Prevalence of Metallo-β-lactamases Producing *Escherichia coli* Isolated from North of Palestine

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ABSTRACT

Objective: This study aimed to determine the prevalence of Metallo-β-lactamases (MBLs) among clinical *E. coli* isolated from North of Palestine.

Methods: A total of 79 of *E. coli* isolates were recovered from hospitals in North of Palestine during February-July 2015. A multiplex PCR was used to determine the prevalence of MBLs among these isolates.

Results: Results showed that the prevalence of MBLs was 87.4%. The *spm* gene was the most prevalent (86.1%) among these isolates. According to the geographical distribution, results showed that the prevalence of MBLs among *E. coli* isolates recovered from Thabet Hospital-Tulkarm was 75.9%. The *Spm* gene was the most common (72.4%) among these isolates, while the *Imp* gene was detected in 41.4% of isolates. For isolates recovered from Jenin Hospitals, the prevalence was 94% and *spm* gene was detected in all MBL producers, 2 other genes were detected, *Imp* and *Sim,* and the prevalence was 12% and 2%, respectively. A total of 26.1% of MPL *E. coli* producers carried 2 genes.

Conclusions: Our results showed high occurrence of MBLs among *E. coli* isolates in Palestine. Based on these results we recommend the continuous monitoring and surveillance of the prevalence, proper control and prevention practices and effective antibiotic use will limit the further spread of MBLs producing isolates within hospitals in this country. Prevalence of MBL producing microorganisms has not been investigated previously.

KEY WORDS: Metallo-β-lactamases
E. coli
MBLs
Palestine

Introduction

Metallo-beta-lactamase (MBL) producing Gram-negative bacteria are being reported with increasing frequency from several countries and have emerged as a most widespread and clinically significant carbapenem resistance mechanism [1]. Metallo-β-lactamase producing pathogens can hydrolyze all types of β-lactams including penicillins, cephalosporins, carbapenems, cephamycins, except monobactams as aztreonam [2]. In addition, their catalytic activities are not affected by available β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam [3]. These enzymes belong to Ambler class B β-lactamases based on their amino acid sequence homology and to group 3 according to the Bush classification based on their substrate and inhibitor profiles [2]. Class B β-lactamase are carbapenemases type which depend on zinc-ions to hydrolyze the β-lactams and due to this, the presence of divalent cation-chelating agents such as EDTA, MBL catalysis is inhibited [4,5].

Metallo-β-lactamases enzymes are either chromosomally encoded (resident MBLs) mainly in environmental bacteria or opportunistic pathogens or clinically relevant metallo-β-lactamases are carried on highly mobile genetic elements allowing easy dissemination (acquired or plasmid mediated MBLs) [6,7]. The MBLs which are produced by environmental bacteria, could be precursors for the efficient MBLs present in clinical pathogens that are exposed to increasing doses of antibiotics [8].

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To date, nine different MBL types have been identified in Gram-negative bacilli, namely, IMP-like, VIM-like, GIM-1, SIM-1, SPM-1, KHM-1, AIM-1, NDM and DIM-1[9]. The most widespread MBLs include IMP, VIM, and NDM [5]. The Indian subcontinent, the Balkans regions, and the Middle East are considered to be the main reservoirs of NDM producing pathogens [10].

Metallo-β-lactamases were common in *Pseudomonas aeruginosa* and *Acinetobacter* spp., recently they have emerged at an increasing rate among different members of

