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Emergence of Klebsiella pneumoniae Carbapenemase (bla_{KPC-2}) in members of the Enterobacteriaceae family in Palestine

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Abstract

Background: The global spread of carbapenem resistant *Enterobacteriaceae* (CRE) has limited the physicians' antimicrobial treatment options of infected patients. CREs' which carry the *Klebsiella pneumonia* Carbapenemase (bla_{KPC}) resistance mechanism have been rapidly spreading in many parts of the world, and have been responsible for high patients' morbidity and mortality.

Methods: Two protocols recommended by the Centers for Disease Control and Prevention (CDC) were followed to detect CREs' in Palestine. In addition, the antimicrobial sensitivity patterns for several antibiotic classes were determined for the isolated CREs' by the disc diffusion method according to the clinical and laboratory standard institute (CLSI) M100-S22 guidelines. The Minimal Inhibitory Concentrations (MICs) of the carbapenem, ertapenem, imipenem and meropenem were determined for all the CREs' by E-test. The isolates β-lactam resistance mechanisms were further investigated by analyzing 31 different types of β–lactamase genes by polymerase chain reaction (PCR).

Results: Four bacterial isolates, three *Enterobacter cloacae* and one *Klebsiella pneumoniae*, were determined to be non-susceptible to one or all of the carbapenems (ertapenem, imipenem and meropenem) tested. All isolates which carried the bla_{KPC-2} gene showed an extreme drug resistance profile. These isolates were resistant to all β-lactam antibiotics, co-trimoxazole and gentamicin, while susceptible to only amikacin and colistin sulfate. Different combination of plasmid encoded β–lactamase genes (bla_{TEM}, bla_{SHV}, bla_{OXA-1}, bla_{MIR-1}, bla_{GES-23} and bla_{KPC-2}) were present in these isolates. Of interest, was the isolation of the first *E. cloacae* strains co-producing the bla_{KPC-2} and a novel bla_{GES-23} β-lactamase.

Conclusions: The presence of all these plasmid encoded β-lactamase in Palestine is alarming and mandates actions to be taken to control antibiotics usage and the activation of hospital infection control programs in order to prevent the spread of these extremely drug resistant bacteria.

Running title: Emergence of bla_{KPC-2} positive *Enterobacteriaceae* family members in Palestine

Key words: $bla_{KPC,}$ $bla_{GES,}$ Carbapenemase, Palestine

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Introduction

The emergence of carbapenem resistant *Enterobacteriaceae* (CRE) has complicated the management of infected or colonized patients (1). Carbapenems are usually the last class of antibiotics used to treat infections caused by resistant bacteria such as the ones carrying the Extended Spectrum β-Lactamases (ESBLs'). Since the description of the class A serine β-lactamases (bla_{NMC}, bla_{IMI}, bla_{SME}, bla_{GES} and bla_{K-PC}), which are mainly encoded on plasmids, *Klebsiella pneumoniae* carbapenemase (bla_{KPC}) has been the only resistance mechanism to rapidly disseminate in many parts of the world.

The resistance mechanism, bla_{KPC}, was first reported in a *K. pneumoniae* isolate detected in a patient residing in the United States of America (USA) in the year 2001 (2). Since then, bacterial isolates carrying this resistance mechanism have rapidly disseminated worldwide (1, 2). This in part could be due to the presence of the bla_{KPC} genes on plasmids with varying sizes and structures (3). Bacterial isolates carrying the bla_{KPC} resistance mechanism have been reported in several countries such as the USA, Switzerland, China, Israel, Canada, France, Colombia, Greece, Brazil and Italy (4-13). Indeed, several countries became "Hot Spots" for such bacterial drug resistance mechanism. These countries include the eastern part of the USA, Israel, Greece and the eastern part of China (1). Interestingly, reports about the isolation of bla_{KPC} prodcuing members of the *Enterobacteriaceae* family in the Arabic speaking Middle Eastern countries were scarce.

