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Molecular Detection of IMP Carbapenemase-Producing Gram-Negative Bacteria Isolated From Clinical Specimens in Ahvaz, Iran

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Abstract

Background: The emergence and spread of carbapenem-resistant *Enterobacteriaceae* (CRE), producing acquired carbapenemases, have created a global public health problem. Carbapenems are important therapeutic agents for the treatment of infections due to multidrug-resistant Gram negative bacteria, particularly those carrying genes for AmpC and extended-spectrum and depressed β-lactamases. Early detection of fecal CRE carriers is essential for effective infection control. The aim of this study was to detect IMP carbapenemases by phenotypic combined disk es and pcr of *IMP* gene in Gram-negative bacteria.

Methods: In this study, 600 *Enterobacteriaceae* clinical isolates were collected and identified by standard biochemical tests. Antimicrobial susceptibility tests were performed using standard disk diffusion method based on guidelines of the Clinical and Laboratory Standards Institute (CLSI). Phenotypic identification of carbapenemases for isolates was done by the combined disk test by ertapenem and imipenem. The carbapenemase bla_{IMP} gene was detected by the Polymerase Chain Reaction (PCR) method.

Results: The results of this study showed that *Escherichia coli* (59.0 %), *Enterobacter* species (21.0%), and *Klebsiella* spp. (10.7%) were the most common clinical isolates among the *Enterobacteriaceae*. The highest and lowest rates of resistance towards ceftriaxone were 37 and 7.5, respectively. Out of 25 isolates, 4.1% were screened positive by the ertapenem and/or imipenem combined- disk tests. None of these 25 isolates were positive for *IMP* Gene.

Conclusions: Our results showed high resistance of *Enterobacteriaceae* isolates to third generation cephalosporin and carbapenem antibiotics. Supervision in antibiogram tests and also prescription of susceptible antibiotics could prevent spread of carbapenem-resistant *Enterobacteriaceae* and the other of extended spectrum beta Lactamase (ESBL)-producing isolates.

Keywords: Antibiotic, Cephalosporins, Carbapenem, Enterobacteriaceae, PCR, Resistance

1. Background

Antimicrobial resistance in bacteria is an important threat to global health, as indicated by the announcement of the world health organization (WHO) on the frequency of deaths that occur annually due to antimicrobial resistance at hospitals (1). *Enterobacteriaceae* are a large part of normal flora in the human gut and a common cause of both health care- and community-associated infections. These species could lead to acute infections such as cystitis, pyelonephritis, septicemia, pneumonia, and meningitis (2-4).

Antimicrobial resistance in *Enterobacteriaceae* is currently on the rise. Bacterial antibiotic resistance has become a problem to public health as indicated by recent reports (2).

Recent reports indicate that bacterial resistance to antibiotics has become a major public health concern bringing the threat of therapeutic impasses (5). Carbapenems are a major class of β-lactam antibiotics for treatment of serious infections in Gram-negative bacteria (6).

Choice of antibiotics for Carbapenems Resistant Enterobacteriaceae (CRE) is especially limited, and the mortality rate in health care-associated infections (HAI) with CRE has exceeded 50% in some case (6).

Carbapenemases are a member of molecular classes A, B and D β-lactamases, which could hydrolyze β-lactam antibiotics. Class B carbapenemases, metallo-B-lactamases (MBLs), are resistant to β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam, but sensitive to inhibition by metal ion chelators such as Ethylene Diamine Tetra-acetic Acid (EDTA), a chelator of Zn^{2+} or other divalent cations. Metallo-B-Lactamases are classified to two major groups, IMP and VIM. Metallo-β-lactamase of the IMP are clinically important and active against many β-lactam antibiotics such as carbapenems (2, 7).

The objective of our study was to describe the resistance pattern of clinical isolates of *Enterobacteriaceae* and

to detect IMP carbapenemses by phenotypic combined-disk test and polymerase chain reaction (PCR) of *IMP* gene.

2. Methods

Enterobacteriaceae clinical isolates, collected from two teaching hospitals of Emam Khomaini and Golestan between July 2014 and January 2014, were transported to Microbiology department of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. The identification of these isolates was done by routine biochemical tests (8), such as triple sugar iron (TSI) agar, urea broth, Simmon's citrate, sodium malonate, and sulfide indole motility (SIM) medium (Merck Co, Germany) (9).