Enterobacteriaceae such as *Escherichia coli* and *Klebsiella pneumoniae* [11,12]. In 2011, outbreaks of NDM-1-producing *E. coli* and *Klebsiella pneumoniae* isolates have been obtained from patients hospitalised in four healthcare facilities in northern Italy [13]. Recently, a study in Nepal showed that the incidence of MBL among *E. coli* recovered from different clinical isolates was 18.98% [12]. Several studies in India showed the prevalence of MBL producing *E. coli* ranged from 1.7%-15% [14-18]. In addition, in India it was shown that 50%-81.8% of *E. coli* resistant/intermediate to carbapenems were MBL producers [19-21]. In Pakistan, it was reported that 71% carbapenem-resistant *E. coli* were MBL producers [22]. In a PCR-Based Nosocomial Surveillance Study in Puerto Rico[23], it was found 5.1% of *E. coli* isolates were MBLs producers. In Nigeria, it was found that the prevalence of MBLs among *E. coli* ranged from 12.5%-41.2% [24,25]. In Iraq, 45.2% of *E. coli* isolated from pregnant and non-pregnant women with genital tract infection were MBL producers [26].

NDM MBL producing *E. coli* was detected in Poland [27]. In Algeria, the first three autochthonous cases of infections caused by NDM-5 MBL-producing *Escherichia coli* strains were recovered from urine and blood specimens, these isolates were coexpressed blaCTX-M-15 with the blaTEM-1 and blaaadA2 genes [28]. In China, the first description of IncX3 plasmids carrying NDM-1 β-lactamases in *E. coli* was reported [29]. In Japan, 90.7% of MBL producing *E. coli* carried IMP-6 gene and 9.3% carried IMP-1 gene, all IMP-6-positive isolates were carried CTX-M, 47% of IMP-6-positive isolates were positive for TEM [30]. VIM-1-β-lactamases-producing *E. coli* has been detected in a university hospital in Greece [31]. The first report of IMP-1-producing *E. coli* in Turkey was described by Aktas [32]. In other study in Turkey, 0.0% of multidrug resistant *E. coli* isolated from a tertiary care training and research hospital were producing MBLs [33]. The first report about detection of the *bla*NDM-1 MBL gene in Australia was described previously [34].

This study was conducted to address part of deficient information in molecular antibiotic resistance characterization and their transmissible potential in Palestine. Therefore, this study aimed to determine the prevalence of MBLs among clinical *E. coli* isolated from North of Palestine using the multiplex PCR technique, as this has not been investigated previously.

Materials and Methods

A total of 79 isolates of *E. coli* were isolated from urine samples from Hospitals in the North of Palestine during February-July 2012. A total of 50 isolates were recovered from Hospitals in Jenin and 29 from Thabet Hospital in Tulkarm. Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method [35]. Antibiotic disks (Oxoid) used were Ceftriaxone (CRO) 30µg, Norfloxacin (NOR) 10 µg, Nalidixic acid (30µg), Ciprofloxacin (CIP) 5 µg, Tetracycline (TE) 30µg, Kanamycin (K) 30µg, Trimethoprim/Sulfamethoxazole (SXT) 1.25/23.75µg, Cefotaxime (CTX) 30 µg, Meropenem (MEM) 10 µg, Imipenem (IMP) 10 µg and Ceftazidime (CAZ) 30µg . The plates were incubated at 37°C for 18-24 hrs. The zone of inhibition were measured in millimetres using a caliper. Strains were classified as resistant or susceptible according to the criteria recommended by CLSI guidelines [35].

E. coli DNA was prepared for PCR according to the method described previously [36]. Briefly, cells were scraped off an overnight nutrient agar plate with a sterile loop, washed with 1 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), then the pellet was re-suspended in 0.5 ml of sterile distilled H_2O, and boiled for 10-15 min. The cells then were incubated on ice for 10 min. The debris was pelleted by centrifugation at 11,500 X *g* for 5 min. DNA concentration was determined using a spectrophotometer and the samples were stored at -20ºC until use for further DNA analysis. Polymerase Chain Reaction (PCR) analysis was performed for VIM, IMP, SPM-1, GIM-1 and SIM-1 according to methods described previously [37]. Sequences of primers and size of amplicons are described in Table 1. The PCR reaction mix with a final volume of 25 µL, was performed with 12.5 µL of PCR premix (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$, Sigma), 0.4 µM of each primer and 2 µL of DNA template. The cycling conditions were as the following: initial DNA denaturation at 94 ºC for 5 min, followed by 36 cycles of 94ºC for 30 s, 52ºC for 40 s and 72ºC for 50 s, followed by a single, final, elongation step at 72 ºC

for 5min. The PCR products were detected by electrophoresis through 1.5% agarose gels to determine the size of amplified fragment after ethidium bromide staining.