Reports from varying parts of the world have shown that bacteria carrying the bla_{KPC} gene are usually non-susceptible to co-trimoxazole, fluoroquinolones and the aminoglycosides, while the majority of the isolates are susceptible to tigecycline and colistin sulfate (1, 4, 12). The variation in susceptibility patterns and the heterogeneous expression of the β-lactamases, have complicated the detection of the bla_{KPC} resistance mechanism in the clinical laboratory (14, 15). Indeed, automated systems have been reported to miss the detection of bla_{KPC} resistance mechanism in 7% to 87% of the bla_{KPC} positive *K. pneumoniae* (15). Not only that, but the utilization of the imipenem and meropenem antibiotics in the disk diffusion assays has also been shown to misidentify carbapenem resistance due to bla_{KPC} in 29% and 52% of the cases, respectively (16). On the other hand, the utilization of the carbapenem; ertapenem, has been shown to consistently identify bla_{KPC} resistance mechanism. Nevertheless, within the *Enterobacteriaceae* family, not all ertapenem resistance is due to bla_{KPC} (17).

The inconsistency in the performance of standardized classical microbiology assays has led to the utilization of molecular assays for the rapid detection and identification of the carbapenem resistant bacteria. Several highly sensitive Polymerase Chain Reaction (PCR) assays and Real-Time PCR assays have been developed and validated for the rapid detection and identification of bla_{KPC} positive bacteria (18-21). However, one of the disadvantages of these assays was the need for specialized equipment and well trained personnel. Consequently, screening agar plates have been developed and utilized for the rapid detection of bla_{KPC} resistant bacteria. Chromogenic agar, CHROMagar^{TM} KPC (CHROMagar, France), has been evaluated for the detection of bla_{KPC} positive bacteria. However, this agar base medium still missed certain strains of bla_{KPC} positive *Enterobacteracae* (1, 22). More recently, the Drigalski agar-based culture medium was reported to have excellent sensitivity and specificity for the detection of bacteria carrying the bla_{KPC} resistance mechanism (1).

In this study, we report the emergence and characterization of bla_{KPC} positive bacteria in Palestine. Antimicrobial susceptibility testing and β-lactamase antimicrobial resistance gene families were also investigated.

Materials and Methods

Patient surveillance samples

Caritas Baby Hospital (CBH) is an 89 bed pediatric hospital located in the district of Bethlehem, Palestine. As a result of the increase in the dissemination of Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant *Enterococcus* (VRE), multi-drug resistant *Acinetobacter baumannii* and carbapenem resistant *Enterobacteriaceae* (CRE), in particular the bla_{KPC} resistance mechanism, CBH infection control unit mandates that all patients referred to CBH from other medical institutions should undergo surveillance for drug resistant bacteria. Since 2008, rectal, nose and umbilical swabs (N = 5,928) were collected on Copan Amies sterile transport swabs (Copan Diagnostics, Corona, CA) by the nursing staff members from all patients upon admission to the hospital sections. All swabs were transported to the clinical laboratory within 30 minutes for analysis.

Detection of carbapenem resistant *Enterobacteriaceae*

Two different protocols were utilized to detect CREs' as reported by the American Centers for Disease Control and Prevention (CDC) (23).

Protocol one: surveillance swabs received by the laboratory personnel were inoculated on MacConkey agar plates (Oxoid, United Kingdom), ensuring that all sides of the swab touched

the initial quadrant. The initial quadrant was plated as a lawn, and a meropenem (10µg) and an ertapenem (10µg) disks were placed in this area using sterile forceps. After overnight incubation at 37°C, inoculated MacConkey agar plates were evaluated for the presence of colonies within the inhibition zone of the meropenem and ertapenem disks.

Protocol two: the same surveillance swabs used in protocol one were inoculated in 5ml trypticase soy broth (TSB) (Oxoid, United Kingdom) after a 10µg meropenem disk was placed into the broth using a sterile forceps. After overnight incubation at 37°C, the inoculated tube was vortexed for 15 seconds and a 100µl aliquot was streaked on MacConkey agar plate and incubated overnight at 37°C in ambient air. All bacterial colonies were identified and sensitivity testing was performed as described below (24, 25).

