Original Article

Evaluation of Carbapenemase Resistance in Pseudomonas Aeruginosa and Enterobacteriaceae Family Isolated from Clinical Specimens by Using Phenotypic Methods

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Abstract

Introduction:
Carbapenems is on second line therapy of multidrug resistant Pseudomonas aeruginosa infections and are the last defense line in critical infections Enterobacteriaceae family but Carbapenems resistance is increased so there are some problems when Carbapenems resistance is increased. Therefore, a new study about carbapenem resistance is necessary.

Materials and Methods:
This study collected 196 isolated bacteria of Pseudomonas aeruginosa and Enterobacteriaceae family from Hospitals patient of Tehran and evaluates them by phenotypic and biochemical methods. Also, the researcher studies the disk diffusion method and use it to determine antibiotic sensitivity of all bacteria according to the CLSI standard table.

Results:
From the total of 196 bacteria collected, the resistance to Meropenem is %39.79 and Imipenem is %45.91 and 30.10 percent to Meropenem and Imipenem resistance so that the percent resistance of bacteria is as follows: Pseudomonas aeruginosa resistance to Meropenem is %39.62 and Imipenem is %43.39. Meropenem and Imipenem resistance in Enterobacteriaceae family are as follows: Salmonella resistance to Imipenem and Meropenem is %0 and %50, Citrobacter koseri the resistance to Imipenem and Meropenem %0 and %50, Citrobacter freundii Meropenem resistance of %28.57 and Imipenem %28.57, Escherichia coli resistance to Meropenem %39.70 and Imipenem %47.05, Klebsiella oxytoca resistance to Meropenem %46.6 and %40 Imipenem and Meropenem resistance Klebsiella pneumoniae %34.14 and Imipenem %53.65, respectively.

Conclusion:
There is an increasing resistance to Meropenem and Imipenem antibiotics in treatment of Pseudomonas aeruginosa and Enterobacteriaceae family in our country. Hence, it should be properly and reasonably use these antibiotics.

Keywords: Carbapenem, Pseudomonas Aeruginosa, Enterobacteriaceae, Phenotypic

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Introduction

Carbapenems are the most successful type of β-lactam antibiotics that contain a beta-lactam ring in their chemical structure. These bactericidal antibiotics act by inhibiting the peptidoglycan synthesis of bacterial cell walls in the cross-linking of peptidoglycan chains as the last synthetic stage of bacterial walls. Carbapenems include imipenem and meropenem as major medications with extensive and stable effectiveness compared to β-lactam antibiotics, and are used to treat drug-resistance particualry in gram-negative bacteria. Although carbapenems were not affected by bacterial resistance, an increasing acquired resistance to these antibiotics has recently caused problems (1).

In fact, antibiotic resistance in bacterial pathogens has turned to a serious threat to humans and affected hospitalized patients across the world (2-3). Furthermore, the changes in the microbial flora caused by antibiotics trigger bacterial and fungal opportunistic invasions, causing the rapid dissemination of multidrug resistant bacteria to emerge as a serious public health concern (4).

The mechanisms of resitance to carbapenems include the production of the hydrolyzing β-lactamase and reduced access of antibiotics to the active site of penicillin-binding proteins (PBPs) caused by mutations. Nevertheless, the most effective mechanism against β-lactam antibiotics is production of β-lactamases by gram-negative bacteria, which inactiavtes β-lactam antibiotics by hydrolizing the β-lactam core. In addition, genes contributing to this resistance can be found on nuclear or plasmid chromosomes (5).

According to the Ambler classification, β-lactamases are divided into four main classes including A, B, C and D, with the A, B and D classes being carbapenem-hydrolyzing. The A and D classes include serine carbapenemases while B encompass zinc-mediated β-lactamases (6).