Table 1. Target genes for PCR amplification, amplicon size, primer sequences and annealing temperature.

Target gene	Primer sequence 5′→3′	Annealing temperature	Amplicon size
Imp	Imp-F GGA ATA GAG TGG CTT AAY TCT C Imp-R CCA AAC YAC TAS GTT ATC T	52ºC	188bp
Vim	Vim-F GAT GGT GTT TGG TCG CAT A Vim-R 5-CGA ATG CGC AGC ACC AG-3	52ºC	390bp
Gim	Gim-F TCG ACA CAC CTT GGT CTG AA Gim-R AAC TTC CAA CTT TGC CAT GC	52ºC	477bp
Spm	Spm-F AAA ATC TGG GTA CGC AAA CG Spm-R ACA TTA TCC GCT GGA ACA GG	52ºC	271bp
Sim	Sim-F TAC AAG GGA TTC GGC ATC G Sim-R TAA TGG CCT GTT CCC ATG TG	52ºC	570bp

ERIC (Enterobacterial repetitive intergenic consensus) PCR was performed using Primer ERIC1: 5′-ATG TAA GCT CCT GGG GAT TCA C-3′ and Primer ERIC2: 5′-AAG TAA GTG ACT GGG GTG AGC G-3′. Each PCR reaction mix was performed in a final volume of 25 µL containing 12.5 µL of PCR premix with MgCl2 (Ready-MixTM Taq PCR Reaction Mix with MgCl2, Sigma), 1 µM of each primer, 3 µL DNA template. In addition, the master mix was modified by increasing the concentration of dNTPs to 0.4 mM , 3 mM MgCl2 and 1.5 U of Taq DNA polymerase. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 2 min at 94°C was followed by 30 cycles of initial denaturation 94°C for 50 s, 50°C for 40 s and 72°C for 1 min, with a final extension step at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1.5 % agarose gel stained with ethidium bromide. The gel images were scored using a binary scoring system that recorded the presence and absence of bands as 1 and 0, respectively. A binary matrix was analyzed by the unweighted pair group method for arithmetic averages (UPGMA), using SPSS statistics software version 20 (IBM).

Results

The antimicrobial susceptibility pattern revealed that the these clinical *E. coli* strains were less resistant (24.1%) to Kanamycine and Ceftriaxone, while showed high resistant to Trimethoprim/Sulfamethoxazle (67.1%) and Tetracycline (60.8%). Data are presented in Table 2.

Table 2. Antibiotic resistance of 79 *E. coli* isolates recovered from different clinical samples-Palestine.

Antibiotic	Resistant strains %
Ciprofloxacin	38.0 %
Trimethoprim/Sulfamethoxazle	67.1%
Ceftriaxone	24.1%
Tetracycline	60.8%
Nalidixic acid	36.7%
Norfloxacin	29.1%
Kanamycin	24.1 %
Cefotaxime	41.8%
Ceftazidime	41.8%
Meropenem	38%
Imipenem	44.3%

Our results showed that the prevalence of MBLs among *E. coli* isolated from North of Palestine was 87.4%. The *spm* gene was the most prevalent (86.1) among these isolates. According to the places, results showed that the prevalence of MBLs genes in *E. coli* isolates recovered from Thabet Hospital Tulkarm using multiplex PCR technique was 75.9%. The *Spm* gene was the most common (72.4%) among these isolates, while the *Imp* gene was detected in 41.4% of isolates. Isolates recovered from Jenin Hospitals, the prevalence was 94% and *spm* gene was detected in all MBL producers. Other 2 genes were detected, *Imp* and *Sim*, and the prevalence was 12% and 2%, respectively. A total of 26.1% of MPL producers carried 2 genes. Data are presented in Table 3 and Figure 1.

