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Frequency Determination of Carbapenem-Resistant Klebsiella Pneumoniae (CRKP) Isolated from hospitals in Isfahan of Iran and Evaluation of Synergistic Effect of Colistin and Meropenem on them

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Abstract: Overuse and misuse of Carbapenems among *Klebsiella pneumoniae* isolates have caused Carbapenem-Resistant *Klebsiella Pneumonia* (CRKP) during recent years. Colistin is one of the last available options, and there are increasing concerns about the dosage and resistance to this agent in long-term monotherapies. This study was designed to identification of carbapenemase producing isolates of *K. pneumoniae* via phenotypic and genotypic methods as well as evaluation of colistin-meropenem combination therapy potential. This study was carried out in Isfahan, of Iran on 100 samples from Alzahra and Khorshid hospitals in 2017. The Modified Hodge Test (MHT) was used to investigate the carbapenemase presence. The minimum inhibitory concentration (MIC) and the Fractional Inhibitory Concentration (FIC) were determined using broth macrodilution and checkerboard assays (respectively) for both meropenem and colistin. The *bla*-KPC gene was studied by polymerase chain reaction (PCR).The highest and the lowest rate of resistance were observed for piperacillin (84%) and ertapenem (50%) respectively. 68 isolates by MHT were CRKP, but None of them were positive for *bla*-KPC gene. 21 isolates from CRKP cases were high resistant to used antimicrobial agents in the study that both MIC and FIC results showed significant synergy for this antibiotics in checkerboard test (p-value < 0.05). 21 resistant isolates from CRKP cases showed statistically significant synergy potential for meropenem and colistin. The meropenem-colistin combination therapy can be applied as a suitable antibiotic synergy but it requires further investigation in clinical assay. Regarding to our findings, Probably other mechanisms of resistance to Carbapenems ,except bla-kpc genes are involved.

Key words: *Klebsiella pneumoniae*; Carbapenemase; *bla*-KPC; Modified Hodge Test; Checkerboard; CRKP.

Introduction

Klebsiella pneumoniae is a gram negative bacillus from the *Enterobacteriaceae* family with its all characteristics including: positive catalase, negative oxidase, lactose fermentative, nonmotile, negative indole, negative Methyl Red (MR), positive Voges-Proskauer (VP), urease and citrate positive. Gastrointestinal tract (GI tract), eyes, respiratory tract and genitourinary system are the common areas of bacterial colonization (1). Ever-increasing resistance to various antibiotics in *K. pneumoniae* isolates is noticeable and worrisome, because it can lead to serious problems such as pneumonia, meningitis, septicemia, high morbidity and mortality, etc. (2). In fact, carbapenems are the last-line therapy for nosocomial infections. They have a broad spectrum of activity and stability in comparison to most β-lactamases. The bipolar structure of these antibiotics helps them to cross easily through the outer membrane proteins in the gram-negative bacterial cell wall and target the penicillin-binding proteins (PBPs) (3). In recent years, carbapenemase producing bacteria especially *K. pneumoniae* have caused many medical problems which require essential considerations. Carbapenemase are a group of enzymes capable of hydrolyzing carbapenems, cephalosporins and broad-spectrum penicillins (4). There are two main groups of carbapenemase: serine carbapenemases, based on the presence of serine residues in their active site, and metallocarbapenemases. Carbapenems are clustered in tetraploid groups of Ambler molecular classification system (5). The enzymes which are grouped in Class A including Imipenemase (IMI), Serratia marcescens enzyme (SME), Serratia fonticola carbapenemase (SFC-1), non-metallocarbapenemase (NmcA), Guiana extended-spectrum β-lactamase (GES) and *K. pneumoniae* carbapenemase (KPC) (6). The discovery and development of novel and more effective antibiotics has been reduced, while antibiotics like colistin have turned to the last therapeutic strategy (7). However, possible side effects of colistin as well as resistance development to it have led to a lot of concerns about the proper dose (8). There are recommendations on the use of colistin along with a carbapenem (9). Considering this point, synergistic therapy was used in current study and the synergistic potential of meropenem and colistin for inhibition of carbapenem-resistant *K. pneumoniae* isolates was investigated. In addition, prevention of complete carbapenems elimination from the treatment period of resistant isolates is another reason to selection of this antibiotic.

