Original Article

Phenotypic and Genotypic Identification of Metallobetalactamase Genes in Resistant *Enterobacteriaceae* Isolated from Medical Centers in Isfahan

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Abstract

Background: The emergence of resistant *Enterobacteriaceae* and the abundance of antibiotic-resistant genes is one of the major problems of the global health system. The present study aimed to determine the phenotypic and genotypic expression levels of metallobetalactamase coding genes (*bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{SIM}$, *bla*$_{SPM}$, and *bla*$_{GIM}$) in *Enterobacteriaceae* isolates (*Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, and *Serratia*) from patients referred to the clinical centers in Isfahan city and typing of these isolates.

Materials and Methods: *Enterobacteriaceae* isolates were identified and isolated after sample collection. Antibiotic sensitivity pattern was investigated by disk diffusion method. MIC was performed in carbapenem-resistant isolates by the E-test method, and the frequency of strains with multidrug resistance was determined. The presence of metallobetalactamase genes was investigated phenotypically using a combined disk test and modified Hodge test. The genotypic identification of the above genes was done by PCR and sequencing techniques. Finally, PCR based on the sequence of repetitive elements was performed for molecular typing of metallobetalactamase-producing *Enterobacteriaceae*.

Results: In the present study, 580 isolates of *Enterobacteriaceae* were isolated by examining 3500 samples. *Klebsiella* and *Escherichia* were the most common isolates, and the frequency of MDR was 60% in *Klebsiella* and 59.53% in *Escherichia*. Moreover, MIC results showed that 33.7% *Klebsiella*, 4.1% *Escherichia*, 5.7% *Enterobacter*, 3.5% *Citrobacter*, and 5.5% *Serratia* were resistant to carbapenems. Frequency of isolates with multidrug resistance in *Escherichia* (MDR 59.53% and XDR 1.5%), *Klebsiella* (MDR 60%, XDR% 3 and PDR 0.8%), *Enterobacter* (MDR 44%), *Citrobacter* (MDR 53.5%) and *Serratia* (MDR 55.5%) were reported. Metallobetalactamase production was confirmed by phenotypic analysis in *Escherichia* (1.8%) and *Klebsiella* (10.4%). Genotypic tests showed that *bla*$_{SIM}$, *bla*$_{SPM}$, and *bla*$_{GIM}$ genes were absent in any *Enterobacteriaceae* isolates. The presence of *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{NDM}$ genes was confirmed in 6.2% of *Klebsiella* isolates and 1.3% of *Escherichia* isolates. The frequency of detected metallobetalactamase genes in *Klebsiella* and *Escherichia* isolates was 4.58% and 1.39% for *bla*$_{VIM}$, 0.83% and 1.39% for *bla*$_{IMP}$ and 0.83% and 1.39% for *bla*$_{NDM}$. The rep-PCR results showed that 11 metallobetalactamase-producing *Klebsiella* isolates are in 4 main groups, and 9 *Escherichia* isolates and 4 *Enterobacter* isolates are classified in two main clusters.

Conclusion: The present study shows the prevalence of *Klebsiella* and *Escherichia* isolates and their resistance to metallobetalactamase-producing *Enterobacteriaceae*. These genes in the horizontal transfer of antibiotic resistance identification of metallobetalactamase-producing isolates in clinical environments are essential to reduce the spread of antibiotic resistance. The high homology of resistant isolates of *Enterobacteriaceae* in

clinical samples indicates the high power of these genotypes in causing infection in hospitalized patients, which can play an important role in increasing antibiotic resistance.

Keywords: *Enterobacteriaceae*, Carbapenem, Metallobetalactamase enzyme, Phenotype, Genotype

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Introduction

The occurrence of antibiotic resistance in bacteria is one of the major challenges to the healthcare system of many societies[1]. Most bacteria resistant to antibiotics belong to the *Enterobacteriaceae* family, considered one of the main causes of hospital infections and severe community-acquired infections[2,3]. Most of these family members are the main pathogens of urinary tract infections (UTI), skin and soft tissue infections, wound and bloodstream infections, meningitis, and ventilator-associated pneumonia[4,5]. By biofilm forming, *Enterobacteriaceae* provide a suitable platform for transferring resistance genes and increasing the incidence of multidrug resistance in bacterial populations[5]. Resistant *Enterobacteriaceae* infections are often treated by prescribing last-line antibiotics, especially betalactams and carbapenems such as meropenem and imipenem[6,7]. Resistance to these antibiotics is often induced by the expression of metallobetalactamase genes and the function of metallobetalactamase enzymes, which is one of the most important mechanisms of inducing antibiotic resistance in the *Enterobacteriaceae* family. So far, several metallobetalactamase enzymes, including Verona integron-encoded metallobetalactamase (VIM), New Delhi metallobetalactamase (NDM), imipenmase (IMP), Seoul imipenmase (SIM), Sao Paulo imipenemase (SPM), and German imipenemase (GIM) has been identified[8]. The spread of *Enterobacteriaceae* producing metallobetalactamase enzymes is now considered almost universal and more studies are needed in this field. Metallobetalactamases are evolving with the expansion of carbapenemase activity and are considered a threat to control the public health situation[9,10].

