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RESEARCH ARTICLE

PREVALENCE AND MOLECULAR CHARACTERIZATION OF B-LACTAMASES IN CLINICAL ISOLATES OF *KLEBSIELLA PNEUMONIAE* FROM NORTH OF PALESTINE

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ABSTRACT

Fifty-one isolates of *Klebsiella pneumoniae* were recovered from different hospitals in Northern Palestine, during September-December 2015. Results showed that the prevalence of ESBLs, MBLs and AmpC β-lactamases using multiplex PCR technique were 92.2%, 9.8 and 3.92%, respectively. For ESBL genes, *TEM* gene was the most dominant (72.5%) among *K. pneumoniae* isolates. Other genes were (0.0%), (17.6%) and (31.4%) for *CTX-M*, *SHV* and *OXA* genes, respectively. For AmpC β-lactamase producing *K. pneumoniae*, only DHA gene was detected and all MBL producing isolates were carried NDM gene. Molecular analysis by construction phylogenetic tree showed that all sequenced *TEM*, *SHV*, *OXA, NDM* and *DHA* genes were belonged to *TEM-1*, *SHV-1*, *OXA-1, NDM-1* and *DHA-1*, respectively. In addition, 51% of *K. pneumoniae* harbored only class 1 integrons, whereas other tested classes were not detected. All class 1 integrons were detected in isolates carried β-lactamase genes. ERIC-PCR profile showed that these isolates were diverse and unrelated clones. This is the first study documented the prevalence and molecular characterization of β-lactamases producing *K. pneumoniae* isolates in Palestine Our results showed high occurrence of β-lactamases among *K. pneumoniae* 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 restrict the further spread of β-lactamases producing *K. pneumoniae* isolates within hospitals in this country.

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INTRODUCTION

Antimicrobial resistance has been identified as a key public health challenge and is often difficult to control. It is obvious that pathogens will continue to develop resistance to multiple classes of antimicrobial drugs through intrinsic and acquired mechanisms (Hammami *et al*., 2011). A major mechanism for antibiotic resistance among Gram-negative bacteria is the production of β-lactamases. These are commonly classified according to two systems; the Ambler molecular classification and the Bush–Jacoby–Medeiros functional classification (Bush and Jacoby, 2010). The former functional scheme classifies β-lactamases into four classes (A-D) according to the protein homology of enzymes. Classes A, C, and D β-lactamases possess an active site serine called serine β-lactamase, whereas class B β-lactamases are collectively known metalloenzymes, usually requiring a zinc molecule for their catalytic activities. The latter one typing β-lactamases according to substrate and inhibitor profiles in an attempt to organize the enzymes in ways that can be correlated with their phenotype in clinical isolates (Bush and Jacoby, 2010). This system grouped the beta-lactamases in 3 major groups and 16 subgroups. The most important β-lactamases are cephalosporinases like, extended spectrum β-lactamases (ESBLs) and the carbapenemases like metallo- β-lactamases (MBLs) (Bush and Jacoby, 2010). 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). Integrons 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

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extended-spectrum β-lactamases (ESBLs) (Luzzaro *et al.*, 2001). ESBLs have emerged worldwide as a significant cause of community and health care associated infections. The overall data on β-lactamase-producing *Enterobacteriaceae* in the countries of the Middle East are extremely worrisome, and this region might indeed be considered one of the major epicenters of the global β-lactamase pandemic (Shaikh *et al*., 2014). A study from Kuwait showed high prevalence of VIM-4 and NDM-1 MBL among carbapenem-resistant *Enterobacteriaceae* including *Klebsiella* spp. It was shown that *K. pneumoniae* isolates produced the MBL NDM-1 and co-produced the plasmid-encoded AmpC CMY-4. The VIM-4-producing isolates co-produced ESBLs including CTX-M-15 and some SHV derivatives (Jamal *et al*., 2013). In Iraq *K. pneumoniae* isolated from vaginal swabs from pregnant and non pregnant patients showed the frequency of ESBLs, MBLs and AmpC β-lactamases were 50%, 35.7% and 0.0%, respectively (Ahmad and Ali, 2014). In United Arab Emirates, results showed that the prevalence of ESBL producing *K. pneumoniae* was 42% (Al-Zarouni *et al.,* 2008). Another report from United Arab Emirates in 2009–2011 revealed the emergence and spread of NDM-1 producer *K. pneumoniae* (Sonnevend *et al*., 2013). In Egypt, during 2004-2005 it was reported that 61.5% *K. pneumoniae* isolates were ESBL producers (Fam *et al*., 2006).

In Bahrain, a retrospective analysis of records during 2005 and 2006, the prevalence of ESBL among *K. pneumonia* was 24.3% (Bindayna *et al*., 2009). In Saudi Arabia in 2007, phenotypic characterization identified a high ESBL rate of 55% of *K. pneumonia* isolates, the prevalence of SHV, TEM and CTX-M β-lactamase genes were 97.3%, 84.1% and 34.1%, respectively, within the CTX-M family, CTX-M-1 and CTX-M-9-like genes were found with prevalence of 60% and 40%, respectively (Al-Agamy *et al*., 2009). In Jordan in 2004, 71.4% were ESBL producers in *K. pneumonia* and 28.6% in *K. oxytoca* (Batchoun *et al*., 2009). A study in Algeria in 2009, reported that the prevalence of ESBL producers in *K. pneumonia* was 17.4%, CTX-M-1 is predominant (Nedjai *et al*., 2012).

