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A study on prevalence of KPC producing from *Klebsiella pneumoniae* using Modified Hodge Test and CHROMagar in Iran

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ABSTRACT

Carbapenems are the last defense resort against Gram-negative bacterial infections that are resistant to extended spectrum antibiotics. Thus the utilization of an appropriate method in order to determine the production of carbapenemase enzyme in infections caused by these organisms is one of the important responsibilities of Microbiology laboratories. The aim of this study was to compare the phenotypic method including CHROMagar KPC and Hodge Test for detection of KPC isolates in Iranian hospitals. Two hundreds and twenty four multiple antibiotic resistant strains of Klebsiella pneumoniae were collected from hospitals located in Tehran and other major cities of Iran. These strains were tested for production of KPC using by Hodge Test and CHROMagar. The results were analyzed to determine the frequency of carbapenemase enzyme. Interpretation of the Hodge Test was achieved according to CLSI recommendations. Chromagar method was applied based on the direction of the manufacture. Thirty six positive strains were identified by Hodge Test and CHROMagar methods respectively. CHROMagar confirmed all the positive strains which were positive by Hodge test method. The sensitivity of the Hodge Test was calculated to be 83.3% in comparison with CHROMagar method. In addition, it was observed that all the positive KPC strains were resistant to other antibiotics including Ampicillin, Amikacin, Gentemycin, Tetracycline, Ceftazidime, Ciprofloxacin and Imipenem Given the findings from the CHROMagar medium in comparison to Hodge Test, the utilization of chromogenic medium seems safe and appropriate. However, gold standard methods is recommended to identify the resistance genes such as bla_{KPC}, bla_{IMP}, bla_{NDM} coding carbapenemase.

Keywords: *Klebsiella pneumoniae*, ESBL, Carbapenemase, Hodge Test, CHROMagar

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INTRODUCTION

Nowadays infections caused by resistant enterobacteriaceae to carbapenems are spreading and causing concerns for health care systems. Enterobacteriaceae resistant to carbapenem are resistant not only to beta-lactam antibiotics but also to many other antimicrobial classes. Carbapenem is the last defense choice against gram-negative bacterial infections which are resistant to extended-spectrum antibiotics (1).

Among carabapenem derivatives, drugs such as Imipenem, Meropenem and Ertapenem can be named that have a wider effect range than other beta-lactam antibiotics and are the best treatment for the infections caused by Enterobacteriaceae that produce beta-lactamase.

Beta-lactamases are the enzymes that hydrolyze beta-lactam antibiotics. EBSLs are beta-lactamases that are resistant to all classes of penicillin, cephalosporins and aztreonams. This resistance, however, is not against cephamycin and carbapenems. These antibiotics are to a great extent restrained by clavulanic acid (2).

Considering the scene and homology, beta-lactamases are classified into four molecule classes A, B, C and D. The produced carbapenamase by *Klebsiella Pneumonia*e belongs to class A that is resistant to penicillin, extended-spectrum cephalosporins, carbapenem and monobactam (3).

In any case, with the increase in the use of carbapenems, the number of resistant bacteria with different resistance mechanisms is rising (4). Some resistance mechanisms include deactivating antibiotics, altering or producing excessive amount of drug target receptors by protein gene mutation, acquiring a new genes that produces insusceptible target, reducing the permeability of cell membrane to antibiotics and also removing active drug from periplasm or from inside of cell (5,6). Among the most important resistance mechanisms against carbapenem is producing carbapenamase that KPC (*Klebsiella pneumoniae* carbapenemase) and VIM are the most common among them in Enterobacteriaceae (1).Resistant Enterobacteriaceae isolates to carbapenems that are isolated from patients have a major role in spreading of the disease.

Carbapenamase was first discovered in the United States in 1996 and later developed worldwide (3,6). Among the other leading countries are Israel and Greece where its production has been endemic (8-12). In 2001, it detected from *Klebsiella Pneumoniae*, and later on it was reported all around European countries such as Finland, Germany, France, Italy, Norway, the Netherland and England (7,13).

Following this trend, some papers from Asian countries like Turkey, Saudi Arabia and Iran were also published (1, 14-16). However, data regarding this issue in our country are limited. In this study, the modified Hodge Test and the method of utilization of chromogenic medium that are two diagnostic procedures for carbapenamase have been examined. Also, it is worth mentioning that these days molecular methods that are high standard methods are also used.