The clinical importance of bacteria with metallobetalactamase genes lies in their ability to hydrolyze the β-lactam ring. Genes encoding these metallobetalactamases are usually present on integrins or transposable plasmids and can be transferred horizontally between bacteria [11]. The increasing rate of acquired resistance to β-lactam antibiotics caused by metallo-β-lactamases has increased dramatically in patients, leading to the ineffectiveness of drugs and inhibitors due to the continuous evolution of β-lactamase variants[12,13]. It is important to determine the pharmacokinetics of their resistance in different populations to optimize the dosage of drugs[14,15]. Infections caused by resistant *Enterobacteriaceae* are often more severe and are associated with increased antibiotic treatment failure and mortality[4,16]. Hence, investigating the prevalence of *Enterobacteria* encoding metallobetalactamase in patients referred to clinical centers in different communities can be very important.

Interestingly, several phenotypic methods have been introduced to identify these enzymes in bacterial species. However, these non-molecular techniques generally do not have sufficient sensitivity and specificity, and molecular techniques are required to confirm the presence of genes encoding metallobetalactamases[17]. Polymerase chain reaction (PCR) is a molecular technique with high specificity and sensitivity for rapidly detecting metallobetalactamase coding genes. In addition, typing methods can help to trace the transmission of pathogens, identify the source of infection, and classify *Enterobacteriaceae* strains by decreasing antibiotic sensitivity[18,19]. Determining the frequency of metallobetalactamases in *Enterobacteriaceae* isolates and their typing in different populations plays a significant role in controlling and improving the

performance of the healthcare system. The results of the study will be important from this point of view. Therefore, according to the mentioned contents, the present study aimed to determine the phenotypic and genotypic expression levels of metallobetalactamase coding genes (*bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{SIM}$, *bla*$_{SPM}$, and *bla*$_{GIM}$) in *Enterobacteriaceae* isolates. (*Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, and *Serratia*) isolated from patients referred to the clinical centers of Isfahan city and the typing of these isolates is important for explaining strategies for more accurate and efficient treatment and control of infections caused by these organisms.

Methods

Isolation of *Enterobacteriaceae* isolates: To determine the frequency of metallobetalactamase genes (*bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{SIM}$, *bla*$_{SPM}$, and *bla*$_{GIM}$) in *Enterobacteriaceae* isolates (*Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, and *Serratia*), 3500 different clinical samples were collected from May 2017 to May 2018 from outpatients and inpatients in three teaching hospitals of Isfahan (Amin, Al-Zahra and Beheshti hospitals of Isfahan). A written consent form was obtained from all participants in this study. *Enterobacteriaceae* isolates were often isolated from urine, blood, cerebrospinal fluid, respiratory samples, wound and abscess samples. *Escherichia*, *Enterobacter*, *Klebsiella*, and *Serratia* isolates were identified using standard API 20E phenotypic and biochemical methods[20]. Also, to confirm the identified bacterial isolates, molecular identification using a specific 16SrRNA gene (universal primers AGAGTTTGATCCTGGCTCAG and GGTTACCTTGTTACGACTT) was performed by PCR and sequencing techniques[21]. Sequence similarity was analyzed and confirmed through Nucleotide Blast software in the GenBank database of the NCBI website.

Determining the resistance of *Enterobacteriaceae* isolates using antibiotic sensitivity test: Antibiotic susceptibility testing was determined using the disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI 2021, M100, 31th edition). Antibiotic discs used (Amikacin, Gentamicin, Netilmicin, Tobramycin, Doripenem, Imipenem, Artapenem, Meropenem, Cefepime, Cefotaxime, Ceftazidime, Ciprofloxacin, Piperacillin/tazobactam, Ticarcillin/clavulanic acid, Ampicillin sulbactam, Amoxicillin clavulanic acid, Colistin, Cefazolin, Cefuroxime, Tigecycline, Trimethoprim sulfamethoxazole, Tetracycline, Doxycycline, Minocycline, Fosfomycin, Aztreonam, Chloramphenicol, Ampicillin, Cefotan and Ceftaroline) were obtained from MAST Group Ltd. In addition, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains. All data were analyzed using WHONET5.6 software. The results were interpreted as sensitive, moderately sensitive, and resistant according to the diameter of the area around each antibiotic disk.

Phenotypic determination of metallobetalactamase-producing *Enterobacteriaceae* isolates using E-test: Minimum inhibitory concentration (MIC) using E-test strips of two antibiotics, imipenem and meropenem (Liofilchem, Abruzzi Rosetodegli) for metallobetalactamase-producing isolates, according to CLSI guidelines (CLSI 2021, M100,31th edition) was investigated to determine carbapenem resistance in *Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, and *Serratia* isolates. In this test, *Klebsiella pneumonia* strain ATCC BAA-1705 was used as positive control, and *Klebsiella pneumonia* strain ATCC BAA-1706 was used as negative control.

