Investigation of VIM, IMP, NDM-1, KPC AND OXA-48 enzymes in *Enterobacteriaceae* strains

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Abstract: Gram-negative bacteria especially *Enterobacteriaceae* species have become an increasing etiologic agent of nosocomial infections. The development of resistance to carbapenems have become an increasing problem in the treatment of nosocomial infections. Especially carbapenamases are common for *Enterobacteriaceae strains.* This study was performed to detect the types of carbapenemases in *Enterobacteriaceae* strains isolated from various clinical samples. *Enterobacteriaceae* species were isolated from urine, blood, tracheal aspirates, wound, and other respiratory samples. Susceptibility of isolates to imipenem, meropenem and ertapenem was tested. Carbapenemase genes were studied using Hyplex SuperBug ID kit. VIM (1-13), IMP (1-22), NDM-1, KPC(1-10) and OXA-48 genes were investigated. Ninety-five isolates of *Enterobacteriaceae* spp. were included in the study. Sixty isolates were resistant to imipenem, meropenem and ertapenem and 20 isolates were found resistant to imipenem or ertapenem while 15 were susceptible to all carbapenems. Among the isolates with carbapenem resistance, 57 were positive for one carbapenemase gene and susceptible isolates did not have carbapenemase gene. OXA-48 was found in 49 of the isolates (86%), NDM-1 in 6 (10.5%) isolates, VIM in 2 isolates. IMP and KPC gene loci were not identified. Carbapenemase genes play a crucial role in the development and spread of resistant strains.

Keywords: *Enterobacteriaceae,* carbapenemase, OXA-48, NDM-1, VIM.

INTRODUCTION

Increased antibiotic resistance among Gram-negative bacteria has become an increasing problem in the treatment of nosocomial infections. Currently, strains with multidrug resistance against more than one antibiotic class as well as pan resistant strains are commonly observed.

Carbapenems are a group of antimicrobial agents with a wide spectrum of activity among beta-lactam class and have a rapid bactericidal action. These agents are known to be stable against almost all of the beta-lactamases, including AmpC and extended-spectrum beta-lactamases (ESBL), which are involved in enzymatic resistance in bacteria (Burak *et al.*, 2012). However, resistance to carbapenems is common among both non-fermentative bacteria and *Enterobacteriaceae* strains as a result of widespread use of carbapenems.

Carbapenem resistance in Gram negative bacteria primarily involves decreased outer membrane permeability or efflux pump system, however beta-lactamases of carbapenemase class have been frequently reported among *Enterobacteriaceae* during the last decade (Nordmann *et al*,. 2002; Nordmann *et al*., 2011). Beta-lactamase enzymes are categorized into four classes (A to D) by Ambler classification based on similarities in their amino acid sequences. Class A, C and D beta-lactamases share a common serine amino acid, whereas class B (metallo-beta-lactamases) contains zinc as the active site (Hirsch *et al*., 2010). Carbapenemases are members of Ambler class A, B and D beta-lactamases. A variety of class A carbapenemases have been described; some are chromosome-encoded (NmcA, Sme, IMI-1, SFC-1), and others are plasmid-encoded (*Klebsiella pneumoniae* carbapenemases [KPC], IMI-2, GES, derivatives), but all effectively hydrolyze carbapenems and are partially inhibited by clavulanic acid (Queenan *et al.*, 2007). Both carbapenemases and metallobetalactamases have been reported with a high global prevalence, each having a different geographical distribution. Specifically OXA-48 beta-lactamase is being reported commonly in our country (Burak *et al.*, 2012). Although, metallo-beta-lactamase enzymes are mainly found in non-fermentative bacteria, they are rapidly spreading among enteric bacteria. VIM and IMP are the most prevalent members of the metallo-beta-lactamase family, which are detected worldwide (Queenan *et al.*, 2007). Resistance associated with New Delphi Metallobeta-lactamase-1 (NDM-1) was firstly detected in 2010 among *Enterobactericeae* spp. which subsequently caused epidemics due to wide intercontinental spread in *K. pneumoniae* and *E. coli* strains (Hsueh 2010).

Carbapenemase-producing pathogens have been associated with high rates of morbidity and mortality, particularly among critically ill patients with prolonged hospitalization (Fukigai *et al.*, 2007; Maltezou 2009). Within *Enterobacteriaceae* family, carbapenem resistance is most common among *K. pneumoniae* isolates. These

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carbepenem-resistant isolates generally have multidrug resistance, which limits therapeutic options (Patel *et al.*, 2009).