MATERIALS AND METHODS

Sample collection:
Two hundred forty four *Klebsiella Pneumoniae* multidrug resistances specimens were collected from the following hospitals in Tehran: Dr. Shariyati, Milad, Imam Khomeini, Baharloo, Iran Mehr and Motahari, Noor and as well as from Kaveh Clinical Laboratories, the Iranian Transplantation Bank. Some of these specimens were also collected from three hospital in Esfehan (Imam Mousa Kazem, Alzahra, Amin and Mahdieh). Received specimens were respiratory secretions, ascetic fluid, urinary tracts samples, CSF, eye discharge and burned tissues and wounds. These specimens were stored at –70 until subclultring. All specimens were cultured on the Blood Agar medium.

The diagnosis of Klebsiella in the mentioned centers was performed by regarding the colony morphology due to the presence of capsule, gram staining and confirmation by biochemical tests including TSI, MR-VP, Simmon Citrate, Urease and SIM. In addition, Indol test was done on all samples to ensure the type of Klebsiella.

Susceptibility testing was performed by disc diffusion method as recommended by CLSI. Brifely a suspention of inoculom prepared equal to 0.5 McFarland turbidity and was streaked on the Mueller Hinton Agar. Antibiotic discs were placed on surface of the medium at an equal distance of 2.4 centimeters. After 16 to 24 hours incubation at 37^{o}C, zone of inhibition was measured and interpreted by

criteria as recommended by CLSI. All the samples were tested by the Hodge Test and KPC CHROMagar and then analyzed.

Modified Hodge Test:
The test was performed according to CLSI instructions as the following:

A bacteria suspension with 0.5 McFarland dilution was prepared from *E.coli* ATCC 25922 bacteria. The dilution of 1.10 with Mueller Hinton Agar medium was supplied from the previous step suspension of 0.5 McFarland. The lawn streak with sterile swab from the 1.10- dilution suspension was done on the Mueller Hinton Agar medium. To dry the mentioned culture, it was incubated for three to five minutes. A 10 µg meropenem disc (Rosco Diagnostika Company, Denmark) was placed in the middle of plate.

The tested bacteria were cultured in a straight line from the edge of the disk to the edge of the plate to examine carbapenamase. This was done carefully to prevent the swab from touching the disk. This was incubated at the temperature of 35±2 for 16 to 24 hours.

After the incubation time, the susceptive zone of in the clover leaf-type indentation in the carbapenamase producing samples was examined. The shape of sensitive zone of the positive control sample was compared to the subject samples to determine the positive cases in the Hodge test. In each plate, depending on its size, two to four samples were cultured in a straight line to do the Hodge Test.

KPC CHROMagar:
KPC CHROMagar was prepared with the proportion of 33 grams per liter of purified water according to the producing company (CHROMagar France). Then it was boiled and autoclaved. The supplementary solution of CHROMagar was prepared with the proportion of 40 milligrams per liter and 10 milligrams of it was added to one liter of the initial medium. The prepared medium was divided in the plates, and after being cooled the samples were cultured in the prepared CHROMagar medium. The growth of bacteria together with the color of the developed colonies, which show the presence of carbapenamase and the type of bacteria producing this enzyme, were examined. E.coli, klebsiella, citrobacter, enterobacter and finally pseudomonas produce dark reddish pink colonies, metallic blue and dark and bright cream respectively.

RESULTS

From the 244 collected and tested strains, 30 (12.3 %) were KPC positive by developing cloverleaf susceptive zone in Hodge Test. From these 30, 18(60 %) cases were for men and 12 (40 %) were for women.

Also, 36 (14.7 %) from the whole samples, by the growth of bacteria in the CHROMagar medium, showed the presence of carbapenamase from which 18 (58.3 %) were men and 15 (41.7%) were women. All the positive cases with the Hodge Test were also tested positive in the CHROMagar method.

