International peer reviewed open access journal

Journal of Medical Pharmaceutical and Allied Sciences

Prevalence and Molecular Characterisation of β- Lactamases among Pathogens Isolated from Surgical Site Infections

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ABSTRACT

Surgical site infections (SSIs) considered a worldwide problem associated with a significant morbidity and mortality, prolonged hospitalization and consequently increasing healthcare expenditures. Thirty bacterial isolates were recovered from SSIs at Rafidia Hospital-Nablus during February-April 2016. The study was conducted to assist the prevalence and molecular characterization β-lactamases and integron classes among the isolated pathogens using multiplex PCR. The results showed that the prevalence of β-lactamase genes was 83.3%. The prevalence of ESBLs, MBLs and AmpC β-lactamases was 83.3%, 23.3 and 13.3%, respectively. The prevalence of ESBLs within isolated bacterial species was 76.5% for *E. coli*; 88.9% for *S. aureus* and 100% for each of *Klebsiells* sp., *Enterobacter* sp. and *Acinatobacter* sp. The most prevalent gene was *bla*TEM (100%). The prevalence of AmpC genes was 11.8% for *E. coli*, 11.1% for *S. aureus*, 100% for *Enterobacter* and 0% for both *Klebsiells* sp. and *Acinatobacter* sp.. MBL genes were detected only in 41.2% of *E. coli* isolates. The most prevalent gene among MBL- producer *E. coli* was *bla*SPM (85.7%). In addition, 30% of the tested isolates harbor at least another type of β-lactamases. With respect to integron classes, 40% of SSIs bacterial isolates harbored class 1 integrons, and none of the isolates expressed classes 2 or 3. All class I integrons expressed in association with β-lactamases, particularly those carrying *bla*TEM genes.

The finding of high level of β-lactamases among pathogens causing SSIs in the area is expected to complicate management protocols of these infections and could be associated with higher SSI rates, prolonged hospitalization and increased number of used antimicrobial agents. Appropriate surgical antibiotic prophylaxis and continuous monitoring systems are absolutely required and can reduce the risk of such infections at surgical sites in the region.

INTRODUCTION

Surgical site infections (SSIs) are considered a common and worldwide problem associated with a significant morbidity and mortality, prolonged hospitalization and consequently increasing healthcare expenditures. Such infections partly results due to the increase in antimicrobial resistance among the etiological pathogens. Despite the technical advances in infection control and surgical practices, SSIs still continue to be considered as a major problem. The incidence of these infections varies depending on different factors such as the surgical procedure, the surveillance criteria used, and the quality of data collection and accordingly, the incidence rate may accounts as high as 20% among surgical patients. In hospitals, approximately 30% to 50% of antibiotics are prescribed for surgical prophylaxis and 30% to 90% of these antibiotics are improperly used. Antimicrobial agents, particularly β- lactams are the most common used types in prophylaxis. The consequences of SSIs are partially referred to increase in antimicrobial resistant bacterial pathogens, which make the choice of therapy more difficult and complex.

It is obvious that pathogens will continue to develop resistance to different classes of antimicrobial agents through intrinsic

