An-Najah National University

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Prevalence and molecular characterization of metallo-β-lactamases in *Pseudomonas aeruginosa* in Palestine

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Dedication

This work is dedicated to…

The memory of my father, who was more than the sky for me.

My mother, for walking with me alongside enlightening the road to be followed.

My sisters and brothers, for giving me the strength to achieve my dream.

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الإقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

Prevalence and molecular characterization of metallo-β-lactamases in *Pseudomonas aeruginosa* in Palestine

أقر بأن ما شملت عليه هذه الرسالة إنّما هو نتاج جهدي الخاص، باستثناء ما تمّت الإشارة إليه حيثما ورد، وأنّ هذه الرسالة ككل، أو أيّ جزء منها لم يقدّم من قبل لنيل أيّ درجة أو لقب علميّ لدى أيّ مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

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Date: **التاريخ:** 17/11/2016

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List of abbreviations

BaCl2	Barium dichloride
CFU	Colony forming unit
CTX	Cefotaxime
CAZ	Ceftazidime
CRO	Ceftriaxone
CIP	Ciprofloxacin
CLSI	Clinical laboratory standards institute
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucloside triphosphate
EDTA	Ethylenediaminetetraacetic acid
ERIC	Enterobacterial repetitive intergenic consensus
ESBL	Extended spectrum beta-lactamase
HCl	Hydrochloric acid
IPM	Imipenem
MBL	Metallo-β-lactamases
MEM	Meropenem
MgCl2	Magniusim chloride
MHA	Mueller Hinton agar
NA	Nalidixic acid
NaOH	Sodium hydroxide
NOR	Norfloxacin
P. aeruginosa	*Pseudomonas aeruginosa*
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
SPSS	Statistical package for the social sciences
SXT	Trimethoprim/Sulfamethoxazole
TAE	Tris-acetate-EDTA buffer
TE	Tetracycline
UPGMA	Unweighted pair group method for arithmetic average

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Abstract

Fifty-one clinical isolates of *Pseudomonas aeruginosa* were obtained from different hospitals and private labs in the West Bank-Palestine, during October 2015- April 2016. Isolates were confirmed in microbiology laboratory at An-Najah National University, Nablus-Palestine. The prevalence of extended spectrum beta-lactamases, metallo-beta-lactamases and integrons was studied by conventional and/or molecular methods. Results showed that 21.6% of *P. aeruginosa* isolates were ESBL producers using conventional methods. The prevalence of metallo-β-lactamases using conventional and molecular methods was 60.8% and 29.4% respectively. The most dominant MBL gene among MBL-producing *P. aeruginosa* isolates was *VIM* gene (60%), while the prevalence of *IMP* and *SPM+VIM* was 33.3% and 6.7% respectively. Neither *GIM* nor *SIM* were detected. The results also showed that 23.5% of *P. aeruginosa* isolates carried class I integrons. Neither class 2 nor class 3 integrons were detected. All isolates harboring MBL genes detected by PCR (15) were analyzed by ERIC-PCR. ERIC-PCR profile, of *P. aeruginosa* isolates harboring MBL genes (15),

showed 4 clones circulating among the hospitals from which isolates were collected.

The present study showed high prevalence of MBLs and ESBLs among clinical isolates of *P. aeruginosa* in the West Bank-Palestine. Based on these results, continuous monitoring and surveillance of the prevalence, proper prevention practices and use of effective antibiotics to limit and restrict the further spread of ESBLs and MBLs producing pathogens including *P. aeruginosa* within and among hospitals in Palestine.

Chapter One
Introduction

1.1. General background

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic, lactose non-fermenting, Gram-negative bacillus of great clinical and epidemiological relevance in hospital-acquired infections. It is a ubiquitous organism present in many diverse environmental settings, and it can be isolated from various living sources, including plants, animals, and humans. In hospitals, *P. aeruginosa* can be isolated from a variety of sources, including respiratory therapy equipment antiseptics, soap, sinks, mops, medicines, and physiotherapy and hydrotherapy pools (Pollack, 1995). *P. aeruginosa* is seldom a member of the normal microbial flora in humans. Representative colonization rates for specific sites in humans are 0 to 2% for skin, 0 to 3.3% for the nasal mucosa, 0 to 6.6% for the throat, and 2.6 to 24% for fecal samples (Morrison and Wenzel, 1984). However, colonization rates may exceed 50% during hospitalization (Pollack, 1995), especially among patients who have experienced trauma to or a breach in cutaneous or mucosal barriers by mechanical ventilation, tracheostomy, catheters, surgery, or severe burns (Ohara and Itoh, 2003; Thuong *et al*., 2003; Erol *et al*., 2004; Valles *et al*., 2004). It is commonly affecting patients with cystic fibrosis, severe burns, neutropenia, and the mechanically ventilated. *P. aeruginosa* infections associated with high case fatality rates (Ajayi *et al*., 2003).

The spread of this pathogen in healthcare settings is often difficult to control, due to the development of intrinsic resistance mechanisms to many antibiotics and acquired mechanisms, which have an ability to develop resistance through mutations in different chromosomal loci or through the horizontal acquisition of resistant genes, which are carried on plasmids, transposons or integrons (Hill *et al*., 2007, Hammami *et al*., 2011). Antipseudomonal β-lactams such as Ticarcillin, Piperacillin, Ceftazidime, Cefepime, Aztreonam, and the carbapenems have an important therapeutic value (Bonomo and Szabo, 2006). Molecular classification based on the amino acid sequence divides β-lactamases into class A, C, and D enzymes, which utilize serine for β-lactam hydrolysis and class B metalloenzymes, which require divalent zinc ions for substrate hydrolysis (Bush and Jacoby, 2010). Class B β-lactamases, termed metallo-β-lactamases, which are the most clinically important carbapenemases because they are capable of hydrolyzing all β-lactam antimicrobial agents, except the monobactam subgroup, and are not susceptible to β-lactamases inhibitors (Walsh *et al.*, 2005). Carbapenem resistance has been observed frequently in *P. aeruginosa* (Gladstone *et al.*, 2005). Currently, the carbapenem hydrolyzing β-lactamases (metallo β-lactamases) belong to the Bush and Jacoby group 3 classification of β-lactamases (Bush and Jacoby, 2010).