2.1. Antimicrobial Susceptibility

The susceptibility of isolates was determined by the disk diffusion method, according to Clinical and Laboratory Standards Institute (CLSI) criteria (10). Used antibiotic disks included Imipenem (10 μg), meropenem (10 μg), ertapenem (10 μg), ceftazidime (30 μg), cefotaxime (30 μg), cefepime (30 μg), ceftriaxone (30 μg), and ceftizoxime (30 μg) (11). All isolates that were resistant to Imipenem (IMP), Meropenem (MER), and Ertapenem (ERT) by disc-diffusion method, were screened for presence of carbapenemases by a combined-disc diffusion test using 10 μg ertapenem and 10 μg imipenem discs. In the combined-disk test, two IPM disks (10 μg), one of them containing 10 μL of 0.1 M (292 μg) anhydrous EDTA and two ERT disks (10 μg), one of them containing 10 μL of 0.1 M (292 μg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO), were used. An increase in zone diameter of $>$ 4 mm around the IPM-EDTA and ERT-EDTA disks compared to IPM and ERT disks alone were considered carbapenemase positive (12).

2.2. Amplification of Carbapenemase Gene by the Polymerase Chain Reaction

DNA extraction of isolates was performed by 10 minutes of boiling of bacterial culture, followed by 1-minute centrifugation at 15000 rpm. The supernatant was collected and used for PCR amplification. The main class B carbapenemase *IPM* genes were amplified using primers and conditions described in the references listed in Table 1 (13).

The PCR condition consisted of an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of DNA denaturation at 95°C for 45 seconds, primer annealing at 51°C for 45 seconds, and primer extension at 72°C for 1 minute, followed by a final extension at 72°C for 1 minute. After the last cycle, the products were stored at 4°C. The PCR products were analyzed by electrophoresis with 1.5% agarose gels in Tris-Borate-EDTA (TBE) buffer. The gels were stained with safe DNA stain (Invitrogen, Portland, OR), and the PCR product bands were visualized in a Gel doc with UV light (15).

3. Results

In this study, 600 *Enterobacteriaceae* species were isolated from clinical specimens. Out of these isolates, 315 species (59%) were identified as *E. coli*, 127 isolates (21%) as *Enterobacter* spp., and 54 isolates (9%) as *K. pneumoniae*. The other isolates and specimen types are mentioned in Table 2.

The highest resistance rates were 37% for ceftriaxone, 35.6% for ceftizoxime, and 31.5% for ceftazidime, respectively, by disk-diffusion method.

Table 3 shows the results of susceptibility tests of *Enterobacteriaceae* isolates towards 8 examined antibiotics.

Out of 55 *Enterobacteriaceae* isolates, which were resistant to meropenem, ertapenem and imipenem by disk-diffusion test, 25 isolates (4.5%) were carbapenemase positive by combined- disk test. Carbapenemase positive isolates in combined-disk test showed an increase of $>$ 5mm in zone of inhibition around the imipenem plus EDTA and ertapenem disks, in comparison to imipenem and ertapenem alone, respectively.

However, the *IMP* gene was not detected in carbapenemase positive isolates by the PCR method.

4. Discussion

The emergence and spread of drug resistance in *Enterobacteriaceae* is confusing the treatment of serious nosocomial infections and threatening to generate drug resistance species.

Antimicrobial resistance interferes with effective treatment of patients with infectious diseases and has caused concern in hospitalized patients by increasing the rate of resistance, especially in cephalosporins and carbapenems (2). About 20% of *Klebsiella pneumoniae* infections and 31% of *Enterobacter* spp. infections at intensive care units of the United States involve resistant strains to third-generation cephalosporins (16). Resistance to these antibiotics in *Klebsiella pneumoniae* is typically caused by the acquisition of genes in plasmids that encode for resistance genes to antibiotics (16).