and acquired mechanisms. One of the major antibiotic resistance mechanisms among pathogenic bacteria is the production of β-lactamases. Beta-lactamase enzymes are classified according to two systems. The Ambler molecular classification system classifies β lactamases into four classes (A- D) according to the protein homology of the 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 Bush-Jacoby- Medeiros functional classification system classifies these 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. The latter system classifies the β-lactamases into 3 main groups and 16 subgroups. The most important β-lactamases are cephalosporinases like (AmpC β- lactamases), extended spectrum β-lactamases (ESBLs) and the carbapenemases like metallo-β-lactamases (MBLs) [1]. Beta-lactamase producing pathogens have become a global problem. The rate of incidence and type of β-lactamase enzymes varies in different countries. In recent study in Tanzania, results showed that 79.3% of Enterobacteriaceae isolated from SSIs were ESBL-producers. Prevalence of ESBLs were 92.3% and 69% among Escherichia coli (E. coli) and Klebsiella pneumoniae (K. pneumoniae), respectively. In recent study in Nigeria, results showed that 73.2% of pathogens isolated from wound and SSIs were β-lactamase producers. The prevalence of β-lactamase producing pathogens were 78.5%, 56.3%, 75%, 50%, 83.3%, 100%, 66.6%, 100% and 100% for S. aureus, coagulase negative staphylococcus (CoNS), Streptococcus pyogenes, Proteus vulgaris (P. vulgaris), P.mirabilis, Klebsiella sp., Pseudomonas, E. coli and Citrobacter freundii, respectively. In Sudan, the prevalence of β-lactamase producing organisms isolated postoperative wound infection were 100% for E. coli, Pseudomonas aeruginosa (P. aeruginosa) and P. vulgaris, while 85.5% and 80% for S. aureus and P. mirabilis, respectively. In India, 43% of E. coli isolated from SSIs reported to carry ESBL genes, the predominant (67%) were carried blaCTX-15, combined ESBL-producers and AmpC hyper producers were 29%, AmpC hyper producers 14% and blaNDM-1 producers were 14%. The prevalence of ESBL genes among K. pneumoniae was 67%. All ESBL-positive isolates carried blaCTX-M-15, and 33% were blaNDM-1 producers. Only one strain of E. cloacae was isolated from SSIs and carried blaNDM-1. In Bangladesh, the prevalence of ESBL-producing pathogens among bacteria recovered from SSI infections was 23.5%. The prevalence of ESBL according to bacterial species were 55%, 33.3%, 11.1%, 6.7% and 0% for E. coli, Klebsiella sp., Proteus sp., Pseudomonas sp. and S. aureus, respectively. In other study from the same country, 26.6% of E. coli isolated from wound

infections had ESBL phenotype. All isolates which were ESBL phenotype positive carried ESBL genes of blaCTX-M group; blaTEM-type and blaOXA-type genes were detected in 41.2% and 88.2% of ESBL phenotype positive isolates, respectively. Class-1 integrons were detected in 41.2% of these isolates. In Poland, the prevalence of ESBL-producing organisms recovered from SSIs was 15.7%.

No information is available about the prevalence and molecular characterization of β-lactamases in pathogens isolated from SSIs in Palestine. The current study was carried out to determine the prevalence and molecular characterization β- lactamases among pathogens isolated from SSIs using molecular techniques; and to evaluate the prevalence of classes 1, 2 and 3 integrons in these isolates.

MATERIAL
Sample collection and Identification

A total of 30 bacterial isolates were collected using sterile cotton swabs from patients clinically diagnosed having SSIs at Rafidia Hospital-Nablus during February- April 2016. The bacterial isolates were 17 *E. coli*, 9 *S. aureus*, 2 *Klebsiella* sp., 1 *Enterobacter* sp. and 2 *Acinatobacter* sp. Antibiotic resistance for these isolates, ERIC-PCR profiles for *E. coli* and *S. aureus* were published previously. For further analysis, DNA of these isolates was stored at -20°C [2].

Detection β-lactamase genes by multiplex PCR
Amplification of ESBL genes

All bacterial isolates were screened for the presence of gene sequences coding for the TEM, SHV, CTX-M and OXA enzymes by multiplex PCR. The oligonucleotide primer sets for these genes were described previously; *bla*SHV genes, *bla*TEM genes, *bla*CTX-M genes, and *bla*OXA genes. Sequence of primers and size of amplicons are described in Table 1. Briefly, PCR reactions were performed in a final volume of 25μl of the amplification mixture containing 12.5μl of PCR premix with MgCl2 (ReadyMix^{TM} Taq PCR Reaction Mix with MgCl$_2$, Sigma), 0.4μM of each primer, 3μl of DNA template. Amplification of genes was performed with a thermal cycler (Mastercycler Personal, Eppendorf) using the following conditions: initial denaturation for 5 min at 94°C; followed by 25 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 2 min; with a final extension step at 72°C for 5 min. PCR products were visualised on 1.5 % agarose gels stained with ethidium bromide.