Active surveillance including contact isolation and rectal swab samples is required when carbepenem-resistant enterobacteria are isolated from hospital units, particularly intensive care unit where critically ill patients are treated. Colonization has been reported to occur within 48 hours in such patients (Percin, 2012). In this context, rapid and accurate identification of carbapenemase-producing enteric bacteria is essential for selection of appropriate antimicrobial therapy and implementation of infection control measures. This is because carbapenemases on mobile genetic elements are able to spread readily among bacteria.

The present study was conducted with the aim to identify carbapenemase producing strains found in our hospital and to investigate whether phenotypically different carbapenem groups (imipenem, meropenem and ertapenem) might be used as an indicator to predict the presence of carbapenemases.

MATERIALS AND METHODS

Identification of bacteria

This prospective study was conducted within a four-month period between April 2012 and July 2012 on enteric bacteria isolated from various clinical samples sent to Gaziantep University Medical Faculty Hospital Microbiology Laboratory. All of these bacteria were isolated from urine, blood, wound, tracheal aspirate and other respiratory samples from different patients. Isolates were mostly from urine (35 strains, 36.8%), blood culture (26 strains, 27.4%) and wound swab (21 strains, 22.1%) samples.

Conventional methodology and Vitek 2 (bioMerieux, France) were used for identification of bacteria showing growth.

Antibiotic susceptibility

Susceptibility to ertapenem (ETP 10µg, Oxoid, United Kingdom), imipenem (IPM 10µg, Oxoid, UK) and meropenem (MEM 10µg, Oxoid, UK) were tested by disc diffusion method according to the CLSI criteria (CLSI 2013). Control strains used in the study were carbapenemase-negative *K. pneumoniae* ATCC 700603 and carbapenemase-positive *K. pneumoniae* ATCC BAA-1705.

Molecular identification of carbapenemase genes

The presence of Carbapenemase genes were investigated using a multiplex, reverse hybridization-based Hyplex® SuperBug ID (Amplex, Germany) kit. VIM (1-13), IMP (1-22), NDM-1, KPC (1-10) and OXA-48 genes were investigated.

DNA extraction was performed from bacteria that grew in the culture. Following amplification, the product was heat-treated to obtain single-stranded DNA. Oligonucleotide primers were loaded onto a coated micro plate and carbapenemase gene loci were investigated in the loaded DNA according to the hybridization reaction principle. The next step of the assay used ELISA principle. Following hybridization, the color formed by chromogenic reaction after further steps of incubation with peroxidase conjugate, washing and substrate addition was measured by a photometer at a wavelength of 450nm.

Approval from local ethics committee was obtained before initiation of the study (03.04.2012/138).

RESULTS

Ninety-five isolates of *Enterobacteriaceae* were included in this study. The distribution of *Enterobacteriaceae* strains according to the clinics and the sample types were shown in table 1, table 2.

Of ninety-five isolates, 80 showed resistance to at least one of the tested carbapenems. Sixty were resistant to all three carbapenems (ertapenem, meropenem and imipenem) (group I). Fifteen isolates were susceptible to all three carbapenems (group II, control group). Fourteen isolates were resistant to only imipenem (group III) and 6 were resistant to only ertapenem (group IV). Distribution of bacterial types into groups are shown in table 3.

PCR results

A gene locus was detected in 57 (60%) of the tested isolates. A breakdown of carbapenemase positive isolates is presented in table 4.

Carbapenemase gene locus was most commonly detected in *K. pneumoniae* (80.5%). None of the *P. mirabilis* and *E. aerogenes* isolates showed carbapenemase gene. Carbapenemase positivity was detected; 16 (45.7%) strains isolated from urine samples, 21 (80.7%) strains isolated from blood culture samples, 12 (57.1%) strains isolated from wound samples, 1 (25%) tracheal aspirate sample and 7 (77.8%) other respiratory samples NDM-1 was detected in 6 isolates, most frequently in *K. pneumoniae* (3 strains). VIM locus was identified in 2 isolates (3.5%) and both were detected in *E. cloacae*. No strain was detected in the KPC and IMP gene.

Fifty-one (89.5%) of the isolates with an identified gene locus were in-group I, the group resistant to all carbapenems (table 5). Carbapenemase gene was identified in 2 (14.3%) of 14 isolates resistant to imipenem (group III) and 4 (66.7%) of 6 isolates resistant to ertapenem (group IV). No gene locus was detected which was susceptible to all of the tested carbapenems (group II).

