilities of *K. pneumoniae* isolates.

Antibiotic	Susceptible %	Intermediate %	Resistant %
Amikacin	68.5	4.3	27.2
Ampicillin/ Sulbactam	3.7	0	96.3
Cefazolin	18.7	0	81.3
Cefepime	26.1	1.1	72.8
Cefotaxime	20.7	3.2	76.1
Cefoxitin	32.6	4.4	63
Ceftaroline	22.7	0	77.3
Ceftazidime	26.1	0	73.9
Chloramphenicol	28.3	43.4	28.3
Ciprofloxacin	26.1	0	73.9
Colistin sulfate	97.8	0	2.2
Gentamicin	39.1	0	60.9
Imipenem	42.4	4.3	53.3
Meropenem	10.9	0	89.1
Nitrofurantoin	11.1	3.7	85.2
Piperacillin/ Tazobactam	34.8	4.3	60.9
Tetracycline	5.4	1.7	92.9
Tigecycline	63	17.4	19.6
Trimethoprim/ Sulfamethoxazole	25.5	7.2	67.3
Aztreonam	1.8	0	98.2

prevalence of *bla*$_{CTX-M}$ gene among ESBL-producing *K. pneumoniae* isolates to be 100% which is similar to our results. In this study, among ESBL-producing isolates the most resistance was observed to aztreonam (100%), followed by tetracycline (97%), ampicillin/sulbactam (97%), cefotaxime (94%), cefazoline (94%), and ceftazidim (91%). Therefore, these results showed that the usage of these agents were not effective in infections caused by ESBL-producing isolates.

Although, today infections caused by PDR *Enterobacteriaceae* are infrequent, but they could result in high mortality rate. In a study that was conducted in Greece from January 2006 to May 2007, it was found that among 28 patients with PDR infections, the rate of mortality was 33.3%. In addition, proportion of MDR and XDR infections were 40.6% and 33.3%, respectively (16). In another study in China in 2012, the rates of MDR, XDR, and PDR isolates were reported as 61.4%, 22.0%, and 1.8%, respectively (27). In USA, 8.4% and 1.2% of *Enterobacteriaceae* have been reported as MDR and XDR during 2011-2012 (29). In our study, we found 85.7% of ESBL-producing isolates had MDR phenotype while 30.3% of these isolates were XDR. This could be explained that the resistance to many antibiotics could have been related to ESBL-positivity phenotype (27). This subject is predictable because the ESBL-encoding genes are usually within the plasmids that can transport other antibiotic resistance genes as well (30).

Table 2. Antimicrobial susceptibilities of ESBL-producing *K. pneumoniae* isolates.

Antibiotic	Susceptible %	Intermediate %	Resistant %
Amikacin	72.2	5.6	22.2
Ampicillin/ Sulbactam	3	0	97
Cefazolin	5.7	0	94.3
Cefepime	9.3	1.8	88.9
Cefotaxime	1.9	3.7	94.4
Cefoxitin	27.8	7.4	64.8
Ceftaroline	2	0	98
Ceftazidime	9.3	0	90.7
Chloramphenico	35.7	46.4	17.9
Ciprofloxacin	13	0	87
Colistin sulfate	98.1	0	1.9
Gentamicin	29.6	0	70.4
Imipenem	38.9	5.5	55.6
Meropenem	14.3	0	85.7
Nitrofurantoin	17.1	2.9	80
Piperacillin/ Tazobactam	27.8	3.7	68.5
Tetracycline	2.8	0	97.2
Tigecycline	61.1	18.5	20.4
Trimethoprim/ Sulfamethoxazol	37.1	2.9	60
Aztreonam	0	0	100

Table 3. Distribution of prevalence of *K. pneumoniae* isolates divided to hospital wards.

Hospital ward	Number of isolates (%)	ESBLs	
		+	-
ICU	59(61.4)	35	24
Internal	16(16.7)	8	8
Emergency	11(11.5)	6	5
Surgery	7(7.3)	7	0
Orthopedic	3(3.1)	2	1
Total	96(100)	58(60.4)	38(39.6)

Table 4. Distribution of prevalence of *K. pneumoniae* isolates divided to clinical specimens.

Specimen type	Number of specimens (%)	ESBLs	
		+	-
Trachea	26(27)	20	6
Urine	42(43.8)	21	21
Catheter	7(7.3)	5	2
Wound	7(7.3)	4	3
Abscess	4(4.2)	1	3
Bronchoalveolar lavage	2(2.1)	1	1
Blood	2(2.1)	2	0
Sputum	2(2.1)	0	2
Cerebrospinal fluid	4(4.1)	4	0
Total	96 (100)	58(60.4)	38(39.6)

According to ERIC-PCR profile, the ESBL-producing isolates were divided in two major clusters. More isolates belonged to cluster two with 73.2%, and the remaining isolates (26.8%) belonged to cluster one. In these clusters, the most isolates were from ICU which means that there was a genetic relationship and clonal spread amongst the *K. pneumoniae* isolates in the hospital's ward. The dendrogram suggested a high rate of nosocomial infections in our study. Likely, the long-term hospitalization and spread of the organism could be through contaminating devices or personnels in the ICU. Our results are similar to the study that was conducted by Xia et al. in China in 2012 (31). They suggested a possible clonal dissemination among *E. coli* isolates with resistance to carbapenems in hospital surgery ward. On the other hand, Ramazanzadeh et al (32), in their study on 187 *E. coli* isolates in Sanandaj hospitals (Northwest Iran), found that 81.3% of isolates had unique profiles and only 18.7% of them showed similar pattern.