Bacterial identification and antimicrobial susceptibility testing

All bacterial isolates suspected of being CREs' were identified by the API-20E (bioMerieux, Inc., France) according to the manufacturer's instructions. Susceptibility testing was performed by disk diffusion method on Mueller Hinton agar as recommended by the Clinical and Laboratory Standard Institute guidelines (CLSI) (25). Susceptibility patterns were determined for amikacin (30µg), amoxycillin/clavulanate (20/10µg), aztreonam (30µg), chloramphenicol (30µg), ceftazidime (30µg), ceftazidime/clavulanate (30/10 µg), ciprofloxacin (5µg), gentamicin (10µg), cefpodoxime (10µg), ceftriaxone (30 µg), cefotaxime (30µg), cefotaxime/ clavulanate (30/10 µg), ertapenem (10µg), nitrofurantoin (300µg), cefepime (30µg), cefoxitin (30µg), imipenem (10µg), meropenem (10µg), norfloxacin (10µg), ofloxacin (5µg), co-trimoxazole (23.75/1.75 µg), tigecycline (15µg), piperacillin/tazobactam (100/10 µg), colistin sulfate (10 µg) (Oxoid, UK). All bacterial isolates that exhibited an intermediate or resistant profile to any of the carbapenems tested (ertapenem, imipenem and meropenem) were further evaluated by the modified Hodge test (25). In addition, carbapenems resistance was confirmed by determining the Minimal Inhibitory Concentration (MIC) of ertapenem, imipenem and meropenem using the Etest® (bioMe´rieux Inc.) on Mueller-Hinton plates (Oxoid, United Kingdom).

Polymerase Chain Reaction (PCR) Amplification of Different β-Lactamase Genes

Analysis of the β-lactamase gene groups, Extended Spectrum β-Lactamase (ESBL) [bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M\text{-}2\ and\ -5}$, $bla_{CTX\text{-}M}$, $bla_{OXA\text{-}1}$, $bla_{OXA\text{-}2\ and\ -20}$, $bla_{OXA\text{-}5\ and\ -10}$, $bla_{PER\text{-}1\ and\ -2}$],and *bla*AmpC ($bla_{ACT\text{-}1}$, $bla_{MIR\text{-}1}$, bla_{CMY}, bla_{LAT}, $bla_{FOX\text{-}1\ to\ 5}$, $bla_{ACC\text{-}1}$, $bla_{DHA\text{-}1\ and\ -2}$), in addition to the different carbapenem resistance molecular classes (class A: bla_{KPC}, $bla_{NMC\text{-}A}$, bla_{SME}, bla_{GES}; class B: bla_{NDM}, bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SPM}, bla_{SIM}; class D: $bla_{OXA\text{-}69}$, $bla_{OXA\text{-}60}$, $bla_{OXA\text{-}55}$, $bla_{OXA\text{-}48}$, $bla_{OXA\text{-}50}$) were performed as previously reported (18, 26, 27). Briefly, suspected bacterial isolates were streaked for isolation on 5% Sheep Blood agar (SBA) (Hy-labs, Rehovot) and incubated at 35°C for 18-24 hours. Well isolated colonies were used to prepare 2.0 MacFarland standard in sterile saline, of which, 100µl aliquot was heated at 100°C for 15 minutes to prepare the bacterial cell lysates that were used for the PCR analysis. PCR amplification was performed in MJ-P-100 Thermocycler using the recommended amplification conditions (18, 26). The primers used to determine the type of the amplified bla_{KPC} PCR product were: forward 5'-atgtcactgtatcgccgtct-'3 and reverse 5'-ttttcagagccttactgccc-'3. In addition, the primers used to determine the type of the amplified bla_{GES} gene were: forward 5'-atgcgcttcattcacgcac-'3 and reverse 5'-ctatttgtccgtgctcagga -'3. The amplified PCR products were visualized on 1.0% agarose gel (Life Technologies, USA) after staining with ethidium bromide.

Determining bla_{KPC} and bla_{GES} types by sequence analysis

Amplified PCR products of the expected size for bla_{KPC} (882bp) and bla_{GES} (864bp) were purified using HighPure PCR product Purification Kit (Roche, Germany) and sequenced using the BigDye® Terminator V1.1 Sequencing Standard Kit (Life Technologies, USA). Sequence analysis was performed on the Applied Biosystems 3130 Genetic Analyzer (Life Technologies, USA) and analyzed using the Sequencher 5.0 (Gene Code Corporation, USA). The obtained nucleotide sequences were compared with previously described bla_{KPC} and bla_{GES} sequences obtained from the GenBank database (http://www.lahey.org/Studies/other.asp).