Table 3. Distribution of MBL genes among *E. coli* isolated from Jenin and Tulkarm hospitals.

	MBL genes n (%)				
Location (N)	*spm*	*Spm+Imp*	*Spm+Sim*	*Imp*	Total
Jenin (50 isolates)	40 (80)	6 (12)	1 (2)	0 (0.0)	47 (94)
Tulkarm (29 isolates)	10 (34.5)	11 (37.9)	0 (0.0)	1 (3.4)	22 (75.9)
Total (79)	50 (63.3)	17 (21.5)	1 (1.3)	1 (1.3)	69 (87.4)

Multiple β-lactamase producing isolates have been detected (data not shown).

ERIC-PCR analysis of 35 isolates from Jenin Hospitals which carried genes for MBLs were genetically diverse and comprised a heterogeneous population with a total 16 ERICPCR clusters at a (50%) similarity level. Data are presented in Figures 2 and 3.

Figure 1. A. Multiplex PCR profile specific for MBLs. L: Lanes ladder; Lanes 1-4 for *Spm* gene (271 bp), lane 5 for *Spm* gene (271 bp) and *Sim* gene (570 bp), lane 6,7 and 9 for *Imp* gene (188 bp) and *Spm* gene (271 bp) and lane 9 for negative control**.** B. It is the same as A but bands are demarcated to be obvious.

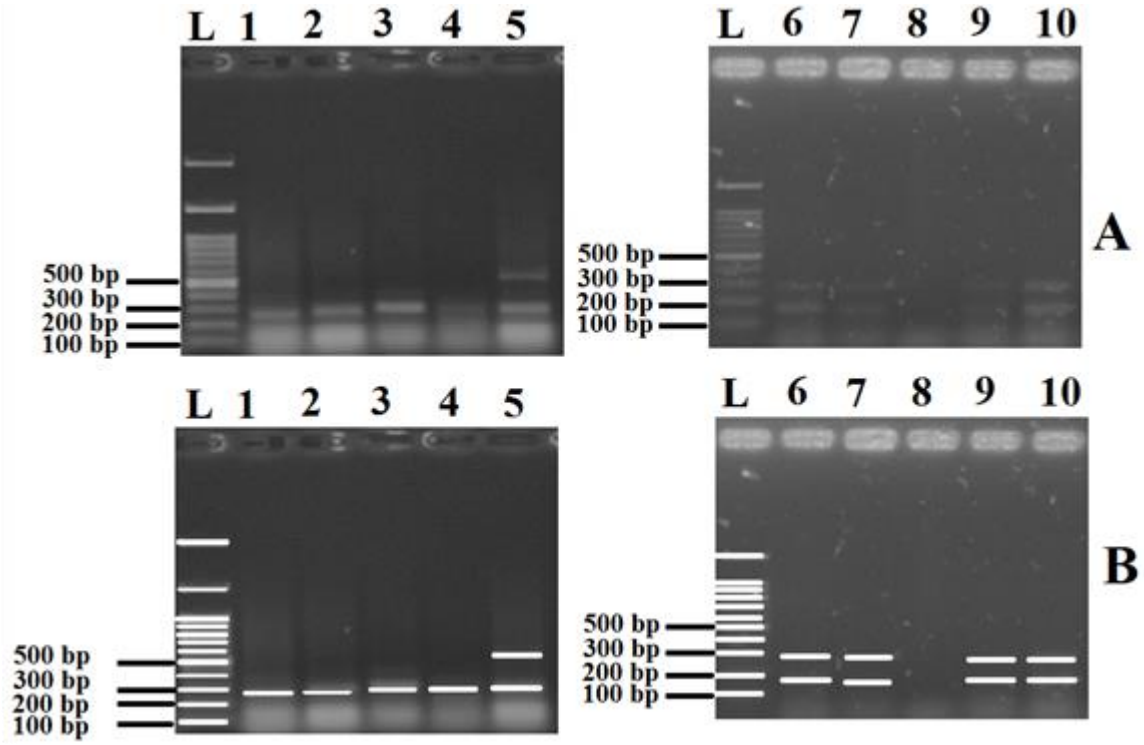
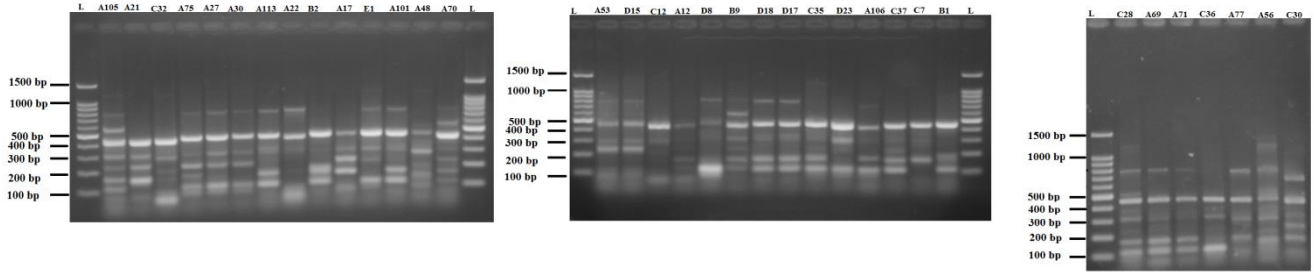