Amplification of AmpC β-lactamase genes

All bacterial isolates were screened for the presence of plasmid-mediated *ampC* gene sequences coding for the MOX, CMY, LAT, BIL, DHA, ACC, MIR-1T, ACT-1 and FOX enzymes. The oligonucleotide primer sets for these genes were described previously. The sequence of primers and size of amplicons are described in Table 1. PCR reactions were performed as well as described in amplification of ESBL genes. Amplification of these genes was carriedout using thermal cycler (Mastercycler Personal, Eppendorf) as follows: initial denaturation for 3 min at 94°C; followed by 25 cycles of denaturation

at 94°C for 30 sec, annealing at 64°C for 30 sec and extension at 72°C for 1 min; with a final extension step at 72°C for 5 min. PCR products were visualized on 1.5% agarose gels stained with ethidium bromide.

Amplification of Carbapenems genes

Detection of gene sequences coding for the VIM, IMP, SPM-1, GIM-1 and SIM-1 enzymes was performed by the multiplex PCR using oligonucleotide primer sets described previously. Detection of other gene sequences coding for the KPC, NDM and DIM enzymes was performed by a different multiplex PCR using oligonucleotide primer sets described previously. Sequence of primers and size of amplicons are described in Table 1. PCR reactions were carried out as described for the amplification of ESBL genes. Amplification of genes was carried out using thermal cycler (Mastercycler Personal, Eppendorf) as follows: initial denaturation for 5 min at 94°C; followed by 36 cycles of denaturation at 94°C for 30 sec, annealing at 52°C for 40 sec and

extension at 72°C for 50 sec; with a final extension step at 72°C for 5 min. PCR products were visualized on 1.5 % agarose gels stained with ethidium bromide.

Amplification of class 1, 2 and 3 integrons

All 30 bacterial isolates recovered from SSIs were screened for the presence of integrase genes *intI1*, *intI2* and *intI3* using primers previously described. Sequence of primers and size of amplicons are described in Table 1. PCR reactions were performed as previously described for the amplification of ESBL genes. Amplification of these genes was carried out using thermal cycler (Mastercycler personal, Eppendorf, Germany) as follows: initial denaturation for 3 min at 94°C followed by 35 cycles of denaturation at 94°C for 40 sec, annealing at 58°C for 40 sec and extension at 72°C for 40 sec; with a final extension step at 72°C for 2 min. PCR products were visualized on 1.5 % agarose gels stained with ethidium bromide [3].

