Original Article

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Evaluation of Carbapenemase-Resistant *Enterobacteriaceae* by Disk Diffusion Test and Polymerase Chain Reaction

Hedieh Moradi Tabriz[1], Sepideh Rastgufar[1], Elham Nazar[1]*, Leyla Pourgholi[2], Mohammad Ali Boroumand[3]

1. Department of Pathology, Sina Hospital, Tehran University of Medical Sciences, Tehran, Iran
2. Clinical Laboratory, Tehran Heart Center, Tehran University of Medical Sciences, Tehran, Iran
3. Department of Pathology, Tehran Heart Center, Tehran University of Medical Sciences, Tehran, Iran

*** Corresponding author**
Elham Nazar, MD
Department of Pathology, Sina Hospital, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 6634 8580
Email: elhamnazar@yahoo.com

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Abstract

Background: Carbapenemase-resistant *Enterobacteriaceae* is a major threat to public health. These microorganisms are resistant to all types of beta-lactam antibiotics.

Methods: This cross-sectional study was conducted on 51 *Enterobacteriaceae* isolates from clinical samples in Sina Hospital and Tehran Heart Center in Iran from 2016 to 2018. Antibiotic susceptibility test was performed by disk diffusion method. Carbapenem-resistant isolates were identified by the Modified Hodge Test (MHT) and Polymerase Chain Reaction (PCR) for surveying the presence of *VIM, NDM, IMMP,* and *OXA-48* genes.

Results: Out of 51 clinical samples, 38 isolates were positive for both MHT and PCR tests, and 5 isolates were negative in both tests. The results of both tests are similar in 84.3% of the isolates.

Conclusion: The MHT is an appropriate and easy method for approving carbapenemase production. Also, a laboratory can detect the carbapenemase production by identification of the KPC genes.

Keywords: Carbapenemase-resistant, Enterobacteriaceae, Polymerase chain reaction

Introduction

Enterobacteriaceae have been reported all over the world in recent years. These microorganisms are often the cause of systemic infections in hospitalized patients (1). *Klebsiella pneumoniae carbapenemase* (KPC)-producing bacteria is more common in the *Enterobacteriaceae* family, but also has been found in *Pseudomonas aeruginosa* (*P. aeruginosa*) (2). The first isolate of KPC-producing bacteria was detected in North Carolina in 1996. The isolate was resistant to all lactamase antibiotics (3). *Klebsiella pneumoniae* (*K. pneumoniae*) is a gram-negative microorganism of the *Enterobacteriaceae* family. It is a non-motile, encapsulated, lactose-fermenting, facultative anaerobic bacilli. It can cause different types of infectious diseases such as pneumonia, urinary tract infections, meningitis, and sepsis (4). The appearance and rapid dissemination of these isolates are considered a dangerous threat to public health. They are resistant to beta-lactams such as Cephalosporines and Carbapenems as well as Fluoroquinolones, Aminoglycosides, and Cotrimoxazole (5). Therefore, *Enterobacteriaceae* can cause invasive infections which result in a high mortality rate. Carbapenems are a choice for beta-lactamase-producing species (6). There are three groups of beta-lactamase with the ability to hydrolyze carbapenems which consist of molecular Amber class A including *IMI, SME, NMCA, KPC-type*, molecular Amber class B consisting of *IMP, VIM*, and class D including *Oxacillinase*. KPC is the most prevalent in class A. KPC enzymes are capable of hydrolyzing Penicillins, Cephalosporines, and Aztreonam in addition to Carbapenems (7). There are several methods for the detection of beta-lactamase-producing species. They include blood agar combined test, Double Disc Test (DDT), Modified Hodge test (MHT), DNA sequencing, and Polymerase Chain Reaction (PCR) amplification (8). MHT shows carbapenemase production in isolates that, if suspected, may produce this enzyme (9). This work aims to compute the prevalence of KPC-producing bacteria in two hospitals in Iran by MHT and PCR for *VIM, IMP, NDM,* and *OXA-48* genes. Knowing the exact prevalence can prevent the inappropriate use of broad-spectrum antibiotics. Preventing the transmission of Carbapenemase-Producing Organisms (CPOs) in healthcare settings requires rapid and accurate laboratory detection methods so that preventive measures can be rapidly implemented for infection control. Since data on this subject is limited in our country, the use of appropriate techniques to identify carbapenemase production is an important practice in the microbiology laboratory.