Phenotypic determination of metallobetalactamase production in *Enterobacteriaceae* isolates using CDST: Simple screening tests were used to detect the production of metallobetalactamase with imipenem-EDTA combined disc test (CDST) in order to check the production of these enzymes. EDTA (Sigma, Germany) is important as an inhibitor of metallobetalactamase enzymes in this test. This test used imipenem discs and 10 µl of 0.5M EDTA. The discs were placed in the Mueller Hinton agar medium cultured with McFarland 0.5 turbidity, and after incubation at 37 ˚C, the diameter of the non-growth halo (inhibition zone) around the two discs was measured. An increase in inhibition zone greater than or equal to 7 mm in the presence of imipenem disc with EDTA compared to imipenem disc alone is considered a positive test for the presence of beta-lactamases. *Klebsiella pneumoniae* strain OA-8053 was used as a positive control[22].

Phenotypic determination of metallobetalactamase production in *Enterobacteriaceae* isolates using

modified Hodge test (MHT): In this test, the standard *Escherichia coli* ATCC 25922 (antibiotic-sensitive strain) was cultured on Mueller Hinton's medium, and an Ertapenem disc was placed in the center of the plate. Then, the investigated bacteria (*Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, and *Serratia*) were cultured in a straight line from the side of the plate to the edge of the disc, and the plate was incubated for 24 hours at 37 ˚C. MHT-positive isolates are identified after 24 hours as a halo of clover leaf growth from the standard strain of *Escherichia coli* 25922 ATCC. The growth of the standard strain of *Escherichia* towards the Ertapenem disk is due to the inhibition of the antibiotic effect of Ertapenem by metallobetalactamase-producing bacteria[23].

Genotypic determination of metallobetalactamase production in *Enterobacteriaceae* isolates using polymerase chain reaction (PCR): The genomic DNA of each isolate was extracted using the Korea GeneAll Exprep kit (GeneAll Biotechnology), according to the manufacturer's instructions to determine the genotypic production of metallo-beta-lactamase in *Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, and *Serratia* isolates. The quality and quantity of the extracted DNA samples were checked using electrophoresis and Nanodrop device (ND-1000; Nanodrop Technologies)[24]. PCR test was performed using specific primers for genes encoding *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{SIM}$, *bla*$_{SPM}$, and *bla*$_{GIM}$ (Table 1).

Table 1: Primers used to perform the PCR reaction.

Target	Primer sequence	size (bp)	Ref.
bla$_{VIM}$	TTTGGTCGCATATCGCAACG CCATTCAGCCAGATCGGCAT	390	58
bla$_{SIM}$	TACAAGGGATTCGGCATCG TAATGGCCTGTTCCCATGTG	570	59
bla$_{SPM}$	AAAATCTGGGTACGCAAACG ACATTATCCGCTGGAACAGG	271	60
bla$_{IMP}$	GGAATAGAGTGGCTTAAYTCTC GGTTTAAYAAAACAACCACC	232	60
bla$_{NDM}$	GAAGCTGAGCACCGCATTAG GAAGCTGAGCACCGCATTAG	761	61
bla$_{GIM}$	TCGACACACCTTGGTCTGAA AACTTCCAACTTTGCCATGC	477	62

Klebsiella pneumonia strain ATCC BAA-1705 was used as the positive control, and *Klebsiella pneumonia* strain ATCC BAA-1706 was used as the negative control. The PCR program included initial denaturation at 95 ˚C for 5 minutes (35 cycles), 94 ˚C for 30 seconds, 58 ˚C for 90 seconds, and 72 ˚C for 10 minutes. Electrophoresis was used to analyze the PCR products. Sequencing of PCR products was also investigated to confirm the production of metallobetalactamase enzymes in *Enterobacteriaceae* isolates.

Molecular typing using PCR based on the sequence of repetitive elements (rep-PCR): Molecular typing and clonal relationship of metallobetalactamase-producing *Enterobacteriaceae* isolates with the PCR technique based on the sequence of repetitive elements (rep-PCR) were used to determine the genotype of these isolates. For this purpose, the primer pair REP 1 (5'-ATGTAAGCTCCTGGGATTCAC-3') and REP 2 (5'-AAGTAAGTGACTGGGGTGAGCG-3') was used[25]. Amplicon fragments were separated using 2% agarose gel electrophoresis with a 100 bp DNA size marker (Sigma Chemicals, Ontario, Canada). After staining, the amplicons were visualized under UV, and the band patterns of each strain were recorded using Gel Doc (Biorad, Hercule, CA, USA). GelCompar (version 6.6) analyzed the rep-PCR patterns and determined their molecular typing relationship. The similarity of strains was determined using the Dice coefficient for comparison and the unweighted paired group method with arithmetic means (UMGMA) for clustering.

Statistical survey: All statistical analysis was done with SPSS version 22 software, GraphPad Prism 8, and Microsoft Excel 2010 software. In this study, the chi-square test was performed to compare qualitative variables. The test of diagnostic value criteria, including sensitivity, specificity, and 95% confidence interval, was calculated. P value less than 0.05 was considered statistically significant. All antibiotic resistance information was analyzed using WHONET software (version 5.6). This study used the chi-square and Fisher test to compare qualitative variables. These two tests were used to check the pattern of antibiotic resistance.