In Tunisia, the prevalence of ESBL-producing *K. pneumoniae* strains was ranged from 32.4%-37.5% (Messai *et al*., 2007; Abbassi *et al*., 2008). *CTX-types*, *SHV*-types, *OXA-1* and *TEM-1* genes have been reported from Tunisia in *K. pneumoniae* strains isolated from humans (Ktari *et al*., 2006; Abbassi *et al*., 2008; Ben Achour *et al*., 2009). In Saudi Arabia in 2011, The prevalence of *NDM-1* and *VIM* genes among *K. pneumonia* isolates was 20% and 1.7%, respectively (Shibl *et al*., 2013). In 2010 and 2011 in Sultanate of Oman, the prevalence of carbapenemase-producing *K. pneumonia* isolates was 81.25%, of these 62.5%, 12.5% and 6.25% for NDM-1, OXA-48 and co-harboured NDM-1and OXA-181, respectively (Dortet *et al*., 2012). In 2008 in Lebanon, CTX-M-15 has been described and the first isolation MBL producing *K. pneumonia* (blaIMP-1) has been detected also (Daoud *et al*., 2008). Little information is available about the prevalence and molecular characterization of β-lactamases in Palestine. The present study was conducted to determine the prevalence and molecular characterization β-lactamase producing *K. pneumoniae* isoates ***using molecular techniques;*** and to assess the prevalence of class 1, 2 3 and 4 integrons in these isolates.

MATERIALS AND METHODS

Sample collection and *K. pneumoniae* identification

A total of 51 isolates of *K. pneumoniae* (11 blood, 18 urine, 5 sputum, 17 swab) were isolated from inpatients and outpatients at An-Najah Natinal University Hospital-Nablus (n=16) , Rafidia Hospital-Nablus (n=2) and Al-Watani Hospital-Nablus (n=2), The Martyar .Dr. Khalil. S. Hospital-Jenin (n=8), Al-Amal Hosptal-Jenin (n=1), AL-Razi Hospital-Jenin (n=1), Al-Shamal Lab-Jenin (n=7), Thabat hospital-Tulkarem (n=15) during September-December 2015. These isolates were identified in laboratories of these hospitals by API 20 E system and also confirmed in Microbiology laboratories at An-Najah National University-Nablus, Palesine. These isolates were cultured on MacConkey, growth at 5°C and 44.5°C was detected, Gram stain, motility test and other biochemical tests were used such as Indole test and Voges-Proskauer test (Brisse *et al*., 2006).

Antibacterial resistance

Antimicrobial susceptibility was determined according to the Clinical and Laboratory Standard Institute (CLSI) using the disk diffusion method (CLSI, 2012). All *K. pneumoniae* isolates were examined using disks (Oxoid) to determine resistance against Ceftazidime (CAZ) 30µg, Cefotaxime (CTX) 30µg, Imipenem (IPM) 10µg, Meropenem (MEM) 10µg, Ceftriaxone (CRO) 30µg and Aztreonam (ATM) 30µg. The plates were incubated at 37°***C*** for 18-24 hrs. Inhibition zones were measured in millimeters and isolates were classified as resistant or susceptible according to the criteria recommended by CLSI guidelines (CLSI, 2012).

DNA isolation

K. pneumoniae genome was prepared for PCR according to the method described previously (Adwan *et al*., 2013). 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 resuspended in 0.4 ml of sterile distilled H_2O, and boiled for 10-15 min. Then, 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.

Multiplex PCR amplification

Detection of MBL genes

Detection of gene sequences coding for the VIM, IMP, SPM-1, GIM-1 and SIM-1 enzymes was performed by the multiplex PCR according to method described previously (Ellington *et al.*, 2007). Sequences of primers and size of amplicons are described in Table 1. Briefly, PCR reactions were performed in a final volume of 25 µl of the amplification mixture containing 12.5 µl of PCR premix with MgCl2 (ReadyMixTM Taq PCR Reaction Mix with MgCl2, Sigma), 0.4µM of each primer, 3µl of DNA template.

Table 1. Target genes for PCR amplification, amplicon size and primer sequences that were used in this study