Table 1: Distribution of *Enterobacteriaceae* clinical isolates according to bacterial types and clinics

Division	Type of bacteria n Percent (%)								Total
	K. pnemoniae	*E. coli*	*P. mirabilis*	*E. cloacae*	*S. marcescens*	*K. oxytoca*	*P. stuartii*	*E. aerogenes*	
ICU[1]	9	5	1	2	1	1	-	-	19 (20)
Adult hematology and oncology	6	7	3	-	-	-	-	-	16 (16.8)
Pediatric	8	2	3	3	-	-	-	-	16 (16.8)
Nephrology	3	4	1	2	-	-	-	-	10 (10.5)
Pediatric hematology and oncology	5	1	1	-	-	-	-	-	7 (7.4)
[2]Surgical Divisions	8	4	1	2	-	-	1	1	17 (17.9)
[3]Other clinics	2	4	3	1	-	-	-	-	10 (10.5)
Total	41 (43.2)	27(28.4)	13 (13.7)	10 (10.5)	1 (1.1)	1 (1.1)	1 (1.1)	1 (1.1)	95 (100)

[1]Intensive Care Unit, [2]Surgical Divisions: Urology, 7 isolates; Orthopedics, 5 isolates; General surgery, 4 isolates; Cardiovascular surgery, 1 isolate; [3]Other Clinical Divisions: Infectious Diseases, 4 isolates; Neurology, 1 isolate; Dermatology, 1 isolate; Adult Gastroenterology, 2 isolates; Adult Endocrinology, 2 isolates.

Table 2: Distribution of *Enterobacteriaceae* clinical isolates according to bacterial and sample types

Sample type	Type of bacteria n Percent (%)								Total
	K. pnemoniae	*E. coli*	*P. mirabilis*	*E. cloacae*	*S. marcescens*	*K. oxytoca*	*P. stuartii*	*E. aerogenes*	
Urine	13	8	8	3	-	1	1	1	35 (36.8)
Blood culture	13	11	-	1	1	-	-	-	26 (27.4)
Wound	7	8	4	2	-	-	-	-	21 (22.1)
Tracheal aspirat	3	-	1	-	-	-	-	-	4 (4.2)
Other respiratory samples	5	-	-	3	-	-	-	-	9 (9.5)
Total	41 (43.2)	27 (28.4)	13 (13.7)	10 (10.5)	1 (1.1)	1 (1.1)	1 (1.1)	1 (1.1)	95 (100)

DISCUSSION

Beta-lactam antibiotics are frequently used for treatment of community and hospital-acquired infections caused by *Enterobacteriaceae* (Nordmann *et al.*, 2011).

The carbapenemases have ability to hydrolyze carbapenems. Most carbapenemase producers are *K. pneumoniae* or *E. coli*. These bacteria are increasingly common in the community and hospital-acquired infections (Nordmann *et al*., 2011; Miriagou *et al.*, 2010). Algorithms were generated for use with established phenotypical and genotypical methods to identify carbapenem resistance. Although there are several phenotypical identification methods such as modified-Hodge test and chromogenic growth media, molecular analysis to show the presence of the enzyme is considered as the gold standard (Nordmann *et al.*, 2012).

Within *Enterobactericeae* spp., the most common carbapenemases are KPC, VIM, NDM and OXA-48 (Walsh, 2010). KPC enzyme, a member of Class A carbapenemases, was first detected in 1996 in eastern USA in a *K. pneumoniae* isolate. Within a few years, KPC producers had spread globally (Nordmann *et al.*, 2002; Yigit *et al.*, 2001). In Turkey, KPC-related carbapenemase resistance has not yet been reported for enteric gram-negative bacteria (Budak *et al.*, 2012).

Although, Class B MBL enzymes are mostly found in non-fermentative bacteria, they were also reported in enteric bacteria (Vatopoulos 2008). The most common MBLs include VIM, IMP, SIM and GIM enzyme families. VIM and IMP are the most prevalent members of the MBL family worldwide (Budak *et al.*, 2012; Walsh *et al.*, 2005). VIM-1 (in *K. pneumoniae*), VIM-2 (in *P. aeruginosa*), VIM-5 and IMP-1 (in *K. pneumoniae*, *E. coli*, *E. cloacae* and *P. aeruginosa*) MBLs have been

Table 3: Distribution of bacterial types into groups

Type of bacteria	Groups n (%)				Total
	Group I	Group II	Group III	Group IV	
K. pneumoniae	37 (61.7)	1 (6.7)	1 (7.1)	2 (33.3)	41 (43.2)
E. coli	14 (23.3)	11 (73.3)	0	2 (33.3)	27 (28.4)
P. mirabilis	0	1 (6.7)	12 (85.7)	0	13 (13.7)
E. cloacae	7 (11.7)	1 (6.7)	0	2 (33.3)	10 (10.5)
S. marcescens	1 (1.7)	0	0	0	1 (1.1)
K. oxytoca	1 (1.7)	0	0	0	1 (1.1)
P. stuartii	0	0	1 (7.1)	0	1 (1.1)
E. aerogenes	0	1 (6.7)	0	0	1 (1.1)
Total	60 (63.2)	15 (15.8)	14 (14.7)	6 (6.3)	95 (100)