The KPCs which are located in the class A enzymes are highly clinically important enzymes with the ability of resistance development and rapid exchange between different bacteria (10). The first case of KPC-producing

bacteria (KPC-2 in *K. pneumoniae*) was reported in the eastern United States in 1996. The KPC-producing bacteria have spread throughout the world over the past decade and now are prevalent in Puerto Rico, Colombia, China, Brazil, Argentina, and recently in the Middle East (11). Lately, *K. pneumoniae* isolates containing *bla*-KPC gene have been responsible for the outbreak of noscomial infections due to their high antibiotic resistancy to routine antimicrobial agents and the isolation or identification of these pathogens are a major challenge for diagnostic laboratories (12).

So this study was designed to identification of carbapenemase producing isolates of *K. pneumoniae* via phenotypic and genotypic approaches as well as evaluation of combination therapy's potential with meropenem and colistin for inhibition of bacterial growth.

Materials and Methods

Patient and samples

This cross sectional study was carried out in Isfahan, of Iran on 100 samples from Alzahra and Khorshid hospitals in 2017. Any clinical specimens such as tracheal aspirate, blood, urine, urethral catheter, wound, etc. were selected to detect *K. pneumoniae*. All samples were cultured on a specific medium and colonies with the characteristics of gram-negative bacteria were isolated.

Antibiotic susceptibility test

All antibiotic disks including ceftazidime (30 µg), imipenem (10 µg), meropenem (10 µg), trimethoprim sulfamethoxazole (30 µg), gentamicin (10 µg), aztreonam (30 µg), ciprofloxacin (5 µg), piperacillin (100 µg), Ertapenem (10 µg), cefotaxime (30 µg), cefipime (30 µg) were purchased from Mast, England. Antibiogram assay was performed using the Kirby-Bauer antimicrobial disk diffusion according to the CLSI standard on the Mueller Hinton Agar plates (Merck, Germany). Standard isolate of *E. coli ATCC25922* was used as a quality control strain.

Modified Hodge Test (MHT)

The Modified Hodge Test (MHT) was performed according to the CLSI recommendation. At first the aliquot of *E. coli ATCC25922* in 5 ml saline was adjusted to 0.5 McFarland standard, and then the suspension was diluted 1:10. Next, the sterile cotton swab was dipped into the suspension and inoculated on Müeller-Hinton agar plate, then a 10 µg meropenem disk was placed in the center of the plate. In a straight line, by a sterile swab, suspected bacteria (resistant or semi-susceptible isolates to one or more antibiotics of the carbapenem family and third generation cephalosporins), were streaked from the edge of the meropenem disc (MEM) to the plate edge. The plate was incubated overnight at 35±2°C in ambient air for 16–24 hours. In negative isolates the clear zones around the disk remains homogeneous, while carbapenemase producing isolates cause cloverleaf like indentation.

The evaluation of minimum inhibitory concentration (MIC)

In order to meropenem and colistin MICs determination in CRKP samples (MHT positive) which were resistant to all used antimicrobial agents in the study (21 samples), macrodilution broth test was carried out as recommended by CLSI protocol. Broth titrations of meropenem (1-256 µg/ml) and colistin (0.0625-32 µg/ml) were performed along with positive and negative controls. After 18-24 hours incubation at 37°C, the MIC was defined as the lowest concentration which inhibits bacterium growth (clear tube) in comparison to the control tubes.

