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Original Article

Antibiograms, toxin profiling and molecular typing of *Staphylococcus aureus* isolates from two tertiary hospitals in Jordan

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Abstract

Introduction: Methicillin-resistant *Staphylococcus aureus* (MRSA) is a major cause of hospital-associated infections. This study was undertaken to investigate toxin profiles as well as antibiotic resistance patterns of S. *aureus* isolates form two tertiary hospitals in Jordan.
Methodology: A total of 250 *S. aureus* isolates from clinical samples of two tertiary hospitals were analyzed for the presence of the *mec*A, *van*A, *van*B, and 16 *Staphylococcus* toxin encoding genes using PCR. The isolates were further tested for antimicrobial sensitivities using the disc diffusion method. DNA from all the isolates were fingerprinted by *coa* gene Restriction Fragment Length Polymorphism (RFLP) to study relationships between isolates from the two hospitals.
Results: 73.2% of the isolates contained the *mec*A gene and thus were designated MRSA. All MRSA isolates showed high levels of resistance to many of the antibiotics compared to those of MSSA. All MRSA isolates were susceptible to vancomycin and teicoplanin while all MSSA isolates were susceptible to nitrofurantoin, teicoplanin, vancomycin, cefoxitin, clindamycin, erythromycin and gentamycin. The isolates exhibited high prevalence of the toxin genes and none of the isolates contained less than 4 genes with one isolate contained 14 genes with no apparent differences in gene profiles among MRSA and MSSA. About 60% of the isolates contained 12 to 13 toxin genes and were isolated either from pus or blood.
Conclusion: Antibiograms of the MRSA isolates were significantly different from MSSA antibiograms while there were no apparent differences in the toxin genes profiles. Further, coagulase gene RFLP of the isolates showed that the isolates are very heterogenic.

Key words: *Staphylococcus aureus*; RFLP; MRSA; MSSA; toxins.

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Introduction

Staphylococcus aureus is one of the most commonly isolated human bacterial pathogen and is responsible for causing a battery of diseases ranging from mild superficial infections such as skin infections to severe life-threatening infections such as, endocarditis, deep-seated abscesses, pneumonia and septicemia [1-5]. The ability of *S. aureus* to cause a spectrum of diseases both in humans and animals is due to a combination of bacterial virulence factors [6]. There are several virulence factors secreted by *S. aureus* including Panton-Valentine Leukocidin (PVL), protein A, toxic shock syndrome toxin 1 (TSST-1), and exfoliative toxins and several other toxins [3].

Clinical isolates of *S. aureus* have the potential to produce a wide array of *Staphylococcus* toxins. In addition to the well characterized *Staphylococcus* toxins encoded by the *sea-see* genes, there are several other toxin encoding genes that have been reported including *seg, she, sei, sej, selm, seln, selo, tst, eta*, *etb* [7]. The most common genes associated with enterotoxin production are *sea, seb, sec, sed* and *see*. Those isolates carrying these genes for these toxins are responsible for 95% of staphylococcal food poisoning outbreaks [8]. Other pathogenic factors include the production of coagulase and although all *S. aureus* strains are coagulase producers, coagulase negative staphylococci (CoNS) have also been reported to harbor toxin genes and thus are potential pathogens [9].

Methicillin-resistant *S. aureus* (MRSA) has become a widely recognized cause of morbidity and mortality throughout the world in rates higher than methicillin sensitive *S. aureus* (MSSA) [10]. MRSA are frequently associated with causing nosocomial infections as well

as community-acquired infections. MRSA exhibit a chromosomal gene designated *mec*A that encodes for a penicillin binding protein with a lower affinity for all beta lactam antibiotics [11] thus allowing the formation of the bacterial cell wall in the presence of high concentrations of beta lactam antibiotics [12].

The increasing use of vancomycin had led to the emergence of a few *S. aureus* strains with reduced susceptibility to vancomycin (VISA) in Japan, USA and Jordan. [13-15].