Results

Detection of carbapenem resistant *Enterobacteriaceae* (CRE)

Four different bacterial strains (3 *Enterobacter cloacae* and 1 *Klebsiella pneumoniae*) were isolated from the rectal swabs of 2 patients upon referral to CBH from local and regional medical institutions (**Table 1**). The 3 *E. cloacae* strains were isolated from a patient with prior hospitalization in an Israeli medical institution while the patient with the *K. pneumoniae* had prior hospitalization in a Palestinian medical institution. All isolates were detected by both protocols used to isolate CRE. The modified Hodge Test confirmed the presence of the carbapenemase enzyme as all four isolates produced a clear clover leaf-type indentation (**Figure 1**).

Table 1. Description of the carbapenemase positive bacterial isolates.

Bacterial Isolate	Isolate Number	Sample Collection Site	Patient Sex / Age	Date of Detection	Referral Hospital	Patients Residency
E. cloacae	1A1	Rectal	F/ 2 months	February 2008	Israeli	Hebron
E. cloacae	1A2	Rectal	F/ 2 months	February 2008	Israeli	Hebron
E. cloacae	1A3	Rectal	F/ 2 months	February 2008	Israeli	Hebron
K. pneumoniae	1A4	Rectal	F/ 6 months	March 2012	Palestinian	Ramallah

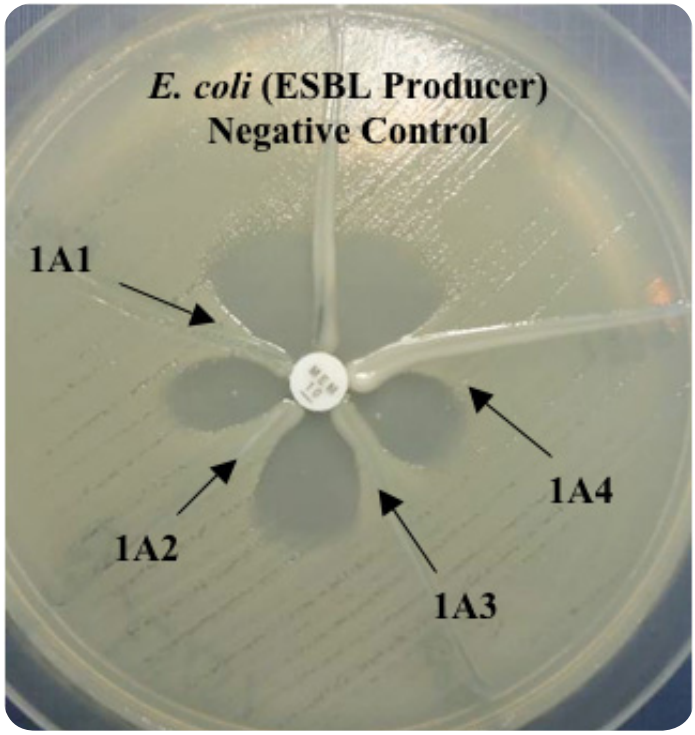

Figure 1: Modified Hodge Test of the four bla_{KPC} positive isolates (1A1, 1A2, 1A3 and 1A4) and an *E. coli* (ESBL producer) as a negative control. All four bla_{KPC} positive isolates produced the characteristic cloverleaf-like indentation, while the negative control, E. *coli* (ESBL), positive did not produce the characteristic indentation.

Antimicrobial susceptibility patterns

Antimicrobial susceptibility testing of the CRE isolates for 24 different antibiotics revealed a multi-drug resistant profile (**Table 2**). Non-susceptible antibiotic profile was noted to all of the β–lactam antibiotics, co-trimoxazole and gentamicin. Determination of the MIC of the carbapenems (ertapenem, imipenem and meropenem) by Etest®, confirmed the resistance profile to the carbapenems. All isolates had carbapenems MIC's greater than 32 µg/ml (**Table 2**). Variable sensitivity patterns were observed for the fluoroquinolones. All isolates had a non-susceptible antibiotic profile for ciprofloxacin, while depending on the isolate; a susceptible and non-susceptible profile was noted for norfloxacin and ofloxacin (**Table 2**). Similarly, a variable sensitivity pattern was noted for the bacteriostatic antibiotic tigecycline. Complete sensitivity patterns were noted only for amikacin and colistin sulfate.