Materials and Methods

In this cross-sectional study, among the 770 samples received, there were 51 samples (one sample from each patient) of Sina Hospital and Tehran Heart Center affiliated with Tehran University of Medical Sciences from 2016 to 2018. All the clinical specimens including tracheal aspirate, blood, urine, central venous line, wound, and stool were examined to detect KPC-producing bacteria. All the samples were cultured and *K. pneumoniae* isolates were detected by the standard biochemical tests including positive for citrate, Voges-Proskauer, urease, ONPG (β-galactosidase), and lactose fermentation. Also, methyl red and motility were negative.

Modified hodge test

All the isolates were tested by MHT to find whether or not they were resistant to Carbapenems. The MHT was performed according to Clinical and Laboratory Standards Institute (CLSI) recommendations. *Escherichia coli* (*E. coli*) ATCC25922 was solved in 5 *mL* saline to make 0.5 McFarland standard, the suspension was then diluted by 1:10. A sterile cotton swab was dipped into the suspension and used to inoculate a Muller-Hinton agar plate. Meropenem disc (10 μg) was placed in the center of the plate. Suspected bacteria (resistant to Carbapenem family and third-generation Cephalosporins) by sterile swab were streaked in a straight line from the edge of the Meropenem disc onto the plate edge. The plate was incubated overnight at 35±2°*C* for 16–24 *hr*. In negative isolates, the clear zones around the disc remained homogeneous, while Carbapenemase-producing isolates caused a clover leaf-like indentation (Figure 1). MHT positive indicates the presence of Carbapenemases.

Molecular study

All the samples were amplified by conventional polymerase chain reaction (PCR) for *OXA-48, NDM,*

Figure 1. Positive MHT test with suspicious isolates.

Table 1. Carbapenemase genes classification

Ambler class	Subclass	Examples
A		*NMC-A IMI-1, IMI-2 All β-lactams SME-1, SME-2, SME-3 KPC-2, KPC-3 All β-lactams GES-2, GES-4, GES-5, GES-6*
B	B1 B2 B3	*- NDM, IMP, VIM, GIM, SPM, Ccr1* *- CphA* *- L1, FEZ-1, GOB-1, CAU-1*
D		OXA-23, OXA-40, OXA-48, OXA-50, OXA-51, OXA-55, OXA-58, OXA-60, OXA-62

VIM, and *IMP* genes (Table 1). Conventional PCR was performed which contained the DNA template, specific forward/reverse primers (synthesized at Active Oligos), and commercial master mix (New England BioLabs). DNA was extracted from the established specimens from microbial culture and eluted in a 60-*μl* volume. PCR was performed in a 25-*μl* final volume containing 5 *μl* of eluted DNA, 200 *μmol/L* of each deoxynucleoside triphosphate, 10 *mmol/L* Tris–HCl (pH=8.3), 50 *mmol/L* KCl, 1.5 *mmol/L* MgCl2, 1.5 *U* of DNA polymerase (Roche Applied Science), and 10 *pmol* of each primer. Primer sequences are shown in table 2.

PCR steps include initial denaturation at 95°*C* for 15 *min*, second denaturation at 95°*C* for 15 seconds, and annealing at 52°*C* for 35 seconds. The final extension step was performed for 5 *min* at 72°*C* using the thermal cycler. The PCR product containing amplicons was analyzed in a 2% agarose gel in ×1 Tris-acetate buffer at 90 *V* for 1 *hr* and was visualized with ethidium bromide using a gel documentation system (Syngene) (Figure 2).