Not all MRSA isolates are genetically the same. They may differ in the arrangement of chromosomal alleles, or the content of variable accessory genetic elements that confer certain resistance traits on the pathogen. Therefore, it is of prime importance to understand the genetic characteristics of these MRSA isolates which could be used to control these pathogens [6].

The detection and typing of MRSA provides essential information to enable appropriate therapy, control, and management of patients especially in case of outbreaks [16]. Numerous methods have been described for the identifying and typing both MRSA and MSSA. Such methods include pulsed field gel electrophoresis (PGFE) [17], analysis of variable number tandem repeat for different genes (VNTR) [18], and Restriction Fragment Length Polymorphism RFLP of the coagulase gene [19,20].

This study aims to characterize a set of clinical *S. aureus* isolates from two major university hospitals in Jordan based on the RFLP of the coagulase gene, and to test the isolates antimicrobial susceptibility as well as study toxin genes presence including the Panton-Vvalentine leukocidin (PVL) exotoxin.

Methodology

Samples Collection

A total of 250 clinical isolates of *Staphylococcus aureus* were randomly collected from different sources (blood, urine, pus, tissue, body fluids, etc.) from two referral university hospitals in Jordan; namely King Abdullah University Hospital (111 isolates) and Jordan University Hospital in Amman (139 isolates) over the period 2008-2009. The isolates included 96 (38%) from females and 154 (62%) from males. The majority of the samples (44.8%) were from pus and other body discharges while 24.4% were from blood samples and the rest of the samples were obtained from multiple body parts or tissues. Table 1 shows the source of the isolates.

Identification of S. aureus

The isolates were identified based on Gram stain, colony morphology, degradation of mannitol, catalase production, and the presence of free coagulase. All the 250 isolates were positive for the above mentioned tests except for 11 isolates that were negative for coagulase based on a latex test kit (Remel, Dartford, UK). The identification of the isolates was confirmed by testing the isolates for the presence of thermonuclease gene (*nuc*) using PCR and all isolates were positive confirming their identity as *S. aureus* [21]. Bacterial cultures were frozen using a mixture of nutrient broth and glycerol at 80:20 v/v and stored at − 80 ± 3°C until use.

DNA isolation and Extraction

DNA extraction was carried out from 100 mL nutrient broth overnight culture using Promega wizard technical genomic DNA purification kit (Promega,

Table1. Sources of the Samples, No. of MRSA +MSSA among each source and their percentages from the total number with the average number of toxin genes for each group.

Sample source	No. of MRSA	No. of MSSA	No. of isolates	%
Pus, aspirate and discharge	87 (9.66)*	25(8.44)	112	44.8
Blood	43(8.79)	18(10.27)	61	24.4
Nasal passage	12(10.17)	9 (10.11)	21	8.4
Urine	12 (8.75)	4 (9.5)	16	6.4
Sputum and Bronchial wash	9 (9.67)	2 (7.5)	11	4.4
Pleural Fluid	6 (8)	2(8)	8	3.2
Foly`s Catheter	4 (10.25)	1(11)	5	2
Corneal scrapping	3 (9.67)	0	3	1.2
Oral cavity	1 (11)	2 (10)	3	1.2
Central line catheter	2 (8.5)	1 (9)	3	1.2
Peritoneal fluid	2 (9.5)	3 (9)	5	2
CSF	1 (10)	0	1	0.4
Tissue	1(9)	0	1	0.4
Total	**183**	**67**	**250**	**100**

The numbers between parentheses denote average number of toxin genes per sample source.

Table2. Oligonucleotide primers used for amplification of staphylococcal genes and the expected PCR products.

