

تعیین الگوی ژنتیکی و تشخیص ژن کارباپنماز کلبسیلا پنومونیه در جدایه‌های مقاوم به کارباپنم خانواده انتروباکتریاسه جدا شده از بیمارستانهای شهر اصفهان

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چکیده

مقدمه: درمان باکتری‌های انتروباکتریاسه مقاوم به کارباپنم (CRE: carbapenem resistant enterobacteriaceae) دشوار است زیرا دارای سطح مقاومت آنتی‌بیوتیک بالایی‌اند که می‌تواند با واسطه آنزیم‌های کارباپنماز مختلف از جمله آنزیم کلبسیلا پنومونیه کارباپنماز (KPC: Klebsiella pneumoniae carbapenemase) باشند. هدف از این مطالعه تعیین الگوی مقاومت، الگوی ژنتیکی و تشخیص ژن KPC در سویه‌های مقاوم به کارباپنم در جدایه‌های انتروباکتریاسه بود. مواد و روشها: در این تحقیق، الگوی مقاومت به آنتی‌بیوتیک و الگوی ژنتیکی جدایه‌های انتروباکتریاسه مقاوم به کارباپنم و فراوانی آنزیم KPC مورد بررسی قرار گرفت. در طول ۱۶ ماه مطالعه (از آذرماه ۱۳۹۵ تا فروردین ۱۳۹۷)، سویه‌های اشریشیاکلی، انتروباکتر و سیتروباکتر از نمونه‌های بالینی مختلف جدا سازی و شناسایی شدند و آزمون حساسیت آنتی‌بیوتیکی در آنها تعیین شد. علاوه بر آن، شیوع آنزیم KPC با استفاده از روش PCR و دو روش فنوتیپی شامل آزمون اصلاح شده هاج (MHT) و آزمون ترکیب با اسید بوریک تعیین شد. برای تعیین شباهت ژنتیکی گونه‌های مقاوم کارباپنم، از روش ERIC-PCR استفاده گردید.

نتایج: نتایج نشان داد که در ۵۲۰، ۱۴۶ و ۵۸ سویه اشریشیاکلی، انتروباکتر و سیتروباکتر به ترتیب، آنتی‌بیوتیک‌های موثر شامل کارباپنمها، پپرازولین/تازوباکتام، آمیکاسین، سفپیم، فسفوماسین، نیتروفورانتوین و جنتامایسین بودند. علاوه بر این، ۱۲ (۲/۳ درصد) سویه‌های اشریشیاکلی، ۴ (۲/۷ درصد) سویه‌های انتروباکتر و ۴ (۶/۹ درصد) سویه‌های سیتروباکتر مقاوم به کارباپنم‌ها بودند. آنزیم KPC با روش فنوتیپی MHT در ۱۸ سویه و با روش فنوتیپی بوریک اسید در ۶ سویه مثبت بود اما نتایج PCR برای ژن آن منفی بود. نتایج ERIC-PCR نشان داد در گونه‌های مقاوم به کارباپنم سیتروباکتر، انتروباکتر و اشریشیاکلی به ترتیب ۳، ۴ و ۹ گروه با شباهت ژنتیکی دیده شدند. بحث و نتیجه‌گیری: این مطالعه نشان دهنده شیوع بالای مقاومت آنتی‌بیوتیک و اختصاصیت پایین روش‌های فنوتیپی استاندارد مورد استفاده برای تشخیص KPC و عدم وجود این آنزیم در سویه‌های وسیع مورد تحقیق در شهر اصفهان بود.

واژه‌های کلیدی: اشریشیاکلی، انتروباکتر، سیتروباکتر، کارباپنم، آنزیم کارباپنماز کلبسیلا پنومونیه (KPC)

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Genotyping and Detection of *Klebsiella Pneumoniae* Carbapenemase (KPC) Enzyme among Carbapenem-resistant Enterobacteriaceae Family isolated from Isfahan Hospitals

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Abstract

Introduction: Carbapenem-resistant Enterobacteriaceae (CRE) bacteria are difficult to treat because of their high antibiotic resistance levels that can be mediated by carbapenemase enzymes such as *Klebsiella Pneumoniae* Carbapenemase (KPC). The purposes of this study were to determine the genetic and resistance patterns and to detect of KPC enzyme in carbapenem-resistant strains of Enterobacteriaceae isolates.