Table No. 1 shows the results from the Hodge Test and CHROMagar Test for the samples

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	Number of Samples	CHROMagar	Hodge Test
1	208	Negative	Negative
2	30	Positive	Positive
3	6	Positive	Negative
4	0	Negative	Positive

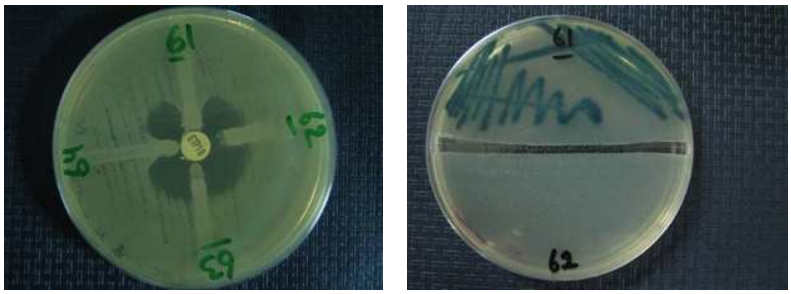

Figure No. 1: Left: Hodge Test with four samples in one plate: samples 61 and 64 are positive and samples 62 and 63 are negative. Right: CHROMagar KPC The growth of sample 61 in this medium shows it to be positive and produce carbapenamase. The fact that sample 62 did not grow in this medium prove it to be negative which was also negative in the Hodge Test.

Table No. 2: Data and results of the positive samples in Hodge test and CHROMagar KPC medium

Sample Data				KPC Tests	
Sample No.	Sex	Sample	s collection'Center	Hodge Test	KPC CHROMagar
2	M	CSF	IranMehr Hospital	Pos	Pos
7	M	Burn Wound	Isfahan burn center	Pos	Pos
8	M	Burn Wound	Isfahan burn center	Pos	Pos
18	F	Burn Wound	center Tehran burn	Pos	Pos
24	F	Urine	Milad Hospital	Neg	Pos
61	F	Burn Wound	Tehran burn center	Pos	Pos
64	M	Burn Wound	Tehran burn center	Pos	Pos
69	M	Burn Wound	Isfahan burn center	Pos	Pos
106	M	Urine	Shariati Hospital	Pos	Pos
134	F	Urine	lShariati Hospita	Pos	Pos
142	M	Respiratory secretions	IranMehr Hospital	Neg	Pos
143	F	Urine	IranMehr Hospital	Neg	Pos
147	M	Urine	Alzahra Hospital	Neg	Pos
155	F	Respiratory secretions	IranMehr Hospital	Neg	Pos
219	M	Urine	IranMehr Hospital	Neg	Pos
220	M	Burn Wound	Tehran burn center	Pos	Pos
221	F	Burn Wound	Tehran burn center	Pos	Pos
222	F	Burn Wound	Tehran burn center	Pos	Pos
223	F	Burn Wound	Tehran burn center	Pos	Pos
224	F	Burn Wound	Tehran burn center	Pos	Pos
225	M	Burn Wound	Tehran burn center	Pos	Pos
226	M	Burn Wound	Tehran burn center	Pos	Pos
227	M	Burn Wound	Tehran burn center	Pos	Pos
231	F	Burn Wound	Tehran burn center	Pos	Pos
233	M	Burn Wound	Tehran burn center	Pos	Pos
234	F	Burn Wound	Tehran burn center	Pos	Pos
235	M	Burn Wound	center Tehran burn	Pos	Pos
236	M	Burn Wound	Tehran burn center	Pos	Pos
237	M	Burn Wound	Tehran burn center	Pos	Pos
238	F	Burn Wound	Tehran burn center	Pos	Pos
239	F	Burn Wound	Tehran burn center	Pos	Pos
240	M	Burn Wound	Tehran burn center	Pos	Pos
241	F	Wound Burn	Tehran burn center	Pos	Pos
242	M	Burn Wound	Tehran burn center	Pos	Pos
243	M	Burn Wound	Tehran burn center	Pos	Pos
244	M	Burn Wound	Tehran burn center	Pos	Pos

The sample data and the results of the tests on them are shown in Table 2. In examining the positive antibiogram samples, the results show that all of them are resistant to the following antibiotics: ampicillin, amikacin, gentamicin, tetracycline, ceftazidime, ciprofloxacin and imipenem.