The global spread of acquired metallo-β-lactamases (MBLs) has raised serious concern because of their ability to confer resistance of Gram-negative bacteria to a broad spectrum of β-lactam antibiotics, including carbapenems. Infections, caused by such bacteria, are believed to result in

high mortality as well as high healthcare costs and a prolonged hospitalization. Thus, regular monitoring of the incidence of the β-lactamase producing organisms become essential. *P. aeruginosa* producing MBLs was first reported from Japan in 1991 (Watanabe *et al.*, 1991), then, MBLs have been reported from various parts of the world including Asia, Europe, Australia, South America and North America, indicating a wide distribution of these resistance determinants (Maltezou, 2009). To date, nine MBL types, namely, *IMP*-like, *VIM*-like, *SPM*-1, *GIM*-1, *SIM*-1, *AIM*-1, *KHM*-1, *NDM*-1 and *DIM*-1, have been identified in Gram negative bacilli (Hammami *et al*., 2011). These are located within a variety of integron structures, where they have been incorporated as gene cassettes. When these integrons become associated with plasmids or transposons, transfer between bacteria is readily facilitated (Queenan and Bush, 2007).

Five classes of integron are known to play a role in the dissemination of antibiotic resistance, and the most extensively studied was class 1 integrons (Mazel, 2006). MBL encoding genes are usually found as gene cassettes in class 1 integrons (Shibata *et al.*, 2003). Integrons have been detected in multidrug resistant *P. aeruginosa* isolates (Dubois *et al.*, 2002a, b). Integrons are genetic elements encoding the components of a site specific recombination system that recognizes and captures mobile gene cassettes, mostly resistance determinants (Recchia *et al.*, 1995). Such elements may be located within transposons, which in turn contribute actively to the dissemination of resistance determinants to aminoglycosides and β-lactams among Gram-negative species (Partridge *et al.*, 2002). In addition, it has

been demonstrated that integrons and transposons are associated with the spread of resistance to third-generation cephalosporins when they encode extended-spectrum β-lactamases (ESBLs) (Luzzaro *et al.*, 2001).

Enterobacterial repetitive intergenic consensus- (ERIC) PCR is a PCR-fingerprinting technique but it is not arbitrary. The ERIC sequences are present in many copies in the genome of different *Enterobacteriaceae*. ERIC elements are highly conserved at the nucleotide level, their positions in enterobacterial genomes vary between different species and has been used as a genetic marker to characterize isolates within a bacterial species. In ERIC-PCR a band pattern is obtained by amplification of genomic DNA located between successive repetitive ERIC elements or between ERIC elements and other repetitive DNA sequences for subtyping different Gram-negative enteric bacteria (Zulkifli *et al*., 2009).

1.2. Literature review

Worldwide, the *IMP* and *VIM* types are the most commonly detected MBLs in *P. aeruginosa* (Toleman *et al*., 2005; Poirel *et al*., 2001). *VIM*-type MBLs are predominant in the Mediterranean region (Walsh *et al*., 2005; Queenan and Bush, 2007). However, *IMP*-type appears to be the dominant MBL in *P. aeruginosa* isolates from Korea (Ryoo *et al*., 2009; Seok *et al*., 2011).

In Tunisia, the prevalence of Imipenem resistant *P. aeruginosa* varies between studies and has ranged from 16% to 37.6% (Kalai *et al*., 2005; Lamia *et al*., 2007; Hammami *et al*., 2010; Ben Abdallah *et al*., 2008).

Since November 2002, *VIM*-2 producing *P. aeruginosa* has been isolated at Charles Nicolle Hospital of Tunisia, mainly in surgery and intensive care unit (Hammami *et al*., 2010). In Saudi Arabia, it was reported that, *VIM*-2 is the dominant MBL gene in MBL-producing isolates, and the prevalence of MBL was 20.57% of *P. aeruginosa* isolates, and 38.57% of isolates were found resistant to Imipenem (Al-Agamy *et al.*, 2011). In Spain, the prevalence of MBL-producing *P. aeruginosa* was 6.9% and all MBL producers showed high-level resistance to carbapenems (Riera *et al*., 2011). In Korea, *P. aeruginosa* isolates harboring *IMP*-6 also exhibited a higher level of resistance to Meropenem than Imipenem (Seok *et al.*, 2011).

In some countries, such as Korea and Brazil, MBL-producing *P. aeruginosa* constitutes nearly 10% (Gales *et al.*, 2003; Lee *et al.*, 2003a), whereas in USA, the prevalence of MBLs (1%) is still comparatively small (Karlowsky *et al.*, 2003; Aboufaycal *et al.*, 2007). High prevalence of MBL using similar clinical isolates were reported in India, were 28.57% of *P. aeruginosa* found to be MBL producers (De *et al.*, 2010).

Outbreaks of *VIM* β -lactamase-producing *P. aeruginosa* have been also reported in Greece (Tsakris *et al*., 2000), Italy (Cornaglia *et al*., 2000) and Kenya (Pitout *et al.*, 2008). Outbreaks of *IMP*-metallo-β-lactamase *P. aeruginosa* have been also reported in Korea (Ryoo *et al.*, 2009).

The emergence of acquired MBLs among *P. aeruginosa* represents an epidemiological problem for many reasons such as: MBLs confer resistance not only to carbapenems but to virtually all β-lactams and are

frequently associated with resistance to aminoglycosides; and, genes encoding for MBL enzymes are most commonly carried on mobile genetic elements (integrons, plasmids, transposons) that can spread horizontally among unrelated strains (Walsh *et al.*, 2005; Hammami *et al*., 2010).

1.3. Aims of the study

This study was conducted to address part of deficient information in molecular antibiotic resistance characterization and their transmissible potential in the West Bank-Palestine.

Therefore, the specific aims of this study were:

A. To determine the levels and patterns of antibiotic resistance of human *P. aeruginosa* isolates in the West Bank-Palestine, including the prevalence of ESBL and MBL-producing *P. aeruginosa* using conventional and molecular techniques.

B. To assess the prevalence of class 1, 2 and 3 integrons in human *P. aeruginosa* isolates.

C. To study the clone identity among MBL-producing *P. aeruginosa* isolates.

To our knowledge, up to date, this is the first study documented the prevalence and molecular characterization of MBL-producing *P. aeruginosa* isolates in Palestine.