Materials and methods: In this study, antibiotic resistant pattern and genotyping of carbapenem-resistant Enterobacteriaceae isolates and frequency of KPC enzyme were investigated. During 16 months of conducting the study (December 2016 until April 2018), strains of *Escherichia coli*, *Enterobacter* spp. and *Citrobacter* spp. were isolated and identified from different clinical specimens and antibiotic susceptibility test was determined. In addition, the prevalence of the KPC enzyme was determined by PCR and two phenotypic methods including Modified Hodge Test (MHT) and the combination test by boronic acid. Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) method was used for the determination of the clonal relationship of carbapenems resistant strains.

Results: The results showed that among 520, 146 and 58 isolated of *Escherichia coli*, *Enterobacter* spp. and *Citrobacter* spp. effective antibiotics were carbapenems, piperacillin/tazobactam, amikacin, cefepime, fosfomycin, nitrofurantoin, and gentamicin, respectively. In addition, 12 strains (2.3%) of *E. coli*, 4 strains (2.7%) of *Enterobacter* and 4 strains (6.9%) of *Citrobacter* were resistant to carbapenems. The KPC investigation results showed the detection of this enzyme in 18 and 6 isolates using MHT and boronic acid methods respectively, but no producer strain was observed using PCR. The result of ERIC-PCR showed in carbapenem-resistant strains of *Citrobacter*, *Enterobacter* and *Escherichia coli*, 3, 4 and 9 distinct main clusters (patterns) were observed respectively.

Discussion and conclusion: This study demonstrates the high antibiotic resistant prevalence and low specificity of standard phenotypic methods that were used for KPC detection in Isfahan City.

Key words: *Escherichia coli*, *Enterobacter*, *Citrobacter*, carbapenem, *Klebsiella Pneumoniae* Carbapenemase (KPC).

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Introduction

Carbapenems have been a major advancement in the treatment of beta-lactam-resistant bacteria because of their wide spectrum of activity and the resistance to hydrolysis by most β -lactamases (1). Carbapenems are selective drugs for the treatment of penicillin-resistant or cephalosporin-resistant gram-negative bacterial infections by inhibiting their cell wall synthesis (1-3). The production of plasmid-mediated carbapenem-hydrolyzing beta-lactamases is increasing among gram-negative bacteria, especially the *Enterobacteriaceae* family. (4, 5). Resistance to carbapenems is accomplished through specific mechanisms that include increasing the activity of antibiotic exit system, reducing the expression of the outer membrane proteins that are essential for the entrance of antibiotics and the production of carbapenem-hydrolyzing enzymes that can make carbapenems inactivate with hydrolysis (1-5).

Beta-lactamase enzymes are grouped into four main groups from A to D based on their amino acid sequence (1). Among the Carbapenemase enzymes, in group A, KPC (an abbreviation for *Klebsiella pneumoniae* Carbapenemase) is found on transportable plasmids and therefore can rapidly exchange among different bacteria and cause high levels of resistance (6). The first member of the KPC family (KPC-1) was reported in a clinical isolate of *Klebsiella pneumoniae* from North Carolina in the United States in 1997 (7). Following the discovery of the KPC-1, another KPC with differences in an amino acid, KPC-2, was quickly found across the United States (8). The KPC-producing bacteria are very important because their therapeutic options are very limited and they are usually sensitive to polymyxin or tigecycline antibiotics, which are

considered to have high side effects (9). Up to now, 17 new subtypes of this gene (KPC1 to KPC17) have been identified and reported by mutations to the original genes in the world (1). Detection of the bacteria resistance and the type of resistance mechanism is important in order to achieve the best solution for their elimination (10). On the other hand, determining the antibiotic susceptibility pattern of these bacteria in order to obtain effective and usable antibiotics against these bacteria is essential in their treatment (11).