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

Group	Targets	Primer sequence 5′→3′	amplicon size (bp)	Primer mix
AmpC β-lactamase (Class C)	*bla*MOX-1, *bla* MOX-2, *bla*CMY-1, *bla*CMY-8 to *bla* CMY-11	MOXM F GCT GCT CAA GGA GCA CAG GAT MOXM R CAC ATT GAC ATA GGT GTG GTG C	520	1
	*bla*LAT-1 to *bla*LAT-4, *bla*CMY-2 to *bla*CMY-7, *bla*BIL-1	CITM F TGG CCA GAA CTG ACA GGC AAA CITM R TTT CTC CTG AAC GTG GCT GGC	462	1
	*bla*DHA-1, *bla*DHA-2	DHAM F AAC TTT CAC AGG TGT GCT GGG T DHAM R CCG TAC GCA TAC TGG CTT TGC	405	1
	*bla*ACC	ACCM F AAC AGC CTC AGC AGC CGG TTA ACCM R TTC GCC GCA ATC ATC CCT AGC	346	1
	*bla*MIR-1T, *bla*ACT-1	EBCM F TCG GTA AAG CCG ATG TTG CGG EBCM R CTT CCA CTG CGG CTG CCA GTT	302	1
	*bla*FOX-1 to *bla*FOX-5b	FOXM F AAC ATG GGG TAT CAG GGA GAT G FOXM R CAA AGC GCG TAA CCG GAT TGG	190	1
ESBL (Carbapenemases, class D)	*bla*OXA	OXA F 5-ATT ATC TAC AGC AGC GCC AGT G OXA R 5-TGC ATC CAC GTC TTT GGT G-3	296	2
ESBL/Carbapenemase (Class A)	*bla*SHV	SHV F 5-ATG CGT TATATT CGC CTG TG-3 SHV R 5-TGC TTT GTT ATT CGG GCC AA-3	747	2
	*bla*TEM	TEM F 5-TCG CCG CAT ACA CTA TTC TCA GAA TGA TEM R5- ACG CTC ACC GGC TCC AGA TTT AT	445	2
	*bla*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	2
	*bla*KPC	KPCM F CGT CTA GTT CTG CTG TCT TG KPCM R CTT GTC ATC CTT GTT AGG CG	789	3
MBL(Carbapenemases, class B)	*bla*NDM	NDM F GGT TTG GCG ATC TGG TTT TC NDM R CGG AAT GGC TCA TCA CGA TC	621	3
	*bla*DIM	DIM F GCT TGT CTT CGC TTG CTA ACG	699	3

RESULT

Prevalence of β-lactamases

The prevalence of β-lactamase genes among bacterial isolates recovered from SSIs was 83.3%. The prevalence of ESBLs, MBLs and AmpC β-lactamases using the multiplex PCR technique was 83.3%, 23.3 and 13.3%, respectively. According to bacterial species, the prevalence of ESBL genes was 76.5% for *E. coli*; 88.9% for *S. aureus*, 100% for *Klebsiella* sp., *Enterobacter* sp. and *Acinetobacter* sp. The most prevalent gene among isolates that carried β-lactamase genes was *bla*TEM (100%). The prevalence of Amp C genes among bacterial species was 11.8% for *E. coli*, 11.1% for *S. aureus*, 100% for *Enterobacter* and 0% for both *Klebsiella* sp. and *Acinetobacter* sp. MBLs genes were only detected in *E. coli*, 41.2% of the isolates of this microorganism were MBL-producers. The most prevalent gene among MBL-producer *E. coli* was *bla*SPM (85.7%). The prevalence of ESBL, AmpC and MBL genes among bacterial isolates recovered from SSIs is presented in Table 2 and Figures 1, 2 and 3. In addition, 9 isolates (30%)

expressed more than one type of β- lactamases.

Prevalence of Integrons

A total of 40% of isolates recovered from SSIs harbored class 1 integrons, whereas class 2 and 3 were not detected. It was also noticed that all class I integrons were detected in β- lactamases positive isolates, particularly those carrying bla_{TEM} genes. The incidence of class I integrons among bacterial species was 29.4%, 66.7% and 50% for *E. coli*, *S. aureus* and *Klebsiella* sp., respectively. None of the isolates of *Enterobacter* sp. and *Acinatobacter* sp. harbor class I integron. Representative results of class 1 integrons are presented in Figure 4

Table 2. Prevalence of β-lactamase genes among 30 bacterial isolates recovered from different SSIs by multiplex PCR technique.

