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Original Article

The Prevalence of Carbapenemase Producing *Klebsiella pneumoniae* Strains Isolated from Clinical Urine Specimens in University Teaching Hospitals, Iran

Amin Dehghan Banadkouki[1]M.Sc., Gilda Eslami[2]Ph.D., Hengameh Zandi[2*]Ph.D., Ali Dehghan Banadkouki[3]B.Sc.

[1]Department of Medical Microbiology, Faculty of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[2]Research Center for Food Hygiene and Safety, Shahid Sadoughi University of Medical Sciences, Yazd, Iran.
[3]Laboratory of Imam Khomeini Hospital, Karaj, Iran.

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Background and Aims: *Klebsiella pneumoniae* is a gram negative opportunistic pathogen of nosocomial infections that causes pneumonia, urinary tract infection (UTI), meningitis, septicemia and diarrhea. The emergence and spread of carbapenemase producing *Klebsiella pneumoniae* often cause failure in the treatment of infections. The aim of this study was to evaluate the prevalence of carbapenemase producing *Klebsiella pneumoniae* strains isolated from clinical urine specimens.

Materials and Methods: In this descriptive-sectional study, from December 2013 to August 2014, 130 *Klebsiella pneumoniae* isolates were collected from hospitalized patients with urinary tract infections and identified by biochemical tests. Antimicrobial susceptibility test was evaluated by the standard disk diffusion method (Kirby-Bauer). E.test method was used for determining of meropenem minimum inhibitory concentration (MIC). Carbapenemase producing was investigated by the Modified Hodge Test (MHT). *bla*$_{KPC}$ was evaluated by conventional polymerase chain reaction (PCR) method using specific primer pair.

Results: The results showed that 61 (46.9%) isolates were non-susceptible to at least one of the carbapenems. 35 (26.9%), 5 (3.8%) and 5 (3.8%) isolates were resistant to imipenem, meropenem and ertapenem, respectively. 45.9% (28 of 61) of isolates were carbapenemase producing and all of them were negative for the presence of *bla*$_{KPC}$.

Conclusions: With respect to the high prevalence of carbapenemase producing *Klebsiella pneumoniae* isolates, it is recommended that MHT is performed in routine laboratories for limiting and controlling the spread of the carbapenem resistant isolates.

***Corresponding Author:** Research Center for Food Hygiene and Safety, Shahid Sadoughi University of Medical Sciences, Yazd, Iran. **Tel:** +989123088324, **Email:** hengameh_zandi@yahoo.com

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Introduction

Klebsiella pneumoniae is a gram negative opportunistic pathogen of nosocomial infections that can survive in hospitals, remain on environmental surface and colonize on human skin and respiratory tract. These bacteria are the cause of pneumonia, urinary tract infection (UTI), meningitis, septicemia and diarrhea [1-4]. As antibiotics have been widely used against *Klebsiella pneumoniae*, resistance to antimicrobial agents has increased and limited the suitable therapeutic choices for the treatment [5, 6]. The most important antibiotics that are used for the treatment of these infections are beta-lactams and aminoglycosides [7]. The carbapenems are generally effective therapeutic choice for the treatment of intensive gram-negative bacterial infections when resistance to other classes of antimicrobials is occurred [8]. Unfortunately, antibiotic resistance toward this class of antibiotics has increased in the last decade [9].

Three mechanisms of resistance to carbapenems include producing carbapenemase enzymes, producing cephalosporinase enzyme with porin loss and expression of efflux pumps [10]. Detection of carbapenemase producing bacteria is difficult by routine antibiotic susceptibility testing, but they can be detected by other phenotypic tests. modified hodge test (MHT) is often being used for detection of carbapenemases in *Enterobacteriaceae* family [11].