The aim of this study was to investigate antibiotic pattern (Resistotyping) and genotyping of carbapenem-resistant *Enterobacteriaceae* family isolates and evaluation of phenotypic and molecular detection of *Klebsiella pneumoniae* carbapenemase (KPC) enzymes in *Enterobacteriaceae* isolates.

Materials and Methods

Isolation and Identification of Bacteria: Different strains of *Citrobacter*, *Enterobacter* and *Escherichia coli* bacteria were isolated and identified from different clinical specimens of the three hospitals of Isfahan during the 16 months. Identification of isolates was carried out using standard phenotypic methods (12).

Determination of Antibiotic susceptibility pattern: Antibiotic susceptibility test was determined in isolated strains according to the 2016 guidance of the Clinical Laboratory Standards Institute (CLSI) by disc diffusion method (13). These antibiotics include imipenem, meropenem, ertapenem, doripenem, gentamicin, ampicillin, amikacin, ciprofloxacin, levofloxacin, cefepime, piperacillin/tazobactam, nitrofurantoin (for urinary tract infections) cefotaxime and ceftazidime (purchased from Mast Company, UK).

Determination of the Minimum Inhibitory Concentration (MIC): Minimum

Inhibitory Concentration (MIC) was used by Etest method for meropenem and imipenem resistant as explained previously (13) by Etest strips (purchased from Biomerieux Company, France).

Phenotypic Detection of KPC Enzyme: According to the proposal of the CLSI Institute, to evaluate phenotypic detection of KPC, resistance to one or more antibiotic in carbapenem family or resistance to one or more antibiotics in third-generation cephalosporins was used for the primary KPC identification (14). The confirmatory assay of the KPC production was performed by Modified Hodge Test (MHT) and the combination test using Boronic acid as described previously (14, 15).

bla_{KPC} Gene Molecular Identification: A pair of specific primers of the bla_{KPC} gene (forward primer: 5-CTGTCTTGTCTCTCATGGCC-3 and reverse primer: 5-CCTCGCTGTGCTTGTCATCC-3) were used to identify bla_{KPC} gene (16). The PCR program was as follows: initial denaturation for 3 minutes at 94°C, then 30 cycles with initial denaturation temperature at 94° C for 30 seconds, annealing step at 56° C for 40 seconds, extension stage at 72°C for 60 seconds, and final elongation step at 72°C for 7 minutes (16). All positive gene products will be sequenced to determine their possible

mutations and the type of subtype of the enzyme present.

Clonal Relationship of Carbapenems Resistant Strains using ERIC-PCR: Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) method was used for the determination of the clonal relationship of carbapenems resistant strains by ERIC1R primer (ERIC1R: ATGTAAGCTCCTGGGGATTAC) as previously was described (17) and dendrograms were drawn by Gelj v.1.3 Software.

Statistical Methods: All calculations were done with SPSS version 22 statistics software (IBM Corp., Armonk, NY, USA) and the Microsoft Excel 2010 software. A chi-square test was performed to compare categorical data. P-values less than 0.05 were considered to be statistically significant.

Results

During 16 months of study, 520, 146 and 58 strains of *Escherichia coli*, *Enterobacter* spp. and *Citrobacter* spp. were isolated and identified respectively. The number of bacteria isolated according to each clinical specimen is given in Table 1. As it shows, most strains were isolated from urine.

Table 1- Distribution of Isolated Bacteria

Bacteria	Urine	Blood	Wound and abscess	Respiratory Samples	CSF	Other	Total	P value
<i>E. coli</i>	334	88	78	10	5	5	520	(p<0.001)
<i>Citrobacter</i> spp.	30	10	15	1	0	2	58	(p<0.001)
<i>Enterobacter</i> spp.	75	40	21	3	3	4	146	(p<0.001)

Antibiotic Susceptibility Pattern of Isolates: In all strains of three bacteria, effective antibiotics were carbapenems, piperacillin/tazobactam, amikacin, cefepime, fosfomycin (for urine isolates of *E. coli*), gentamicin and nitrofurantoin (for urine isolated strains) and most resistance was observed to ampicillin and trimethoprim/sulfamethoxazole (Table 2).