A type of carbapenemases is located in the Ambler Class B or Metallo-β-lactamase (MBLs). These enzymes have a clinical significance around the word (8, 10). Furthermore, MBLs can lead to carbapenems resistant and all β-lactam except Aztreonam in clinical isolates (17). The *IMP*

Table 1. Primers Used in This Study

Class	Target	Sequence (5′-3′)	Size (bp)	Refrerence
Class B	*IMP*	Forward: ATGGTTTGGTGGTTCTTGT	488	(14)
		Reverse: ATAATTTGGCGGACTTTGGC		

Table 2. Enterobacteriaceae Recovered

Species	Urine	Wound	Discharge	Tracheal	Abscess	Blood	Totale, %
Klebsiella pneumonia	14	7	4	25	3	4	57 (9.5)
Enterobacter cloacae	35	25	8	2	1	8	79 (13.1)
Escherichia coli	263	16	19	8	3	7	316 (52.6)
Enterobacter aerogenes	30	4	13	7	3	8	65 (10.8)
***Citrobacter* spp**	20	2	4	-	-	-	26 (4.3)
Serratia marcescens	3	4	3		2		12 (2.0)
Klebsiella oxytoca	5	3	3	8	1	-	20 (3.3)
***Proteus* spp**	13	2	-	3	2		20 (3.3)
Salmonella	-		-	-	-	5	5 (0.8)
Total isolates	383	63	54	53	15	32	600 (100)

Table 3. In Vitro Activities of Eight Antimicrobials Against All Species Combined[a]

Antibiotics	Resistant	Semi-Sensitive	Sensitive
Ertapenem (10 μg)	48 (8.0)	10 (1.6)	542 (90.4)
Imipenem (10 μg)	45 (7.5)	11 (1.8)	544 (90.7)
Meropenem (10 μg)	55 (9.2)	13 (2.1)	532 (88.7)
Ceftizoxime (30 μg)	214 (35.6)	27 (4.5)	359 (59.9)
Ceftazidime (30 μg)	189 (31.5)	20 (3.3)	391 (65.2)
Cefepime (30 μg)	158 (26.3)	25 (4.2)	417 (69.5)
Ceftriaxone (30 μg)	222 (37.0)	26 (4.3)	352 (58.7)
Cefotaxime (30 μg)	182 (30.3)	31 (5.1)	387 (64.6)

[a]Value are expressed as mean number percent.

gene was first identified in a Japanese *Pseudomonas aeruginosa* isolate in 1988. The first report of this gene in *Enterobacteriaceae* was from a different hospital in Japan, within 5 years of the previous report (18).

According to Huang et al.'s report, from January 2007 to April 2011, there was an evolution in epidemiology of Carbapenem Non-Susceptible *Enterobacteriaceae* (CNSE), including CRE in Belgium. Furthermore, compared to years 2007 to 2009, significantly higher numbers of CNSE and CPEs were detected in 2010 to 2011 (19).

Regarding the wide spread and importance of *Enterobacteriaceae* in hospitalized patients, the transfer of resistant bacteria between patients, and the mobility of resistance factors as plasmids and transposons between strains of diverse species, surveillance of drug susceptibilities to all classes of agents is necessary (2). According to other studies, *Escherichia coli* was the major agent involved in Urinary Tract Infections (UTIs) (74.6%), followed by *Klebsiella* spp. (11.7%) (20). Shahcheraghi reported the prevalence of isolates from clinical specimens as 67.7% for *E. coli*, 12.5% for *K. pneumoniae* and 9.4% for *Enterobacter* spp. in five hospitals of Tehran. In this study, the rate of resistance was as follows, meropenem 6.3%, ertapenem 3%, and imipenem 1.1%.In the present study, antibiotic susceptibility tests indicated high prevalence of resistance to cephalosporins, such as cefotaxime, ceftriaxone and ceftazidime.

As the results of examinations on antibiogram isolates collected in this study indicate, resistance to cephalosporins, such as cefotaxime, ceftriaxone, and ceftazidime was high (21). In this study, we examined the prevalence of *Enterobacteriaceae* isolates from various clinical specimens, and the rate of resistance to important classes of antibiotics, especially carbapenemase. Also *IMP* gene carbapenemase was assessed in the current investigation.

According to the findings, it should be noted that many of the broad spectrum antibiotics did not eliminate carbapenem resistant enterobacteriaceae and mortality rate of patients due to these bacteria are a significant concern.

Management of these infections is complex. Therefore,

identification of carbapenemase-producing isolates is essential for empirical antibiotic therapy. It also helps in monitoring the development of antibiotic resistance and use of prospering drugs, and effective strategies for control of spread of these resistant strains.

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