Chapter Two
Materials and Methods

2.1. Bacterial strains and identification

A total of 51 isolates of *P. aeruginosa* were collected from different clinical samples during 2015-2016. The clinical samples included 12 urine, 18 wound swabs, 6 sputum, 1 sputum trap, 4 ear swabs, 1 nasal swab, 3 rectal swabs, 4 skin swabs, 1 blood and 1 abscess. Medical centers and number of isolates from each included in this study were: An-Najah National University Hospital-Nablus (n=12), Rafidia Hospital-Nablus (n=8), Medicare Labs-Nablus (n=1), Patient's Friends Society-Nablus (n=2), Palestinian Medical Complex- Ramallah (n=19), AL-Amal Hospital-Jenin (n=1), Al-shamal Laboratory for Medical Analysis-Jenin (n=1), Ghannam Laboratory for Medical Analysis-Jenin (n=1), Al-Zakat Hospital-Tulkarem (n=1), Thabet Hospital-Tulkarem (n=2), Al-Hussein Hospital-Bet Jala (n=2) and Martyr Yasser Arafat Hospital-Salfeet (n=1). All of these isolates were identified by API 20 E system in laboratories of these centers. The isolates were subcultured in the microbiology laboratory at An-Najah National University on MacConkey agar and Cetrimide agar. Gram stain and oxidase production test were performed for all isolates.

2.2. Media preparation

MacConkey agar (BioMaxima, Poland, catalog number: E081 A), Cetrimide agar (Acumedia, USA, code number: 7222), Muller Hinton agar (Acumedia, USA, code number: 7101) and Tryptone soy broth (Oxoid,

England, code number: CM0129) were prepared according to the manufacturer instructions.

2.3. Chemicals preparation

2.3.1. Eethylenediaminetetraacetic Acid (EDTA 0.5 M, pH 8)

Fifty ml of 0.5M (EDTA, pH 8) was prepared by adding 7.31 gram of EDTA (disodium ethylenediamine tetraacetate•$2H_2O$, Mwt=372.2) powder to 35 ml distilled water and the pH was adjusted to 8 by adding NaOH crystals. Then, the volume was adjusted to 50 ml by adding distilled H_2O. After that, the solution was autoclaved at 121˚C for 15 minute and sorted at room temperature.

2.3.2. Tris-acetate-EDTA buffer (TAE buffer 50X, pH 8)

A total of 100 ml of 50X TAE buffer was prepared by adding 24.2 g Tris base, 5.7 ml glacial acetic acid, 10 ml of 0.5M EDTA (pH=8) and distilled H_2O up to 100 ml. The mixture was stirred until all components were dissolved. The solution was sorted at room temperature and 1X of this solution was used as a working buffer in gel electrophoresis.

2.4. Antibacterial resistance

2.4.1. Preparation of McFarland turbidity standard No. 0.5

The preparation of McFarland 0.5 turbidity standard was carried out by adding 0.05 ml of a 1.175% (w/v) barium chloride dihydrate ($BaCl_2.2H_2O$) solution to 9.95 ml of 1% (v/v) sulfuric acid. To prevent evaporation,

McFarland standard tube was sealed well and stored in dark at room temperature. The accuracy of the density of a prepared McFarland standard was checked by using a spectrophotometer; the absorbance at a wavelength of 625 nm and water was used as a blank standard for the 0.5 McFarland standard must be 0.08 to 0.13. Before use, the 0.5 McFarland standard was vigorously mixed the turbidity using a vortex mixer. A 0.5 McFarland standard is comparable to a bacterial suspension of 1.5 X 10^8 colony-forming units (CFU)/ml (Andrews, 2006). A 0.5 McFarland standard of *P. aeruginosa* suspension was used for detection of phenotype of ESBL and MBL producing isolates.

2.4.2. Antibacterial susceptibility test

Antimicrobial sensitivity testing was carried out according to instructions determined by the clinical and laboratory standard institute (CLSI) using the disk diffusion method (CLSI, 2014). All *P. aeruginosa* isolates were examined using disks (Oxoid) to determine resistance against Ciprofloxacin (CIP) 5µg, Nalidixic acid (NA) 30µg, Cefotaxime (CTX) 30µg, Meropenem (MEM) 10µg, Norfloxacin (NOR) 10µg, Ceftazidime (CAZ) 30µg, Tetracycline (TE) 30µg, Ceftriaxone (CRO) 30µg, Imipenem (IPM) 10µg and Trimethoprim/Sulfamethoxazole (SXT) 1.25/23.75 µg. Mueller Hinton agar plates were seeded with a 6-8 h old culture of the bacterial strains; antibiotic disks were placed on the Muller-Hinton agar plates containing the inoculum. Then, the plates were incubated at 37°C for 24 h. The zones of inhibition were measured and the isolates were

classified as resistant or susceptible according to the criteria recommended by CLSI guidelines (CLSI, 2014).

2.4.3. Detection of ESBL production by phenotypic test

All *P. aeruginosa* isolates showed resistance to 3rd generation cephalosporins were tested for the presence of ESBLs by combination double disk test (CDDT). Normal saline suspensions of all *P. aeruginosa* isolates were adjusted to McFarland's 0.5 standard and heavily subcultured on MHA plates. Four discs namely Ceftazidime (CAZ) 30µg, and Ceftazidime + Clavulanic acid (30/10µg), Cefotaxime (CTX) 30µg, and Cefotaxime + Clavulanic acid (30/10µg) were placed at a distance of 20 mm (centre to centre) on seeded Muller-Hinton agar plates. The plates were incubated for 24 h at 37°C. The presence of an ESBL-producing *P. aeruginosa* was confirmed if an increase in the zone diameter greater than or equal to 5 mm for both Ceftazidime and Cefotaxime tested in combination with Clavulanic acid *vs.* its zone diameter when tested alone (Ibrahim *et al*., 2013).

2.4.4. Detection of MBL production

2.4.4.1. Combined disc diffusion test (CDDT)

Normal saline suspensions of all *P. aeruginosa* strains were adjusted to the McFarland 0.5 standard and used to inoculate MHA plates with zinc sulfate at a final concentration 70µg/ml. Two discs of Imipenem (10µg), Meropenem (10µg) and Ceftazidime (30µg) were placed on inoculated

MHA plate. EDTA solution was added to one of Imipenem (10 µg), Meropenem (10µg) and Ceftazidime (30µg) discs to obtain a concentration of 750µg. Then, the plates were incubated at 37°C for 24 h, the MBL-positive isolates were distinguished from the MBL-negative isolates on the based on the criterion of more than 7 mm increase in the inhibition zone for Imipenem and/or Meropenem or ≥ 4 mm increase in the inhibition zone for Ceftazidime with the discs to which EDTA was added (Yong *et al*., 2002; Franklin et *al*., 2006).

