Original Article

Detection of the *Klebsiella pneumoniae* carbapenemase (KPC) in *K.pneumoniae* Isolated from the Clinical Samples by the Phenotypic and Genotypic Methods

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ABSTRACT

Background and Objective: **The production of carbapenemases especially *Klebsiella pneumoniae* carbapenemase (KPC) is the most important mechanism of enzymatic resistance in isolated Enterobacteriaceae such as *K. pneumoniae*. The purpose of this study was detected of the carbapenemase producer *K.pneumoniae* strains with phenotypic and genotypicmethods.**

Method: **Out of 800 strains, 270 *K. pneumoniae* strains (33.7%), were obtained. Antibiotic susceptibility test was performed by disk diffusion method in accordance with CLSI guidelines. Carbapenem resistant strains were identified by the Modified Hodge Test based on CLSI instruction and PCR for surveying the presence of bla-KPC gene.**

Results:**A total 270 *K. pneumoniae* strains were collected. Antibiotic susceptibility test results showedthe highest and lowest resistance was related to piperacillin (60.6%) and carbapenems (14.6%) respectively.80.5%(33 of 41) isolates were positive by MHT, but all of them (100%)were negative for amplification of the bla-KPC gene in the PCR method.**

Conclusion:**The MHT was an appropriate method for approving carbapenemase production. Moreover, a laboratory could accept the carbapenemase production with PCR method for the bla-KPCgene, which has the additional profit of validating which KPC is present.**

***Keywords: Klebsiella pneumoniae*, Carbapenem Resistance, *bla-KPC* gene, MHT**

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Introduction

Klebsiella pneumoniae is a non-motile, encapsulated, lactose-fermenting, facultative anaerobic, rod-shaped and gram-negative opportunistic pathogen that common cause of nosocomial infections. These bacteria, particularly in children are the cause of pneumonia, meningitis, sepsis, diarrhea and bacteremia (1, 2). The increasing appearance of multidrug resistance (MDR) among *K. pneumoniae* nosocomial isolates has confined the suitable therapeutic choices for the treatment of this infections (3). Newly, the emergence of new β-lactamases such as NDM-1 (New Delhi metallo-β-lactamase-1), OXA-48 (Oxacillinase-48), OXA-181 (oxacillinase-181), KPC (*K. pneumoniae* carbapenemase) and CTX-M-15 (Cefotaxime-M-15) admit resistance to the most antibiotics for example penicillins, carbapenems, cephalosporins, and sulfamethoxazole (4,5).

These resistant genes are located on transferable plasmids and can be freely transferred between bacteria, from one region to another and from one country to the other and then spread all over the world (6). Carbapenems are a class of β-Lactam antibiotics with a broad spectrum of antibacterial activity (7). Misuse, overuse and abuse of the carbapenems can increase resistance in the *K. pneumoniae* carbapenemase (KPC)-producing bacteria (8).

The mechanisms of resistance to carbapenems may be related to the combination of decrease in bacterial outer membrane permeability, increasing production of Extended-Spectrum Beta-Lactamases (ESBLs), AmpC beta-lactamasesand expression of betalactamases like Carbapenemases (9,10).The production of carbapenemases especially KPC is the most important mechanism of enzymatic resistance in isolated *Enterobacteriaceae* such as *K.pneumoniae* (11).KPCs are encoded by the gene *bla-KPC*, whose potential for different species and universal spreading is mainly elucidated by its location within a Tn3-type transposon, Tn4401. This transposon is able to inserting into varied plasmids of Gram-negative bacteria. Plasmids carrying *bla-KPC* are related to resistance factors for other antibiotics (6, 12). Although *K.pneumoniae* remains the most prevalent bacterial species carrying KPCs, the enzyme has been identified in several other Gram-negative bacilli (13).

KPC-producing bacteria have initially been reported from the New York City area; however, *bla-KPC* is present among *Enterobacteriaceae* isolates in the worlds (14-16).KPCs do not demonstrate the first or the only mechanism of carbapenem resistance, they are notable because they are often not detected by routine susceptibility screening tests and possess an exceptional potential for distribution. As well as, the infection control challenges that have arisen because of limited antibiotic options (17-19). Therefore, detection of KPC-producing bacteria may be difficult based on routine antibiotic susceptibility testing (20). It is essential to implement efficient infection control actions to limit the spread of these pathogens (21).

Considering the fact that information on the subject is limited in our country so utilization of a suitable method for determinationof the carbapenemaseproduction is an important pointin themicrobiology laboratory(8).Therefore, the aim of this study was determination of the KPC-producing *K. pneumoniae* isolates by phenotypic and genotypic methods.