Organism (n)	ESBL genes* n (%)			Total n (%)
	bla_{TEM}	bla_{TEM} and bla_{OXA}	bla_{TEM}, bla_{OXA} and bla_{SHV}	
E. coli (17)	11 (64.7)	1 (5.9)	1 (5.9)	13 (76.5)
S. aureus (9)	8 (88.9)	0 (0)	0 (0)	8 (88.9)
Klebsiella sp. (2)	2 (100)	0 (0)	0(0)	2 (100)
Enterobacter sp. (1)	1(100)	0 (0)	0(0)	1 (100)

Figure 1. Multiplex PCR profiles specific for ESBL. Lanes L represent the ladder; lane1 represents bla_{TEM} and bla_{OXA} genes; lanes 2,3,4 and 5 represent bla_{TEM} gene and lane 6 represents bla_{SHV}, bla_{TEM} and bla_{OXA} genes.
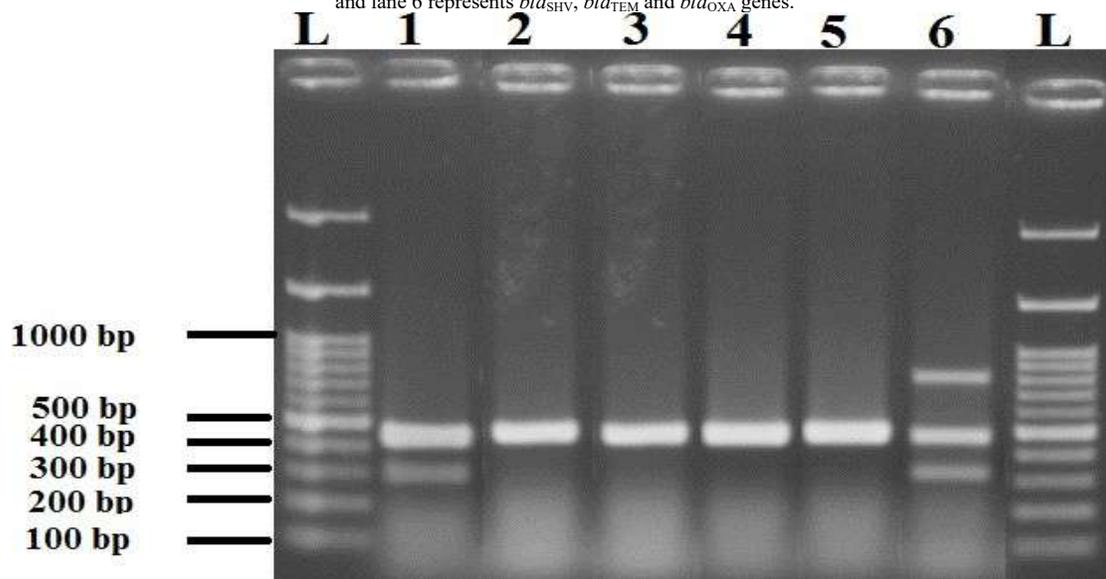

