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ORIGINAL ARTICLE

Identification of *Klebsiella pneumoniae* Carbapenemase-producing *Klebsiella oxytoca* in Clinical Isolates in Tehran Hospitals, Iran by Chromogenic Medium and Molecular Methods

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Abstract
Objectives: Production of carbapenemase, especially *Klebsiella pneumoniae* carbapenemases (KPC), is one of the antibiotic resistance mechanisms of Enterobacteriaceae such as *Klebsiella oxytoca*. This study aimed to investigate and identify KPC-producing *K. oxytoca* isolates using molecular and phenotypic methods.
Methods: A total of 75 isolates of *K. oxytoca* were isolated from various clinical samples, and were verified as *K. oxytoca* after performing standard microbiological tests and using a polymerase chain reaction (PCR) method. An antibiotic susceptibility test was performed using a disc diffusion method according to the Clinical and Laboratory Standards Institute guidelines. CHROMagar KPC chromogenic culture media was used to examine and confirm the production of the carbapenemase enzyme in *K. oxytoca* isolates; in addition, PCR was used to evaluate the presence of bla_{KPC} gene in *K. oxytoca* strains.
Results: Of a total of 75 *K. oxytoca* isolates, one multidrug resistant strain was isolated from the urine of a hospitalized woman. This strain was examined to assess its ability to produce carbapenemase enzyme; it produced a colony with a blue metallic color on the CHROMagar KPC chromogenic culture media. In addition, the bla_{KPC} gene was confirmed by PCR. After sequencing, it was confirmed and deposited in GenBank.
Conclusion: To date, many cases of KPC-producing Enterobacteriaceae, in particular *K. pneumoniae*, have been reported in different countries; there are also some reports on the identification of KPC-producing *K. oxytoca*. Therefore,

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to prevent the outbreak of nosocomial infections, the early detection, control, and prevention of the spread of these strains are of great importance.

1. Introduction

According to Ambler classification, β-lactamases are categorized into four classes (A−D) based on their molecular structure (sequence of amino acids and nucleic acids). Class A includes the genes for TEM, SHV, CTX-M, and *Klebsiella pneumoniae* carbapenemases (KPC) β-lactamase enzymes. KPC can hydrolyze a range of β-lactams including third-generation cephalosporins, monobactams, and carbapenems. *Klebsiella pneumoniae* carbapenemase (KPC), which is classified as a molecular class A β-lactamase, is a serious clinical challenge. In addition, KPC-producing Enterobacteriaceae isolates, especially KPC-producing *K. pneumoniae* isolates, are rapidly spreading all over the world. The worldwide emergence and spread of carbapenem-resistant Enterobacteriaceae isolates is a challenge for physicians and clinical microbiologists [1]. Therefore, early diagnosis and prevention of strain dissemination is a medical emergency [2].

KPCs are not just limited to the *K. pneumoniae* strain, and nowadays KPCs are also found extensively in other bacteria such as *Escherichia coli*, *Enterobacter* spp., *Serratia* spp., *Morganella morganii*, *Citrobacter freundii*, *Salmonella enterica*, *Klebsiella oxytoca*, *Acinetobacter* spp., *Pseudomonas aeruginosa*, and *Pseudomonas putida* [3,4]. To date, a total of 24 genotypes have been identified [5], and KPC-2 and KPC-3 are the most common genotypes isolated from clinical specimens. Among the identified genotypes, KPC-2 is the most dominant genotype worldwide [6,7]. The *KPC* gene is on transposon Tn 4401, which facilitates the transmission between plasmids and bacterial species from a bacterium to other bacteria, from one patient to another, and even from one country to another; it can also generate multidrug-resistant strains [8]. KPC-producing *K. pneumoniae* isolates have high levels of antibiotic resistance; because of the plasmid transfer of the *KPC* gene to other genes and species of Enterobacteriaceae, they have been responsible for many outbreaks of nosocomial infections in the world in recent years. In addition, there have been many reports on the identification of KPC-producing strains of Enterobacteriaceae, in particular *K. pneumoniae*, in different countries [9].