This study was approved by the Ethics Committee of the Islamic Azad University of Shiraz, Shiraz, Iran (IR.IAU.SHIRAZ.REC.1299.037).

Results

Bacterial isolates: Of 3500 collected clinical samples, 580 were culture-positive for *Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, and *Serratia* isolates. A number of 215 (37%) *Escherichia* isolates, 240 (41.3%) *Klebsiella* isolates, 70 (12%) *Enterobacter* isolates, 28 (4.8%) *Citrobacter* isolates, and 18 (3.1%) *Serratia* isolates were isolated. Nine (1.5%) isolates included other members of the *Enterobacteriaceae* family. In this study, 350 *Enterobacteriaceae* isolates were isolated from men and 230 isolates from women. The highest frequency of isolated samples was from patients with an average age of more than 60 years. The frequency of *Enterobacteriaceae* isolates collected according to the type of clinical sample shows that 297 (51.2%) isolates from urine, 139 (23.9%) isolates from respiratory samples, 76 (13.1%) isolates from blood, 15 (2.5%) isolated from cerebrospinal fluid, 38 (6.5%) isolated from wound or abscess and 12 (2%) isolated from other clinical samples. The distribution of bacteria had significant differences depending on the type of sample ($P<0.001$). Among these samples, the highest number of isolates of *Enterobacteriaceae* was isolated from the urine sample (51.2%). Also, *Escherichia* was the most isolated *Enterobacteriaceae* isolated from urine samples.

Antibiotic sensitivity test: The antibiotic sensitivity test results of *Escherichia*, *Enterobacter*, *Citrobacter*, *Serratia*, and *Klebsiella* isolates show that the most effective antibiotics against *Escherichia* isolates include amikacin (95.8%). Also, the highest resistance of *Escherichia* isolates to ampicillin (94.9%) was reported. The most effective antibiotics against *Enterobacter* included imipenem (97.8%) and meropenem (97.6%). The highest resistance was also seen against cefuroxime (95.9%) and amoxicillin (97.2%). In addition, the most effective antibiotics against *Citrobacter* include meropenem (92.6%), and the most resistance against ampicillin and cefuroxime (96.4%) and ticarcillin/clavulanic acid (82%) was determined. Similarly, the most effective antibiotics against *Serratia* include meropenem (93.4%) and the most resistant against ampicillin and cefuroxin (94.4%). The results of the resistance pattern of *Klebsiella* isolates also showed that the highest level of resistance was observed against ampicillin (98%) and piperacillin (98%), followed by cefazolin (97.9%), aztronam (91.8%), amoxicillin/clavulanic acid (91.7%). Fisher's exact and chi-square tests showed a significant relationship ($P<0.001$) between the resistance and the type of bacteria.

Results of MIC determination using E-test: MIC test for carbapenem family antibiotics, including imipenem and meropenem, was performed on isolates of *Escherichia*, *Enterobacter*, *Citrobacter*, *Serratia*, and *Klebsiella* producing metallobetalactamase using E-test strips. The results showed insensitivity to imipenem and meropenem antibiotics (range 6 to more than 32 µg/ml) in 9 (4.1%) *Escherichia* isolates. Eighty-one isolates of *Klebsiella* (33.7%) and four isolates of *Enterobacter* (5.7%) also showed complete resistance to carbapenem family antibiotics (*Klebsiella* in the range of 0.064 to 64 µg/ml, *Enterobacter* in the range of 8 to more of 32 µg/ml). 3.5% and 5.5% showed complete resistance to carbapenem family antibiotics in one isolate of *Citrobacter* and *Serratia*, respectively. The distribution of the range of MIC value for imipenem and meropenem in terms of micrograms per milliliter in the case of *Citrobacter* and *Serratia* strains has shown that it is variable in the range of 24 to more than 32 micrograms per milliliter. In general, according to the results extracted from the WHONET software, the prevalence of MDR, XDR and PDR *Enterobacteriaceae* isolates related to *Escherichia* (59.53% MDR and 1.5% XDR), *Klebsiella* (60% MDR, 3% XDR, and PDR 0.8%), *Enterobacter* (MDR 44%), *Citrobacter* (MDR 53.5%) and *Serratia* (MDR 55.5%) which were resistant to carbapenems, has been shown according to the CLSI model.

Production of metallobetalactamase enzymes in *Enterobacteriaceae* isolates based on phenotypic investigation: The results of the imipenem-EDTA combined disc test (CDST) for the phenotypic detection of metallobetalactamase enzyme production confirm the presence of these enzymes in four *Escherichia* isolates (1.8%) and 25 *Klebsiella* isolates (10.4%). In addition, the results of the modified Hodge test (MHT) showed the phenotypic production of metallobetalactamase enzymes for 47 *Escherichia* isolates (1.8%), two *Enterobacter* isolates (2.8%) and 25 *Klebsiella* isolates (10.4%).