Target Gene	Primer sequence	Running conditions	Product size (bp)	Reference
mecA	F: 5'GCAATCGCTAAAGAACTAAG R; 5'GGGACCAACATAACCTAATA	Pre-denaturation 94°C for 3 min for the first cycle; 94°C for 10 s (denaturation) and 53°C for 20 s (annealing) for the next 30 cycles, and a final extension step at 72 °C for 5 min	222	[52]
coa	F 5'ATA GAG ATG CTG GTA CAG G3' R 5'GCT TCC GAT TGT TCG ATG C3'	Pre-denaturation 94 C for 45 sec for one cycle; 94 °C 20 seconds denaturation ; annealing at 57 °C for 15 sec for 30 cycles; final extension step at 72 °C for 2 min	Variable	[20]
nuc	F5' GCGATTGATGGTGATACGGTT R5'AGCCAAGCCTTGACGAACTAAAGC	Pre-denaturation at 95°C for 5 min for one cycle; denaturation for 94 C for 30 sec; annealing at 53°C for 45 sec, for 30 cycles; extension at 72°C for 10 min.	270	[53]
vanA	F5'CATGAATAGAATAAAAGTTGCAATA3' R5' CCCCTTTAACGCTAATACGATCAA 3'	Initial denaturation for 10 min at 94 C; 30 cycles at 30 Sec denaturation at 94 C; annealing for 45 sec at 50 C; 30 sec extension step at 72 C and last at 72 C for 10 min	1030	[54]
vanB	F 5' GTGACAAACCGGAGGCGAGGA3' R 5' CCGCCATCCTCCTGCAAAAAA3'	Same as *van A*	433	[54]

Madison USA) according to manufacturer's instructions. The purity and integrity of the extracted DNA was evaluated using spectrophotometer (Biomate 3, Thermoscientific, Madison, USA) and agarose gel electrophoresis (Cleaver Scientific, UK). The extracted DNA from all isolates was tested for the presence of *nuc, mec*A, c*oa*, *van*A and *van*B, resistance genes. All the isolates were tested for the presence of 16 toxin genes (*sea-see, seg, she, sei, sej, selm, seln, selo, tsst, eta, etb* and *pvl*) using PCR using different protocols and primers listed in the Tables 2 and 3.

Confirmation of the S. aureus identity using PCR amplification of the nuc gene

PCR amplifications of the *nuc* gene was performed in tubes containing; 5 µLBacterial DNA template, 1 µL 25 mM $MgCl_2$, 25 µL ready mix TM Taq, 1 µL from each primer (10 pm/µL) (Table2). Nuclease free water was added to a final volume of 50 µL. All the PCR amplifications in this manuscript were performed in the Mygenie 96 Thermal Block cycler (Bioneer, Daejeon, South Korea). The identityof the *S. aureus* was confirmed using the primers and the running conditions outlined in Table 2.

Detection of mecA gene by PCR

Amplification of *mec*A gene was performed in tubes containing; 2.5 µLBacterial DNA template, 12.5 µL Master mix, 1 µLfrom each primer (10 pm/µL) (Table2). Nuclease free water was added to a final volume of 25 µL. The amplification was performed using primers as well as the running conditions outlined in Table 2.

Testing for vanA and vanB genes using PCR

All isolates were tested for the presence of *van*A and *van*B genes using the primers listed in Table 2 and the corresponding running conditions outlined in Table 2. Amplifications were performed in tubes containing; 2.5 µLBacterial DNA template, 12.5 µL Master mix, 1 µL from each primer (10 pm/µL) (Table 2). Nuclease free water was added to a final volume of 25 ul. Amplified samples were held at 4° C until they were analyzed.

Antibiotic resistance profiles of the isolates

Antimicrobial susceptibility of the isolates was performed using the disk diffusion method (Kirby-Bauer method) on Muller Hinton Agar plates (Oxoid, Basingstoke, UK) as described in the Clinical and Laboratory Standards Institute guidelines [22]. The antibiotics that were used for susceptibility testing of *Staphylococcus* isolates in this study were; penicillin (P), nitrofurantoin (F), teicoplanin (TEC), levofloxacin (LEV), linezonid (LZD), vancomycin (VA), cefoxitin (FOX), ceftriaxone (CRO), erythromycin (E), clindamycin (DA), rifampin (RD), trimethprime / sulfa (SXT), gentamycin (GN) and oxacillin (OX). All the antibiotics used in this study were obtained from Oxoid, (Basingstoke, UK).