Pseudomonas aeruginosa emerged in the mid-twentieth century as a major nosocomial pathogen. This opportunistic gram-negative bacterium constitutes the third most common cause of nosocomial infections and the second cause of burn infections (7). Reports suggest a 50% mortality rate in patients afflicted by this bacterium, which is highly resistant to various types of antibiotics (8-9). Strains of Pseudomonas aeruginosa seem to present multidrug resistance to antibiotics such as imipenen as the second-line treatment for the associated infections (10). The Enterobacteriaceae are abundant in nature and can be found in animals, particularly mammals, human's intestine, contaminated vegetables and some food products. The high incidence of infections caused by these bacteria manifests itself in 5-10% of inpatients, 30-40% of septicemic patients and over 70% of those with urinary tract infections (11). Carbapenems are the last-line defense and treatment for serious infections caused by these pathogens. The resistance of the Enterobacteriaceae to carbapenems can thus be alarming for the treatment of infections caused by these organisms (12).

Although the Hodge test is a quick carbapenemase detection technique with high sensitivity and specificity that can be effective on the preliminary Carbapenemase assessments, possible false negatives should be considered (13).

Given the effectiveness of Carbapenems on the treatment of infections caused by Pseudomonas aeruginosa and the Enterobacteriaceae, the present study was conducted to investigate the necessity of being aware of the prevalence of resistance to Carbapenems in Pseudomonas aeruginosa and the Enterobacteriaceae isolated from clinical specimens.

Materials and Methods

The present descriptive cross-sectional study was condcuted to investigate the pattern of resistance to Carbapenemas in

the specimens of the Enterobacteriaceae and Pseudomonas aeruginosa isolated from patients presenting to Shariati Hosptial and Imam Khomeini Hospital in Tehran, Iran. Sampling was conducted in February-June 2014. Clinical samples included wounds (20.4%), urine (63.2%) and blood (16.4%) and those suspected of being contaminated with the Enterobacteriaceae and Pseudomonas aeruginosa were collected and classified based on gender and age of the subjects and then transferred to the microbiology laboratory. The colonies were macroscopically and microscopically investigated for identification. The samples collected were cultured in blood agar and MacConkey's agar and incubated for 24 hours at 37 ˚C. The bacteria were preliminarily detected by investigating the colonies in terms of size, shape, color, hemolysis and pigments followed by diagnostic tests such as Simmons Citrate Agar, MRVP, TSI and SIM. The specimens were then identified using the table of biochemical reactions of the Enterobacteriaceae and Pseudomonas aeruginosa and ultimately approved using gram stain under microscopes.

The disk diffusion test was used based on Mueller-Hinton Agar and the halo diameter of the growth inhibition zone was accurately measured in mm and compared with the CLSI standard table to report the sensitivity of 196 specimens of the Enterobacteriaceae and Pseudomonas aeruginosa to antibiotics including imipenem (10 µg), ceftazidime (30 µg), meropenem (10 µg), cefotaxime (30 µg), piperacillin (100 µg), ticarcillin (75 µg), aztreonam (30 µg), gentamicin (10 µg), amikacin (30 µg), ofloxacin (5 µg) and levofloxacin (5 µg). These bacteria had been identified using biochemical tests. The turbidity of the bacterial suspension was adjusted to be equivalent to the 0.5 McFarland (1.5×10^8 CFU/ml). A sterile swab was used to perform streak culture three times on the plate containing the Mueller-Hinton medium. The disks were taken out of freezers and placed on the culture medium surface and fixed in place using forceps one hour later. The plates were then incubated for 24 hours at 37 ˚C. An accurate ruler was used to measure the halo size of the growth inhibition zone in mm and the results were reported as sensitive, semi-sensitive and resistant based on the associated table of standards.