Production of metallobetalactamase enzymes in

***Enterobacteriaceae* isolates based on the genotypic investigation:** The results of the PCR test to check the presence of *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{SIM}$, *bla*$_{SPM}$, and *bla*$_{GIM}$ genes in all carbapenem-resistant *Enterobacteriaceae* isolates showed that the three *bla*$_{SIM}$, *bla*$_{SPM}$ genes, and *bla*$_{GIM}$ were not present in any of the examined isolates. In contrast, in *Klebsiella* isolates the presence of *bla*$_{VIM}$ genes in 11 (4.58%) isolates (GI:1812714301, GI:1812714303, GI:1812714305, GI:1812714306, VGI:1812714307, GI:1812714308, GI:1812714310, GI:1812714310). 2714312, GI:1812714314 and GI:1812714318), *bla*$_{IMP}$ in 2 (0.83%) isolates (GI:1784624133, GI:1784624134), and *bla*$_{NDM}$ in 2 (0.83%) isolates (GI:1784624137, GI:1784624138) were identified and registered in the NCBI gene database. Also, in *Escherichia* isolates, *bla*$_{VIM}$, *bla*$_{NDM}$, and *bla*$_{IMP}$ genes were identified in 3 (1.39%) isolates and deposited in the gene database. No metallobetallactamase gene was detected in the rest of the *Enterobacteriaceae* species.

Molecular typing of *Enterobacteriaceae* isolates producing metallobetalactamase genes: The results of rep-PCR for 15 *Klebsiella* isolates producing metallobetalactamase genes showed that the dominant fragments in the gel with sizes of 300, 600, and 1500 bp were determined in *Enterobacteriaceae* isolates and the size of the bands varied from 300 to 1500 bp. The results showed that 11 *Klebsiella* isolates producing metallobetalactamase gene are in 4 main groups. In this way, two isolates producing the metallobetalactamase gene are in the same clonal group, and nine isolates containing the metallobetalactamase gene are in a separate clonal group. Also, in other isolates of *Enterobacteriaceae* resistant to metallobetalactamase, the results of the rep-PCR method showed that nine isolates of *Escherichia* and four isolates of *Enterobacter* were classified into two main clusters and had 100% homology. The homology of *Citrobacter* with *Enterobacter* was 65%, *Escherichia* 55%, and *Serratia* 50%. *Serratia* had the least homology with the rest of the isolates and was placed in the branch of separation (50% homology). The homology of *Escherichia*, *Enterobacter*, and *Citrobacter* was equal to 55%. The clonal correlation of carbapenem-resistant isolates showed distinct clusters that indicate the different nature of these isolates compared to similar isolates. Therefore, according to these results, two main clusters were obtained. Dominant fragments in the gel with sizes of 300, 600, and 1500 bp were determined in *Enterobacteriaceae* isolates, and the size of the bands varied from 300 to 1500 bp. Band sizes for *Escherichia* were 300, 600, and 800 bp. *Serratia* and *Citrobacter* species showed 300 bp. *Enterobacter* species showed bands of 300, 600, 800, and 1500 bp sizes. The gel electrophoresis image of rep-PCR is shown in Figure 1.

Discussion

Infections caused by *Enterobacteriaceae* have long been considered one of the severe challenges of the healthcare system. These organisms quickly become resistant to a wide range of antibiotics, and the infection caused by them is associated with high morbidity and mortality in many cases[26]. Currently, the β-lactam ring of carbapenem antibiotics is considered one of the main options for treating multidrug-resistant bacterial infections[27]. However, the emergence of resistant *Enterobacteriaceae*-producing carbapenemase enzymes, especially metallobetalactamases, has greatly reduced the effectiveness of carbapenems, and the rapid spread of these resistant organisms is a significant threat to the clinical use of all beta-lactam antibiotics[28]. In several studies, the importance of the relationship between nosocomial infections and the frequency of *Enterobacteriaceae* during sudden outbreaks in the hospital has been pointed out.

In many cases, the hospital environment has been a source of infection, and investigating the role of the environment in causing infection is important and necessary to provide strategies that lead to the reduction of contamination and the spread of pathogens[28]. One of the important strategies to prevent the spread of resistant *Enterobacteriaceae* is the accurate and rapid identification of people carrying these bacteria in the hospital and community[29,30]. Therefore, determining the frequency of metallobetalactamases in *Enterobacteriaceae* isolates and their typing in different populations can play a vital role in improving the performance of the healthcare system. Therefore, the present study aimed to determine the phenotypic and genotypic expression levels of metallobetalactamase coding genes (*bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{SIM}$, *bla*$_{SPM}$, and *bla*$_{GIM}$) in