Group	Targets	Primer sequence 5′→3′	Expected amplicon size (bp)	Primer mix	References
Metallo-β-lactamases (Class B)	*Imp*	Imp-F GGA ATA GAG TGG CTT AAY TCT C Imp-R CCA AAC YAC TAS GTT ATC T	188bp	1	Ellington *et al*., 2007
	Vim	Vim-F GAT GGT GTT TGG TCG CAT A Vim-R CGA ATG CGC AGC ACC AG	390bp	1	Ellington *et al*., 2007
	Gim	Gim-F TCG ACA CAC CTT GGT CTG AA Gim-R AAC TTC CAA CTT TGC CAT GC	477bp	1	Ellington *et al*., 2007
	Spm	Spm-F AAA ATC TGG GTA CGC AAA CG Spm-R ACA TTA TCC GCT GGA ACA GG	271bp	1	Ellington *et al*., 2007
	Sim	Sim-F TAC AAG GGA TTC GGC ATC G Sim-R TAA TGG CCT GTT CCC ATG TG	570bp	1	Ellington *et al*., 2007
	NDM	NDM F GGT TTG GCG ATC TGG TTT TC NDM R CGG AAT GGC TCA TCA CGA TC	621	2	Poirel *et al*., 2011a
	DIM	DIM F GCT TGT CTT CGC TTG CTA ACG DIM R CGT TCG GCT GGA TTG ATT TG	699	2	Poirel *et al*., 2011a
extended spectrum β-lactamases (Class A)	KPC	KPCM F CGT CTA GTT CTG CTG TCT TG KPCM R CTT GTC ATC CTT GTT AGG CG	789	2	Poirel *et al*., 2011a
	SHV	SHV F 5-ATG CGT TATATT CGC CTG TG-3 SHV R 5-TGC TTT GTT ATT CGG GCC AA-3	747	3	Paterson *et al*., 2003
	TEM	TEM F 5-TCG CCG CAT ACA CTA TTC TCA GAA TGA TEM R5- ACG CTC ACC GGC TCC AGA TTT AT	445	3	Monstein *et al*., 2007
	CTX	CTX-M F ATG TGC AGY ACC AGT AAR GTK ATG GC CTX-M R TGG GTR AAR TAR GTS ACC AGA AYC AGC GG	593	3	Boyd *et al*., 2004
extended spectrum β-lactamases (Class D)	*OXA*	OXA F 5-ATT ATC TAC AGC AGC GCC AGT G OXA R 5-TGC ATC CAC GTC TTT GGT G-3	296	3	Kim *et al*., 2009
AmpC β-lactamase (Class C)	*MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11*	MOXM F GCT GCT CAA GGA GCA CAG GAT MOXM R CAC ATT GAC ATA GGT GTG GTG C	520	4	Pérez-Pérez and Hanson, 2002
	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITM F TGG CCA GAA CTG ACA GGC AAA CITM R TTT CTC CTG AAC GTG GCT GGC	462	4	Pérez-Pérez and Hanson, 2002
	DHA-1, DHA-2	DHAM F AAC TTT CAC AGG TGT GCT GGG T DHAM R CCG TAC GCA TAC TGG CTT TGC	405	4	Pérez-Pérez and Hanson, 2002
	ACC	ACCM F AAC AGC CTC AGC AGC CGG TTA ACCM R TTC GCC GCA ATC ATC CCT AGC	346	4	Pérez-Pérez and Hanson, 2002
	MIR-1T, ACT-1	EBCM F TCG GTA AAG CCG ATG TTG CGG EBCM R CTT CCA CTG CGG CTG CCA GTT	302	4	Pérez-Pérez and Hanson, 2002
	FOX-1 to FOX-5b	FOXM F AAC ATG GGG TAT CAG GGA GAT G FOXM R CAA AGC GCG TAA CCG GAT TGG	190	4	Pérez-Pérez and Hanson, 2002
Integrases	*intI1*	intI1 F GCA TCC TCG GTT TTC TGG intI1 R GGT GTG GCG GGC TTC GTG	457bp	5	Shibata *et al*., 2003
	intI2	intI2 F CAC GGA TAT GCG ACA AAA AGG T intI2 R GTA GCA AAC GAG TGA CGA AAT G	789bp	5	Shibata *et al*., 2003
	intI3	intI3 F ATT GCC AAA CCT GAC TG intI3 R CGA ATG CCC CAA CAA CTC	922bp	5	Shibata *et al*., 2003
	Intl4	Intl4 F: CGG TAT GTC TAA TTG CTC TTG Intl4 R: TGG CCA CAA AGA CTC AAT CAC	696bp	5	Goldstein *et al*., 2001

Table 2. Antibiotic resistance of 51 *K. pneumoniae* isolates recovered from different clinical samples

Antibiotic	Resistant strains	
	No.	%
Cetriaxone	27	52.9
Cefotaxime	28	54.9
Imipenem	0	0
Ceftazidime	21	41.2
Aztreonam	23	45.1
Meropenem	35	68.6

Table 3. Prevalence of β-lactamase among 51 clinical *K. pneumoniae* isolates recovered from different clinical samples by multiplex PCR technique

β-Lactamases														
Extended spectrum β-lactamases No. (%)					AmpC β-lactamase No. (%)					Metallo-B-lactamases No. (%)				
Class (A)				Class (D)	Class (C)					Class (B)				
SHV	TEM	CTX-M	TEM and OXA	OXA	MOX, CMY, BIL	DHA	ACC	MIR-1T, ACT-1	FOX	Imp	Vim	Gim	Spm	NDM
9 (17.6)	22 (43.1)	0 (0)	15 (29.4)	1 (1.96)	0 (0)	2 (3.9)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	5(9.8)
Total 47 (92.2)					Total 2 (3.92)						Total 5 (9.8)			