According to the Ambler scheme, carbapenemases belong to class A, class B, and class D [9]. One of this carbapenemases is *Klebsiella pneumoniae* carbapenemase (KPC). Firstly, KPC was reported in the United States in 1996 [12]. KPC enzymes belong to Ambler class A and Bush functional group 2f beta-lactamases [13]. Since these genes are found as mobile genetic elements like transposons and plasmids, transmission to other gram-negative bacteria is an important issue [9]. In some countries such as the north-eastern USA, Greece, Israel, Columbia and Puerto Rico, KPCs are endemic [14]. France, Sweden, Norway, Scotland, China, Colombia, Brazil, Trinidad and Tobago, and Poland are the countries that have been reported pathogens harbouring KPCs [13]. However, information on this issue is limited in our country. The aim of this study was to evaluate the prevalence of carbapenemase producing *Klebsiella pneumoniae* strains isolated from clinical urine specimens.

Materials and Methods

Bacterial isolates

In this descriptive-sectional study, from December 2013 to August 2014, 130 *Klebsiella pneumoniae* isolates were collected from urine specimens of hospitalized patients with UTIs from Yazd and Karaj university teaching hospitals. Urine samples were cultured on blood agar and eosin methylene blue agar (EMB) and after 24 hours of incubation at 37°C, mucoid colonies were identified by convectional biochemical tests, including fermentation of sugars in triple sugar iron (TSI) medium, indole production and motility in sulfide indole motility (SIM) medium, the way of glucose fermentation in methyl red voges proskauer (MR-VP) medium, growth in simmon citrate medium and urease production in

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urea medium (all media from Merck, Germany) [15].

Antibiotic susceptibility test

Antibiotic susceptibilities were determined using standard disk diffusion method (Kirby-Bauer) according to the clinical and laboratory standards institute (CLSI) recommendations [16]. The tested antibiotics were: ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg), cefepime (30 µg), imipenem (10 µg), meropenem (10 µg) and ertapenem (10 µg) (all antibiotic disks from Mast, UK). *Escherichia coli* ATCC 25922 was used for quality control.

The evaluation of minimum inhibitory concentration (MIC)

MIC of meropenem was determined for the isolates that were non-susceptible to at least one of the carbapenems using the E-test method (all E-test strips from Liofilchem, Italy) [17]. The results were interpreted according to the CLSI guidelines [16]. *Escherichia coli* ATCC 25922 was used for quality control.

Modified Hodge Test (MHT)

The strains that were non-susceptible to at least one of the carbapenems were investigated by the MHT based on CLSI instruction [16]. Briefly, a 0.5 McFarland standard suspension was prepared in saline and diluted 1:10 in saline. Afterward, the diluted suspension was inoculated on Mueller-Hinton agar medium. The plate was allowed to dry incubating for three to five minutes. A 10 µg meropenem disc (Mast, UK) was placed in the middle of the plate. The tested bacteria were cultured in a straight line from the edge of the disk to the edge of the plate and incubated at the temperature of 37°C for 16 to 20 hours. After the incubation time, an inhibition zone in the form of clover leaf represented the carbapenemase production.

DNA extraction

Salting out method was used for DNA extraction [6, 18]. Briefly, bacteria was cultivated in tryptic soy broth (Merck, Germany) 18 hours prior to the extraction procedures. 1 ml of fresh bacterial suspension in tryptic soy broth was poured in 1.5 ml microtube, then washed for three times with phosphate-buffered saline (PBS). The bacterial pellet was suspended with 500 µl NET buffer and sodium dodecyl sulfate (SDS) with end concentration of 1% and incubated at 56°C for one hour. For DNA purification, 300 µl of 6M NaCl was added and centrifuged. The supernatant was transferred into a 1.5 ml microtube and absolute ethanol was added for precipitation. The washing step was performed by using ethanol 70%. The pellet was diluted in 100 µl sterile distilled water and stored at -20°C.