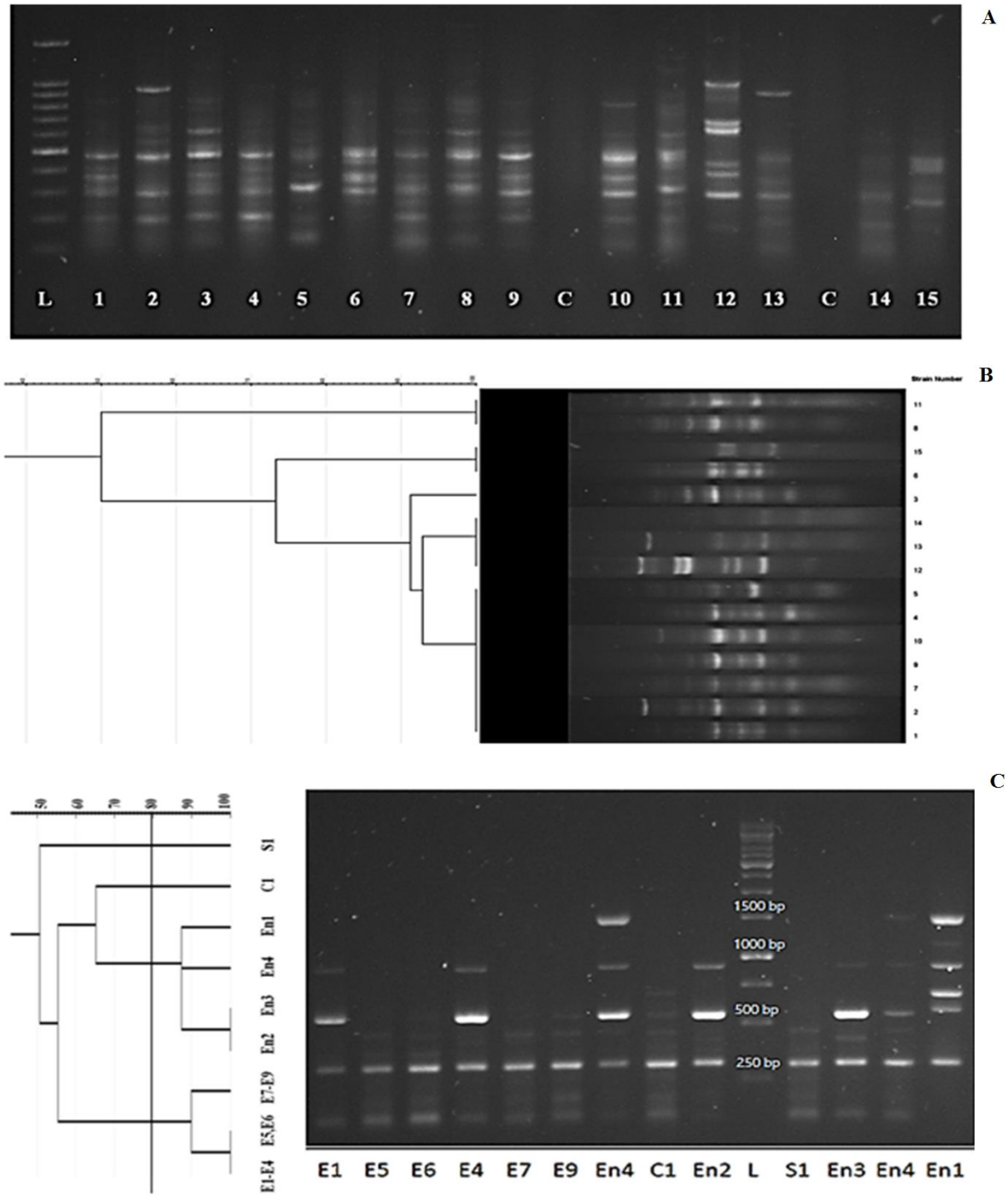

Figure 1. rep-PCR typing for *Enterobacteriaceae* isolates producing metallobetalactamase genes. A) The number of bands and their location similarity between *Klebsiella* isolates; B) Dendrogram drawn for rep-PCR performed in *Klebsiella* isolates containing metallobetalactamase genes; C) rep-PCR patterns and dendrograms drawn for rep-PCR performed in *Escherichia*, *Serratia*, *Citrobacter*, and Enterobacter isolates. E: *Escherichia*; En: *Enterobacter*; C: *Citrobacter*; S: *Serratia*; L: Ladder 250 bp.

Enterobacteriaceae isolates (*Escherichia*, *Enterobacter*, *Citrobacter*, *Klebsiella*, and *Serratia*) isolated from patients referred to the clinical centers of Isfahan city and typing of these isolates, which is important to explain strategies for more accurate and efficient treatment and control of infections caused by these organisms and to reduce the spread of their resistance. For this purpose, it is essential to determine

the pattern of antibiotic resistance to commonly prescribed antibiotics in *Enterobacteriaceae* isolates isolated from patients in different populations[28].