PCR amplification of β-lactamase genes and sequence analysis of bla_{KPC} and bla_{GES} PCR products

PCR amplification of 31 different β-lactamase genes representing members of the ESBL's, AmpC, and the carbapenemases revealed that carbapenem resistance in the 4 isolates was due to the presence of the bla_{KPC} carbapenemase (**Table 3**). In addition, the three *E. cloacae* isolates also co-produced the Guiana extended-spectrum β-lactamases (bla_{GES}) (**Table 3**). Sequence analysis of the bla_{KPC} (760 base pair) and bla_{GES} (823 base pairs) PCR products revealed that all 4 bacterial isolates carried the bla_{KPC-2} gene while the 3 *E. cloacae* isolates co-produced a novel type of the bla_{GES} called bla_{GES-23} gene. The bla_{GES-23} had a point silent mutation at nucleotide position 159 where the nucleotide cytosine was replaced by an adenine in all three *E. cloacae*. All 3 bla_{GES-23} sequences were deposited in the Genbank under the accession numbers JX437086-88. With regard to the ESBL genes, all isolates carried the bla_{TEM} and bla_{SHV} genes, while none carried any of the bla_{CTX} genes. Moreover, three of the isolates (2 *E. cloacae* and 1 *K. pneumoniae*) carried the bla_{OXA-1} gene (**Table 3**). As for the *bla*AmpC β-lactamases, the bla_{MIR-1} gene was detected in the 3 *E. cloacae* while it was absent in the *K. pneumoniae* isolate (**Table 3**).

Discussion

The emergence of carbapenem resistant *Enterobacteriaceae* (CRE) has exerted an unprecedented pressure on the health

Table 2. Antimicrobial susceptibility patterns of the different *bla*$_{KPC}$ positive isolates was determined and interpreted by the disk diffusion (DD) method, while the MIC's for the carbapenems (ertapenem, imipenem and meropenem) were determined by the E-test. The interpretation of the sensitivity patterns were: S = Sensitive; I = Intermediate; R = Resistant.

Antibiotic (concentration)	1A1	1A2	1A3	1A4
	E. cloacae DD / E-Test	*E. cloacae* DD / E-Test	*E. cloacae* DD / E-Test	*K. pneumoniae* DD / E-Test
Amikacin (30µg)	S	S	S	S
Gentamicin (10µg)	R	R	R	R
Amoxycillin / Clavulanate (20/10µg)	R	R	R	R
Piperacillin / Tazobactam (100/10µg)	R	R	R	R
Aztreonam (30µg)	R	R	R	R
Ceftazidime (30µg)	R	R	R	R
Ceftazidime / Clavulanate (30/10µg)	R	R	R	R
Cefotaxime (30µg)	R	R	R	R
Cefotaxime / Clavulanate (30/10µg)	R	R	R	R
Cefpodoxime (10µg)	R	R	R	R
Ceftriaxone (30µg)	R	R	R	R
Cefepime (30µg)	R	R	R	R
Cefoxitin (30µg)	R	R	R	I
Ofloxacin (5µg)	I	I	S	S
Ciprofloxacin (5µg)	R	R	R	I
Norfloxacin (10µg)	R	I	I	S
Nitrofurantoin (300µg)	S	S	S	I
Chloramphenicol (30µg)	R	R	R	S
Co-trimoxazole (23.75/1.75µg)	R	R	R	R
Ertapenem (10µg)	R / >32 µg/ml	R/ >32 µg/ml	R/ >32 µg/ml	R/ >32 µg/ml
Imipenem (10µg)	R / >32 µg/ml	R/ >32 µg/ml	R/ >32 µg/ml	R/ >32 µg/ml
Meropenem (10µg)	R / >32 µg/ml	R/ >32 µg/ml	R/ >32 µg/ml	R/ >32 µg/ml
Tigecycline (15µg)	I	I	I	S
Colistin sulfate (10µg)	S	S	S	S

care provider's ability to manage infected or colonized patients. This in part was due to the limited antimicrobial options available for treating infected patients and to the recommendations for isolating patients with CRE. Of interest was the rapid dissemination of *bla*$_{KPC}$ resistant bacteria in the Eastern part of the USA, China, Greece and Israel (4, 10, 12, 17, 28). Other countries in the world have reported sporadic isolation of *Enterobacteriaceae* family members carrying the *bla*$_{KPC}$ resistance mechanism (1). However, reports about the isolation of *bla*$_{KPC}$ producing members of the *Enterobacteriaceae* family in any of the Arabic Middle Eastern countries are infrequent.