Determination of the Fractional Inhibitory Concentration (FIC) by Checkerboard assay

96 well microplates were used to evaluation of synergistic effect of two antibiotics and a total of 150 µl of Mueller-Hinton broth was distributed into each well. 150 µl of each antibiotic stock solution (colistin and meropenem purchased from Sigma–Aldrich) was added to the first well, and then the serial dilutions of each antibiotic were prepared along the ordinate and abscissa. Finally, 150 µl of bacterial suspension was added to each well. Positive control (bacterial suspension plus culture medium) and negative control (a mixture of antibiotic dilutions and culture medium) were included in each plate. After incubation at 37 ° C for 18 to 24 hours, the lowest concentration which completely inhibited the bacterium growth (compared to control wells) was considered as MIC(Combination). To further confirmation, the agar plates were inoculated by a suspension of MIC(Combination) and its adjacent wells. The obtained results were plugged in the formula 1. The combination is considered synergy when the FIC index ≤0.5, indifferent when the FIC index is >0.5 to <4, and antagonistic when the FIC index ≥4 (13).

Formula1.

$$FICI_{A/B} = \frac{MIC_{A(combination)}}{MIC_{A(alone)}} + \frac{MIC_{B(conbination)}}{MIC_{B(alone)}}$$

Molecular detection of bla-KPC by PCR

The forward primer 5' -TCTGGACCGCTGGGAGCTGG-3' and reverse primer 5'- TGCCCGTTGACGCCCAATCCC-3' were used to amplify 399 bp of bla-KPC gene in MHT positive samples. Polymerase chain reaction (PCR) was conducted in a final reaction volume of 30 µl as follow: initial activation at 95°C/10 minutes, 36 cycles of 94°C/60 seconds, 63°C/60 seconds, 72°C/60 seconds and a final extension step of 72°C/5 minutes. The final products of PCR were electrophoresed on agarose gel. The extracted acid nucleics of *K. pneumoniae* ATCC BAA-1705 and *K. pneumoniae* ATCC BAA-1706 were used as positive and negative controls (respectively).

Results

100 cases of *K. pneumoniae* were isolated and identified from different clinical samples. The population study was included 62% females and 38% males (p=0.01). The urine specimens with 46 (46%) isolates were the prevalent clinical cases; while blood and cerebrospinal fluid derived samples each with 2 (2%) were the rare ones. The clinical profile of *K. pneumoniae* isolates are

Table 1. Prevalence of *Klebsiella pneumoniae* isolates by Specimen Type and Clinical Ward.

Specimen Type	Number of Patients(%)	Clinical Ward				
		ICU	Internal	Surgery	Emergency	Infant
Urine	46(46%)	36(36%)	5(5%)	0	0	5(5%)
Tracheal	16(16%)	9(9%)	5(5%)	1(1%)	1(1%)	0
Catheter	8(8%)	0	0	8(8%)	0	0
Wound	7(7%)	0	2(2%)	3(3%)	2(2%)	0
Bronchial	7(7%)	0	3(3%)	0	4(4%)	0
Abdominal fluid	5(5%)	0	3(3%)	2(2%)	0	0
Abscess	4(4%)	4(4%)	0	0	0	0
Sputum	3(3%)	2(2%)	0	0	1(1%)	0
Cerebrospinal fluid	2(2%)	1(1%)	0	0	0	1(1%)
Blood	2(2%)	1(1%)	0	0	0	1(1%)
Total	100(100%)	53(53%)	18(18%)	14(14%)	8(8%)	7(7%)

demonstrated in Table 1. Based on the results of Table 1, the ICU ward with 53 (53%) and the infant ward with 7 (7%) samples were the most and least frequent cases (respectively).

Results of antibiotic susceptibility test and identification of carbapenemase producing isolates of K. pneumoniae via phenotypic and genotypic methods

The profile of antibiotic susceptibility was determined by disc diffusion method. As it is shown in Table 2, The highest and the lowest rate of resistance were observed for piperacillin (84%) and ertapenem (50%) respectively. The modified Hodge test was performed for suspected carbapenemase producing isolates (Figure. 1). 68 (68%) isolates of *K. pneumoniae* were positive for the phenotypic test of carbapenemase KPC (CRKP samples). In MHT positive isolates, urine samples (64.7%) accounted for the majority of cases, while abdominal and cerebrospinal fluids (0%) were the lowest groups. In addition, the ICU wards with 47 (69.1%) and the emergency units with 4 (5.9%) samples, were the most and least frequent cases in MHT positive group (respectively).