The first case of KPC-producing *K. pneumoniae* was identified and introduced in North Carolina in 2001 through Project Intensive Care Antimicrobial Resistance Epidemiology [10]. From 1990 to 2015, there were many studies that reported the identification of the first cases of KPC-producing *K. pneumoniae* isolates in different countries such as USA, Canada, Italy, Poland, France, Spain, UK, Colombia, India, Argentina, China, Ireland, Greece, Brazil, Turkey, Japan, South Korea, and Iran [9−17]. KPC-producing *K. oxytoca* isolates are rarely reported [18]. *K. oxytoca* is an opportunistic pathogen and is now identified and introduced as an important clinical pathogen that is associated with nosocomial infections in hospitalized patients, including children, newborns, and individuals with immune deficiency [19]. A few studies have reported the identification of the *KPC* gene in *K. oxytoca* strains in some countries such as Austria [18], Brazil [20], and Venezuela [21]. Identification of this gene plays an important role in the study of *K. oxytoca* antibiotic resistance. This study aimed to evaluate the use of phenotypic method (CHROMagar KPC chromogenic culture media) and a polymerase chain reaction (PCR) method for the identification of KPC-producing *K. oxytoca* isolates. In this study, we report the first detection of the bla_{KPC} gene among isolates of *K. oxytoca* in Iran.

2. Materials and methods

2.1. Bacterial isolates

A total of 75 *K. oxytoca* strains were collected from several hospitals in Tehran between 2013 and 2014. Clinical strains were isolated from stool, blood, urine, sputum, and wounds.

2.2. Microbiological methods

Using standard microbiological tests in the laboratory, all bacterial isolates were identified as *K. oxytoca*. In addition, all *K. oxytoca* isolates were also identified and confirmed by PCR method, which was performed through the amplification of galacturonase specific gene (*pehX*).

PCR was performed through the amplification of 344 base pairs (bp) specific to *K. oxytoca*. To amplify *pehX* gene we used the forward primer PEH C (5′-GAT ACG GAG TAT GCC TTT ACG GTG-3′) and reverse primer PEH D (5′-TAG CCT TTA TCA AGC GGA TAC TTG-3′) [22].

2.3. Antimicrobial susceptibility testing

Susceptibility testing of isolates was performed by disc-diffusion method, using antibiotic discs manufactured by MAST Company (Bootle, Merseyside, UK) and according to criteria recommended by the Clinical and Laboratory Standards Institute [23]. Antibiotic discs used were: getamycin (10 μg), imipenem (10 μg), meropenem

(10 μg), cefotaxime (30 μg), ciprofloxacin (5 μg), ampicillin (10 μg), amoxicillin (25 μg), aztreonam (30 μg), ceftriaxone (30 μg), cefepime (30 μg), cefuroxime (30 μg), ticarcillin (75 μg), ceftazidime (30 μg), amikacin (30 μg), ampicillin/sulbactam (20 μg), cephalothin (30 μg), and trimethoprim/sulfamethoxazole (25 μg).

2.4. Molecular detection of bla$_{KPC}$ gene

Genomic DNA of *K. oxytoca* isolates was obtained through boiling two or three colonies in 500 μL of distilled water for 10 minutes and centrifugation for 10 minutes at 10,000 rpm. The supernatants were then used as a template for amplification [24]. To identify the *bla*$_{KPC}$ gene, KPC-Forward and KPC-Reverse primers, with the following sequences, were used: KPC-Forward: 5′-CAG CTC ATT CAA GGG CTT TC-3′, KPC-Reverse: 5′-AGT CAT TTG CCG TGC CAT AC-3′ [25]. PCR reaction was performed in a final volume of 20 μL. To perform each PCR reaction, we used 10 μL Master mix (Ampliqon, Odense, Denmark), 0.5 μL forward primer 10 pmol (Bioneer, Daejeon, Korea), 0.5 μL reverse primer 10 pmol (Bioneer), 8.5 μL distilled water, and 50 ng of bacterial DNA.

PCR reaction was performed in a thermocycler (PEQLAB, Erlangen, Germany) with an initial denaturation at 95°C for 5 minutes; and 35 cycles including denaturation steps at 94°C for 45 seconds, the annealing at 52°C for 45 seconds, the extension at 72°C for 45 seconds, and final extension at 72°C for 10 minutes. The electrophoresis of the PCR products was performed in a 1% agarose gel. The gel was stained using ethidium bromide (50 mg/L) and DNA detected by Gel Doc (GVM20 model; Syngene, Cambridge, UK). The positive PCR product for sequencing was submitted to Macrogen, Inc. (Seoul, Korea). A KPC-producing *K. pneumoniae* strain with accession number JX966417 was used as a positive control strain.