Molecular detection of *bla*$_{KPC}$ by polymerase chain reaction (PCR)

The conventional PCR assay was performed to determine the presence of *bla*$_{KPC}$ in the carbapenemase producing strains using thermo cycler (Quant Biotech, UK) and Taq DNA polymerase 2X master mix (Ampliqon, Denmark). The *bla*$_{KPC}$ specific primer pair includes forward 5′-ATGTCACTGTATCGCCGTCT-3′ and reverse 5′-TTTTCAGAGCCTTACTGCCC-3′were used for amplification and the amplicon size was 893 bp [19]. The reaction was performed with end concentrations of the 1X PCR master mix, 8 pmol of each primer and 100 ng of template DNA in a total volume of 20 µl. PCR conditions were as follows: initial

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denaturation step at 94°C for 5 min, 30 cycles of denaturation at 94°C for 60s, annealing at 54°C for 60s, extension at 72°C for 60s and a final extension at 72°C for 5 min. PCR products were analyzed on 1% agarose gel alongside of 50 bp DNA ladder by electrophoresis. The study was approved by the Ethics Committee of Shahid Sadoughi university of medical sciences, Yazd, Iran.

Results

In this cross-sectional study, 130 *Klebsiella pneumoniae* isolates were collected from urine specimens of the patients with urinary tract infections. The results showed that 61 (46.9%) isolates were non-susceptible to at least one of the carbapenems. 35 (26.9%), 5 (3.8%) and 5 (3.8%) isolates were resistant to imipenem, meropenem and ertapenem, respectively (Table 1).

Table 1. Susceptibility pattern of *Klebsiella pneumoniae* isolates.

Antibiotic	Resistant No (%)	Intermediate No (%)	Sensitive No (%)	Total No (%)
Cefotaxime	54 (41.5)	9 (6.9)	67 (51.5)	130 (100)
Ceftriaxone	52 (40)	1 (0.8)	77 (59.2)	130 (100)
Ceftazidime	52 (40)	-	78 (60)	130 (100)
Cefepime	44 (33.8)	2 (1.5)	84 (64.6)	130 (100)
Imipenem	35 (26.9)	26 (20)	69 (53.1)	130 (100)
Ertapenem	5 (3.8)	7 (5.4)	118 (90.8)	130 (100)
Meropenem	5 (3.8)	5 (3.8)	120 (92.3)	130 (100)

The MIC of meropenem was investigated for the isolates that were non-susceptible to at least one of the carbapenems and the results showed that 6.5% (4 of 61) of them were resistant (MIC≥4) (Table 2 and Fig. 1).

Table 2. MIC of meropenem for carbapenem non-susceptible isolates.

Antibiotic	**Meropenem**		
MIC range	**0.002- 32 µg/ml**		
Sensitivity	Resistant	Intermediate	Sensitive
MIC (µg/ml)	≥ 4	2	≤ 1
No (%)	4 (6.5)	4 (6.5)	53 (87)

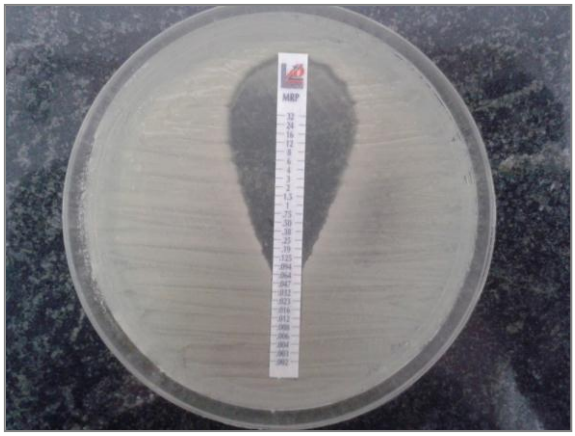


Fig. 1. Detection of meropenem MIC by E. test method, the sample MIC is ≤ 1 µg/ml.

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Out of 61 (46.9%) isolates that were non-susceptible to carbapenems, 45.9% (28 of 61) of them were positive as carbapenemase producing in MHT by developing clover leaf inhibition zone (Fig. 2).

