



Investigation of New Delhi Metallo-Beta-Lactamase 1 (NDM-1) in Clinical *Enterobacteriaceae* Isolates in Southwest Iran

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ABSTRACT

NDM-1 producing bacteria are highly resistant to beta-lactams, including carbapenems. So the emergence of these microorganisms becomes a problem for the health system. The aim of this study was to investigate the presence of NDM-1 by PCR in clinical isolates of Carbapenem-resistant *Enterobacteriaceae* (CRE) in the university-associated hospitals in Ahvaz city, southwest Iran. Between June 2014 to July 2015, 726 non duplicate isolates of *Enterobacteriaceae* species collected from different clinical samples from two university-affiliated hospitals in Ahvaz, southwest Iran. All isolates were subjected to antibiotic susceptibility testing according to CLSI 2014 principles and combined-disk test to phenotypic evaluation of MBL production. Gene encoding NDM-1 was screened by PCR. Of the 726 *Enterobacteriaceae* isolates, 72 isolates (9.9%) were resistant to carbapenems. Combined-disk test (CDT) was positive in 62 (86.1%) of the 72 carbapenem-resistant isolates suggesting Metallo- β -lactamase production, of which, *E. coli* had highest rate ($n=28$, 45.2%) followed by *K. pneumoniae* ($n=19$, 30.7%). None of MBL producer *Enterobacteriaceae* was positive for the NDM-1 gene by PCR assay. This investigation performed on the NDM-1 gene in clinical isolates of *Enterobacteriaceae* in southwest Iran. Although the prevalence of CRE was low and NDM-1 positive *Enterobacteriaceae* were not detected in southwest Iran, it should be monitored regularly. Other possible Metallo-Beta-Lactamase should also be screened for better characterization of Carbapenem-resistant *Enterobacteriaceae* isolates.

Key words: Carbapenem-resistance *Enterobacteriaceae*, Metallo- β -lactamase, NDM-1

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INTRODUCTION

To date, the bacterial resistance to antibiotics is one of the most important global health threat introduced by the World Health Organization (WHO) and most annual mortality due to hospital infections occurs because of this challenge [1]. According to the US Centers for Disease Control and Prevention (CDC) report, carbapenem-resistant *Enterobacteriaceae* (CRE) is one of the three main antibiotic resistance threats [2]. *Enterobacteriaceae* are Gram-negative bacilli which cause a wide range of infections including septicemia, pneumonia, urinary tract infection, meningitis and abscess in the different organs [3,4].

Different antibiotics are used to treat infections caused by these bacteria, but carbapenems such as imipenem and meropenem are a selective treatment for progressive

infections caused by Gram-negative bacteria resistant to fluoroquinolones and Extended-spectrum beta-lactams (ESBL) [5,6]. Resistance to carbapenems has been reported in *Enterobacteriaceae* in different parts of the world and has created a lot of worries in developing and developed countries [7-10]. Carbapenemase enzymes are one of the most important causes of resistance to carbapenems antibiotics in gram-negative bacteria [11]. A large number of carbapenemase has been identified in recent years, which are classified into three classes, A (*K. pneumoniae* carbapenemase or KPC), B (New Delhi Metallo-beta-lactamase or NDM) and C (oxacillinase or OXA-48) [12].

Metallo- β -lactamase (MBL) is the most diverse class of carbapenemases that represent an important clinical threat [6]. The MBLs belong to class B according to the Ambler classification and present hydrolytic activity against β -lactam antibiotics (except for monobactams); they are inhibitable by divalent cation chelators such as ethylene di-amine tetra acetic acid (EDTA) and sodium mercaptoacetate (MAS), and escape the action

of all β -lactamase inhibitors for clinical use, such as clavulanic acid and sulbactam [13]. NDM-1 is a strong metallo-beta-lactamase that was discovered for the first time in 2008 in *K. pneumoniae* and *Escherichia coli* isolates from a Swedish patient who had traveled to New Delhi, India [14]. The NDM-1 coding gene is located on mobile plasmids that capable of being transmitted to different bacterial strains and promotes the spread of drug resistance throughout the world [15]. To date, the isolates carrying MBL have been found almost exclusively in health institutions, in patients with prolonged hospitalization and exposed to multiple antimicrobial therapies [16]. Hence, the early diagnosis of these carbapenemases among clinical isolates is important for controlling and preventing their spread.