Statistical analysis

For the statistical analysis, the results were presented as mean±standard deviation (SD) for quantitative variables and were summarized by absolute frequencies and percentages for categorical variables. For the statistical analysis, the statistical software SPSS version 19 for Windows (IBM Corp. Armonk, New York, USA) was used.

Results

The examination of 51 clinical samples yielded the

Table 2. The sequence of the primers for *OXA-48, NDM, VIM,* and *IMP* genes

Primer	Sequence (5 ⟹ 3)	Gene	Product size, *bp*
OXA-48-F	CCA AGCATT TTTACC CGCATC KACC	*BlaOXA48*	438
OXA-48-R	GYTTGACCATACGCTGRCTGCG		
NDM-F	GGTTTGGCGATCTGGTTTTC	*blaNDM*	621
NDM-R	CGGAATGGCTCATCACGATC		
VIM-F	GATGGTGTTTGGTCGCATA	*blaVIM*	390
VIM-R	CGAATGCGCAGCACCAG		
IMP-F	GGAATAGAGTGGCTTAAYTC	*blaIMP*	232
IMP-R	TCGGTTTAAYAAAACAACCACC		

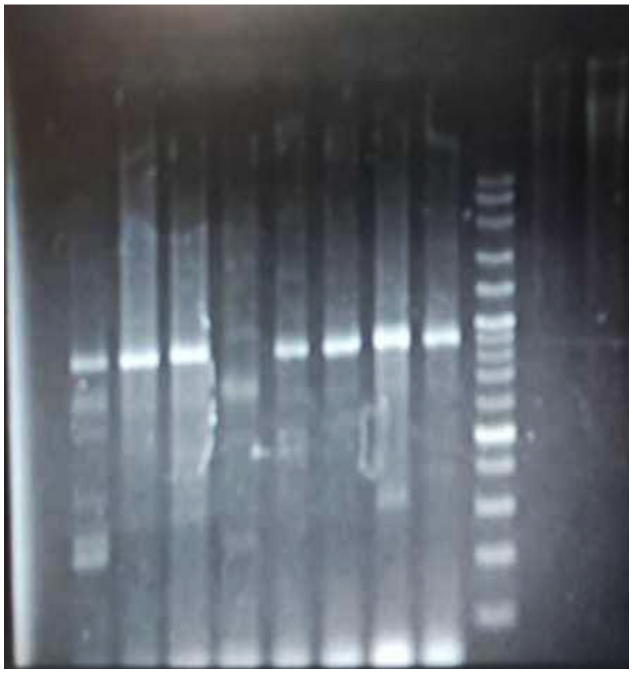

Figure 2. Conventional PCR for OXA-48, NDM, VIM, and IMP genes in suspicious isolates.

Table 3. Demographic information of the patients

Sex	
Male	32 (62.7%)
Female	19 (37.3%)
Inpatient department	
ICU	23 (45.1%)
Non-ICU	28 (54.9%)
Age	
20-40 years	12 (23.5%)
40-60 years	39 (76.5%)
Source of samples	
Urine	24 (47.1%)
Blood	2 (3.9%)
Wound	7 (13.7%)
Stool	1 (2.0%)
Tracheal	15 (29.4%)
Central vein catheter	2 (4.0%)