Testing the isolates for the toxin genes using PCR

The DNA of all isolates was tested for the presence of toxin genes using a set of published primer sequences listed in Table 3. All PCR tests were performed as uniplex reactions. PCR reaction mixtures were performed in a total volume of 25 µL, containing 0.8 µL primer 1 (10 pmol/µL), 0.8 µL primer 2 (10 pmol/µL), 12.5 µL i-MAX II Master Mix (iNtRON Biotechnology, SungNam, South Korea) or Quick-Load Taq 2X Master Mix (New England Biolabs, Hitchin, UK) and 9.7 µL nuclease-free water. Finally, 1.2 µL DNA template was added to each reaction tube.

Amplification conditions consisted of: Initial denaturation at 94°C for 5 min, then 35 cycles: Denaturation 94°C for 45s, annealing 55°C for 45s (except for *sej*; 62°C for 45s), extension 72°C (or 68°C when Quick-Load Taq 2X Master Mix was used) for 45s. The final extension was performed at 72°C (or 68°C when Quick-Load Taq 2X Master Mix was used) for 7 min, and the amplified products were preserved at 4°C. The PCR products were separated by electrophoresis on 2% agarose.

Genotyping of S. aureus by PCR-RFLP analysis of the coa gene

Amplification of the coagulase gene was performed in tubes containing; 5.0 µLBacterial DNA template, 20 µLMaster mix, 2 µLfrom each primer (10 pm/ul) (Table 2). Nuclease free water was added to a final volume of 50 µL. PCR products were held at 4° C or -20° C until they were used.

Approximately 10 µL of PCR product were digested with 2 µL of (10 unites/µL) restriction endonuclease *Alu*I and *Cfo*I (Fermentas, ThermoFisher, Waltham, USA) using the following conditions and parameters; 10 µL of PCR reaction mixture, 2 µL 10X buffer, and 16 µL free nuclease water. The mixture was centrifuged for 2 min at 15,000 xg, and then it was

Table3. Oligonucleotide primers used for amplification of staphylococcal toxin genes, the expected PCR products and the numbers and percentages of positive isolates.

Enterotoxigenic gene	Primer and the oligonucleotides sequence (5'→3')	Reference	PCR Product (bp)	Number of Positive Isolates and % [a]
sea	**SEA-1** AAAGTCCCGATCAATTTATGGCTA **SEA-2** GTAATTAACCGAAGGTTCTGTAGA	[55]	219 bp	221 (88.4)
seb	**SEB-1** TCGCATCAAACTGACAAACG **SEB-2** GCAGGTACTCTATAAGTGCC	[56]	476 bp	162 (64.8)
sec	**SEC-1** GACATAAAAGCTAGGAATTT **SEC-2** AAATCGGATTAACATTATCC	[56]	257 bp	240 (96)
sed	**SED-1** CTAGTTTGGTAATATCTCCT **SED-2** TAATGCTATATCTTATAGGG	[56]	317 bp	4 (1.6)
see	**SEE-1** TAGATAAAGTTAAAACAAGC **SEE-2** TAACTTACCGTGGACCCTTC	[56]	169 bp	51 (20.4)
seg	**SEG-1** AATTATGTGAATGCTCAACCCGATC **SEG-2** AAACTTATATGGAACAAAAGGTACTAGTTC	[57]	642 bp	199 (79.6)
seh	**SEH-1** CAATCACATCATATGCGAAAGCAG **SEH-2** CATCTACCCAAACATTAGCACC	[57]	376 bp	175 (70)
sei	**SEI-1** CTCAAGGTGATATTGGTGTAGG **SEI-2** AAAAAACTTACAGGCAGTCCATCTC	[57]	577 bp	140 (56)
sej	**SEJ-1** CATCAGAACTGTTGTTCCGCTAG **SEJ-2** CTGAATTTTACCATCAAAGGTAC	[58]	192 bp	105 (42)
selm	**SEM-1** TCTTAGGAACTATTATGGTAGC **SEM-2** CCTGCATTAAATCCAGAA	[59]	471 bp	225 (90)
seln	**SEN-1** GGAGTTACGATACATGATGG **SEN-2** ACTCTGCTCCCACTGAAC	[59]	292 bp	194 (77.6)
selo	**SEO-1** TGATGATTATATAAATAATCGATTTACG **SEO-2** ATATGTACAGGCAGTATCC	[59]	249 bp	246 (98.4)
tst	**TSST-1** ATGGCAGCATCAGCTTGATA **TSST-2** TTTCCAATAACCACCCGTTT	[56]	350 bp	75 (30)
eta	**ETA-1** CTAGTGCATTTGTTATTCAA **ETA-2** TGCATTGACACCATAGTACT	[56]	119 bp	113 (45.2)
etb	**ETB-1** ACGGCTATATACATTCAATT **ETB-2** TCCATCGATAATATACCTAA	[56]	200 bp	0 (0)
pvl	**luk-PV-1** ATCATTAGGTAAAATGTCTGGACATGATCCA **luk-PV-2** GCATCAASTGTATTGGATAGCAAAAGC	[60]	433 bp	249 (99.6)