Table 3. Determination of the presence of several β-lactamase genes in the carbapenem resistant isolates by PCR.

Group	β-lactamase Genes	1A1	1A2	1A3	1A4
		E. cloacae	*E. cloacae*	*E. cloacae*	*K. pneumoniae*
ESBL	bla_{TEM}	Positive	Positive	Positive	Positive
	bla_{SHV}	Positive	Positive	Positive	Positive
	$bla_{CTX\text{-}M\text{-}2\ and\ 5}$	Negative	Negative	Negative	Negative
	$bla_{CTX\text{-}M}$	Negative	Negative	Negative	Negative
	$bla_{OXA\text{-}1}$	Negative	Positive	Positive	Positive
	$bla_{OXA\text{-}2\ and\ 20}$	Negative	Negative	Negative	Negative
	$bla_{OXA\text{-}5\ and\ 10}$	Negative	Negative	Negative	Negative
	$bla_{PER\text{-}1\ and\ 2}$	Negative	Negative	Negative	Negative
*bla*AmpC	$bla_{ACT\text{-}1}$	Negative	Negative	Negative	Negative
	$bla_{MIR\text{-}1}$	Positive	Positive	Positive	Negative
	$bla_{CMY\ and\ LAT}$	Negative	Negative	Negative	Negative
	$bla_{FOX\text{-}1\ and\ 5}$	Negative	Negative	Negative	Negative
	$bla_{DHA\text{-}1\ and\ 2}$	Negative	Negative	Negative	Negative
	$bla_{ACC\text{-}1\ and\ 2}$	Negative	Negative	Negative	Negative
Class A	bla_{KPC}	Positive	Positive	Positive	Positive
	bla_{NMC}	Negative	Negative	Negative	Negative
	bla_{SME}	Negative	Negative	Negative	Negative
	bla_{GES}	Positive	Positive	Positive	Negative
Class D	$bla_{OXA\text{-}69}$	Negative	Negative	Negative	Negative
	$bla_{OXA\text{-}55}$	Negative	Negative	Negative	Negative
	$bla_{OXA\text{-}48}$	Negative	Negative	Negative	Negative
	$bla_{OXA\text{-}50}$	Negative	Negative	Negative	Negative
	$bla_{OXA\text{-}60}$	Negative	Negative	Negative	Negative
Class B	$bla_{IMP\text{-}1}$	Negative	Negative	Negative	Negative
	$bla_{IMP\text{-}2}$	Negative	Negative	Negative	Negative
	$bla_{VIM\text{-}1}$	Negative	Negative	Negative	Negative
	$bla_{VIM\text{-}2}$	Negative	Negative	Negative	Negative
	$bla_{SPM\text{-}1}$	Negative	Negative	Negative	Negative
	$bla_{GIM\text{-}1}$	Negative	Negative	Negative	Negative
	$bla_{SIM\text{-}1}$	Negative	Negative	Negative	Negative
	bla_{NDM}	Negative	Negative	Negative	Negative

Because of medical tourism, a large number of Palestinians living in occupied Palestine seeks the medical services in the Israeli hospitals. Since bla_{KPC} positive *Enterobacteriaceae* has disseminated in the majority of Israeli medical institutions, Palestinians who were hospitalized in Israel and are returning back to the Palestinian hospitals have a high probability of being colonized with bla_{KPC} positive *Enterobacteriaceae* (10, 20). Indeed, this report describes the first isolation of bla_{KPC} positive *E. cloacae* isolated from a patient upon admission to Caritas Baby Hospital right after hospitalization in an Israeli hospital. In addition, what was more alarming was the detection of bla_{KPC} positive *K. pneumoniae* in a patient

with no prior travel history outside occupied Palestine. Thus, indicating the emergence of this resistance mechanism in the Palestinian hospitals.