Figure 2. Multiplex PCR profiles specific for AmpC β-lactamases. Lanes L represent the ladder; lanes 1 and 2 in A1 represent bla_{LAT} or bla_{CMY} or bla_{BIL-1} and bla_{FOX} genes in *E. coli*; lane 1 in B2 represent bla_{LAT} or bla_{CMY} or bla_{BIL-1} and bla_{FOX} genes in *S. aureus* and lane 2 in B2 represents bla_{MIR-1T} or bla_{ACT-1} gene *Enterobacter* sp. Figure 2. A1 and B1: They are the same as A2 and B2, respectively, but bands are demarcated for clarity.
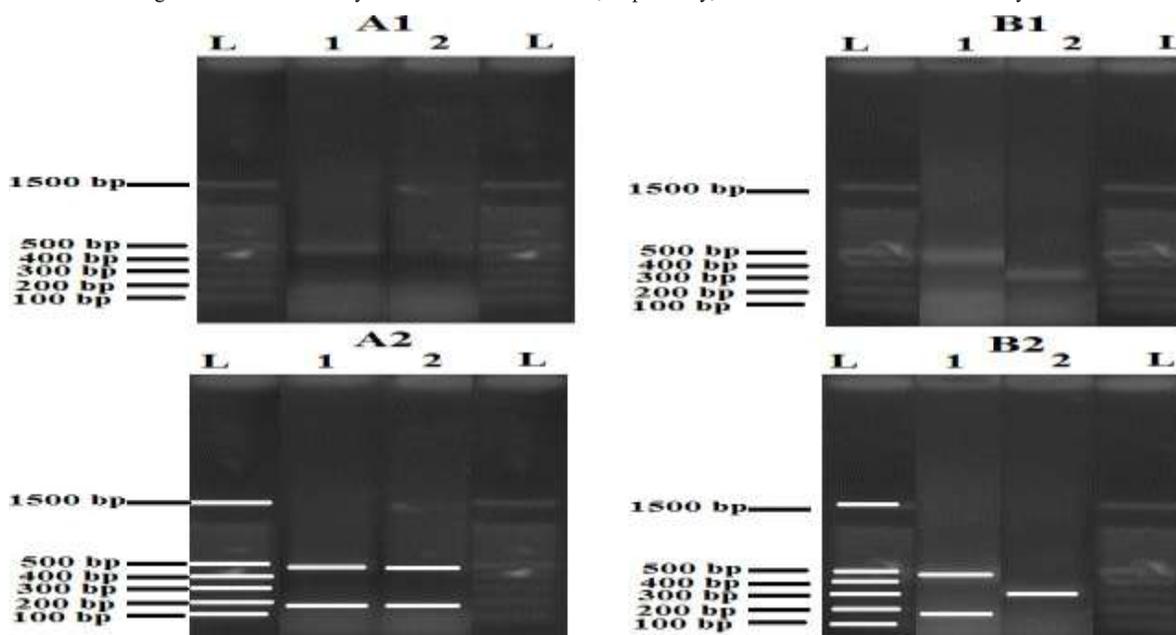
Figure 3. A1: Multiplex PCR profiles specific for MBLs. Lanes L represent the ladder; lane 1   represents *bla*SPM and *bla*IMP genes; lanes 2, 4, 5 and 6 repe *bla*SPM gene; lane 7 represents *bla*SIM gene; lane 8 represents *bla*SIM and *bla*SPM genes and lane 3 represents negative control. Figure 3. A2: It is the same as A1 but bands are demarcated for clarity.
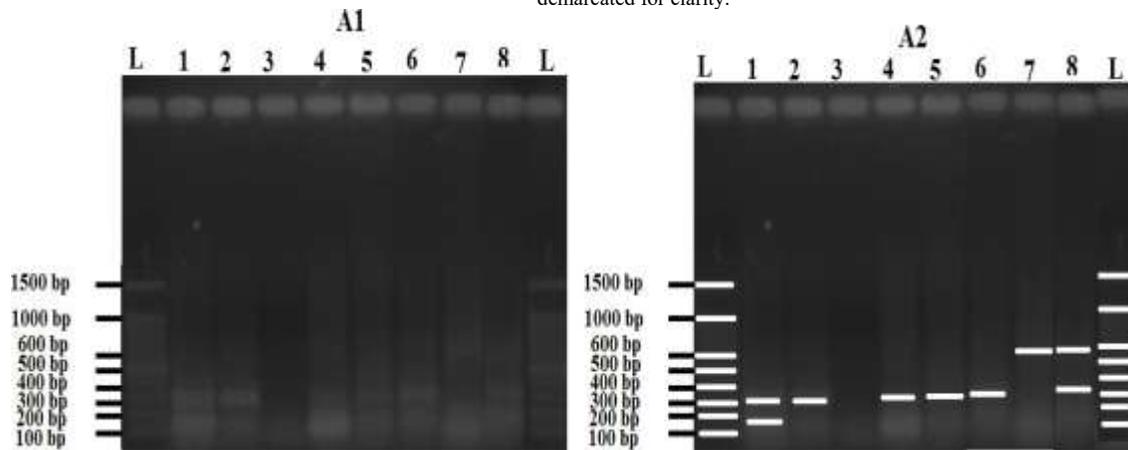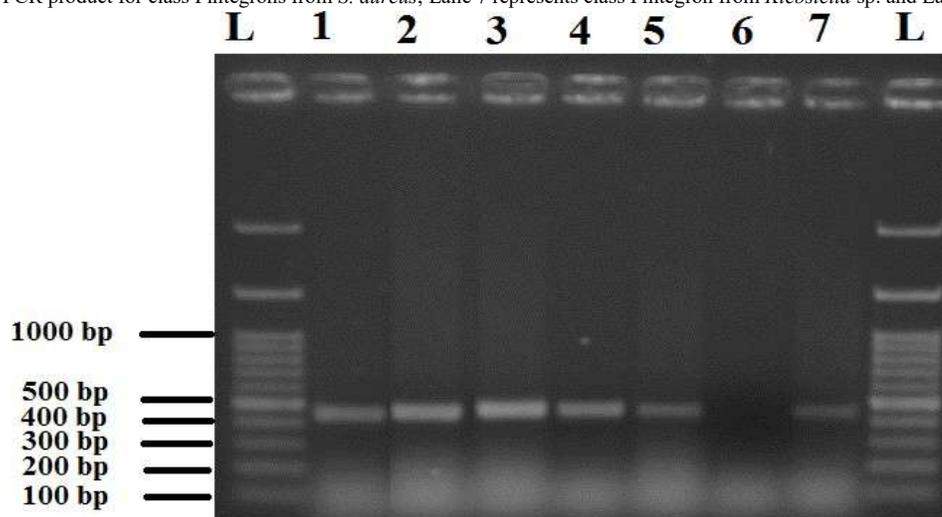