2.4.4.2. Double disc synergy test (DDST)

Normal saline suspensions of all *P. aeruginosa* isolates were adjusted to the McFarland 0.5 standard and used to inoculate MHA plates with zinc sulfate at a final concentration 70µg/ml. Ceftazidime disc (30µg) was placed on MHA medium inoculated with test *P. aeruginosa* strain and 20 mm apart, a blank filter study disc impregnated with 10µl of 750µg EDTA solution was added, another Ceftazidime disc was placed on the far side of the medium. Then, the plates were incubated at 37°C for 24 h. Enhancement of the zone of inhibition in the area between Ceftazidime disc and the EDTA disc in comparison with the zone of inhibition on the far side was interpreted as a positive result. The same was conducted with Imipenem (10µg) and Meropenem (10µg) discs (Lee *et al.*, 2003b).

2.5. DNA isolation

Pseudomonas aeruginosa genome was prepared for PCR according to the method described previously with some modifications (Adwan *et al*.,

2013). Briefly, cells were scraped off an overnight MHA plate, washed with 800µl of 1X Tris-EDTA buffer (10mM Tris-HCl, 1mM EDTA [pH 8]), and then the pellet was re-suspended in 400µl of sterile double distilled H_2O, and boiled for 10-15 min. Then, the cells 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.

2.6. PCR amplification

2.6.1. Detection of class 1, 2 and 3 integrons

All *P. aeruginosa* isolates were tested for the presence of integrons *intI1*, *intI2* and *intI3* using primers described previously (Shibata *et al.*, 2003). Primers sequences and amplicons size are presented in Table 2.1. The PCR reaction mix with a final volume of 25µl, was performed with 12.5µl of PCR premix (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$, Sigma), 0.4µM of each primer and 150-300ng of DNA template. The amplification process was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation at 94ºC for 2min followed by 30 cycles of denaturation at 94ºC for 40s, annealing at 56ºC for 40s and extension at 72ºC for 40s, were followed by a final extension step at 72ºC for 5 min. Amplified PCR products were detected by agarose gel electrophoresis (1.5% agarose gel) to determine the size of amplified fragments after staining with a final concentration 0.5µg/ml of ethidium bromide dye.

2.6.2. Detection of MBL genes by multiplex PCR assay

Polymerase chain reaction was performed for detection of *VIM*, *IMP*, *SPM*-1, *GIM*-1 and *SIM*-1 genes according to methods described previously (Ellington *et al.*, 2007). Sequences of primers and size of amplicons are described in Table 2.1. The PCR reaction mix with a final volume of 25µl, was performed with 12.5µl of PCR premix (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$, Sigma), 0.4µM of each primer and 3µl of DNA template. The cycling conditions were: initial denaturation at 94 ºC for 5 min, followed by 36 cycles of 94ºC for 40s, 50ºC for 50s and 72ºC for 1 min, were followed by a final elongation step at 72 ºC for 5 min. PCR products were detected and analyzed by electrophoresis using 1.5% agarose gels stained with ethidium bromide staining (0.5µg/ ml).

2.6.3. ERIC-PCR

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 (25µl) composed of 10mM PCR buffer pH 8.3; 3mM MgCl2; 0.4mM of each dNTP; 0.8µM of each primer; 1.5U of Taq DNA polymerase and 3µl of DNA extract solution. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: initial denaturation for 3 min at 94ºC was followed by 40 cycles of denaturation at 94ºC for 50s, annealing at 50ºC for 60s and extension at 72ºC for 2min,

were followed by extension step at 72ºC for 5min. The PCR products were analyzed by electrophoresis on 1.5% agarose gels. Gel images were scored using binary scoring system that recorded the absence and presence of bands as 0 and 1, respectively. A binary matrix was analyzed by the unweighted pair group method for arithmetic averages (UPGMA), using SPSS statistical software version 20 (IBM). The number of different bands in each fingerprint was considered for comparison of bacterial species as previously described (Moosavian and Darban, 2010), based on the following criteria: identical clones (no different band), "closely related clones" (have 1 different band), "possibility different clones" (have two different bands), "different clones" (have three or more different bands).

Table 2.1:Target genes for PCR amplification, amplicon size and primer sequences used in the study.

Group	Targets	Primer sequence 5′→3′	Annealing temperature	Size (bp)	Primer mix	References
Metallo-β-lactamases (Class B)	*Imp*	Imp-F 5-GGAATAGAGTGGCTTAAYTCTC-3 Imp-R 5-CCAAACYACTASGTTATCT-3	52ºC	188bp	1	Ellington *et al*., 2007
	Vim	Vim-F 5-GATGGTGTTTGGTCGCATA-3 Vim-R 5-CGAATGCGCAGCACCAG-3	52ºC	390bp	1	Ellington *et al*., 2007
	Gim	Gim-F 5-TCGACACACCTTGGTCTGAA-3 Gim-R 5-AACTTCCAACTTTGCCATGC-3	52ºC	477bp	1	Ellington *et al*., 2007
	Spm	Spm-F 5-AAAATCTGGGTACGCAAACG-3 Spm-R 5-ACATTATCCGCTGGAACAGG-3	52ºC	271bp	1	Ellington *et al*., 2007
	Sim	Sim-F 5-TACAAGGGATTCGGCATCG-3 Sim-R 5-TAATGGCCTGTTCCCATGTG-3	52ºC	570bp	1	Ellington *et al*., 2007
Integrases	*intI1*	intI1 F 5-GCATCCTCGGTTTTCTGG-3 intI1 R 5-GGTGTGGCGGGCTTCGTG-3	58ºC	457bp	2	Shibata *et al*., 2003
	intI2	intI2 F 5-CACGGATATGCGACAAAAAGG T-3 intI2 R 5-GTAGCAAACGAGTGACGAAATG-3	58ºC	789bp	2	Shibata *et al*., 2003
	intI3	intI3 F 5-AT TGCCAAACCTGACTG-3 intI3 R 5-CGAATGCCCCAACAACTC-3	58ºC	922bp	2	Shibata *et al*., 2003

Chapter three
Results

3.1. Identification of bacteria

All of *P. aeruginosa* isolates were identified by API 20 E system in laboratories of hospitals from where the isolates were collected. In microbiology laboratory at An-Najah National University, all *P. aeruginosa* isolates inoculated on MacConkey agar had Brown colonies while on Cetrimide and Mueller Hinton agar had pyocynin pigment (Blue-Green) and pyoverdine (Yellow-Green fluorescent). Gram stain showed that *P. aeruginosa* was Gram negative bacteria and had rod shape. Biochemically, all isolates were oxidase positive.