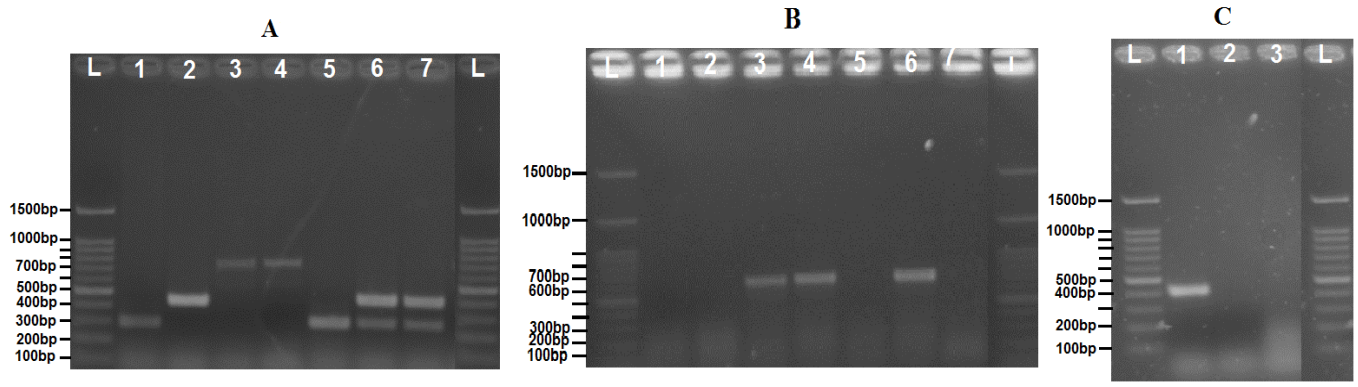

Figure 1. Multiplex PCR profiles specific for ESBLs, MBLs and/or AmpC β-lactamases. L represented the ladder Figure A for ESBLs: Lanes 1 and 5 for *OXA* gene; Lane 2 for *TEM* gene; lanes 3 and 4 for *SHV* gene and Lanes 6 and 7 for *TEM* and *OXA* genes. Figure B for MBLs: Lanes 3, 4 and 6 for *NDM* genes. Figure C for AmpC β-lactamases: Lane 1 for *DHA* gene

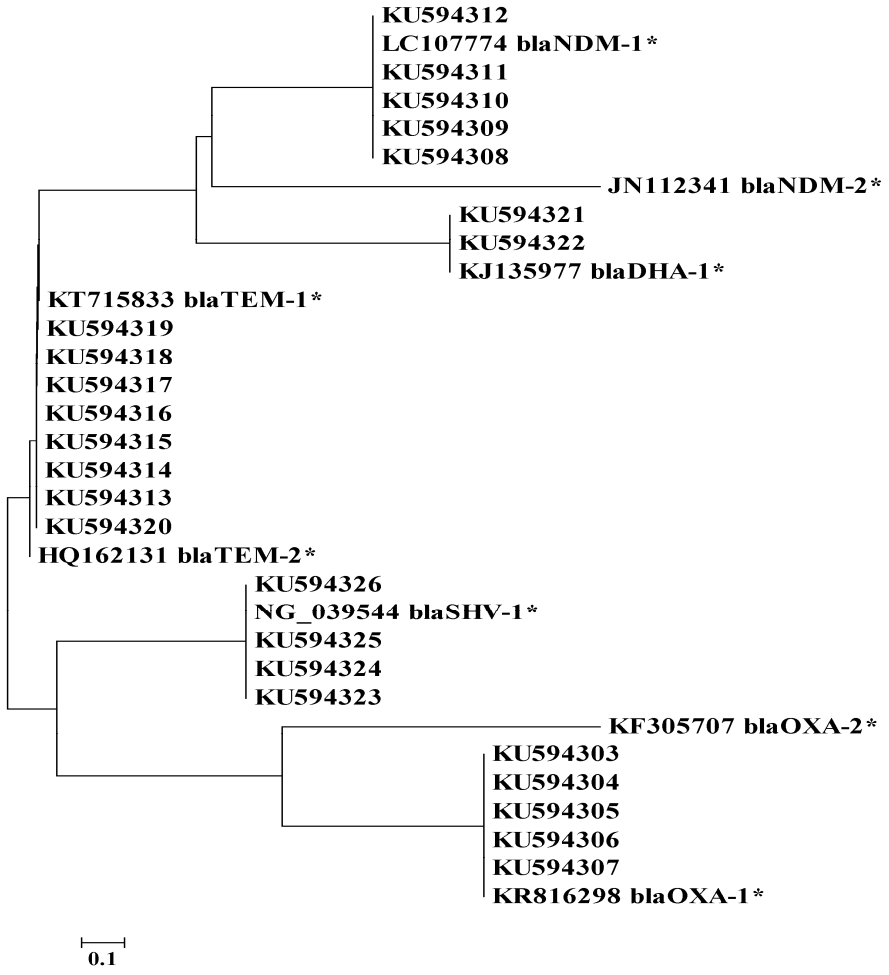

Figure 2. Phylogenetic analysis constructed by Neighbor-Joining method based on the partial *NDM-1*,*DHA-1*, TEM-1, *SHV-1* and *OXA-1* and gene nucleotide sequences. Reference sequences for the *NDM-1, NDM-2, DHA-1*, *TEM-1*, *TEM-2*, *SHV-1*, *OXA-1 and OXA-2* gene nucleotide sequences were denoted by asterisk. The tree was bootstrapped with 1000 replicate, and the genetic distance corresponding is shown by the bar. The evolutionary distances were computed using the Maximum Composite Likelihood method. All positions containing gaps and missing data were eliminated. There were a total of 264 positions in the final dataset. Evolutionary analyses were conducted using MEGA version 5