Due to the limited data on the prevalence of NDM-1 in *Enterobacteriaceae* from our region, this study was conducted to investigate the presence of this enzyme by amplification of *bla*_{NDM-1} gene using polymerase chain reaction (PCR) in clinical isolates of carbapenem-resistant *Enterobacteriaceae* from two university-affiliated hospitals in Ahvaz, southwest Iran.

MATERIALS AND METHODS

Ethical consideration

As the bacterial isolates were collected as part of routine patient care investigation in the hospital, ethical approval was not required.

Study region, sample collection and bacteria identification

This cross-sectional study was performed on 726 non duplicate isolates of *Enterobacteriaceae* species collected from different clinical samples including urine (n=461, 63.4%), blood (n=39, 5.4%), tracheal aspirate (n=60, 8.3%), wound (n=86, 11.9%), discharge (n=64, 8.8%) and abscess (n=16, 2.2%) of hospitalized patients at two university-affiliated hospitals Golestan and Emam Khomeini in Ahvaz, southwest Iran from June 2014 to July 2015. These two hospitals are the main hospitals of the Ahvaz city. Ahvaz is one of the major metropolises in Iran, which is considered to be the seventh most populated city in Iran. All isolates were identified by conventional microscopic and biochemical standard tests such as: Gram stain, lysin iron agar (LIA), triple sugar iron agar (TSI), sulfur indol motility medium (SIM), Simmon's citrate agar and urea broth (Merck Co, Germany) in the microbiology department of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran [17]. After the definitive diagnosis of bacteria, some of the pure colonies were suspended in Tryptic Soy Broth (TSB) (Merck Co, Germany) containing 15% glycerol (Sigma-Aldrich, St. Louis, MO) and placed in -80°C for prolonged storage.

Antimicrobial susceptibility testing (AST)

Antibiotic susceptibility testing was carried out against three carbapenems, Imipenem (IMP 10 μg), Meropenem (MER 10 μg) and Ertapenem (ERT 10 μg), by the Kirby-

Bauer disk diffusion method using Mueller-Hinton agar (Merck Co, Germany) and results were interpreted based on the Clinical and Laboratory Standards Institute (CLSI) principles [18,19]. All isolates that showed resistant pattern against one or more carbapenem were included for phenotypic detection of MBL using combined-disk test. All antibiotics were purchased from Mast, Merseyside, United Kingdom. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC BAA-2470 was used for quality control as carbapenem-susceptible and carbapenem-resistant strain, respectively.

Phenotypic evaluation of MBL producers

Phenotypic detection of metallo-beta-lactamase was performed by combined-disk test (CDT) according to Franklin et al. method [20]. Initially, a bacterial suspensions equivalent to the 0.5 McFarland standard inoculum were prepared and then inoculated onto Mueller-Hinton agar (Merck Co, Germany). Subsequently, two imipenem disks one of which impregnated with 10 μL of 0.1 M (292 μg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO) were placed on medium 25 mm apart. An increase in the diameter of the inhibition zone by more than four millimeters with IPM-EDTA disk compared to that of the IPM disk alone, after 18-24 h incubation at 37°C was interpreted as MBL positive.

DNA extraction

Bacterial DNA was extracted from colonies of all isolates by boiling method as described elsewhere [21]. Five colonies of an overnight growth (24 h) on a blood agar plate were suspended in 500 μL of sterile Tris-EDTA buffer. The colonies were lysed by heating at 95°C for 10 minute using Incublock microtube incubators (Denville Scientific, USA), and centrifuged at 15,000 rpm for 10 min at 4°C . The supernatant was stored at -20°C and used as DNA template for polymerase chain reaction (PCR) assay. The concentration and quality of extracted DNA were assessed by measuring the absorbance of A260 and A280 nm with NanoDrop (Thermo Scientific, USA) and agarose gel electrophoresis, respectively.