The Hodge test:

This test was performed based on the CLSI procedure. The bacterial suspension turbidity was adjusted to equal 0.5 McFarland using the E. coli ATCC 25922 standard method. The 1:10 concentration of the bacterial suspension of E. coli ATCC 25922 was based on the 0.5 McFarland in the Mueller Hinton Broth medium. The streak culture of the suspension was conducted with a 1:10 concentration using a sterile swab on the Mueller Hinton Agar medium. The culture was dried for 3-5 minutes at room temperature. A meropenem disk (10 µg) was placed on the plate center and the bacteria studied in terms of containing Carbapenemases were linearly cultured from the meropenem disk edge at the center towards the plate edge and then incubated for 24 hours at 35 ˚C. The growth inhibition halo of positive control samples were then compared with the studied samples to detect positive cases in the Hodge test. Samples confirmed to produce Carbapenemases were identified as being resistant to meropenem and imipenem based on the Hodge test.

The results obtained from the present research were statistically analyzed in SPSS16 using descriptive statistics.

The authors committed themselves to observing all research ethical principles and confidentiality of the information.

Results

A total of 196 bacteria were isolated from the clinical samples. Male samples comprised 122 (62%) cases and female ones 74 (38%), suggesting a high

proportion of presenting men compared to women. The highest prevalence of positive cases was respectively observed in the age group of 70-80 years (33.17%) and 30-40 years (14.3%) and the lowest was reported in 90-100-year-old group (1.5%) (Diagram 1).

Of the 196 study samples, the highest relative frequency of resistance to Carbapenems was respectively associated with Escherichia coli (34.69%) in the Enterobacteriaceae, Pseudomonas aeruginosa (28%), Klebsiella pneumoniae (20.9%), Klebsiella oxytoca (7%), Citrobacter freundii (3.5%), Citrobacter koseri (3%), Salmonella (2%) and Proteus mirabilis (1%) (Table 1).

In terms of antibiotic resistance, 36.22% were found to be meropenem-resistant and 45.91% imipenem-resistant. Moreover, 30.10% were resistant to both meropenem and imipenem (Table 2); the resistance to meropenem and imipenem was found to be respectively 39.62% and 43.39% in Pseudomonas aeruginosa. In the Enterobacteriaceae, the resistance to meropenem and imipenem was respectively as follows: Salmonella 0% and 50%, Citrobacter koseri 0% and 50%, Citrobacter freundii 28.57% and 28.57%, Escherichia coli 39.70% and 47.05%, Klebsiella oxytoca 46.6% and 40% and Klebsiella pneumonia 34.14% and 53.65% (table 1). The highest resistance was observed in the collected samples against amikacin (94%), followed by gentamicin (92%) and piperacillin (91%), while the lowest resistance was associated with levofloxacin (68%) (Table 3).

According to the Hodge test, the growth of the study bacteria (Pseudomonas aeruginosa and the Enterobacteriaceae) cultured was compared with that of the bacteria (Escherichia coli) which was sensitive to the antibiotic disk of meropenem or ertapenem, and the growth inhibition halo of the clover leaf of the sensitive bacteria confirmed the production of Carbapenemases by the study bacteria (Figure 1).