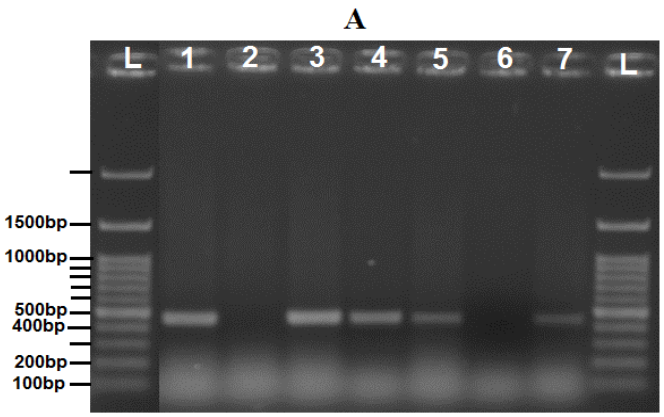

Figure 3. Multiplex PCR profile specific for integrons. L represented the ladder, Lanes 1, 3, 4 and 5 represent class I integron

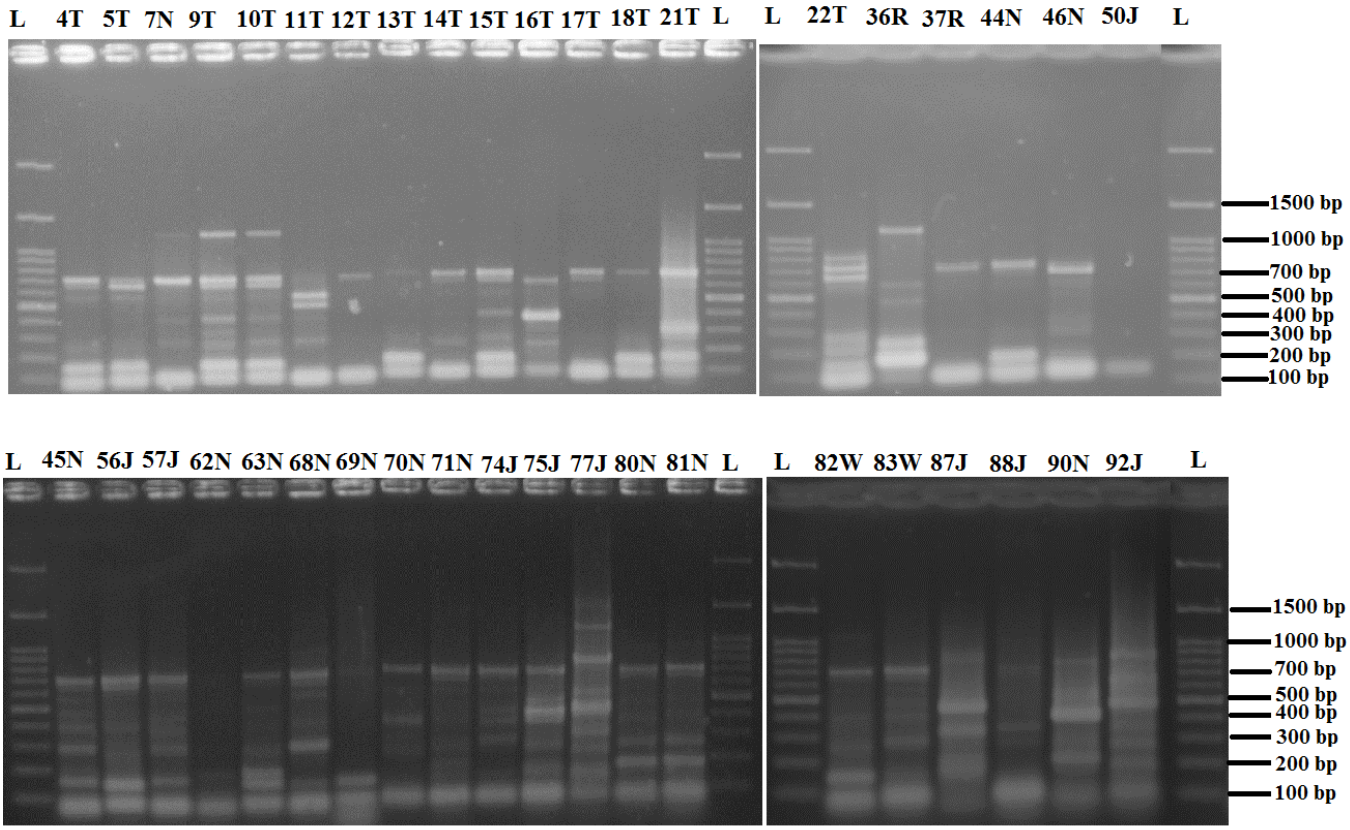

Figure 4. DNA fingerprints generated by ERIC-PCR typing of 40 clinical K. pneumoniae **isolates carried genes for ESBLs, MBLs and/or AmpC β-lactamases recovered on 1.5% agarose gel. Lanes L represented the ladder, while other lanes for ERIC-PCR products**

Gene amplification was carried out with a thermal cycler (Mastercycler Personal, Eppendorf) using the following conditions: 94 °C for 5 min; 94 °C for 30 sec, 52 °C for 40 sec and 72 °C for 50 s for 36 cycles; with a final extension at 72 °C for 5 min. PCR products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

Detection of KPC, *NDM* and *DIM* genes

Detection of gene sequences coding for the *KPC*, *NDM* and *DIM* genes was performed by the multiplex PCR according to method described previously (Poirel *et al*., 2011a).