3.2. Antibiotic susceptibility

Results of this study showed that all *P. aeruginosa* isolates were resistant to Trimethoprim/Sulfamethoxazole; most of isolates were resistant to Nalidixic acid, Ceftriaxone and Cefotaxime. The most effective tested antibiotics against these clinical *P. aeruginosa* isolates were Norfloxacin, Ciprofloxacin and Ceftazidime. Results of antibiotic resistance against the clinical *P. aeruginosa* isolates are presented in Table 3.1. In addition, all of the isolates were multi-drug resistant.

3.3. Detection of β-lactamase producing *P. aeruginosa* and integrons

Results of the current study showed that 21.6% of the tested clinical *P. aeruginosa* isolates were ESBL-producers using conventional techniques.

Table 3.1: Antibiotic resistance of 51 *P. aeruginosa* isolates collected from different clinical samples.

Antibiotic	Resistant strains	
	No.	%
Trimethoprim / Sulfamethoxazole	51	100
Tetracycline	38	74.5
Nalidixic acid	50	98
Ceftriaxone	49	96.1
Meropenem	23	45.1
Imipenim	25	49
Cefotaxime	49	96.1
Ciprofloxacin	10	21.6
Norfloxacin	11	19.6
Ceftazidime	13	25.5

The prevalence of MBL genes in these isolates were 60.8% and 29.4% by the conventional techniques and multiplex PCR technique, respectively. The prevalence of detected genes was 17.6%, 9.8%, 2% for *VIM*, *IMP*, *SPM* and *VIM*, respectively. Neither *GIM* nor *SIM* was detected in these isolates. In this study it was shown that *VIM* gene was the most common (60%) among *P. aeruginosa* isolates that carried MBL genes. There was 22 isolates tested positive for MBL using conventional tests only, but negative with multiplex PCR assay. Six of the examined *P. aeruginosa* isolates were also found carrying MBL genes using PCR assay only, and these isolates were found to be non MBL producers using conventional tests. In addition, ESBL and MBL enzymes were coexisted in 9 of *P.*

aeruginosa isolates. Data on prevalence of MBLs is presented in Table 3.2, Table 3.3 and Figure 3.1. Antibiotic profile of the MBLs positive isolates were summarized in Table 3.3.

Table 3.2: Prevalence of MBL genes among 51 isolates of *P. aeruginosa* detected by multiplex PCR and conventional techniques (CDDT and DDST).

Technique	**Metallo-β-lactamase (MBL) genes No. (%)**				
	VIM	*IMP*	*SPM+VIM*	*GIM*	*SIM*
PCR technique	9 (17.6)	5 (9.8)	1 (2)	0 (0)	0 (0)
	Total 15 (29.4)				
Conventional techniques (CDDT and DDST)	31 (60.8)				
Distribution according to methods	Positive by both techniques: (n=9) Positive by PCR only: (n=6) Positive by conventional only: (n=22)				

Table 3.3: Summary of the isolates phenotype and genotype.

Sample#	Antibiogram										Detected
	SXT	TE	NA	CIP	NOR	CRO	CAZ	CTX	IPM	MEM	MBL gene
1	R	R	R	S	S	R	S	R	S	S	*VIM*
2	R	S	R	S	S	R	R	R	R	R	*IMP*
3	R	R	R	R	R	R	S	R	S	R	*IMP*
4	R	R	R	R	R	R	R	R	R	R	*IMP*
5	R	R	R	S	S	R	R	R	S	S	*VIM*
6	R	R	R	S	S	R	S	R	R	S	*SPM+VIM*
7	R	S	R	S	S	R	R	R	S	S	*VIM*
8	R	R	R	S	S	R	R	R	R	R	*VIM*
9	R	S	R	R	S	R	R	R	R	R	*IMP*
10	R	S	R	S	S	R	S	R	S	S	*VIM*
11	R	R	R	S	S	R	S	R	R	R	*VIM*
12	R	R	R	S	S	R	S	S	S	S	*VIM*
13	R	R	R	S	S	R	S	R	S	S	*VIM*
14	R	R	R	R	R	R	S	R	R	R	*VIM*
15	R	R	R	R	R	R	R	R	R	R	*IMP*

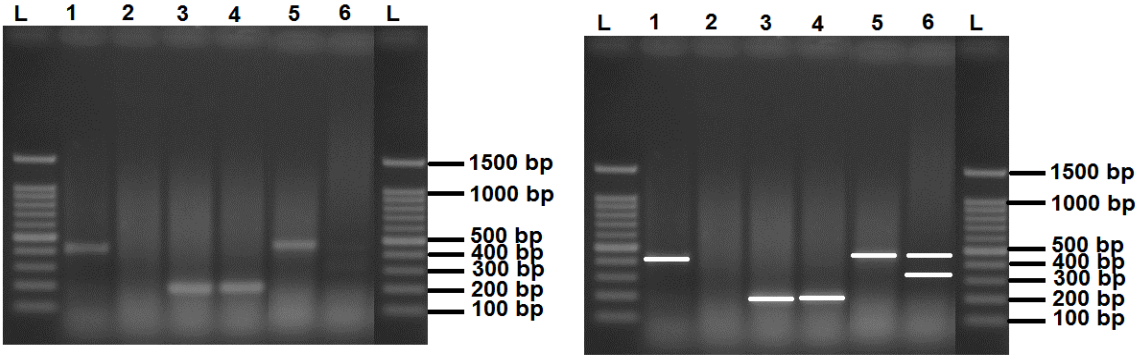

Figure 3.1: Multiplex PCR profile specific for metallo-β-lactamase genes detected in the clinical isolates of *P. aeruginosa* by multiplex PCR**.**

For clarification, the same figure was highlighted on the right side. Lanes L donate for DNA ladder; lanes 1 and 5 donate for *VIM* gene; lane 2 represents a negative sample; lanes 3 and 4 donate for *IMP* gene and lane 6 donates for both *VIM* and *SPM* genes.