following results. The distribution of samples in men and women was 62.7% and 37.3%, respectively. Demographic data is summarized in table 3. In total, 45 (88.2%) cases showed positive results in MHT, and the prevalence of KPC by MHT was 88.2% (Table 4). The *VIM* gene by PCR was positive in 17 cases (33%). These cases were also MHT-positive. Sensitivity and specificity for the *VIM* gene by MHT were 94.4% and 15.2%, respectively. The prevalence of the *VIM* gene was determined at 35.3%. PCR for the *IMP* gene demonstrated positive results in 17 cases (33%), all of which were MHT positive. Sensitivity and specificity for *IMP* gene by MHT was 100% and 17.6%, respectively. The prevalence of the *IMP* gene was determined 33.3%. MHT and *OXA-48* gene by PCR were positive in 30 (58.8%) cases. Sensitivity and specificity for *OXA-48* gene by MHT was 100% and 28.6%, respectively. The prevalence of *OXA-48* gene was determined 58.8%. In 11 (21.6%) cases, MHT and *NDM* gene by PCR showed positive results. Sensitivity and specificity for *NDM* gene by MHT was 97.7% and 12.8%, respectively. The prevalence of the *NDM* gene was determined 23.5%. Between these genes examined by PCR, *OXA-48* had high prevalence (58.8%) in the isolated strains. In 38 (74.5%) cases, MHT and PCR methods had both positive results. Also, 5 (9.8%) cases showed negative results in both tests. In 7 (15.7%) cases, MHT was

Table 4. Frequency of KPC detection by PCR and MHT

			MHT		Total
			Negative	Positive	
PCR	Negative	Count	5	7	12
		% of Total	9.8%	13.7%	23.5%
	Positive	Count	1	38	39
		% of Total	2.0%	74.5%	76.5%
Total		Count	6	45	51
		% of Total	11.8%	88.2%	100.0%

positive while PCR was negative. Therefore, a sensitivity of 97.4%, specificity of 41.7%, positive predictive value of 84.4%, and negative predictive value of 83.3% are used for MHT for KPC detection. Out of the 51 clinical samples examined, 23 (45.1%) isolates were mostly from critically ill patients admitted to intensive care units. The frequency of the urine sample was the highest (47.1%) and the stool sample and central venous line were the lowest (2%). Tracheal aspirate, wound discharge, and blood had 29.4%, 13.7%, and 3.9% frequencies, respectively.

Discussion

The increase in the prevalence of carbapenemase species results in higher morbidity and mortality rates as well as an increase in extended-spectrum antibiotic prescriptions (10). These strains are both hypervirulent and multidrug-resistant and may also be highly transmissible and able to cause severe infections in both the hospital and the community (11). Carbapenems may thus become inefficient for treating enterobacterial infections with KPC-producing bacteria, which are, in addition, resistant to many other non-β-lactam molecules, leaving few available therapeutic options (7). To prevent and treat these infections, we need to better understand how to discover new ways to predict and detect infections. Therefore, the latest molecular and biochemical techniques are suitable for early detection of KPC (12). The purpose of this study is to estimate the efficacy of KPC detection by MHT and investigate *OXA-48, IVM, NDP,* and *IMP* genes in these isolates by PCR. MHT is a phenotypic method, approved by CLSI and accepted as a sensitive method. However, MHT cannot be a confirmatory method due to the possibility of false positive results (13). Tsakris *et al*'s study on 163 organisms showed all 57 KPC producers provided positive results (sensitivity:100%), while all 106 non-KPC producers were negative (specificity:100%) by using the CLSI method and disks having imipenem, meropenem, or cefepime (14). But, false detection of Carbapenemase production was observed by the MHT possibly as a result of Extended-Spectrum β-Lactamase (ESBL) production coupled with porin loss as reported before (15). According to da Silva KE *et al*'s study, the false positive results observed using MHT probably occur due to low-level hydrolysis of Ertapenem by ESBLs, particularly those of the *CTX-M* type (16). Also, MHT is easy to implement and of low cost for the detection of Carbapenemase producers in any clinical setting (17). The current study showed that all the isolates had positive genetic profiles that could be detected by MHT. Thus, there are both phenotypic and molecular methods available for KPC detection (18). However, the detection of isolates producing carbapenemases can be unreliable by automatic methods and often demands confirmatory tests (19). Sensitive PCR-based techniques for the detection of KPC have been developed as an alternative to culture-based methods. As PCR accelerates isolation and provides the opportunity for preventive measures in colonized cases, its use should be implemented promptly during outbreaks (20). Hindiyeh *et al* study suggested that PCR had high sensitivity and specificity for detection (21). Zee *et al* represented that PCR is a reliable and rapid method for the detection of the most prevalent carbapenemases (22). Francis *et al* demonstrated that higher sensitivity, specificity, and positive and negative predictive values of the PCR assay when compared with MHT (23). Also, the production of KPCs recognized by the MHT revealed a strong connection with the existence of the blaKPC gene by PCR ($p<0.0001$) (24). Therefore, for other genes, a supplementary study is required. Also, precise consideration should be paid to accurate inoculum preparation for MHT methods. In addition, using ertapenem or meropenem disc will develop detection for class reporting of carbapenem susceptibility (25). Patients with *NDM-1* positive gene CRE have a higher rate of mortality in comparison to patients with *NDM-1/OXA-48* positive CRE treated with either a carbapenem-containing regiment or colistin-containing regiment (26).