PCR amplification of *bla*_{NDM-1} gene

PCR assay was performed on all isolates to investigate the presence of the *bla*_{NDM-1} gene by using the target specific primer set, NDM-Fm (5'-GGTTTGGCGATCTGGTTTTTC-3') and NDM-Rm (5'-CGGAATGGCTCATCACGATC-3') as previously described [22]. PCR master mix was prepared in a final volume of 25 μL containing 10X PCR Buffer (2.5 μL), MgCl_2 50 mM (0.8 μL), dNTPs 10 mM (1 μL), each primer 10 μM (1 μL), Taq DNA Polymerase 5 U/ μL (0.2 μL), 5 μL of extracted DNA and 13.5 μL of distilled water. The PCR assay was carried out in a thermocycler (Eppendorf, Germany), with following Protocol: initial denaturation at 94°C for 5 min, 34 cycles of denaturation at 94°C for 45 sec, annealing at 54°C for 40 sec, extension at 72°C for 50 sec and a final extension at 72°C for 5 min. In every PCR run, *K. pneumoniae* ATCC BAA-2470 and distilled water were used as the positive and negative controls, respectively.

The 621bp PCR product was separated by electrophoresis (100 V, 45 min) on a 1.5% agarose gel in 1X Tris-Borate-EDTA buffer (TBE) containing 0.5 µg/ml ethidium bromide (Sinaclone, Iran). The bands were analysed using a Gel doc UV illuminator system (Proteinsimple, USA). A DNA size marker of 100bp (Sinaclone, Iran) was used for comparative analysis.

Statistical analysis

The results were analyzed using the SPSS software (Version .22 IBM, Chicago, IL, USA). The variables were reported as the number and percentage. The results were expressed as descriptive statistics.

RESULTS

A total of 726 clinical isolates were diagnosed as *Enterobacteriaceae* by standard biochemical tests. Of the 726 recovered isolates, 376 isolates (51.7%) identified as *Escherichia coli*, 79 isolates (10.9%) as *Klebsiella pneumoniae*, 72 isolates (9.9%) as *Enterobacter aerogenes*, 100 isolates (13.8%) as *Enterobacter cloacae*, 31 isolates (4.3%) as *Citrobacter Spp*, 12 isolates (1.7%) as *Serratia marcescens*, 45 isolates (12.5%) as *K. oxytoca*, 26 isolates (3.6 %) as *Proteus Spp* and 8 isolates (1.1%) identified as *Salmonella Spp*.

Of the isolates, 72 isolates (9.9%) were resistant to ertapenem, 51 isolates (7.1%) were resistant to imipenem and 65 isolates (8.9%) were resistant to meropenem (Table 1). All of these isolates were considered as carbapenem-resistant.

Table1: Carbapenems resistance pattern of studied *Enterobacteriaceae* isolates

Antibiotics	Resistant	Semi-sensitive	Sensitive
	No (%)	No (%)	NO (%)
Ertapenem (10 µg)	72 (9/9)	14 (1.9)	542 (88.2)
Imipenem (10 µg)	51 (7.1)	7 (0.9)	667 (92.0)
Meropenem (10 µg)	65 (8.9)	16 (2.2)	645 (88.9)

Combined-disk test (CDT) was positive in 62 (86.1%) of the 72 carbapenem-resistant isolates suggesting Metallo-β-lactamase production. Among the 62 phenotypically confirmed MBL-producing isolates, *E. coli* had highest rate (n=28, 45.2%) followed by *K. pneumoniae* (n=19, 30.7%), *E. cloacae* (n=7, 11.3%), *K. oxytoca* (n=3, 4.8%), *E. aerogenes* (n=2, 3.2%), *Citrobacter Spp* (n=2, 3.2%), and *Proteus Spp* (n=1, 1.6%).

Gene encoding NDM-1 was not detected in *Enterobacteriaceae* isolates (Figure 1).

DISCUSSION

Currently, the emergence of carbapenemase-resistant *Enterobacteriaceae* (CRE) especially NDM producers in the hospital setting, represents a real global public health problem [23]. The most frequent subtype of this enzyme is NDM-1 that encoded by the plasmidic *bla_{NDM-1}* gene [24].

In Asian countries, this enzyme is reported for the first



Figure 1: Agarose gel electrophoresis of PCR amplified product of *bla_{NDM-1}* gene: lane 1, 100bp DNA ladder (Sinaclone, Iran), lane 2 to 10, clinical samples, lane 11: negative control (no template DNA added), lane 12, *bla_{NDM-1}* positive amplicons (621bp), lane 14, 100bp DNA ladder

time, in the year 2009 in New Delhi, India [14]. Since then, several researches have been conducted on this subject worldwide [25,26].