About half percentages of the strains of three bacteria were resistant to third generation cephalosporins, including cefotaxime and ceftazidime. In addition, 12 strains of *E. coli* (2.3%), 4 strains of *Enterobacter* (2.7%) and 4 strains of *Citrobacter* (6.9%) were resistant to carbapenems (Table 2).

Table 2- Percentages of Susceptible (S), Resistant (R) and Intermediate (I) to different antibiotics in isolated bacteria.

Antibiotic name (Concentration: mcg)	<i>Escherichia coli</i>			<i>Enterobacter</i> spp.			<i>Citrobacter</i> spp.		
	S%	I%	R%	S%	I%	R%	S%	I%	R%
Gentamicin (10)	75.5	0.4	24.1	68.4	0.7	30.9	62	1.7	36.3
Amikacin (30)	93.5	0.4	6.1	74.2	0.4	25.4	78.2	0	21.8
Meropenem (10)	97.7	0	2.3	96.6	0.7	2.7	93.1	0	6.9
Doripenem (10)	97.5	0.2	2.3	97.3	0	2.7	93.1	0	6.9
Imipenem (10)	97.5	0.2	2.3	95.9	1.4	2.7	91.4	1.7	6.9
Ertapenem (10)	97.5	0.2	2.3	96.6	0.7	2.7	93.1	0	6.9
Piperacillin/Tazobactam (100/10)	63.6	0	36.4	72.6	0.7	26.8	74.6	0	25.4
Cefepime (30)	65	0	35	59	0.4	40.6	55	0.7	44.3
Cefotaxime (30)	52.2	0.8	47	45	1.4	53.6	49	0	51
Ceftazidime (30)	50	0.8	49.2	44	1.4	54.6	46	0	54
Ciprofloxacin (5)	49.1	0.8	50.21	51	0.4	48.6	55	0	45
Levofloxacin (5)	58	0	42	59	1.4	39.6	61	0	39
Ampicillin (10)	4.2	0	95.8	6	0	94	5.1	0	94.9
Fosfomycin (200)	82.4	0	17.6	-	-	-	-	-	-
Trimethoprim/Sulfamethoxazole (1.2)	37	1.2	61.8	41.2	0.7	58.1	39	0	61
Nitrofurantoin (100)	89.2	0.2	10.6	76.4	0	23.6	72	0	28

Phenotypic and Molecular Detection of KPC Enzyme: The result of phenotypic and molecular detection of KPC enzyme among strains that were resistant to third generation cephalosporins and carbapenems are shown in Table 3. This results showed

that by MHT method, 12, 1 and 5 strains of *E. coli*, *Citrobacter* spp. and *Enterobacter* spp. were KPC producers, respectively, and these results by Boronic acid were 4, 0 and 2 strains. No bla_{KPC} gene was observed using PCR method in these isolates.

Table 3- The result of phenotypic and molecular detection of KPC enzyme among strains that were resistant to third generation cephalosporins and carbapenems.

Method	Positive number of Bacteria			P value
	<i>E. coli</i>	<i>Citrobacter</i> spp.	<i>Enterobacter</i> spp.	
MHT	12	1	5	(p<0.01)
Boronic acid	4	0	2	(p<0.01)
PCR	0	0	0	(p<0.01)

Clonal relatedness of carbapenem-resistant strains: The clonal relatedness dendrogram using ERIC-PCR of carbapenem-resistant strains are shown in Figure A1, A2, and A3. As shown in Figure A1, in four carbapenem-resistant *Citrobacter* strains, three main clusters were observed. The strain 1 and strain 4 that were isolated from two different hospitals were in the distinct clusters and had a different genetic relationship (less than 80% relationship) and strains 2 and 3 isolated from the same hospital had a close genetic similarity (94% relationship). The four carbapenem-resistant strains of *Enterobacter*

isolated from two different hospitals had four main clusters and were not genetically related (less than 70% relationship) as it is shown in Figure A2. In addition, in carbapenem-resistant strains of *Escherichia coli* 9 main clusters were observed. As showed in figure A3, there was no genetic relationship between strains No. 1, 2, 5, 8, 9 and 10. Strains 3 and 4 with 96% genetic relationship and strains 6, 7 and 11, 12 strains with 92% genetic relationship were isolated from different hospitals but were similar in antibiotic resistance pattern (Table 2).