Table 4: Distribution of PCR-positive carbapenemase gene loci according to the bacteria types

Genes	Type of Bacteria n (%)								Total
	K. pneumoniae	*E. coli*	*E. cloacae*	*S. marcescens*	*K. oxytoca*	*P. stuartii*	*P. mirabilis*	*E. aerogenes*	
OXA-48	30 (73.2)	14 (51.8)	4 (40)	0	0	1 (100)	0	0	49 (86)
NDM1	3 (7.3)	0	1 (10)	1 (100)	1 (100)	0	0	0	6 (10.5)
VIM	0	0	2 (20)	0	0	0	0	0	2 (3.5)
Total	33 (80.5)	14 (51.8)	7 (70)	1 (100)	1 (100)	1 (100)	0 (0)	0 (0)	57 (100)

demonstrated in Turkey (Aktas *et al.*, 2006; Aktas *et al.*, 2012).

NDM-1, another interesting MBL, was first defined as an intercontinental epidemic strain in 2010 among *K. pneumoniae* and *E. coli* isolates. NDM-1 resistance is carried via a plasmid and accounts for more than 90% of resistance to carbapenems. Although NDM-1 is most commonly identified in *K. pneumoniae* strains, it is rapidly spreading among other enteric gram-negative bacteria and has been reported in other members of enterobacteria including *C. freundii*, *M. morganii* and *E. cloacae*. While all NDM-1-bearing isolates detected in the United Kingdom were *E. coli* strains, PFGE profiles was found to be similar to that of *K. pneumoniae* strains isolated in India. The common profile as shown by PFGE results has been attributed to plasmid-related clonal spread (Budak *et al.*, 2012). Poirel *et al.* (Poirel *et al.*, 2012) have reported the first NDM-1-carrying *K. pnemoniae* strain from Turkey. Also, Aksu *et al.* (Aksu *et al.*, 2013) reported another NDM-1-positive *K. pnemoniae* strain isolated from a blood culture from Turkey.

Class D carbapenemases are OXA-type carbapenemases and mainly found in *P. aeruginosa* and *Acinetobacter* spp. and to a lesser extent, in EGNB (enteric gram-negative bacteria). A few regional studies have shown OXA-48 carbapenemase activity related with carbapenem resistance among EGNB isolates (Budak *et al.*, 2012). OXA-48 beta-lactamase enzyme is mainly identified in *Enterobactericeae* spp. in Turkey. However, OXA-48-bearing *K. pneumoniae* isolates have recently been reported from several other countries (Aktas *et al.*, 2012).

Use of different molecular assays seems to be appropriate while investigating carbapenemases. Primarily, multiplex PCR, melting curve analysis, hybridization or microarray techniques are used for this purpose. In the present study, we utilized hybridization method, which reportedly has 99% specificity for OXA-48 and 100% for other enzymes (Kaase *et al.*, 2012).

In the present study, one carbapenem resistance gene was detected in 57 of (60%) 95 isolates. Among identified resistance genes, 49 were (86%) OXA-48, 6 (10.5%) were NDM-1 and 2 (3.5%) were VIM. IMP and KPC gene loci were not identified.

One gene locus was detected in 51 of 60 (85%) group I isolates, 2 of 14 (14.3%) Group III isolates and 4 of 6 group IV isolates (66.7%). No gene locus was detected in-group II which was susceptible to all tested carbapenems. In the light of these findings, we might suggest that there is a correlation between phenotypical resistance results and resistance gene and it is not necessary to carry out

genotypical investigation for isolates with no resistance; also, isolated ertapenem resistance should be regarded cautiously to investigate the presence of a genotypical resistance gene.

Table 5: Susceptibility to carbapenem and distribution of carbapenemase genes

Groups	Genes, n (%)			Total
	OXA-48	NDM1	VIM	
I	44 (89.8)	5 (9.8)	2 (3.9)	51 (89.5)
III	2 (4.1)	-	-	2 (3.5)
IV	3 (6.1)	1	-	4 (7.0)
Total	49 (86)	6 (10.5)	2 (3.5)	57 (100)

Carbapenemases were mostly identified in *K. pneumoniae*. Carbapenemases were reported to have a diverse geographical distribution (Girlich *et al*., 2012; Swayne *et al*., 2011). In the present study, OXA-48 (86%) was most prevalent, followed by NDM-1 (10.5%) and VIM (3.5%). Although KPC enzyme was a prevalent carbapenemase as identified in many studies (Vrioni *et al*., 2012; Wilkinson *et al.*, 2012). We did not detect KPC in our isolates.