Figure 4. Multiplex PCR profile specific for class I integrons. Lanes L represent the ladder; Lanes 1, 2, and 3 represent PCR product for class I integrons from *E. coli*; Lanes 4 and 5 represent PCR product for class I integrons from *S. aureus*; Lane 7 represents class I integron from *Klebsiella* sp. and Lane 6 represents negative control.

DISCUSSION

Beta-lactamases are the enzymes produced by a variety of microorganisms including Gram-posistive and Gram-negative bacteria. Failure or lack to detect these types of enzymes in clinical isolates of pathogenic bacteria has contributed to their uncontrolled spread and therapeutic failure. World- wide, SSI is considered to be the third most common hospital acquired infection preceded only by urinary tract and pneumonia infections. Therefore, β-lactamase producers could become a serious issue in the future from the point view of the prevention and control of SSIs.

In this research, the prevalence of β- lactamase genes among bacterial isolates recovered from SSIs was too high. Result of this research is in agreement with previous study, were the prevalence of β- lactamases among pathogens isolated from SSIs was 73.2%. In this research, the prevalence of β-lactamases according to the bacterial species was 76.4%, 88.9%, for E. coli and S. aureus, respectively. The prevalence of β-lactamases among Klebsiella sp., Enterobacter sp. and Acinatobacter sp. was 100%. Such findings were in agreement with previously published studies were the prevalence was 100% for E. coli and Klebsiella sp., 78.5%-85% for S. aureus. The prevalence of ESBLs

using multiplex PCR technique was 83.3%. This result is in contrast to other studies previously published, which showed that the prevalence of ESBL- producing pathogens isolated from SSIs ranged from 15.7%-50%. In the current study, the prevalence of ESBLs among different bacterial species, isolated from SSIs, was 76.5% for E. coli; 88.9% for S. aureus, 100% for Klebsiells sp., Enterobacter sp. and Acinatobacter sp. In other published studies, the prevalence of ESBL ranged from 26.6%- 100% for E. coli, 0%-69% for Klebsiella sp., 79% for Enterobacter sp. and 0% for S. aureus. The finding of all isolates recovered from SSIs were ESBL-producers carried blaTEM is in contrast to other studies which showed that blaCTX-types were the dominant.