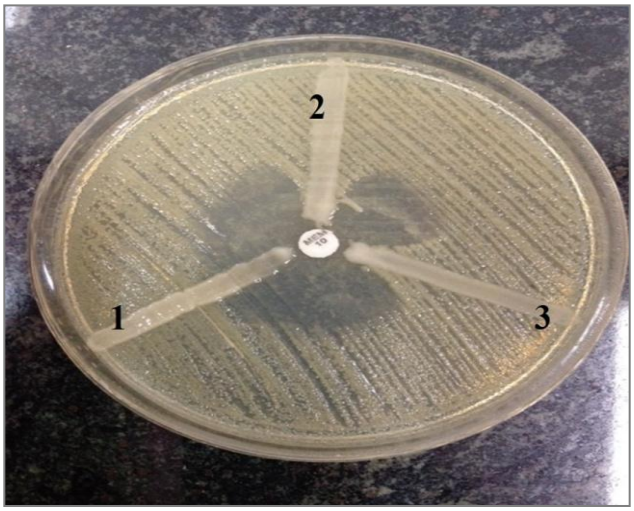


Fig. 2. Modified Hodge Test Method. Isolates 2 and 3 are carbapenemase producing.

The PCR products obtained in this work showed that all carbapenemase producing isolates were negative for the presence of bla_{KPC} on gel electrophoresis.

Discussion

Carbapenems were first introduced in 1980. Carbapenems such as imipenem and meropenem are commonly used for severe infections caused by extended spectrum β lactamases producing bacteria. The emergence and global outbreak of carbapenemase producing *Enterobacteriaceae* cause a serious concern about controlling nosocomial infections [20]. The phenotypic detection of carbapenemase producing bacteria is based on reduced susceptibility to carbapenems that should be confirmed by MHT, but this test cannot discriminate between KPCs and other carbapenem-hydrolyzing enzymes, like metallo β-lactamases (MBLs) [21]. Boronic acid compounds can be used in disk potentiation tests or differentiating KPC producers from MBL producers [22]. In this study, 45.9% (28 of 61) of isolates were positive as carbapenemase producing, while Bina et al. reported that 80.5% (33 of 41) of isolates were positive by MHT [23]. In Italy, 84% (32 of 38) of clinical isolates showed the production of carbapenemase [24]. In another study done in Brazil, thirty-six of the forty-four carbapenem-nonsusceptible *Klebsiella pneumoniae* isolates were phenotypic carbapenemase producers as determined by the MHT [25]. Azimi et al. reported that MHT was positive in all of carbapenem-resistant *Klebsiella pneumoniae* strains isolated from wound infections of the burned patients [26]. In another study, phenotypic detection of carbapenemase producing among burned patients showed that

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that all of carbapenem-resistant *Klebsiella pneumoniae* were positive by MHT [27]. So, these differences show that staying in hospitals for a long time and the widespread use of broad-spectrum cephalosporins and carbapenems, especially in burned patients can increase carbapenemase producing *Klebsiella pneumoniae* infections.

In this study, all carbapenemase producing isolates were negative for the presence of bla_{KPC}. Similar to our findings, in Iran, Bina et al. reported that bla_{KPC} was not identified in any carbapenemase producing isolates [23]. Azimi et al. showed that bla_{KPC} was absent in all carbapenemase producing isolates [26]. Eftekhar et al reported that none of the isolates harbored the bla_{KPC} [28]. In the USA, Bratu et al reported that 24% of *Klebsiella pneumoniae* isolates collected in Brooklyn were found to possess bla_{KPC} [29]. A study was carried out in Greece showed that 53% of *Klebsiella pneumoniae* isolates were positive for bla_{KPC} [30]. So, these differences show that KPCs are not common in Iran and they are endemic in the countries such as the USA and Greece. It should be noted, a number of carbapenemases have been reported, including Ambler class A (GES, SME and IMI), Ambler class B (NDM, VIM and IMP) and Ambler class D (OXA) can also produce a positive results in MHT [31, 32].

Conclusions

The results of this study and other similar studies show that resistance to carbapenems and the amount of carbapenemase production has increased. These enzymes can hydrolyze carbapenems and most antimicrobial agents, so therapeutic options are limited for these infections. For limiting and controlling the spread of the carbapenem resistant isolates, it is recommended that MHT is performed for detection of carbapenemase producing isolates in routine laboratories and the results are reported to the clinicians and infection control committees.

Conflict of Interest

All authors of this manuscript declare that there is no conflict of interest.

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