A total of 12 of clinical isolates of *P. aeruginosa* (23.5%) were carried class I integrons. Nine of class I integrons positive isolates were detected in either MBL producers or/and ESBL producers *P. aeruginosa* isolates. Results are presented in Figure 3.2. Class II and III integrons were not detected in these isolates.

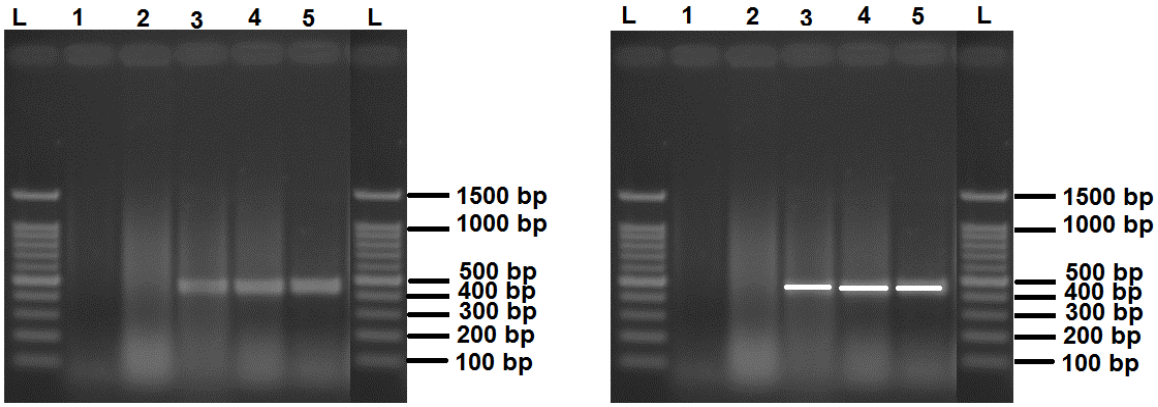

Figure 3.2: PCR profile specific for integrase genes detected in clinical isolates of *P. aeruginosa.*

For clarification, the same figure was highlighted on the right side. Lanes L donate for DNA ladder, Lanes 1 and 2 represent negative control and negative integrase sample, respectively, while Lanes 3, 4 and 5 represent positive for integrase 1.

3.4. ERIC-PCR analysis

ERIC-PCR typing of 15 clinical isolates of *P. aeruginosa* carried different metallo-β-lactamase genes were congregated into 4 ERIC-PCR profiles (clusters) at a 75% similarity level. Results of ERIC-PCR typing showed that at least there are 4 clones circulating among these hospitals. These clones are C1CL1 (isolates 9 and 14); C2CL1 (isolates 2 and 3); C3CL1

(isolates 5 and 12) and C4CL1 (isolates 4, 7 and 11). Results of ERIC-PCR analyses are presented in Figures 3.3 and 3.4.

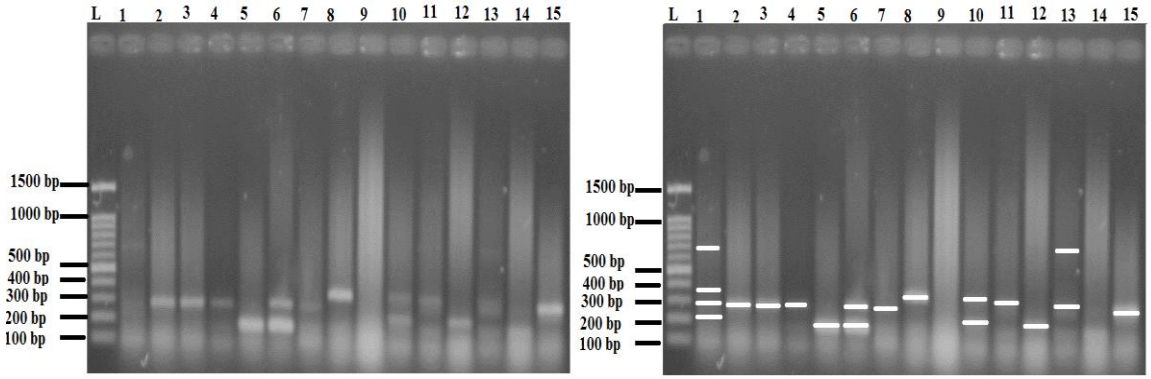

Figure 3.3: ERIC-PCR profile of 15 clinical isolates of MBL-producers *P. aeruginosa* recovered on 1.5% agarose gel.

For clarification, the same figure was highlighted on the right side. Lanes L represent DNA ladder, while other lanes represent ERIC-PCR products.