The prevalence of AmpC β-lactamases among SSIs isolated pathogens of the current study using multiplex PCR technique was 13.3%. The incidence of AmpC genes among E. coli was 11.8% (2/17), both are coexisted with ESBL genes. Results of our study was in contrast to recent study and showed that prevalence of AmpC alone and in combination with ESBL among E. coli isolated from SSIs was 14% and 29%.

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B-lactamases genes of classes ESBL and AmpC have been detected in S. aureus by primers designed for β-lactamases genes in Gram- negative bacteria, this may indicate that S. aureus has β-lactamase genes similar to that in Gram negative bacteria or these staphylococcal β-lactamase genes have sequences complementary to these primers [4].

Staphylococci are the major pathogens which produce β-lactamases. Four types (A to D) of closely related staphylococcal β-lactamases have been identified. With exception type D, all types are inducible and are excreted extracellularly into the surrounding medium and destroy the beta-lactam molecules before they have a chance to enter the cell. The genes determining the staphylococcal β- lactamases usually located on plasmids which can transferred by transduction or conjugation.

The prevalence of MBLs using multiplex PCR technique 23.3% among all pathogens recovered from SSIs. Metallo-β-lactamase genes were detected only in E. coli isolates and 41.2% of this pathogen were MBL- producers. The most prevalent gene among MBL-producer E. coli was blaSPM (83.3%). In recent study, 14%, 33% and 100% of pathogens isolated from SSI were blaNDM-1 producers, these pathogens were E. coli, K. pneumoniae and E. cloacae, respectively.

Coexistence of two or three types of β- lactamase genes in single isolates was observed in 30% of β-lactamase-producing pathogens isolated from SSIs in this study, this may seriously limited the options of treatment, create diagnostic challenge and may lead to use an inappropriate antimicrobial therapy and the result may be fatal. These results are in agreement with other studies conducted recently in Palestine, which showed coexistence of β-lactamase genes in single isolates was detected. These results are also in agreement with study previously published, which showed that coexistence β-lactamase genes was observed in 29% of isolates recovered from SSIs [5].

This study showed that the prevalence of β- lactamase genes among bacteria isolates recovered from SSIs is too high. Results of this study are in agreement with other studies carried out recently in Palestine which showed that prevalence of β-lactamase genes among bacterial isolates recovered from other sample sources were also high. This high level of β-lactamases detected in this study could be referred, at least in part, to inappropriate and misuse of antimicrobial agents, thus favoring the emergence of resistant bacteria. The implication of the wound infections caused by β-lactamase- producing microorganisms, that they make treatment expensive, since it could not be possible with β-lactam antimicrobial agents [6].

A total of 40% of bacterial isolates recovered from SSIs harbored class 1 integrons, whereas class 2 and 3 were not detected. It was also noticed that all isolates carried class 1 integrons were detected in isolates with β- lactamases, particularly carried blaTEM genes.

These results are in consistent with that published previously, which showed that class-1 integrons were detected in 41.2% of E. coli isolated from SSIs, while none of the isolates contained class 2 integron. These results are in agreement with other studies conducted recently in Palestine, which showed that prevalence of class I integrons among bacterial isolates recovered from other sample sources were about 50%, all integrons were detected only in β-lactamase-producing pathogens whereas classes 2 and 3 were not detected. High prevalence of class 1 integrons supports the process of rapid dissemination of β-lactamase genes and other multiple antibiotic resistance determinants among bacterial species in hospitals [7].

CONCLUSION

The high level of β-lactamases among pathogens causing SSIs, complicates the management of these infections at Palestinian hospitals, and could be associated with higher SSI rate, prolonged hospitalization and increased number of antimicrobial agent's usage. Appropriate surgical antibiotic prophylaxis can reduce or prevent the risk of the SSIs, but additional antibiotic use also increases the selective pressure favouring the emergence of antimicrobial resistance. In addition, high prevalence of β-lactamase- producing bacteria in SSIs indicating that continuous monitoring systems and effective control measures are absolutely required.

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