Iran is among the countries confronting the problem of the emergence of NDM-producing *Enterobacteriaceae* [27,28]. There is constrained information on the carbapenemase-producing *Enterobacteriaceae* in Iran and most studies have been done only on carbapenem-resistant *K. pneumoniae* in our country. Several studies in different regions of Iran have been conducted in this regard. However, little studies have been carried out in the southern regions. To best of our knowledge, this survey provides the first epidemiological data of carbapenem-resistant *Enterobacteriaceae* and presence of NDM-1 gene among them in Southern area of Iran.

The first detection of *bla_{NDM-1}* gene in Iran was reported by Shahcheraghi et al. in multiple drug-resistant strain of *K. pneumoniae* isolated from the urine sample of a 52 year-old male patient from Tehran (North of Iran) in 2013 [29]. The second report on the detection of *bla_{NDM-1}* in Iran was raised by Fazeli et al. [30]. Also, Nobari et al. reported the presence of the NDM-1 gene in three isolates of 42 carbapenem-resistant *K. pneumoniae* collected from Tehran hospitals between 2009 and 2012 [27]. The first report on emerging of *bla_{NDM-1}* producing *E. coli* in Iran was expressed by Eyvazi et al. in 2 isolates from 2 burn wounds of patients in the Motahari hospital, Tehran [31].

In the present study, we investigated the metallo-beta-lactamase production and presence of *bla_{NDM-1}* gene in CRE isolates with phenotypic and PCR methods, respectively. Despite report of the presence of NDM-1 in carbapenem-resistant *K. pneumoniae* in the southern region of Iran by Hosseinzadeh et al. [32], none of MBL-producing *Enterobacteriaceae* isolates in our study were found to possess NDM-1 gene, so their MBL producing property may be due to other metallo-beta-lactamase genes such as VIM that were not investigated in this study. Differences in the results of our study compared to the findings of the Hosseinzadeh et al. may be due to the lack of uniformity in the type of the bacteria, as they

were only investigate the *K. pneumoniae* strains in their study. Our results suggest that the presence of NDM-1 in our country is not yet widespread. Recently, similar to our results, from Thailand no NDM-type metallo-beta-lactamase was detected in 181 CRE isolates [33].

In this research, based on AST findings, most of our *Enterobacteriaceae* isolates showed high sensitivity (>85%) to carbapenem drugs tested which was in concordant to previous studies in Iran [29,34]. Furthermore, our results showed the high frequency (86.1%) of MBL production among carbapenem-resistant *Enterobacteriaceae* isolates by CDT that was in line with findings of Firoozeh et al. who reported 77.7% of imipenem-resistant *K. pneumoniae* as MBL producer strains. Contrary to our findings, in a research performed by Fazeli et al. in Isfahan 10.2% of carbapenem-resistant *K. pneumoniae* isolates have been reported as MBL producers [30].

According to the findings from the present study, it seems that most carbapenems used in our region are appropriate antibiotics for the treatment of *Enterobacteriaceae* infections because of their low resistant rate to these drugs. However, since the NDM-1 gene was not found in any of our CRE isolates, the presence of other carbapenem-resistant genes should be investigated.

Limitations of the study

It is necessary to point out potential limitations to this study. First, in this study we only investigated the *bla*_{NDM-1} gene in *Enterobacteriaceae* isolates which have resulted in a lack of data for other carbapenem-resistant genes. Although we failed to detect the *bla*_{NDM-1} gene, our findings were consistent with results from the abstract of a study published in Turkey [35]. Second, we included all *Enterobacteriaceae* family in study design, which may have led to the heterogeneity of the results compared to other study in different countries that have emphasized on NDM-1-producing *K. pneumoniae*.

CONCLUSION

We performed the first investigation for the emergence of CRE at the two largest university-affiliated hospitals in Ahvaz city, Southwest Iran. Although the prevalence of CRE was low, it should be monitored regularly. None of the isolates had NDM-1 gene but the rate of MBL production among phenotypically confirmed CRE was significant. Therefore, investigating carbapenem resistant pattern of *Enterobacteriaceae* is necessary for help to identify and prevent the spread of carbapenemase producing bacteria.

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CONFLICT OF INTEREST

The authors' declares that they have no conflict of interest.

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