In this study, 3500 clinical samples were collected from three hospitals of Isfahan City (Al-Zahra, Beheshti, and Amin Isfahan) during 12 months of study, 580 samples were cultured positive for *Enterobacteriaceae* (37% *Escherichia*, 41.3% *Klebsiella*, 12% *Enterobacter*, 8.4% *Citrobacter*, and 1.3% *Serratia*). Examining the antibiotic resistance pattern of these isolates showed that in the isolates of *Escherichia*, *Enterobacter*, *Citrobacter*, *Serratia*, and *Klebsiella*, the most effective antibiotics include meropenem and the most resistance is also against cefuroxime, ampicillin, and amoxicillin. Many studies in Iran have investigated antibiotic resistance patterns in *Enterobacteriaceae*. The resistance pattern identified in this study aligns with the results of various studies that have confirmed the effectiveness of these antibiotics for treating *Enterobacteriaceae* infections. For example, the high resistance of *Enterobacteriaceae* to ampicillin, cefuroxime, ceftazidime, and cefotaxime was reported in a study from Tehran, where 100% sensitivity to colistin was determined in *Klebsiella* strains resistant to carbapenems[31]. As mentioned, the prevalence of resistant *Enterobacteriaceae*-producing carbapenemase enzymes, especially metallobetalactamase, greatly reduces the effectiveness of carbapenems (one of the most important prescribed antibiotics for the treatment of multidrug-resistant bacterial infections), and examining the prevalence of these organisms is important to provide solutions for more efficient control in communities[28].

Investigating resistance to carbapenem family antibiotics, including imipenem and meropenem in *Escherichia*, *Enterobacter*, *Citrobacter*, *Serratia* and metallobetalactamase-producing *Klebsiella* isolates in this study showed that there is high resistance to these antibiotics and the frequency of *Enterobacteriaceae* isolates with multiple resistances Drug (MDR, XDR and PDR) is significant among members of this family. In other words, in *Escherichia* isolates, the frequency of MDR is 5.59%, and XDR is 5.1%. MDR 60%, XDR 3%, and PDR 0.8% were observed in *Klebsiella* isolates, and MDR frequency was reported in *Enterobacter*, *Citrobacter*, and *Serratia* isolates as 44%, 53.5%, and 55.5%, respectively. Similarly, many studies have investigated the prevalence of multidrug-resistant *Enterobacteriaceae*. A study examining 3248 clinical samples showed that the frequency of MDR strains among 109 *Citrobacter* isolates was 89 cases[32]. The frequency of MDR *Escherichia* strains 45.5%, MDR *Klebsiella* 98.6%, and MDR *Enterobacter* 53% has been reported[33-36]. The difference in the pattern of antibiotic resistance and the prevalence of multidrug-resistant *Enterobacteriaceae* in different communities is largely influenced by personal and social health conditions, geographic conditions of regions, lifestyle, and the amount of use of broad-spectrum antibiotics. In addition, the differences can be partly related to the difference in infection control committee programs and surveillance systems to identify and treat carriers and differences in race and geographic region. Therefore, it is essential to study the prevalence of resistant *Enterobacteriaceae* with different phenotypic and genotypic techniques for better diagnosis and more accurate control of infections caused by these organisms in different communities[37,38]. As mentioned before, the production of metallobetalactamase enzymes is one of the most important mechanisms of *Enterobacteriaceae* resistance to carbapenems, such as imipenem and meropenem[39].

Phenotypic detection of the presence of metallobetalactamase enzymes in *Enterobacteriaceae* isolates by CDST and MHT methods has been used in laboratories in the past. This study, CDST results showed that 1.8% of *Escherichia* isolates and 10.4% of *Klebsiella* isolates produce metallobetalactamase enzymes. In addition, MHT test results confirmed the production of metallobetalactamase enzymes for 1.8% of *Escherichia* isolates, 2.8% of *Enterobacter* isolates, and 10.4% of *Klebsiella* isolates phenotypically. Most studies in Iran have investigated the prevalence of metallobetalactamases in *Klebsiella* strains, and some have investigated the frequency of these enzymes in *Escherichia* strains, and other *Enterobacteriaceae* have received less attention. For example, using phenotypic tests, the frequency of metallobetalactamase-producing *Klebsiella* and *Escherichia* isolates has been reported as 7.9% and 7%, respectively, in Iran[40,41]. It has been seen that the use of phenotypic methods to identify metallobetalactamase-producing *Enterobacteriaceae*

isolates may increase the probability of a false positive result[42,43]. Therefore, the use of molecular techniques for faster and more accurate identification of the prevalence of metallobetalactamase enzymes is important. In this study, in order to investigate the presence of *bla*$_{VIM}$, *bla*$_{NDM}$, *bla*$_{IMP}$, *bla*$_{SIM}$, *bla*$_{SPM}$, and *bla*$_{GIM}$ genes in all carbapenem-resistant *Enterobacteriaceae* isolates, genotypic tests were performed using PCR and sequencing. The three genes *bla*$_{SIM}$, *bla*$_{SPM}$, and *bla*$_{GIM}$ were absent in any examined *Enterobacteriaceae* isolates. However, the presence of *bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{NDM}$ genes was confirmed in *Klebsiella* and *Escherichia* isolates. The rest of the *Enterobacteriaceae* isolates did not show the presence of any metallobetallactamase genes. In recent years, many reports of different *Enterobacteriaceae* strains producing metallobetalactamase enzymes carried on plasmids have been presented worldwide. Although these enzymes are mostly found in *Klebsiella* and *Escherichia* strains, there are also reports of these enzymes in other bacteria of the *Enterobacteriaceae* family, including *Enterobacter*, *Citrobacter*, and *Serratia*[44,45]. In India, in 108 *Enterobacteriaceae* isolates, including 59 *Klebsiella* isolates, 52 *Escherichia* isolates, 19 *Enterobacter* isolates, and 3 *Citrobacter* isolates, the prevalence of *blaNDM* was reported as 45.3% [46]. In addition, a study reported 18 isolates of *Enterobacteriaceae* producing *bla*$_{NDM}$. Six of 18 isolates encoding *bla*$_{NDM}$ belonged to *Klebsiella*, five to *Escherichia* isolates, four to *Acinetobacter* isolates, and three to *Enterobacter* isolates[47].