[a] number of positive isolates and percentages were based on 250 S. aureus isolates used in the study.

incubated at 37° C for 90 min. Digested PCR product (20 µL) were analyzed by electrophoresis using 3 % agarose gels. Band sizes were estimated using specialized software and were scored as 1 for the presence of the band and 0 for the absence of the band [20].

Statistical analysis and phylogenetic tree development

Results of the RFLP analysis were subjected to cluster analysis. Similarity analysis was performed from a combined binary matrix based on the absence or presence of an amplified DNA band (Binary 0/1) using the Jaccard coefficient and strain clustering was performed by the unweighted pair group method with arithmetic mean (UPGMA) analysis. The statistical program SPSS, version 20 (IBM, USA) was used for the analysis. A 0.17 and 0.30 similarity was calculated for *Alu*I and *Cfo*I respectively, using the method of Naffa *et al*. [23], and was used as a discriminating threshold to define homologous clusters.

Results

Antimicrobial susceptibility testing

Table 4 shows that 158 *S. aureus* isolates (63.2 %) exhibited resistance to oxacillin, while only 154 of the isolates (61.6%) were resistant to cefoxitin. The resistance to other antibiotics ranged from 0 % for vancomycin and teicoplanin to 86.8 % for penicillin.

The isolates were classified as MRSA or MSSA based on the presence of the *mec*A gene using PCR and then the isolates antibiograms were evaluated for each group separately. 183 isolates were MRSA while 67 isolates were MSSA.

Among the 183 MRSA isolates, there were 157 isolates (86%) which showed resistance to oxacillin while 154 isolates (84%) exhibited resistance to cefoxitin thus confirming the usefulness of using these two antibiotics to evaluate the presence of MRSA. Similarly, about 95% (173) of the isolates were resistant to penicillin (Table 4).

When antibiograms of MSSA isolates were evaluated separately, resistance to penicillin dropped significantly to 65.7 % indicating a lower correlation between penicillin resistance and MSSA. Similarly, no isolates among the MSSA showed any resistance to clindamycin, Cefoxitin, nitrofurentoin, vancomycin, Teicoplanin. Likewise, only one single isolate showed resistance to oxacillin while the resistance to the other antibiotics ranged from 1.5% for Rifampin (RD) and Linezolid (LZD) to 19.4% for the Trimethoprim-sulphamethoxazole (STX) (Table 4).

Presence of vanA, vanB resistance genes and coa in the S. aureus isolates

When the isolates were tested for the presence of *van*A and *van*B genes, none of the isolates were positive. *S. aureus* are usually coagulase positive, however, when the isolates were tested for the presence of *coa* gene, 239 (95.6%) isolates were positive while 11 isolates (4.4%) did not have *coa* gene although they were confirmed as *S. aureus* by the presence of the *nuc* gene. Therefore, they were confirmed as coagulase negative *S. aureus.*

Table 4. Antimicrobial Susceptibility of MRSA and MSSA isolates.