2.5. Detection by use of chromogenic medium

To study the production of carbapenemase enzyme in isolates of *K. oxytoca*, we used the CHROMagar chromogenic media (CHROMagar, Paris, France) [6,7,26]. The *K. oxytoca* strains were isolated from clinical specimens cultured in the prepared CHROMagar medium. *K. oxytoca* strains that produce carbapenemase enzyme are detected when they produce a colony with a blue metallic color on this chromogenic culture media.

3. Results

A total of 75 *K. oxytoca* isolates from clinical specimens were collected from stool (9.3%), blood (14.7%), urine (68%), sputum (5.3%), and wounds (2.7%) of patients in four hospitals in Tehran between 2013 and 2014. The primary assessment of isolated strains showed that all strains had a galacturonase-specific gene and they were identified as *K. oxytoca* isolates. Antibiotic susceptibility test was performed for all the examined isolates (Table 1) and we identified a multidrug-resistant phenotype.

The PCR confirmed the presence of *bla*$_{kpc}$ gene; in addition, the presence of carbapenemase enzyme was confirmed by the production of a colony with a blue metallic color on CHROMagar culture media (Figure 1). Hence the isolate was identified as a strain of KPC-producing *K. oxytoca.* This strain was isolated from a urine sample of a 73-year-old female patient admitted to a women's surgery ward. First, using standard microbiological tests and using PCR, it was identified as a *K. oxytoca* strain. Antibiotic susceptibility test which was performed using a disc-diffusion method showed that this isolate was resistant to imipenem, cefepime, cefotaxime, ceftazidime, amikacin, kanamycin, ciprofloxacin, aztreonam, cefazolin, and sulfamethoxazole/trimethoprim. A molecular method then confirmed the presence of the *bla*$_{KPC}$ gene in this isolate (Figure 2).

Sequence analysis of the *bla*$_{kpc}$ gene revealed that it was 99% identical to the other of the *bla*$_{KPC}$ genes deposited in GenBank database. The nucleotide sequence of the *bla*$_{KPC}$ gene, which was determined in our study, was assigned to the GenBank nucleotide sequence database under the accession number KU057943.

4. Discussion

Many studies have reported the identification of Gram-negative bacteria producing class A, B, C, D carbapenemases in the Middle East [27]. In recent years, many cases of KPC-producing Enterobacteriaceae, in particular *K. pneumoniae*, have been reported in different countries [7,9,26,28]. KPC-producing *K. oxytoca* isolates are rarely reported in various parts of the world [28]. In this study, first the *K. oxytoca* isolates were examined in terms of the presence of KPC gene by using molecular and phenotypic methods. In study by Samra et al [26], chromogenic medium CHROMagar KPC was shown to have a sensitivity of 100% and specificity of 98.4% relative to PCR. The CHROMagar chromogenic KPC culture media was used as a KPC screening method. One isolate was identified as a positive KPC. After sequencing, it was confirmed and deposited in GenBank. This is the first case of KPC-producing *K. oxytoca* ever detected and reported in Iran, although others studies have detected KPC-producing *P. aeruginosa* and *Acinetobacter baumannii* isolates [29,30]. In July 2012, Nobari et al [17] identified a strain of *K. pneumoniae bla*$_{KPC}$ gene that was isolated from the urine of a female patient hospitalized

Table 1. Antimicrobial susceptibility patterns of all *Klebsiella oxytoca* isolated in this study.

Antibiotic	Susceptible, *n* (%)	Intermediate, *n* (%)	Resistant, *n* (%)
GM	67 (89.3)	1 (1.3)	7 (9.4)
AK	66 (88)	7 (9.3)	2 (2.7)
IMI	74 (98.7)	0 (0)	1 (1.3)
MEM	74 (98.7)	0 (0)	1 (1.3)
AP	14 (18.7)	2 (2.7)	59 (78.6)
A	14 (18.7)	3 (4)	58 (77.3)
SAM	58 (77.3)	3 (4)	14 (18.7)
TC	9 (12)	5 (6.7)	61 (81.3)
ATM	72 (96)	0 (0)	3 (4)
CRO	56 (74.7)	5 (6.7)	14 (18.6)
CTX	53 (70.)	7 (9.3)	15 (20)
CPM	64 (85.3)	2 (2.7)	9 (12)
KF	55 (73.3)	3 (4)	17 (22.7)
CAZ	61 (81.3)	6 (8)	8 (10.7)
CXM	66 (88)	0 (0)	9 (12)
TS	48 (64)	2 (2.7)	25 (33.3)
CIP	56 (74.7)	6 (8)	13 (17.3)