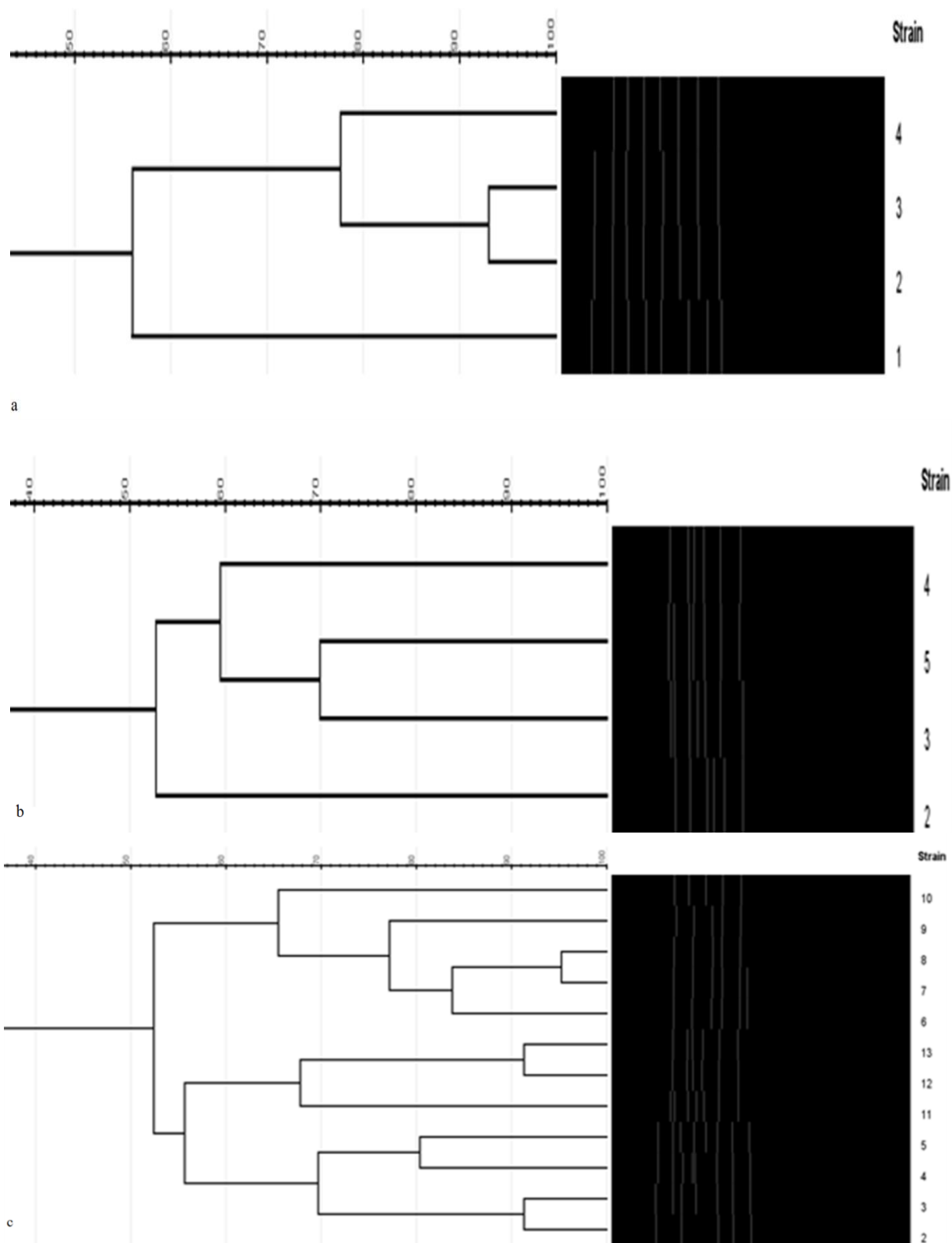


Fig. 1- Clonal relatedness dendrogram using ERIC-PCR of carbapenem-resistant strains; a three main clusters in *Citrobacter* strains, b four main clusters in *Enterobacter* strains, c nine main clusters in *Escherichia coli* strains.