68 isolates by MHT were CRKP, but None of them were positive for *bla*-kpc gene.

Table 2. Antimicrobial resistance profile of *Klebsiella pneumoniae* isolates

Antibiotic name	Susceptible	Intermediate	Resistant
Cefepime	29%	0%	71%
Ceftazidime	29%	1%	70%
Cefotaxime	17%	7%	76%
Gentamicine	37%	1%	62%
Ciprofloxacin	24%	6%	70%
Meropenem	28%	6%	66%
Imipenem	24%	12%	64%
Ertapenem	44%	6%	50%
Piperacillin	9%	7%	84%
Sulfa-methoxazole-Trimethoprim	44%	3%	53%
Aztreonam	22%	7%	71%

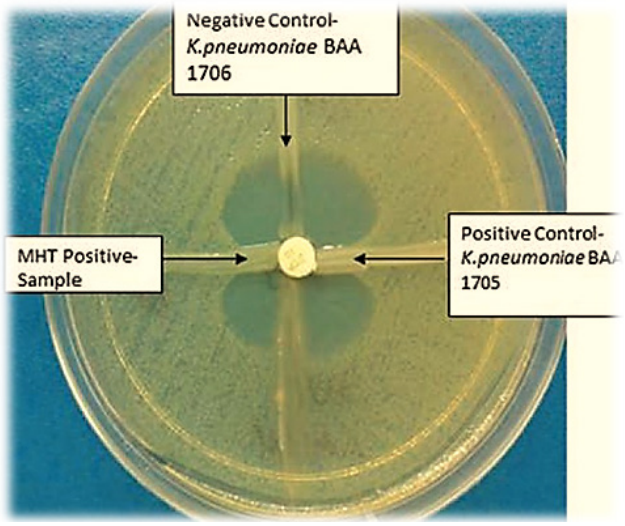

Figure 1. Isolates tested for KPC production through MHT.

Meropenem-colistin in vitro synergy testing

In CRKP samples which were resistant to all used antimicrobial agents in the study (21 samples), urine samples (85/7%) and ICU wards (66.7%) cases had the highest prevalence . The meropenem and colistin MICs were defined by mentioned methods and the results are demonstrated in formula 1 and Table 3. The FIC results showed Synergy potential for meropenem and colistin was found in 57.15% of resistant isolates (p-value < 0.05).

Discussion

Carbapenems with a broad spectrum of activity are considered as the last line agents in treating infections caused by *Enterobacteriaceae* family especially *K. pneumoniae*. However, the widespread use of carbapenems has led to resistance development against these antibiotics. Resistance to carbapenems can have various reasons, however Carbapenem-hydrolyzing β-lactamase are the most important parameter in inactivation of these agents (14). KPCs as the most clinically important enzymes were originally reported from the United States, but now have the worldwide distribution (15).

In Deshpande et al. study 51 out of 8885 *Enterobacteriaceae* cases, were positive for carbapenemase

Table 3. Results of MICs and FICIs.

Isolate Number	MIC_{MEM} (mg/l)	MIC_{CST} (mg/l)	$FICI_{MEM/CST}$
1	32	1	0.25
2	16	1	0.5
3	32	1	0.375
4	32	0.125	0.37
5	16	4	0.75
6	16	0.25	0.5
7	32	2	1
8	64	0.5	0.375
9	32	8	0.5
10	64	4	0.5
11	256	4	1
12	32	2	0.25
13	32	1	0.375
14	32	4	1
15	64	2	0.5
16	32	16	0.625
17	16	4	0.5
18	16	8	1
19	16	1	0.75
20	16	0.25	1
21	16	2	0.625

MEM:Meropenem, CST: Colistin, MIC: Minimum Inhibitory Concentration ,FIC: Fractional Inhibitory Concentration.