Sequences of primers and size of amplicons are described in Table 1. PCR reactions and conditions were performed as well as described in detection of MBL genes. PCR products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

Detection of AmpC β-lactamase genes

Detection of plasmid mediated AmpC β-lactamase (*ampC*) genes in all isolates was carried out by multiplex PCR as described previously (Pérez-Pérez and Hanson, 2002). Primer nucleotide sequences and expected sizes of amplicons for

AmpC β-lactamase (*ampC*) genes are presented in Table 1. PCR reactions were performed as well as described in detection of MBL genes. Gene amplification was carried out with a thermal cycler (Mastercycler Personal, Eppendorf) using the following conditions: 94 °C for 3 min; 94 °C for 30 sec, 64 °C for 30 sec and 72 °C for 1 min for 25 cycles; with a final extension at 72°C for 7 min. PCR products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

Detection of ESBL (TEM, SHV, CTX-M and OXA) genes

Detection of gene sequences coding for the TEM, SHV, CTX-M and OXA enzymes was performed by the multiplex PCR. The oligonucleotide primer sets and expected amplicon sizes (bp) specific for the SHV, TEM, CTX-M and OXA genes are presented in Table 1 (Paterson *et al*., 2003; Boyd *et al*., 2004; Monstein *et al*., 2007; Kim *et al*., 2009).

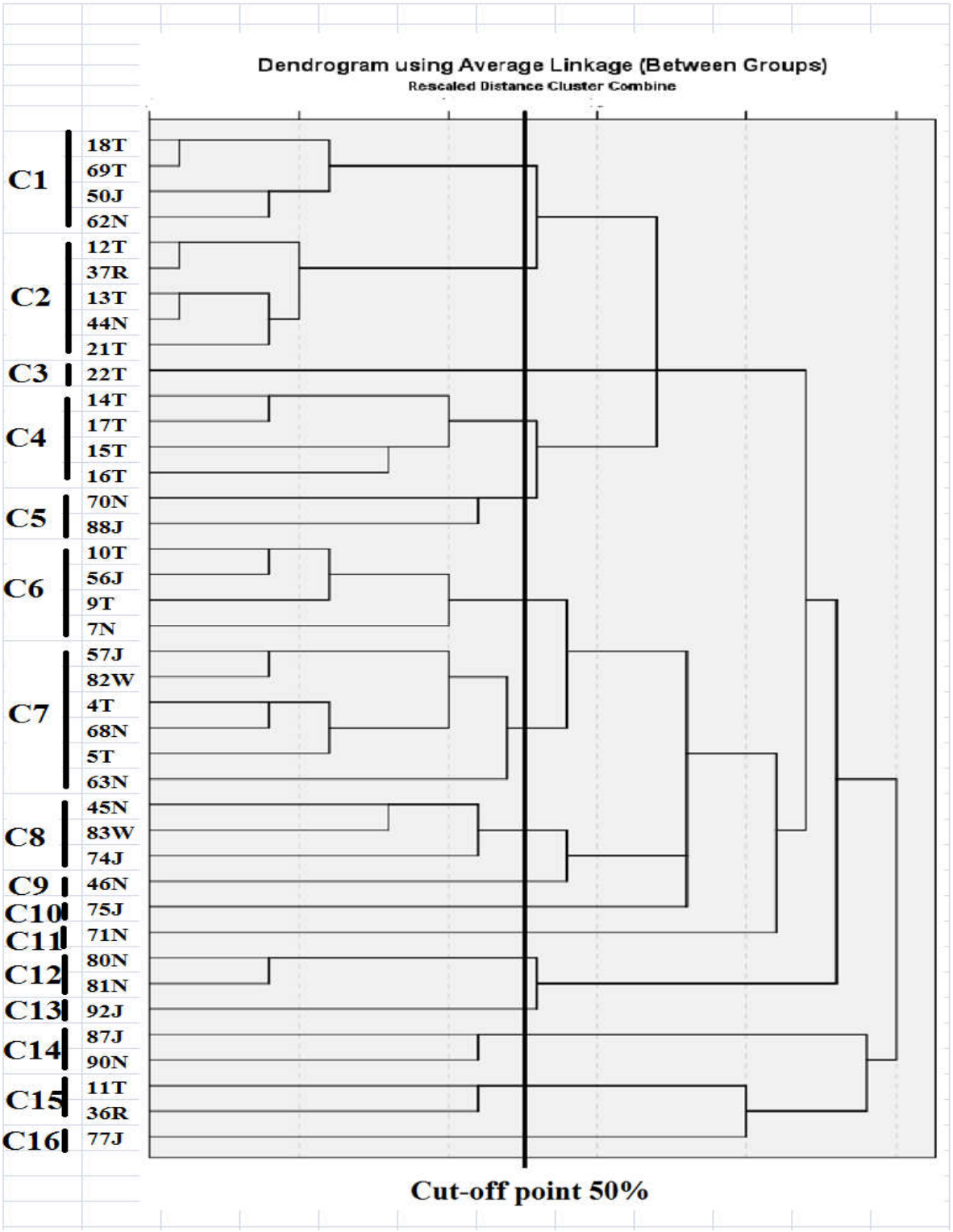

Figure 5. Dendrogram of 40 K. pneumoniae **isolates carried genes for ESBLs, MBLs and/or AmpC β-lactamases based on the UPGMA method derived from analysis of the ERIC-PCR-profiles at a 50% similarity level. C: Cluster**

PCR reactions were carried out as well as described in detection of MBL genes. Gene amplification was performed with a thermal cycler (Mastercycler Personal, Eppendorf) using the following conditions: 94 °C for 5 min; 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 2 min for 25 cycles; with a final extension at 72 °C for 5 min. PCR products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