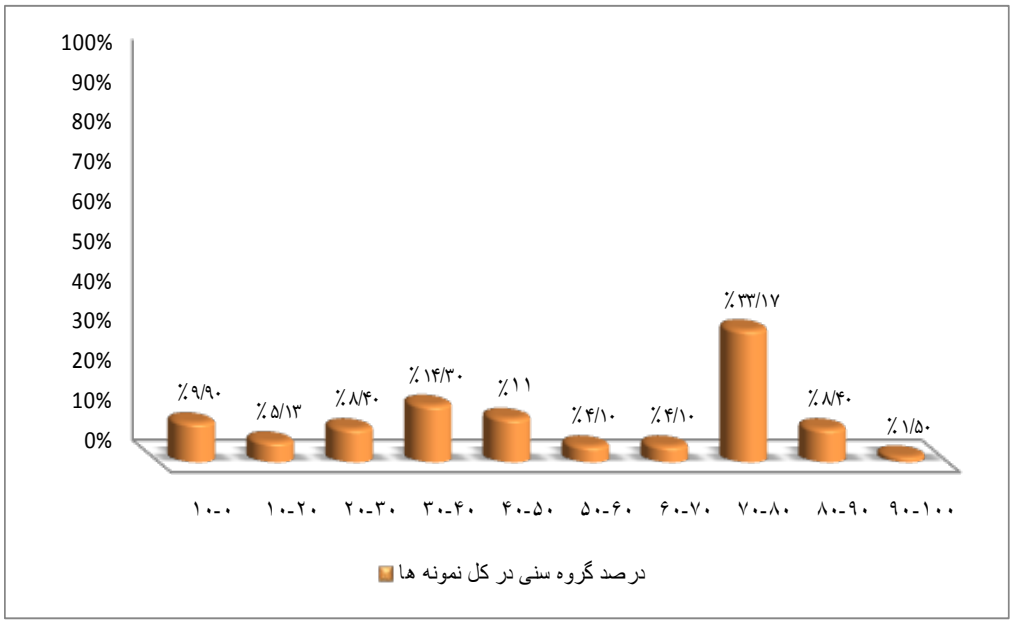

Diagram 1: Relative frequency of age groups in all samples

Table 1: Relative frequency and resistance to meropenem and imipenem in the study bacteria

Bacteria		Relative frequency	Resistance to meropenem	Resistance to imipenem
Klebsiella	pneumonia	20.9%	34.14%	53.65%
	oxytoca	7%	46.6%	40%
Escherichia coli		34.6%	39.70%	47.05%
Citrobacter	freundii	3.5%	28.57%	28.57%
	koseri	3%	0%	50%
Salmonella		2%	0%	50%
Pseudomonas aeruginosa		28%	39.62%	43.39%
Proteus mirabilis		1%	0%	0%

Table 2: Resistance of all the samples to Carbapenems reported as sensitive, resistant and semi-sensitive

Resistant to Carbapenems			Semi-sensitive to Carbapenems		Sensitive to Carbapenems		
Resistant to meropenem	Resistant to imipenem	Resistant to both meropenem and imipenem	Semi-sensitive to meropenem	Semi-sensitive to imipenem	Sensitive to meropenem	Sensitive to imipenem	Sensitive to both imipenem and meropenem
36.22%	45.91%	30.10%	18.87%	13.77%	41.32%	40.30%	27.04%

Table 3: Resistance of all the samples to other antibiotics reported as sensitive, semi-sensitive and resistant

Antibiotic	Resistant	Semi-sensitive	Sensitive
Ceftazidime	80%	11%	9%
Cefotaxime	71%	5%	24%
Piperacillin	91%	3%	6%
Ticarcillin	76%	8%	16%
Aztreonam	79%	16%	5%
Gentamicin	92%	8%	0%
Amikacin	94%	4%	2%
Ofloxacin	74%	14%	12%
Levofloxacin	68%	9%	23%

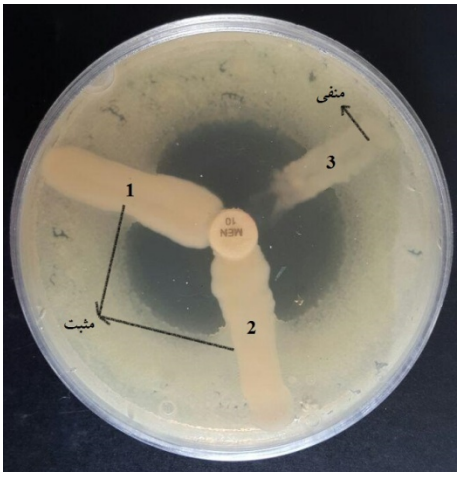

Figure 1: A typical performed Hodge test

Discussion

As of the introduction of antibiotics, recent years have witnessed their indiscriminate use which caused plasmid-mediated resistant strains to emerge and disseminate among gram-negative bacteria and therefore bring about numerous problems. According to the US Centers for Disease Control and Prevention, Pseudomonas aeruginosa is the fifth major nosocomial pathogen among hospital microorganisms and accounts for 10% of hospital-acquired infections. Bacteriological studies found imipenem to be the most effective antibiotic, particularly in critical conditions, for the treatment of Pseudomonas infections; the widespread use of imipenem has, however, increased the bacterial resistance to it (14). Carbapenems are the mainstay of treatment for gram-negative bacterial infections which tend to acquire resistance to widely effective antibiotics. The emergence of resistance to Carbapenems in the Enterobacteriaceae, as the most common cause of human bacterial infections, can seriously jeopardize the treatment (15).