(16). Shanmugam et al. reported a frequency of 82% (38/46) in carbapenemase producing cases which *K. pneumoniae* were the predominant isolate and Krishnappa et al. study indicated that 95% of all Klebsiella isolates were carbapenem resistant (17,18). In current study , similar to the Husseini's study, urinary specimens from the ICU wards were the most cases and like Bina's results the highest resistance was to piperacillin. In comparison to Bina and Rudbari studies, our findings indicate higher prevalence of carbapenem resistant *K. pneumoniae*, it can mean that the frequency of carbapenemase is higher in Isfahan and needs further investigation and consideration (19-21). As it mentioned, the study of Seyed Hosseini in Kashan, of Iran indicated that among 181 *K. pneumoniae* isolates, 26.5% cases were imipenem resistance. The isolates showed high resistance to ampicillin, cefalotin, and cefotaxime, while the low resistancy was found to ertapenem and doripenem. The urinary and respiratory samples from ICU departments accounted for the most frequent infections . In another study by Rudbari, 20 out of 280 isolates of *K. pneumoniae* from Qa'im and Imam Reza hospitals of Mashhad in Iran were positive for carbapenemase. Bina et al. reported that, the highest and the lowest resistancy was to piperacillin and imipenem . In a study that was conducted by Shokri, 87% of *K. pneumoniae* isolates were carbapenem resistant (22).

In our study 68 isolates by MHT were CRKP, but None of them were positive for *bla*-KPC gene. Similar results had been published by Nagaraj in India, Shib and Zaman in Saudi Arabia, Flont, and Anderson (23-27). Bina et al. reported, all isolates were negative for *bla*-KPC gene (21). In several other publications like Zare, Eftekhar, and Azimi studies in Iran all carbapenemase producing strains were *bla*-KPC negative (28-30). In Bratu study, the KPC gene was found in 24% of carbapenem-resistant *K. pneumoniae* isolates (31). The *bla*-KPC positive isolates were reported 51% and 70.6% by Castanheira and Chen (respectively) (32,33). There is a dramatic increase in carbapenem resistant isolates in Iran, despite the absence of bla-KPC gene. These findings indicate that other carbapenemase encoding genes must be considered for future studies.

Antibiotic resistance has led to a major clinical and public health challenge and there are only few therapeutic options to deal with carbapenem resistant *K. pneumoniae* infections. Thus, identification of appropriate antibiotics with minimal side effects as well as determination of best and lowest dose regimen is essential. Infections with carbapenem resistant strains are difficult to treat and there is less hope to develop new and more powerful antibiotics. Colistin is the last therapeutic option, although there are worldwide concerns about the dosage and resistance to this agent. The study of Miyakis et al. in 2011, There are recommendations on the use of colistin along with another antibiotics (9).

Here we used colistin-meropenem combination therapy to help reduce matters regarding dose and adverse effects of colistin in monotherapies, in addition to prevention of complete deletion of carbapenems in treating resistant strains. The findings are promising and the synergistic effect of combined antibiotics was 57.15% (p-value < 0.05).

According to our findings there is a raise in the resistant *klebsiella pneumoniae* isolates, especially carbapenem resistant strains, in the country. Our results indicate that colistin-meropenem combination therapy is an effective strategy to treat gram-negative resistant bacterial infections. The results of previous and current studies suggest that *bla*-KPC resistant gene has low prevalence in the country, so other encoding gene for carbapenem hydrolyzing enzymes must be responsible for resistance to carbapenem family in *Enterobacteriaceae* isolates. The results of this study contribute to the information on the status of antimicrobial resistance in the country.

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Interest conflict
The authors declare that there is no conflict of interest.

Author's contribution
Study concept and design: Hossein Fazeli
Acquisition of data and sampling: Leila Gheitani
Analysis and interpretation of data: Leila Gheitani
Drafting of the manuscript: Leila Gheitani
Critical revision of the manuscript for important intellectual content: Leila Gheitani
Study supervision: Hossein Fazeli

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