Consistent with earlier reports that *bla*$_{KPC}$ positive *Enterobacteriaceae* are usually multidrug resistant organisms, all isolates reported here showed multidrug resistance profile (12, 17, 29). Indeed, all isolates were resistant to all β–lactam antibiotics, co-trimoxazole, and gentamicin. High carbapenems (ertapenem, imipenem and meropenem) with MIC's (>32μg/mL) confirmed the disk diffusion results, while the presence of carbapenemase enzymes was confirmed by the modified Hodge test. Variable sensitivity patterns were noted for the fluoroquinolones (ofloxacin, ciprofloxacin and norfloxacin), tigecycline, and nitrofurantoin which further complicate patient's infection management. Complete susceptible patterns were noted only to amikacin and colistin sulfate, thus leaving the health care providers with limited options for how to treat patients infected with *bla*$_{KPC}$ positive bacteria (**Table 2**). *E. cloacae* and *K. pneumoniae* isolates positive for the *bla*$_{KPC}$ gene and with similar drug resistance profile as in occupied Palestine have been previously reported from several Israeli medical institutions and several countries worldwide (1, 10, 30-37).

Eleven different types of *bla*$_{KPC}$ genes have been reported in different bacterial genuses worldwide, of which *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ are endemic in Israel (26). Sequence analysis of the four Palestinian bacterial *bla*$_{KPC}$ genes revealed that they all belong to *bla*$_{KPC-2}$ type. *Bla*$_{KPC-2}$ which has been reported in Israel and in several countries worldwide differ from the widely disseminated *bla*$_{KPC-3}$ by only one amino acid difference at position 272, where histidine was replaced by tyrosine (H272Y) (35).

The extremely drug resistance profile that the Palestinian *bla*$_{KPC-2}$ isolates exhibited was further investigated by screening the bacterial cell lysates for the presence of different β–lactamase genes by PCR. Of the 31 different β–lactamase genes investigated, all isolated lysates contained the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes. Several *E. cloacae* and *K. pneumoniae* isolates carrying the β–lactamase genes; *bla*$_{KPC-2}$, *bla*$_{TEM}$ and *bla*$_{SHV}$ have been previously reported from several countries (35, 38-40). On the other hand, the *bla*$_{OXA-1}$ was detected in the *E. cloacae* isolates 1A2, 1A3 and the *K. pneumoniae* isolates 1A4, while the *bla*AmpC (*bla*$_{MIR-1}$) was detected in all *E. cloacae* isolates 1A1, 1A2 and 1A3. The presence of these resistance mechanisms in combination with the *bla*$_{KPC}$ is extremely rare and at the same time alarming as these plasmids encoded β-lactamase genes, ESBL's and *bla*AmpC, can easily spread from one bacteria to another in the hospital setting (41, 42).

What was more disturbing was the detection of the plasmid encoded *bla*$_{GES}$ gene in the three *E. cloacae* isolates. The *bla*$_{GES}$ enzymes possess an unusual phenotypic plasticity, since they have activities against carbapenems, extended-spectrum β-lactams, cephamycins and monobactams (43). The presence of the *bla*$_{GES-23}$ enzyme which might have some carbapenemase activity with the *bla*$_{KPC-2}$ is a strong reminder about the seriousness of this emerging problem. This is of extreme importance since it represents, to our knowledge, the first report of the presence of both *bla*$_{KPC}$ and *bla*$_{GES}$ genes in a member of the *Enterobacteriaceae* family. Indeed, the co-production of *bla*$_{KPC}$ and *bla*$_{GES}$ has been previously reported in an *Aeromonas* spp. isolated from hospital sewage in Brazil (44).

The presence of all these plasmids encoded β–lactamase (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{OXA-1}$, *bla*$_{MIR-1}$, *bla*$_{GES-23}$ and *bla*$_{KPC-2}$) genes is the Palestinian bacterial isolates is alarming and mandates the Palestinian Ministry of Health (Pal-MOH) to take practical steps in order to help the Palestinian hospitals in detecting these extremely drug resistant pathogens. In addition, the Pal-MOH must take action in controlling the misuse of antibiotics and must mandate activation and monitoring of the Palestinian hospitals infection control programs in order to prevent the spread of these extremely drug resistant bacteria. In Palestine, the presence of these resistance mechanisms does not preclude the presence of other rapidly spreading carbapenemases in the *Enterobacteriaceae* family such as the recently reported *bla*$_{OXA-48}$ in Lebanon (45).

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