Detection of class 1, 2, 3 and 4 integrons

All *K. pneumoniae* isolates were screened for the presence of integrase genes *intI1*, *intI2*, *intI3* and *intI4* using primers previously described (Goldstein *et al*., 2001; Shibata *et al.*, 2003). Sequences of primers and size of amplicons are described in Table 1. PCR reactions were performed as well as described in detection of MBL genes. The amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following thermal conditions: denaturation at 94 ºC for 30 s, annealing at 58 ºC for 30 s, extension at 72 ºC for 1 min (30 cycles), with a final extension step at 72ºC for 2 min. PCR products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

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 (25 µL) was carried out using 12.5 µl of PCR premix with $MgCl_2$ (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$, Sigma), 1 µM of each primer, 3 µl DNA template. In addition, the master mix was modified by increasing the concentration of dNTPs to 400 µM, 3 mM MgCl2 and 2 U of Taq DNA polymerase for each reaction. 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 94ºC was followed by 40 cycles of denaturation at 94ºC for 50 s, annealing at 40ºC for 40 s and extension at 72ºC for 1 min, with a final extension step at 72ºC for 5 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel. The 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).

Sequence homology and phylogenetic analysis

Amplified PCR products of some β-lactamases genes were purified by the ChargeSwitch®-Pro PCR Clean-Up Kit (Invitrogen, USA). PCR products were sequenced by dideoxy chain termination method using 3130 Genetic Analyzer (Applied Biosystems®,USA), Bethlehem University, Bethlehem, Palestine. Sequence information for these genes were further submitted to Genbank for accession numbers. The comparison of the continuous sequences was made with previously available sequences of the β-lactamases genes in NCBI (National Center for Biotechnology Information) using BLAST system. Multiple sequence alignment was carried out using ClustalW of the computer program MEGA software (version 5). The evolutionary distances were computed using the Maximum Composite Likelihood method. Phylogenetic analyses were based on alignments obtained from ClustalW of a 264 bp sequence. Phylogenetic tree was constructed using the program Neighbor-Joining in the same software. The robustness of the groupings in the Neighbor Joining analysis was assessed with 1000 bootstrap resamplings.

RESULTS

Antibiotic resistance

The resistance pattern of 51 *K. pneumoniae* isolated from different hospitals against six chosen antimicrobial agents is presented in Table 2. Results of the current study showed that all *K. pneumoniae* isolates were sensitive to Imepenem (100%), while 68.62% and 54.9% of the isolates were resistant to Meropenem and Cefotaxime, respectively.

Detection of β-lactamases and integrons

Among the 51 tested strains of *K. pneumoniae*, the prevalence of ESBLs, MBLs and AmpC β-lactamases using multiplex PCR technique was 92.2%, 9.8 and 3.9%, respectively. The majority of isolates producing a single ESBL harbored the TEM gene (43.1%) and the remaining isolates carried the following genes SHV (17.6%) and OXA (1.96%). Fifteen isolates were found to carry TEM and OXA with the prevalence (29.4%). TEM gene is considered the most common (n=37; 72.5%) detected among *K. pneumoniae* isolates. For AmpC β-lactamases, only DHA gene was detected and the prevalence was (3.92%). Five MBL producing isolates were identified among all the collected isolates. In this study the NDM gene was detected in all of these isolates with the prevalence (9.8%). In addition, six isolates (11.76%) were coexisted with at least another type of β-lactamases in the same isolate. Results are presented in Table 3. and Figure 1. Molecular analysis by construction phylogenetic tree showed that all sequenced *TEM*, *SHV*, *OXA, NDM* and *DHA* genes were belonged to *TEM-1*, *SHV-1*, *OXA-1, NDM-1* and *DHA-1*, respectively . Results are presented in Figure .2. The nucleotide sequences reported in this study were further deposited at the Gene Bank database under the accession numbers KU594303-KU594326. A total of 26 of *K. pneumoniae* isolates (51%) harbored class 1 integrons, whereas class 2, 3 and 4 were not detected. Representative result of class 1 integrons is presented in Figure 3. All integrones detected in isolates with β-lactamases.

ERIC-PCR analysis

ERIC-PCR typing of 40 *K. pneumoniae* isolates which harbored genes for ESBLs, MBLs and/or AmpC β-lactamases were genetically diverse and consisted of a heterogeneous population with a total of 16 ERIC-PCR profiles (clusters) at a 50% similarity level. Results of ERIC-PCR profiles are presented in Figures 4 and 5.

DISCUSSION

Bacterial infection complications are considered as an important factor of outcome for patients requiring hospital