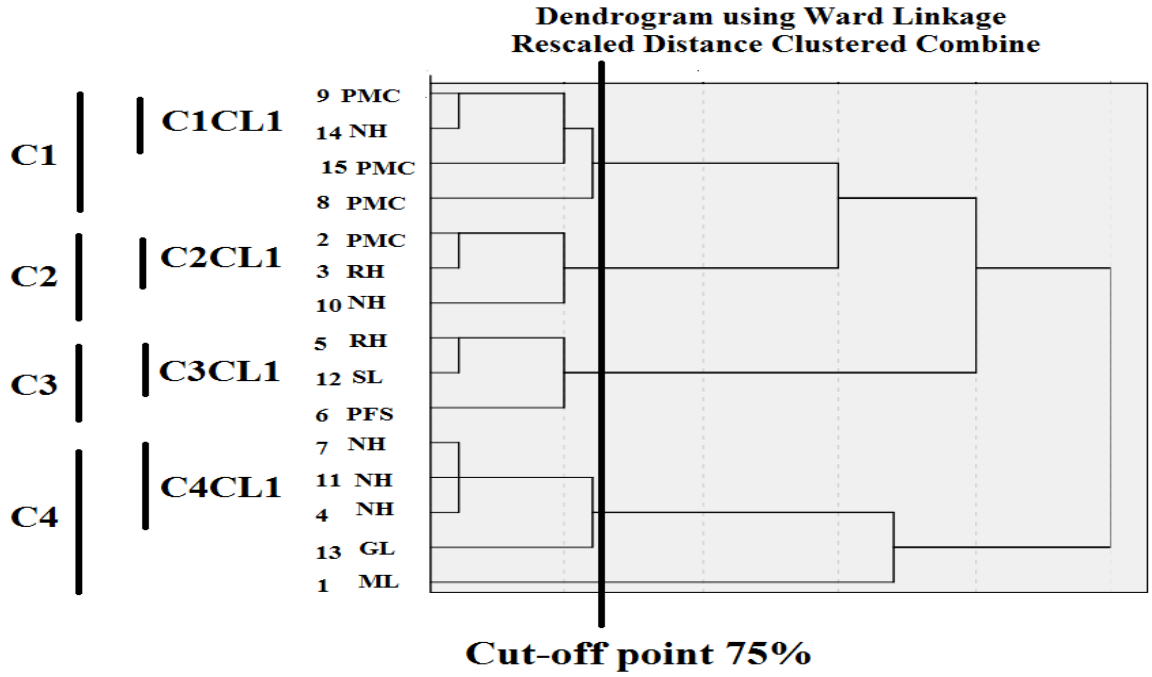

Figure 3.4. Dendrogram of cluster analysis of 15 clinical *P. aeruginosa* isolates harbor MBL-genes based on UPGMA method derived from analysis of ERIC-PCR-profiles at a 75% similarity level.

C: Cluster, CL: Clone, PMC: Palestinian Medical Complex, RH: Rafidia Hospital, NH: An-Najah National University Hospital, SL: Al-Shamal Laboratory for Medical Analysis, PFS: Patient's Friends Society, GL: Ghannam Laboratory for Medical Analysis, ML: Medicare Labs.

Chapter Four
Discussion

Pseudomonas aeruginosa is one of the most common life-threatening bacteria that cause several complications to the hospitalized patients. The great clinical relevance of this pathogenic microorganism is its ability to develop resistance to many classes of antibiotics via different mechanisms including intrinsic and acquired resistance mechanisms. One of these mechanisms is producing MBL enzymes, which could hydrolyze β-lactam ring in β-lactam antibiotics. Nowadays, one of the great challenges is developing of resistance against carbapenem derivatives (Imipenem, Deripenem, Meropenem and Ertapenem), which are considered the drugs of choice for the treatment of infections caused by multi-drug resistant Gram negative rods (Chika *et al.*, 2015; Al-Agamy *et al.*, 2011; Elligton *et al.*, 2007). This is due to the stability of these agents against majority of beta lactamases and their high rate of permeation through bacterial outer membranes (Devi *et al.*, 2015).

Results of the current study showed that the prevalence of ESBL among *P. aeruginosa* isolates in Palestine is high and it was 21.6% using phenotypic test. In different countries of the Middle East, the prevalence of ESBL producing *P. aeruginosa* ranged from 7.4%-39.2% (Zafer *et al.*, 2014; Hayajneh *et al.*, 2015; Al-Marjani *et al.*, 2013; Shaikh *et al.*, 2015; Rafiee *et al.*, 2014) while the prevalence of ESBL in *P. aeurginosa* was ranged between 22%- 30.8% in other countries around the world including

European regions (Okesola and Oni 2012; Chika *et al*., 2015; Velvizhi *et al*., 2013; Peshattiwar and Peerapur 2011).

The present study showed that the Prevalence of MBLs in Palestine is very high and it was 29.4% and 60.8% using multiplex PCR amplification and conventional methods, respectively. In different countries in Middle East, the prevalence of MBLs in clinical *P. aeruginosa* isolates ranged from 20%-37.3% (Al-Marjani *et al*., 2013; Al-Agamy *et al*., 2011; Zafer *et al*., 2014; Rafiee *et al*., 2014) while in other regions in the world including European countries it was ranged between 6.9%- 36% (Riera *et al*., 2011; Peshattiwar and Peerapur 2011; Velvizhi G *et al*., 2013; Devi *et al*., 2015). The used methodologies and type of detected MBL genes may account for the observed variation in the prevalence rates between different studies. Worldwide, the *IMP* and *VIM* types are the most commonly detected MBLs in *P. aeruginosa* (Toleman *et al*., 2005; Poirel *et al*., 2001). Based on the findings of this study, *VIM* gene was the most common gene among MBL-producing *P. aeruginosa* isolates. This finding is in agreement with other published reports which showed that *VIM*-type MBLs are predominant in the Mediterranean region and other parts of the world (Walsh *et al*., 2005; Queenan and Bush, 2007; Zafer *et al*., 2014; Labarca *et al*., 2016). However, this result was in contrast to other studies, were *IMP* or *SPM-1* gene were the most common genes found in MBL-producers *P. aeruginosa* isolates (Ryoo *et al*., 2009; Seok *et al*., 2011; Labarca *et al*., 2016). Among the tested clinical *P. aeruginosa* isolates using phenotypic tests, 22 were found to be MBL producers. These

isolates were MBL negative by multiplex PCR assay. This proposed that negative amplification in these phenotypic positive *P. aeruginosa* isolates may be due to the fact that these isolates carried other MBL genes, which could not be amplified by the used primers or these isolates may have MBL genes on the chromosomes and not on plasmids. On the other hand, 6 of the *P. aeruginosa* isolates were negative using conventional methods and were positive using PCR technique. Such finding emphasizes the need for the use of other substrates in conventional methods in order to increase the sensitivity of conventional tests. Additionally, the ESBL and MBL co-production was observed in this study. These results were in agreement with other reports (Oberoi *et al*., 2013; Salimi and Eftekhar, 2013; Kotwal *et al*., 2016). The coexistence of different classes of β-lactamases in a single *P. aeruginosa* isolate may pose diagnostic and treatment challenges for this pathogen (Oberoi *et al*., 2013).