Materials and Methods

Bacterial strains

We accomplished a cross-sectional study during,"Sep 2011" to "Jun 2013", at three large teaching hospitals of Tehran, Iran (Milad, Motahari and Aliasghar). Clinical specimens counting urine, blood, stool, burn wound, sputum, cerebrospinal fluid (CSF), skin lesion, eye discharge, abscess, trachea and catheter were achieved. Identification of *K. pneumoniae*doubtful grown colonies were,according to the Gram stainingand standard biochemical tests such as indole test,

motility, lactose fermentation, citrate andurease test, lysine decarboxylase and MR-VP.

Antimicrobial susceptibility testing and Modified Hodge Test

Susceptibility testing was performed by the using of disk diffusion method with the antibiotic discs MAST Company, UK according to Clinical and Laboratory Standards Institute (CLSI)guideline (22). Antibiotic tested comprise: ertapenem (10µ), imipenem (10µ), meropenem (10µ), cefotaxime (30µ), ceftazidime (30µ), cefepime (30µ), cefoxitine (30µ), ceftriaxone (30µ), gentamicine (10µ), piperacillin (100µ), aztreonam (30µ). *Klebsiella pneumoniae* ATCC 13883 was used as a control strain for disk susceptibility testing. Ertapenem resistant strains were tested for KPC producing by the Modified Hodge Test (MHT) according to the CLSI recommendation using *E. coli* ATCC 25922 and the ertapenem disk (Mast, UK).MHT- Positive *K. pneumoniae* ATCC BAA-1705was used as a positive control.

DNA Extraction

DNA extraction was carried out by boiling method (23). Briefly, *K. pneumoniae* strains were grown overnight at 35°C on MacConkey agar (Merck Co., Germany). Two or three colonies of each culture were harvested from the surface of the agar plates and re -suspended in 200 µl of sterile distilled water. The cell suspension was heated for 15 min at 100°C and then centrifuged at 10,000 gfor 10 min and used supernatant as a source of template DNA for *bla-KPC* gene amplification by polymerase chain reaction (PCR).

Genotypic detection of bla-KPC by PCR

Detection of resistant ertapenem strains for the surveying presence of the *bla*-KPCgene was performed by PCR usingspecific primers (24).The *bla-KPC* specific primer pairs includeforward,5'-CGTCTAGTTCTGCTGTCTTG -3', and reverse 5'-CTTGTCATCCTTGTTAGGCG -3'(24). A volume of 1.5 µL of ready DNA (0.5 µg) was added to a final volume of 30 µL PCR mixture comprising 12.5 µL of 2× Master Mix (Ampliqon, Denmark), including 1× PCR buffer, 1.5 mmol/L MgCl2, 0.15 mmol/L dNTP, and 1.25 IU Taq DNA polymerase, (Ampliqon Co., Denmark), 0.7 µL of 0.8 µmol/L each primer and 14.6 µL of sterile distilled water. PCR program was performed as follows, 94 °C for 3 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 31 s and 73 °C for 1 min, with a final extension at 72°C for 5 min. The PCR products were visualized by electrophoresis in 1.5% agarose gels stained with ethidium bromide.

Results

Overall, 800 clinical samples, 270 *K. pneumonia* were obtained. The distribution of the *K.pneumoniae* was, 57% and 43% were collected from the women and men, respectively. The highest and lowest resistance was related to piperacillin (60.6%) and imipenem (13.9%), respectively. Intermediate resistance to meropenem was not observed (0%) and intermediate resistance inhibition zone was similar (1.6%) for cefepime, cefoxitin, ceftriaxone and gentamicine. The highest frequency of susceptibility among *K. pneumonia* was associated to meropenem with 85.5%, and the lowest rate of sensitivity was related to piperacillin with 28.2% (Table 1).Out of, 15.1% (41 of 270) strains resistant to carbapenems, 87.8% (36 of 41) strains were isolated from the burn wound and 80.5% (33 of 41) of them were resistant to all tested antibiotics, they could therefore be considered as being multidrug-resistant (MDR) strains. Therefore, 33 strains were positive as KPC producing in MHT by developing clover leaf shape (Fig.1). PCR method was done for identification of KPC producing strains by amplification of *bla-KPC* gene in forty one carbapenem resistant isolates. The PCR products obtained in this work, revealed that all carbapenem resistant isolatesdid not carry a *bla-KPC*gene.