Of 196 bacterial specimens collected, including Pseudomonas aeruginosa and the Enterobacteriaceae, 36.22% were found to be resistant to meropenem, 45.91% to imipenem and 30.10% to both meropenem and imipenem. Pseudomonas aeruginosa presented a resistance of 39.62% to meropenem and 43.39% to imipenem. The resistance of the Enterobacteriaceae to meropenem and imipenem was respectively as follows: Salmonella 0% and 50%, Citrobacter koseri 0% and 50%, Citrobacter freundii 28.57% and 28.57%, Escherichia coli 39.70% and 47.05%, Klebsiella oxytoca 46.6% and 40% and Klebsiella pneumonia 34.14% and 53.65%.

Altoparlak et al. (2005) found 30.8% of 120 Pseudomonas aeruginosa isolates collected from Turkish patients with burn infections to be resistant only to imipenem (16). Moreover, the study conducted by Yoo et al. (2012) in South Korea showed that 244 (37.8%) of 644 strains of Pseudomonas aeruginosa were resistant to imipenem (17), which is lower than the figure reported in the present study.

In studies respectively conducted in Tabriz and Tehran, Iran, Nahaei et al. (2006) and Shahcheraghi et al. (2008) reported a 2% and 6% resistance to imipenem in Pseudomonas aeruginosa (18-19), suggesting lower resistance to imipenem compared to the present research and indicating the emergence of antibiotic resistance.

Akhavan-Tafti et al. (2013) respectively found a 66% and 74% prevalence of resistance to meropenem and imipenem in 180 Pseudomonas aeruginosa specimens isolated from burn wounds in Yazd, Iran (20). In addition, Doosti et al. (2013) reported a prevalence of 62.8% (n=44) for resistance to imipenem in 70 clinical isolates of Pseudomonas aeruginosa in Zanjan, Iran (21). The pattern of resistance to imipenem seems to vary depending on the drug consumption pattern in different cities and countries.

Mohajeri et al. (2011), who studied the frequency of Escherichia coli in urine samples in Kermanshah, Iran, found a 100% sensitivity to imipenem in 200 Escherichia coli samples isolated from urinary tract infections (22). Abdollahi Kheirabadi et al. reported an 11.1% resistance to imipenem in 234 Escherichia coli strains isolated from patients in Fasa, Iran (23). Furthermore, the study conducted by Shokri et al. (2015) in

Isfahan, Iran, respectively suggested a 3.3% and 8.9% resistance to Carbapenems in 300 clinical strains of Escherichia coli and Enterobacteriaceae (24). The present study, however, suggests higher prevalence and thus increasing antibiotic resistance in the bacteria compared to the studies cited.

Rastegar Lari et al. (2011) used a phenotypic approach and found 19 (54.28%) in 35 Klebsiella pneumoniae isolates of burn patients in Tehran, Iran to be resistant to Carbapenems (25), which is fairly consistent with the present study. It is worth noting that the diverse bacterial species studied in the present research, including Pseudomonas aeruginosa and the Enterobacteriaceae (Salmonella, Citrobacter koseri, Citrobacter freundii, Escherichia coli, Klebsiella oxytoca and Klebsiella pneumonia), has paved the way for obtaining more accurate results as to the prevalence of antibiotic resistance to imipenem and meropenem in the region.

Conclusion

The results obtained in the present study and similar ones suggest an increasing resistance in Iran to meropenem and imipenem, as the treatment for Pseudomonas aeruginosa and Enterobacteriaceae. These antibiotics are therefore recommended to be properly and rationally restricted so as to reduce antibiotic resistance.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this article.

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