In this study, only class I of integrons was detected among tested *P. aeruginosa* isolates and none of these isolates carried other tested classes of integrons. This result was consistent with other reports previously published, which showed that the class 1 integrons was the only detected class among clinical isolates of *P. aeruginosa* (Gu *et al*., 2007; Poonsuk *et al.*, 2012; Odumosu *et al*., 2013; Nikokar *et al*., 2013). In the current research, 23.5% of tested *P. aeruginosa* isolates carried integrons. This prevalence is much less than that reported in previously published data, were the prevalence of class 1 integrons in *P. aeruginosa* isolates was too high and ranged from 40.8%-69.3% (Gu *et al*., 2007; Poonsuk *et al.*, 2012;

Odumosu *et al*., 2013; Nikokar *et al*., 2013). Presence of class 1 integrons among multi-drug resistant *P. aeruginosa* isolates or other types of pathogens might be responsible for the dissemination of antibiotic resistance gene (Shibata *et al.*, 2003; Nikokar *et al*., 2013).

Frequency of Imipenem and Meropenem resistance among *P. aeruginosa* isolates in this study was 49% and 45.1% respectively. This indicates a high level of resistance for carpabenems in Palestine and most of this resistance is due to MBLs production. Our study showed that 84% of Imipenem resistant and about 81% of Meropenem resistant isolates were MBL producers. Other mechanisms may be responsible for Imipenem and Meropenem resistance. Worldwide, carpabenems resistance is increasing. Reports from Middle East region showed percentages ranged from 16-98% (Hammami *et al.*, 2011; Al-Agamy *et al.*, 2011; Zafer *et al.*, 2014; Rafiee *et al.*, 2014). High percentages of resistance to carpabenems (23%-66%) were also reported in other regions of the world including European countries (Velvizhi *et al.*, 2013; Riera *et al.*, 2011; Labarca *et al.*, 2016). High prevalence of antibiotic resistance and high prevalence of MBL-producing *P. aeruginosa* in Palestine may be due to selective pressure of antibiotic imposed by the high rate and misuse of antimicrobial agents, particularly cephalosporins in the country could be the only major cause.

ERIC-PCR analysis was carried out to study the genetic relationship among 15 *P. aeruginosa* isolates harboring MBL genes. ERIC-PCR profiles for these isolates at 75% similarity level showed that there were 4 clusters with 4 identical bacterial clones circulating among the studied hospitals.

Dendrogram analysis indicates that strains C1CL1, C2CL1 and C3CL1 clones were circulating in different hospitals, this could be likely due to the fact that these medical centers are medical referrals medical centers in the area. The finding that strains of C4CL1 clone were circulating only at An-Najah National University Hospital-Nablus is a strong indication of nosocomial outbreak in this hospital. In previous studies, pulsed field gel electrophoresis (PFGE) was the common technique used to study the genetic relationship among *P. aeruginosa* isolates (Seok *et al.*, 2011; Shibata *et al.*, 2003; Riera *et al.*, 2011; Hammami *et al.*, 2011). Three methods were used in genotyping of *P. aeruginosa* isolates including multi-locus sequence typing, PFGE and ERIC-PCR, the least discriminatory methods was observed by ERIC-PCR (Kidd *et al.*, 2011).

The finding of high prevalence of MBL-producing *P. aeruginosa* in Palestine underline the necessity to adopt effective control measures to limit the spread of MBL-producing *P. aeruginosa* and other β-lactamase-producing pathogens. Correct diagnosis of β-lactamase-producing bacteria including MBL-producing *P. aeruginosa* in due time is obligatory for optimal patient management and for immediate institutional appropriate infection control measures to prevent the spread of these organisms.

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كلية الدراسات العليا

الوصف الجزيئي ومدى انتشار الزائفة الزنجارية المنتجة لانزيمات البيتا-لاكتاميزيز الفلزية في نابلس-فلسطين

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قدمت هذه الأطروحة استكمالاً لمتطلبات نيل درجة الماجستير في برنامج العلوم الحياتية بكلية الدراسات العليا، في جامعة النجاح الوطنية، في نابلس- فلسطين.

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الملخص

تم الحصول على 51 عينة من الزائفة الزنجارية من مستشفيات ومختبرات مختلفة في الضفة الغربية- فلسطين في الفترة الواقعة بين شهر تشرين أول 2015 وحتى نيسان 2016 , بهدف دراسة مدى انتشار أنزيمات البيتا-لاكتاميز المحللة للمضادات الحيوية باستخدام الطرق التقليدية وأنزيمات البيتا لاكتاميز الفلزية باستخدام كل من الطرق التقليدية والجزيئية في عزلات بكتيريا الزائفة الزنجارية من فلسطين.

أظهرت النتائج أن 21.56% من العينات تنتج إنزيمات البيتا- لاكتاميز المحللة للمضادات الحيوية من نوع ESBL باستخدام الطرق التقليدية. بينما أظهرت النتائج أن نسبة انتشار إنزيمات البيتا- لاكتاميز الفلزية باستخدام الطرق التقليدية وتقنية تفاعل البلمرة المتسلسلة (PCR) هي 60.8%و 29.4% على التوالي. كما أظهرت النتائج أن جين *VIM* هو الذي كان سائدا في عزلات الزائفة الزنجارية المنتجة لإنزيمات البيتا- لاكتاميز الفلزية بنسبة 60% بينما نسبة انتشار الجينات الأخرى *SPM+VIM, IMP* كانت 33.3%, 6.7% في عزلات الزائفة الزنجارية المنتجة لإنزيمات البيتا- لاكتاميز الفلزية على التوالي . فيما لم يظهر أي من الجينين *GIM* و *SIM* في هذه العزلات.

الكشف عن وجود الانتجرونات في عزلات الزائفة الزنجارية تم باستخدام تقنية ال PCR وأظهرت النتائج أن 23.5 % من العزلات تحتوي على الانتجرونات نوع 1 أما الأنواع 2 و 3 لم تكن موجودة في هذه العزلات.

أظهرت نتائج ال ERIC-PCR وجود 4 سلالات منتشرة في المستشفيات الفلسطينية.

أظهرت هذه الدراسة أن نسبة انتشار ال MBLs و ال ESBLs في عينات الزائفة الزنجارية في فلسطين عالية وهذا يؤكد على ضرورة المراقبة المستمرة لأن مراقبة الانتشار والرقابة السليمة وممارسات الوقاية واستخدام المضادات الحيوية الفعالة بطريقة صحيحة سوف يحد من انتشار المزيد من السلالات المنتجة لإنزيمات ال ESBL وإنزيمات ال MBL داخل وبين المستشفيات في فلسطين.