Damjanova *et al*. (Damjanova *et al*., 2012) among 122 carbapenemase-producing *Enterobactericeae* strains, KPC enzyme were detected in 12 *K. pneumoniae* isolates (9.8%) and VIM in 110 isolates (90%). In that study, VIM-type carbapenemase was identified in the majority of the isolates. In a similar study, among 127 carbapenemase-producing *Enterobactericeae* isolates, KPC was found in 53 (41.7%), NDM in 44 (34.6%), VIM in 13 (10.2%) VIM, IMP in 11 (8.6%) and OXA-48 in 6 (4.7%) (Bereksi *et al.*, 2012). Cuzon *et al*. (Cuzon *et al*., 2011) found in France that all of 17 carbapenem-resistant *K. pneumoniae* strains, isolated mostly from ICU, contained OXA-48 gene.

A common finding reported by studies in Turkey is that among *Enterobactericeae* spp., carbapenemase enzyme prevailed in *K. pneumoniae* isolates and the most prevalent enzyme was OXA-48, with no detection of KPC (Aktas *et al.*, 2012; Percin *et al.*, 2012; Us *et al.*, 2010). These findings are consistent with ours; however, we believe that, identification of NDM-1 (10.5%) and VIM-type carbapenemases (3.5%) in the present study is important and relevant.

NDM-1 has been reported to be increasingly detected over time. In their multinational study conducted in 13 countries from 2008 to 2010, Struelens *et al*. (Strulens *et al.*, 2010) reported that the number of patients with NDM-1 was 8 in 2008, 30 in 2009 and 39 during the first 9 months of 2010. Identified strains were similar to those found in our study and generally included *K. pneumoniae* (54%). Poirel *et al*. (Poirel *et al*., 2012) were the first to report NDM1 from Turkey. Aksu *et al*. (Aksu *et al*., 2013) investigated carbapenemases in a *K. pneumoniae* strain isolated from blood culture using multiplex PCR analysis. They identified blaNDM1 gene but none of the other gene loci (VIM, IMP, KPC or OXA-48). That was the second case of NDM-1-bearing *K. pnuemoniae* reported from Turkey. Both of these studies presented their cases individually. However, in the present study, NDM-1-positivity (10.5%) was identified for the first time in 6 isolates in our country. We anticipate that NDM-1 would become a prevalent carbapenemase with increased detection rate in Turkey over time.

In the present study, 2 isolates (3.5%) were found to contain VIM gene and both of them were *E. cloacae*. In literature, there is one study of VIM reported in *E. cloacae* isolates from Turkey (Gacar *et al.*, 2005).

With regard to our grouping based on phenotypical resistance to carbapenems, no gene locus was detected in any of the group II isolates, which were susceptible to all of the three carbapenems. This suggests that investigation of resistant genes would have no added value if phenotypical resistance is absent. Nine of 60 bacteria in group I, 12 of 14 bacteria in-group III, 2 of 6 bacteria in group IV no gene locus were identified although seen phenotypically resistant. We believe that this might have resulted from the inability to identify the presence of other carbapenemases due to using a commercially available kit and non-carbapenemase resistance such as GSBL/AmpC or some other mechanisms, which led to carbapenem resistance including porin loss.

In the current study, a carbapenemase enzyme was identified at a high rate (66.7%) in Group IV which showed phenotypical resistance to ertapenem. Anderson *et al*. (Anderson *et al*. 2007) investigated carbapenem susceptibility among 31 KPC-positive *Enterobacteriaceae* isolates using various test methods and found that ertapenem was the most susceptible indicator. Consistent with our results, they reported that ertapenem was a good predictor for the presence of a carbapenem resistance gene.

In conclusion, similar to what was reported by studies across Turkey, OXA-48 was the most frequently identified enzyme in carbapenemase-producing *Enterobactericeae* strains in local hospital. Surprisingly, this was the first study to detect NDM-1 simultaneously in six isolates for the first time in Turkey. Another important finding of the present study is the detection of VIM and no IMP or KPC. We believe that each hospital should construct their own diagnostic algorithm to identify resistant strains based on the resistance patterns observed in their setting. Accurate analysis of susceptibility results is needed in order to implement contact isolation and other infection control measures at

an early stage. We also believe that molecular analysis of individual resistance genes detected in hospitals at regular intervals would be valuable for epidemiological purposes.

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