The incidence of the positive samples is reported as follow: The infection of burn wounds at the highest percentage: 27 cases (75 %), Urine samples: 6 cases (16.7%), Lung samples: 2cases (5.6 %), CSF sample: 1 case (2.8 %)

Regarding the positive incidence in the Hodge Test, the sensitivity of this method is calculated to be 83.3%.

DISCUSSION

Nowadays, multiple bacterial resistances have caused a lot of problems in health care centers. Therefore extensive studies to identify them to choose proper treatment are applied. Since enterobacteriaceae infections and *Klebsiella Pneumoniae* are among the frequently reported infections, extensive study and research are required. Selecting the most effective antibiotic with the least side effect as well as the minimum amount of applied antibiotic is really crucial (17). Obviously determination MIC and also selecting suitable antibiotic agent can make it possible to prescribe the right antibiotic.

Reported studies confirm that resistant of *Klebsiella pneumoniae* to routinely used antibiotics is one of the important problems in our country. In a report from Emam Khomaini hospital from Iran, prevalence of antibiotic resistance on 145 *Klebsiella pneumoniae* isolates was as follows (1): Carbenicillin 94%, Piperacillin 55%, Cefotaxime 33 percent, Ceftazidime 31%, Ceftriaxone 27%, Ceftizoxime: 22%, Ciprofloxacin 18%, Gentamicin 17%, Amikacin 14%, Piperacillin 12%, Imipenem was reported with the lowest resistant rate in comparison to ther antibioticts.

In another study by Amir Mozafari (et al. 2007), multidrug resistant rate in 303 *lebsiella pneumonia* was 60.2% of cases and were 100% resistant to cephalosporins (cephalothin, cefixime, ceftriaxone, cefotaxime, ceftazidime, and ceftizoxime). The resistance to tetracycline, nalidixic acid, cotrimoxazole, imipenem ,ciprofloxacin, nitrofurantoin were 80 ,56.4,53.2,40.3, 24.1 and 23.3 percent respectively(13).

In a similar study, antibiotic resistance was tested for 7655 urine samples.(Nastaran Langari Zadeh,2010). Sewventy two isolates of resistant *Kebsiella pneumoniae* were identified. The highest antibiotic resistance rate was belonged to Amoxicilin (98.6 %) and was followed by cotrimoxazole, nitrofurantoin , ceftazidime , cephalothin , gentamicin , tetracycline , nalidixic acid , chloramphenicol , norfloxacin ,amikacin, ciprofloxacin and imipenem with 95.8, 94.4, 80.5, 77.7, 73.6, 72.2, 58.3, 48.6, 43 and 20.8 percent respectively(18).

Resistant to Carbapenems is one of the important problems in the treatment of infections caused by multidrug resistant strains. Although KPC strains are sensitive to Colistin, but its prescription is not recommended because of various side effects. Therefore, identifying KPC strains is one of main duty of diagnostic laboratories that are carried out by phenol-typing and genotyping methods. Each method has some advantages and disadvantages. The limitation of these methods has been studied. Samra (et al 2008) has compared CHOROM agar with Hodge test. In this study, it is reported 43 (35.2%) of *Klebsiella pneumonia*e isolates were positive in CHROMagar plate while 38 isolates (31.1%) has a positive results with Hodge Test (17).

In another study CHROMagar was compared with McConkey agar plates supplemented with imipenem (1 mg/L). Panagea (et al 2011) reported specificity and sensitivity were 100 and 98.8 percent for CHROMagar while it 94.7 and 88.6 percent for McConkey procedures (19).

Nowadays there is a lot of attention to the study of the molecules of resistance genes of bacteria due to the presence of low MIC in them. The high sensitivity and specificity of this method and requirement of less time for doing the tests in comparison to the mentioned phenotype methods are the other reasons that make molecular test most popular among diagnostic tests such as determination of resistance genes in bacteria .In *Klebsiella pneumoniae* bacteria, the bla_{KPC-2}, bla_{IMP-1}, bla_{NDM-1} genes are studied (6,14,20).

Although molecular methods provide higher sensitivity and specificity, these protocols are not available in each laboratory and needs to special skill and standardization as well.

CONCLUSION

In addition to molecular methods, which belong to Golden Standards Methods, using KPC CHROMagar can be an appropriate method in diagnosing cabapenamase producing resistant bacteria.

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