In another study, the frequency of carbapenem-resistant *Enterobacteriaceae* in hospitalized patients was reported to include *Klebsiella* 71.4%, *Escherichia* 23.8%, and *Enterobacter* 4.8% respectively, and 20 isolates were metallobetalactamase producers, of which 10% 25% and 5% were positive for *bla*$_{IMP}$, *bla*$_{VIM}$ and *bla*$_{NDM}$, respectively[48]. In another study, using the disk diffusion method, it was determined that all isolates of *Enterobacteriaceae* investigated, except for one isolate, were resistant to at least three classes of antibiotics (cephalosporin, carbapenem, and fluoroquinolone) and were reported as MDR. In addition, 29 of 32 isolates had at least one metallobetalactamase gene, with *bla*$_{NDM}$ being the most abundant[49]. Also, by examining 160 *Escherichia* isolates, five imipenem-resistant isolates that did not encode any of the *bla*$_{IMP}$, *bla*$_{VIM}$, and *bla*$_{NDM}$ genes were identified[50]. In a survey of 83 medical centers in Europe and America, the prevalence of *Escherichia* strains producing metallobetalactamase was reported as 0%[51]. The difference in the prevalence of antibiotic resistance coding genes, especially metallobetalactamase genes, can be influenced by factors such as the difference in the source of isolation of organisms, the studied population, the pattern of use of antibiotics prescribed by doctors in Different countries and the availability of non-prescription antibiotics[52]. Therefore, investigating the clonal relationship of different isolates of resistant *Enterobacteriaceae* with typing techniques to investigate the source of infection and types circulating in the community can be very important for improving communities' health monitoring and control system.

This study also performed a rep-PCR typing technique to investigate the genetic relationship of *Enterobacteriaceae* isolates producing metallobetalactamase. The results of this technique for 15 *Klebsiella* isolates producing metallobetalactamase genes showed that 11 *Klebsiella* isolates belong to four main groups. Also, in other isolates of *Enterobacteriaceae* resistant to metallobetalactamase, investigation by rep-PCR technique showed that nine isolates of *Escherichia* and four isolates of *Enterobacter* are classified in two main clusters and have 100% homology. The homology of *Citrobacter* with *Enterobacter* was 65%, *Escherichia* 55%, and *Serratia* 50%. *Serratia* had the least homology with the rest of the isolates and was placed in the branch of separation (50% homology). The homology of *Escherichia*, *Enterobacter*, and *Citrobacter* was equal to 55%. Clonal correlation of carbapenem-resistant isolates identified distinct clusters that indicate the different nature of these isolates compared to similar isolates. The simultaneous presence of carbapenemase genes in bacteria has caused many concerns for the healthcare system because most beta-lactam antibiotics hydrolyze as one of the main treatment options. In addition, the transmission of these genes between carriers and hospitalized patients increases their prevalence, which makes control strategies difficult[53,54]. Until now, other studies have been done using this method in order to classify *Escherichia* isolates. Investigating the genetic relationship of 98

*Escherichia i*solates with the rep-PCR technique showed 70% homology in six groups of these isolates[55,56]. In general, according to the results of the previous studies and present studies' results, the rep-PCR technique has an acceptable discriminative power in the genotyping of *Enterobacteriaceae* isolates, especially *Escherichia* and *Klebsiella*[57].

Conclusion

The results of the present study show the prevalence of *Klebsiella* and *Escherichia* isolates as metallobetalactamase-producing *Enterobacteriaceae* in clinical samples collected from three hospitals in Isfahan (Amin Isfahan, Al-Zahra, and Beheshti). In addition, the results show the abundance of MDR strains in the most common isolates of *Enterobacteriaceae*, *Klebsiella*, and *Escherichia*. On the other hand, it confirms the importance of metallobetalactamase genes bla_{IMP}, bla_{VIM}, and bla_{NDM} in resistance to carbapenems in *Klebsiella* and *Escherichia*. Therefore, these genes are essential in the horizontal transfer of antibiotic resistance identification of isolates producing metallobetalactamase in clinical environments to prevent these resistant isolates further, reduce the spread of antibiotic resistance, reduce hospitalization rates, and reduce treatment costs. The high homology of resistant isolates of *Enterobacteriaceae* in clinical samples also indicates the high power of these genotypes in causing infection and colonization in hospitalized patients, which can play an important role in increasing antibiotic resistance and the quality of the country's healthcare system. Therefore, it is necessary to pay special attention to monitoring, identification, and screening protocols to prevent the spread of these important isolates in different communities.

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Conflict of interest

The authors further declare that they have no conflict of interest.

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