admission specially in the intensive care unit. This is particularly true in the current period due to increasing in antimicrobial resistance that affects many bacterial pathogens. *K. pneumoniae* is one of opportunistic pathogens that frequently cause nosocomial infections. Beta-lactam antibiotics are broadly used in the treatment of bacterial infections; this may lead a significant increase in the incidents of β-lactamases-associated infections throughout the world (Shaikh *et al*., 2014). Results of this study showed that the prevalence of ESBL among *K. pneumoniae* in Palestine is too high. In countries of the Middle East, the prevalence of ESBL producers *K. pneumoniae* ranged from 17.4%-71.4% (Fam *et al*., 2006; Al-Agamy *et al*., 2009; Batchoun *et al*., 2009; Nedjai *et al*., 2012). Results of current research showed that TEM type ESBL was most common in *K. pneumoniae* isolates, which is in agreement with other studies (Ghasemi *et al*., 2013; Chelliah *et al*., 2014). In contrast, other studies showed that CTX-M-type or SHV-type ESβL was most common (Daoud Z., 2008; Al-Agamy *et al*., 2009; Nedjai *et al*., 2012; Khosravi *et al*., 2013). Sequence analyses of PCR products from amplification of TEM, SHV and OXA genes showed that these genes were homologues to TEM-1, SHV-1 and OXA-1 genes, and this result was consistent with various studies (Khosravi *et al*., 2013; Sugumar *et al*., 2014). In this study, coexistence of 2 ESBL genes (*TEM* and *OXA*) was noticed in 29.4% of *K. pneumoniae* isolates. Presence of coexistence of ESBL genes in a single isolate was reported (Kiratisin *et al*., 2008).

Results of this study showed that 3.9% of *K. pneumoniae* isolates carried AmpC β-lactamase genes. The prevalence of *AmpC* β- lactamases among *K. pneumoniae* isolates varied among countries and had a range from 0%- 79% (Ahmad and Ali, 2014; Gajul *et al*., 2015). This low prevalence of *AmpC* β-lactamases among *K. pneumoniae* isolates was due to that primers in this research used to detect plasmid encoding genes only, while conventional methods have ability to detect both plasmid and non-plasmid-derived (chromosomal) AmpC activity (Adwan and Abu Jaber 2016). This research showed that only the DHA-1 subtype of AmpC β-lactamases was detected among *K. pneumoniae* isolates. These results were in agreement with other studies (Lagha *et al*. 2014; Matsumura *et al*., 2015). Whereas, studies from various parts of the world reported the presence of other different subtypes (Yan *et al*., 2004; Ktari *et al*., 2006; Fam *et al*., 2013).

The increasing and rapid spread of MBL producing *Enterobacteriaceae*, particularly *K. pneumoniae* constitutes a serious medical problem to public health worldwide. Results of the current study showed that 9.8% of *K. pneumoniae* isolates were produce MBLs. Our result is in contrast to other studies from Middle East which showed that the prevalence has ranged from 21.7% to 35.7% (Shibl *et al*., 2013; Ahmad and Ali, 2014). Isolates which carried MBL genes revealed the presence of NDM-1type gene, this report is considered the first about detection of NDM gene in Palestine. Emergence of NDM-1 producing *K. pneumoniae* isolates has been reported from Middle East (Poirel *et al* 2011b; Jamal *et al*., 2013; Dortet *et al*., 2012; Shibl *et al*., 2013; Sonnevend *et al*., 2013). On the other hand, many other studies reported the presence of different subtypes of MBLs (Shibl *et al*., 2013; Sonnevend *et al*., 2013). According to NDM-producing pathogens, India and Pakistan are considered to be the major reservoir for NDM-producing isolates, it has also been suggested that the Middle East countries might be a secondary reservoir for the spread of blaNDM-1 isolates as there is a high frequency of population movement. NDM-1, has now been isolated in Europe, Asia, North America, and Australasia, NDM-producers are of particular concern as they also harbor multiple chromosomally and plasmid-encoded resistance genes resulting in a multidrug resistant phenotype (Shibl *et al*., 2013). Coexistence of two or three types of β-lactamase genes in single isolates was observed in this study., this may seriously restrict the options of treatment, create diagnostic challenge and may lead to use an unsuitable antimicrobial therapy and the result may be fatal (Oberoi *et al*., 2013). Coexistence of more than one of β-lactamase classes or multiple genes of ESBLs, MBLs or AmpC β-lactamases has been reported from different species of bacterial pathogens including clinical *K. pneumoniae* isolates (Yan *et al*., 2004; Jamal *et al*., 2013; Oberoi *et al*., 2013). In this study, approximately half of the of *K. pneumoniae* isolates carried class 1 integrons and none carried other tested classes of integrons. This results is in agreement with other reports recently published, which showed that the class 1 integrons was the most common or the only detected among clinical isolates of *K. pneumoniae* (Derakhshan *et al*., 2014; Lima *et al*., 2014; Mobarak-Qamsari *et al*., 2014). High prevalence of class 1 integrons supports high genetic variability and rapid dissemination of β-lactamase genes among clinical isolates of *K. pneumoniae* in hospitals (Lima *et al*., 2014).

Genotyping of β-lactamases-producing *K. pneumoniae* isolates by the ERIC-PCR method showed different DNA banding profiles. A 50% similarity cut-off value analysis, showed that there were a total of 16 clusters among the tested 40 *K. pneumoniae* isolates. These findings demonstrate that isolates included in our study were genetically diverse and multiple clones of β-lactamase-producing *K. pneumoniae* isolates were prevalent in these hospitals. 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. This supporting that the high prevalence of β-lactamases-producing *K. pneumoniae* isolates may be due to selective pressure of antibiotic imposed by the high rate and misuse of antimicrobial agents especially cephalosporins in the country could be the only major cause (Adwan and Abu Jaber, 2016). To our knowledge, up to now, this is the first study documented the prevalence and molecular characterization of β-lactamases producing *K. pneumoniae* isolates in Palestine. In conclusion, our results showed high occurrence of β-lactamases among *K. pneumoniae* 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 restrict the further spread of β-lactamases producing *K. pneumoniae* isolates within hospitals in this country.

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