Figure 2. DNA fingerprints generated by ERIC-PCR analysis of 35 clinical *E. coli* isolates carried genes for MBLs recovered on 1.5% garose gel. L: Lanes contained ladder, while other lanes for ERIC-PCR products.



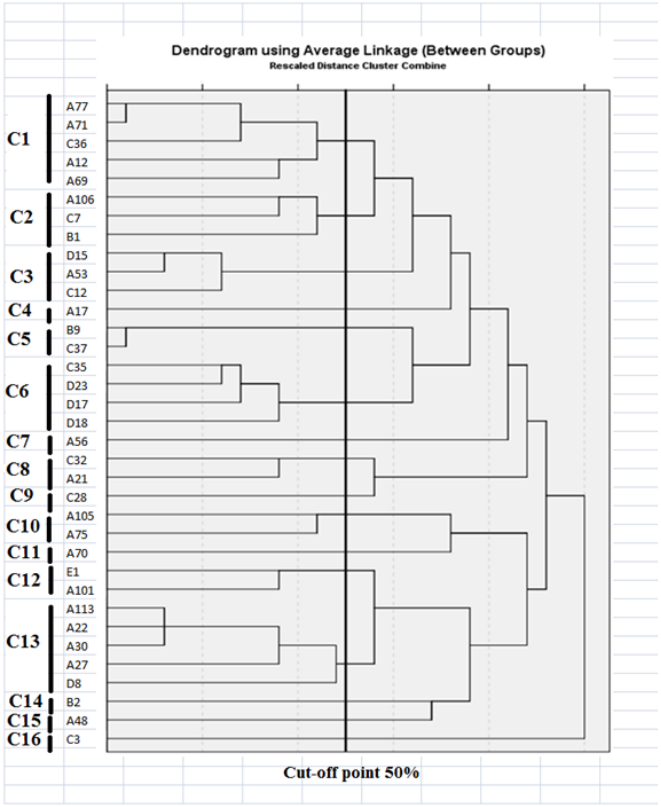
Discussion

The increase in the rates of antibiotic resistance is a major cause for concern in isolates of the *Enterobacteriaceae* family. Beta-lactamses antimicrobial agents are considered one of the main treatment for serious infections. Carbapenems are considered the most active of these antibacterials, which can be used for the treatment of infections caused by ESβL-producing microorganisms, particularly *Escherichia coli* [7]. The early detection of β-lactamase producing microorganisms would be important to avoid the intrahospital dissemination of such strains and to reduce death rates among patients [16].