MDR-GNB infection treatment may have several challenges and limitations: 1. As long as there are restricted choices for effective treatments, there is a considerable worrisome to increase resistance to effective antibiotics (27,28); 2. Nephrotoxicity risk and suboptimal dose usage (29); 3. The effectiveness and optimal use of novel treatment options should be evaluated in the long term (30). Before novel antibiotic agent availability, several antibiotics were used often in combinations including polymyxins,

tigecycline, and aminoglycosides. Recently, more effective agents with *in-vitro* anti-Cre activity are available such as meropenem/tazobactam, ceftazidime/avibactam, eravacycline, and plazomicin (31). PCR is not available in all microbiological laboratories and is also an expensive method. Our study established MHT that was a reliable method for KPC detection when PCR was unavailable with a sensitivity of 97.4% and specificity of 41.7%. *OXA-48* type carbapenemase-producing bacteria are very common in many countries (32) and in our study, the *OXA-48* gene among other examined genes had a high prevalence (58.8%). Hence, we examined *OXA-48* in genetic profile in the isolated strains. Also, Amy *et al* recommended that the best phenotypic test for the detection of KPC is MHT (9) which was similar to our study. A rapid effective and accessible method for detecting KPC is needed to avoid therapeutic failures (33). The expanding geographic spread of KPC carbapenemases highlights the significance of the detection of these enzymes (34). Rapid and reliable recognition of KPC-producing *Enterobacteriaceae* is critical for infected patients' management and interferences for their spread limitation (35). The MHT is an appropriate technique for the prediction of carbapenemase existence. Moreover, a laboratory could support the carbapenemase production with the PCR method which has the extra yield of validating in which KPC is existent (4). Detection of carbapenem resistance is generally according to phenotypic methods in routine laboratory, but these methods often have technical challenges and are time-consuming (36). PCR is prompt and easy to do, with a high degree of sensitivity and specificity, and may help find new variants (37). The main limitation of this study is that it included isolates of a single *Enterobacteriaceae* species, *K. pneumoniae*, which carries only certain carbapenemases.

The MHT test has some limitations; The main problem among them is that they are not accurate in producing KPC. Molecular techniques, including PCR, are the only method to demonstrate the existence of genes encoding KPC resistance factors (38). Additionally, KPCs only reduce sensitivity to carbapenems, they do not induce intentional resistance. To achieve complete carbapenem resistance, it is often necessary to reduce the ability to penetrate the outer membrane. Overall, it can be stated that the assessment of KPC by the MHT test is reliable. One of the limitations of this study is the small sample size. Therefore, we strongly recommend repeating the study with a larger sample in a multicenter setting to confirm our findings.

Conclusion

KPC production in gram-negative bacilli is a growing problem worldwide. In this study, the MHT results and PCR results for KPC detection were compatible. Therefore, we can conclude that MHT is a suitable, inexpensive, and simple method for KPC screening in the microbiology laboratory. Since there is a large variation between countries in the incidence and mortality of KPC, further studies in Iran on a larger scale are recommended.

Ethical Approval

All items of the project were approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.MEDICINE.REC.1396.3091).

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Conflict of Interest

There is no conflict to be declared.

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