Table1: Antimicrobial resistance profile of *Klebsiella pneumoniae*isolates

Result	% of Antibiotic resistance										
	ERTA	IMP	MER	CTX	CFT	CP	CFX	CF	GM	PIP	AZ
Resistance	15.5	13.9	14.5	50	44.2	36.4	20.9	50	41.3	60.6	48.8
Intermediate	4.9	2.0	0.0	0.8	3.0	6.1	1.6	1.6	1.6	11.0	4.0
Sensitive	79.5	85.2	85.5	49.1	52.7	57.3	77.4	48.3	56.9	28.2	50.8

ERTA; Ertapenem, IMP; Imipenem, MER; Meropenem, CTX;Cefotaxime,CFT; Ceftazidime, CP; Cefepime, CFX;Cefoxitin, CF; Ceftriaxone, Gm; Gentamicine, PIP; Piperacillin, Az; Aztreonam.

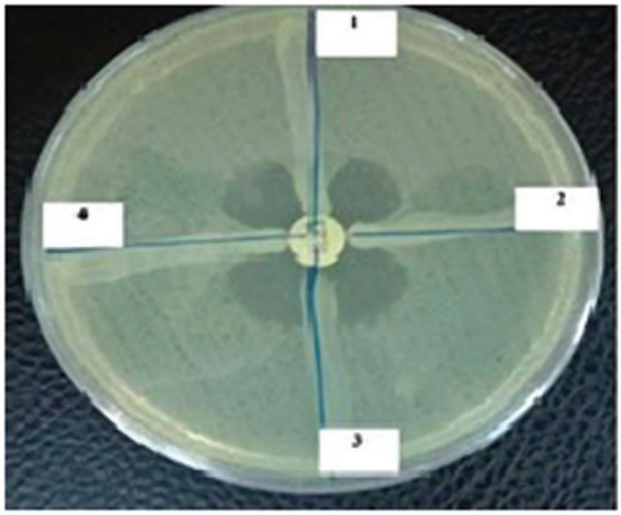

Fig.1: Isolates tested for KPC production through MHT

Discussion

The gold standard to approve the attendance of a KPC is the spectrophotometry methodand then PCR of the *bla-KPC*gene. This genotypic assay, is time consuming for a clinical microbiology laboratory and commonlyneeds isolates to be referred to reference laboratories for confirmation. For the recognition of KPCs, have been established several phenotypic tests (12, 25). The method presentlyapprove by the CLSI is the modified Hodge test (MHT)and this method isaccepted as a specificand sensitive method for detection of carbapenemase(26, 27). Because of thedifficult elucidation and false positive results, MHT cannot be used as a confirmatory test for recognition of the KPCs (28, 29). False-positive results are a more common in isolates producing AmpC and CTX-M b-lactamase(30).

In this study, 14.65%of the *K. pneumoniae* strains were resistant to carbapenems.The antibiotic susceptibility test results showed that the highest resistance to the antibiotic were related to gentamicin and cefepime, whereas, the lowest rate was associated to imipenem, meropenem, ertapenem and ceftazidime, that is agreement with the result of Brato et al. study (31). The highest resistance to the antibiotics was related to gentamicin (26).In the MHT,80.5% (33 of 41) strains were positivefor KPC that wasconsistent with Cury et al.(MHT 71% positive) (26). In two studies, 84% (32 of 38) and 12.3% (30 of 244) strains showed the production of carbapenemase (8, 17).We showed that all 41 carbapenem – resistant *K. pneumoniae* isolates were negative for *bla-KPC*gene.These results are consistent with Flonta et al.(13) and Anderson and his coworkers(19)studies. Whereas, other studies from the USA (32), China (27) and Italy (17) confirm the presence of the*bla*-KPCgene by PCR, that having contrast with our study. This contrast can be due to reduced susceptibility to at least one extended-spectrum cephalosporin (22) and another mechanism such as of carbapenem resistance as a result of a combination of an ESBL or AmpC-type enzyme with porin loss (33,34).Woodford and his colleague declare that non-susceptibility to ertapenem is not reason for carbapenemase production, particularly when carbapenemase production is unusual (35).

In this study, the MHT was a suitable method for approving carbapenemase production. Moreover, a laboratory could approve the carbapenemase production with PCR method for the *bla-KPC*gene, which has the added profit of endorsing which KPC is present.

Conclusion

The accuratecure of infections caused by KPC-producing isolates is uncertain. The evolution of antibiotic resistancemechanisms and the lack of antimicrobial agents against gram negative bacteria is a main treatment problem for clinicians.It is vital for laboratories are alert about the recognition of appearance KPC resistance. Approaches to laboratory recognition of KPC resistance will probably have to be revised and adjusted as a more studied.We hope the results of this studybe usefulfor application of an effective infection disease control approachtoavoid and decrease the prevalence of KPC-producing *K. pneumoniae*in Iran.

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Conflict of interest

the authors declare that there is no conflict of interests.

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