A = amoxicillin; AK = amikacin; AP = ampicillin; ATM = aztreonam; CAZ = ceftazidime; CIP = ciprofloxacin; CPM = cefepime; CRO = ceftriaxone; CTX = cefotaxime; CXM = cefuroxime; GM = gentamycin; IMI = imipenem; KF = cephalothin; MEM = meropenem; SAM = ampicillin/sulbactam; TC = ticarcillin; TS = trimethoprim/sulfamethoxazole.

in an Intensive Care Unit ward; they reported it as the first case of *K. pneumoniae* bla_{KPC} gene in Iran. Some cases of *K. oxytoca* have also been identified and reported in Brazil [20] and Venezuela [21]. A study in Austria reported the identification of 31 strains of KPC-producing *K. oxytoca* isolated from five patients hospitalized in Intensive Care Unit wards [1]. In the present study, one carbapenem-resistant isolate of *K. oxytoca* was recovered. Antibiotic susceptibility test results showed that highest and lowest resistances were related to ticarcillin (81.3%) and meropenem and imipenem (1.3%), respectively.

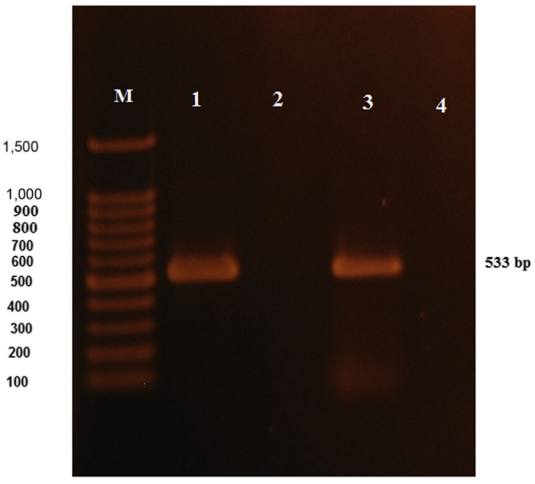

Figure 1. Electrophoresis of PCR products amplified from KPC gene in 1% agarose gel. Lane 1, positive control; Lane 2, negative isolate; Lane 3, positive isolate; Lane 4, negative control; M, 100 bp ladder.

Because of high antibiotic resistance, the location of the *KPC* gene on Tn 4401 transposons, and plasmid transfer of the genes to other species of Enterobacteriaceae, KPC-producing *K. pneumoniae* and *K. oxytoca* isolates are often associated with nosocomial infections [8]. These isolates can rapidly disseminate and lead to widespread resistance; in recent years, they have been responsible for outbreaks of nosocomial infections around the world [3,7,9]. Since the identification and prevalence of KPC-producing Enterobacteriaceae is considered a medical emergency, timely diagnosis, control, and prevention the spread of bacteria is of great importance. The mortality rate associated with KPC-producing bacterial infections is 22–59% [7]; thus, after the emergence of KPC-producing bacteria, the main challenge is to treat infections caused by these bacteria. However, the treatment is very difficult and therefore the mortality rate is high [8]. Given the increasing prevalence of carbapenem-resistant Enterobacteriaceae isolates, KPC-producing bacteria are spreading in the community and in the hospitals; therefore, it is going to become a treatment challenge that requires some necessary measures to control the disease and successfully treat the infections.

To date, many cases of KPC-producing Enterobacteriaceae, in particular *K. pneumoniae*, have been reported in different countries; there are also some reports about the identification of KPC-producing *K. oxytoca* in some countries. In this study, we reported the first detection of the bla_{KPC} gene among isolates of *K. oxytoca* in Iran. This study showed that there is an urgent need for the implementation of strategies to control the dissemination of KPC-producing *K. oxytoca* isolates.

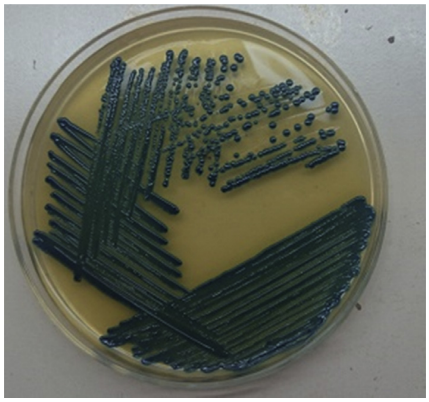

Figure 2. The growth of *K. oxytoca* in CHROMagar KPC medium and produce carbapenamase by produce metallic blue colonies.

Conflicts of interest

The authors declare no conflicts of interest.

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