Discussion and Conclusion

Carbapenems are commonly used for the treatment of superbug and high-risk multidrug-resistant (MDR) bacterial infections and so resistant to these agents are catastrophic (18). In this study antibiotic pattern (Resistotyping) and genotyping of carbapenem-resistant *Enterobacteriaceae* isolates were investigated and frequency of *Klebsiella pneumoniae* carbapenemase (KPC) enzyme in these isolates was determined. In isolated bacteria, only extended-spectrum antibiotics were effective antibiotics including carbapenems, piperacillin/tazobactam, amikacin, cefepime, and gentamicin. Additionally, nitrofurantoin that is for urine isolated strains had a very good effect that showed old antibiotics that their use is restricted and can be effective now. Very frequented used antibiotics including ampicillin and trimethoprim/sulfamethoxazole had high resistance rate to fluoroquinolones including ciprofloxacin and levofloxacin was moderate (near to half). These results showed increasing rates of antibiotic resistance in Isfahan, Iran. Fosfomycin was very effective against urine isolates of *E. coli* especially for extended-spectrum beta-lactamases (ESBLs) isolates that showed it can be a good and an effective alternative for these resistant bacteria. This effect can be as rare use of this antibiotic in Iran because it is addet to local pharmacopeia recently. About half percentages of the strains were resistant to third generation cephalosporins, suggesting that these strains are potentially ESBLs producers. Resistance to carbapenems in investigated bacteria was less than 10 percentages. The results of this study, like other studies such as Zhang et al (18) and Pasteran et al (9), showed that carbapenem-resistant strains had high resistance to other antibiotics such as amikacin, ampicillin, ciprofloxacin,

cefepime, piperacillin/tazobactam (tazocin), ceftazidime and cefotaxime, which can be due to the presence of several antibiotic-resistant genes together in these isolates.

KPC-producing strains of *Enterobacteriaceae* have been reported in several studies and there have reports of the presence of this enzyme in other bacteria of this family in addition to *Klebsiella pneumoniae*, including *Enterobacter*, *Salmonella*, *E. coli* and *Serratia* spp. (16, 18). For example, Urban et al. (16) reported nine cases of *E. coli* producing KPC enzymes in New York City at Queens' Hospital between 2004 and 2007. Also, Zhang et al. (18) reported in Hangzhou, China, the presence of the KPC2 enzyme in *Serratia marcescens*. In our study, KPC investigation results showed the detection of this enzyme by two phenotypic methods (MHT and Boronic acid methods) but no producer strain was observed using PCR. Although Boronic acid methods showed less false positive result comparing to MHT method, these two methods had false positive results. False positive results were observed in other carbapenemase genes in previous studies (9, 16); therefore, MHT and Boronic acid methods had low specificity for KPC detection.

The result of the clonal relationship of carbapenem-resistant strains using the ERIC-PCR method showed that there was no genetic correlation with *Enterobacter* strains in addition to *Citrobacter* strains and their antibiotic pattern was not similar. This result was predictable regarding different location of these strains isolation. However, in six strains of *E. coli* that were isolated from different hospitals, three similar genetic (clusters) and antibiotic resistance patterns were observed, indicating the possibility of spreading hazardous bacteria among hospitals in a city and even in a country that is of particular importance and special attention

should be paid to prevent this spread. Accurate molecular characterization in addition to molecular typing of these isolates can be carried out for comparing to other clones in the world with other methods such as Multi-Locus Sequence Typing (MLST).

In general, although no real KPC-producing strain was detected in this study, in the most investigated isolates; high drug resistance was observed especially in carbapenem-resistant strains that it showed an urgent need to control antibiotic use for preventing the emergence of these resistant strains.

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