In the last decade, drug companies have attempted to make of new antibacterial agents which have not been very successful. Unfortunately, development of novel antibiotics may not be the solution for defeating and or decreasing MDR microorganisms. For example, decreased sensitivity to colistin (one of the last-resort antibiotics for MDR *K. pneumoniae*) and tigecycline (a new antibiotic) have been reported from many parts of the world (33).

Undoubtedly, applying stringent programs to control nosocomial infections could prevent advent of novel antimicrobial resistances. For example, screening for ESBLs in routine laboratories is a useful approach in prescription of antibiotics by clinicians that could be avoid from under dosing and prolong treatment, as well as preventing antibiotic over consumption. There is rare data on blood bacteremia caused by *Staphylococcus* spp. in hospitals of Iran. Mohammadi et al. (2014) studied neonatal bacteremia isolates and their antibiotic resistance pattern in Sanandaj, Iran. They reported 7.6% positive for bacterial growth amongst 355 blood cultures from which 74% were *Staphylococcus* spp. (16).

In addition our results showed that the ERIC-PCR could be used as a molecular typing technique to study the clonal diversity of bacterial isolates such as *K. pneumoniae* (32, 33) which is easy, rapid and low cost than other typing techniques (33).

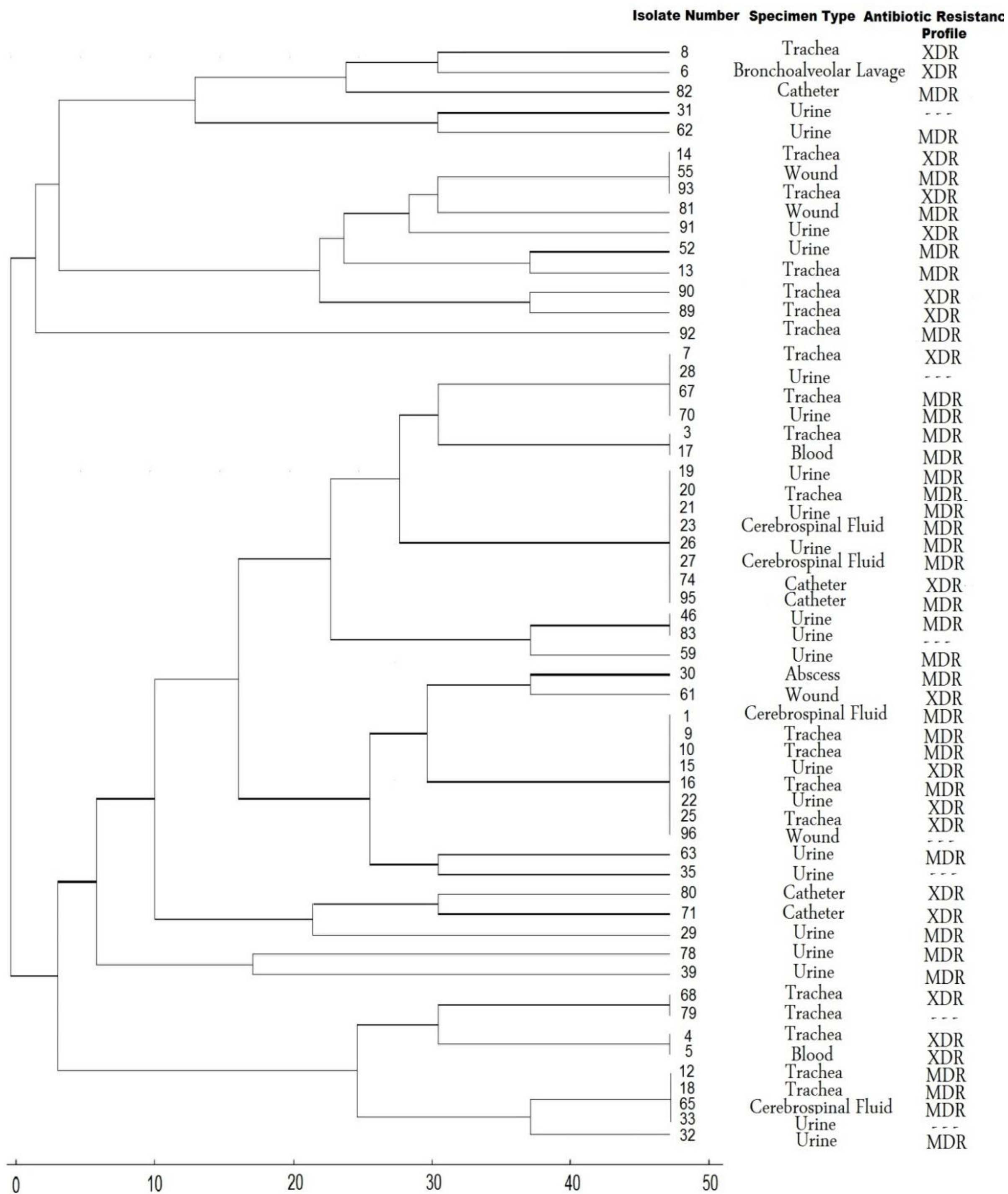

Figure 1. Dendrogram that determind by ERIC-PCR method indicated the genetic related and none-related in ESBLs-producing *K. pneumoniae* isolates.

Conclusion

In conclusion our results showed that the ERIC-PCR could be used as a molecular typing technique to study the clonal diversity of bacterial isolates such as *K. pneumoniae* (32, 34) which is easy, rapid and low cost than other typing techniques (34).

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Conflict of interest

None declared conflicts of interest.

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