Studies on carbapenemase producing *E. coli* were very limited when compared to those on non-fermenters. In Palestine there are no studies about MBL producing microorganisms till now. Results of this study showed that the prevalence of MBLs among *E. coli* in North of Palestine using multiplex PCR technique was 87.4%. In other countries, the prevalence of MBL producers *E. coli* ranged from 1.7%-45.2% [12,14,18,24,26]. Finding of this re-

search showed that *Spm* -type MBL was the most common in *E. coli* isolates and this result is inconsistent with other studies in Japan [30], which showed that 100% of MBL producing E. coli carried IMP gene. The most widespread MBLs in other bacterial species include IMP, VIM, and NDM [5].

Figure 3. Dendrogram of 35 *E. coli* isolates carried genes for MBLs based on the UPGMA method derived from analysis of the ERIC-PCR profiles at a 50% similarity level. C: Cluster



The high prevalence of MBLs producers among *E. coli* isolates in Palestine may be due to several risk factors such as long term exposure to antibiotics in hospitals, prolonged hospitalization, incorrect therapy, nursing home residency, severe illness, catheterization and movement of health staff in the hospital leading to dissemination of these pathogens throughout the hospital [38,39]. Results of the study showed a differences between the prevalence of MBLs between the 2 governorates, Tulkarm and Genin, which are both located in North of Palestine. Geographical variation in the occurrence rate of β-lactamases production have been detected from different countries and even from hospital-to-hospital within the same country [40-42]. Multiple β-lactamase has been detected in many of these isolates (data not shown) and a total of 26.1% of MPL producers carried 2 genes, this was an alarming finding. The coexistence of different classes of β-lactamases in a single bacterial isolate may pose therapeutic challenges, this will seriously limited treatment options. In addition, may pose diagnostic challenge that high-level expression of certain β-lactamases such as the AmpC β-lactamases may mask the recognition of the ESβLs and it may result in a fatal and an inappropriate antimicrobial therapy [43]. The presence of AmpC β-lactamases and ESβLs in a single isolate decreases the effectiveness of the β-lactam-β-lactamase inhibitor combinations, while MBLs and AmpC β-lactamases confer resistance to carbapenems [44]. In addition, coexistence of different classes of β-lactamases in a single bacterial isolate poses a serious concern for infection control management, associated with increases in length and cost of hospital stays [23]. Coexistence more than one type of β-lactamases was reported from different species of *Enterobacteriaceae* including *E. coli* [28,30,44,45,46].

The ERIC-PCR typing of MBLs-producing isolates showed various DNA banding profiles. This clonal diversity suggests that most of the strains have been unable to be maintained or spread in different settings of hospital. This observation challenges many conventional thoughts about the nosocomial epidemiology of antibiotic resistance including β-lactamase. These isolates recovered mostly from urine of patients treated mainly in hospitals, sharing significant patient demographics (all isolates used in ERIC-PCR typing are from patients from Jenin Governorate) and isolate characteristics including antibiotic resistance profiles differed. It is clearly indicates that multiple clones of these β-lactamases producing isolates were widespread in these hospitals but not sporadic. This supporting the suggestion that the high rate and extensive inappropriate use of antibiotic especially cephalosporins in the country could be the only major cause [46].

MBLs producing Gram-negative bacteria are an increasing public health problem worldwide because of their resistance to all β-lactams except Aztreonam. In conclusion, our results showed high occurrence of MBLs among *E. coli* isolates in Palestine. Based on these results we recommend the continuous monitoring and surveillance of the prevalence, proper control and prevention practices and effective antibiotic use will limit the further spread of MBLs producing isolates within hospitals in this country.

Conflict of Interest

We declare that we have no conflict of interest.

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