	Number of isolates					
	MRSA (N:183)			MSSA(N:67)		
Antibiotics	**S**	**I**	**R**	**S**	**I**	**R**
P	10	-	173	23	-	44
F	152	9	22	67	-	-
TEC	183	-	-	67	-	-
LEV	141	2	40	65	-	2
LZD	167	2	14	64	2	1
VAN	183	-	-	67	-	-
FOX	29	-	154	67	-	-
CRO	78	15	90	64	1	2
DA	126	20	37	64	3	-
E	82	1	100	64	-	3
RD	171	3	9	64	2	1
SXT	113	6	64	54	-	13
GM	121	-	62	67	-	-
OX	24	2	157	66	-	1

P: penicillin; F: Nitrofurantoin; TEC: teicoplanin; LEV: levofloxacin; LZD: Linezolid; VAN: vancomycin; FOX: Cefoxitin; CRO: Ceftriaxone; DA: Clindamycin; E: erythromycin; RD: rifampin; SXT: Trimethoprim-sulfamethoxazole; GM: gentamicin; OX: oxacillin; S: sensitive; I: intermediate; R: resistant; N: numbers of isolates.

Table 5. Number of isolates positive for each tested toxin gene.

Number of isolates N = 250	2	2	17	13	26	43	56	60	24	6	1	0	0
Percentage (%)	<1	<1	6.8	5.2	10.4	17.2	22.4	24	9.6	2.4	<1	0	0
Number of toxin genes	4	5	6	7	8	9	10	11	12	13	14	15	16

Toxin genes distribution among the isolates

The prevalence of the total number of toxin genes present in the isolates are shown in Table 5. Irrespective of the isolation site, all of the 250 isolates were found to be positive for at least 4 toxin genes. One isolate from a pus sample exhibited genes for 14 toxins while the other 249 isolates tested positive for toxin genes in the range of 4 to 14. Among those, 24.5% were positive for 11 toxin genes while 22% were positive for 10 toxin genes. The most commonly found toxin genes were *pvl, selo, sec, selm* and *sea*, with 99.6% (249), 98.4% (246), 96% (240), 89.2% (223) and 88.4% (221) of the isolates respectively. Interestingly, *etb* gene was not detected in any of the isolates while the *sed* was only detected in 4 isolates. The *eta* gene was detected in 113 (45.2%) of isolates while the rest of the toxins were detected in varied number of isolates (Results are presented in Table 6).

When the average number of toxin genes were calculated for isolates from different infection sites, there was no major differences observed with approximately an average of 9 toxin genes present for the whole number of samples (Table 1). Further, the isolates were separated into MRSA and MSSA and the average toxin genes were calculated for different groups based on sample site and there were no major differences observed. There was no observed trend between the RFLP pattern, the toxin genes or the site of infection (data not shown).

RFLP Analysis

239 *coa* positive PCR-amplified coagulase gene products were digested with *Alu*I and *Cfo*I, and the resulted fragments were separated using 3% agarose gel electrophoresis.

Upon digestion with *Alu*I enzyme, six isolates were not digested, while ten isolates were not digested upon restriction with *Cfo*I enzyme.

The 233 isolates which were digested with *Alu*I enzyme yielded 88 different RFLP patterns with a number of fragments varying between 2-5 and fragment sizes varying between 85 to 710 bp. The 229 isolates which were digested with *Cfo*I yielded 56 different RFLP patterns with a number of fragments varying between 2-5 and fragment sizes varying between 80 to 550 bp.

The different RFLP fragments produced after cutting with the two enzymes were used for cluster analysis. The genetic similarity indices were calculated based on Jaccord coefficients. The cutoff distance index was made based on the average mean of similarity matrix which was 0.17 and 0.30 for *Alu*I and *Cfo*I enzymes, respectively.

Table 6. Distribution of toxin genes of *S. aureus* among MRSA (n = 183) and MSSA (n = 67) and the total positive in the total 250 isolates.

Toxin gene	MRSA +ve (%)	MRSA –ve (%)	MSSA +ve (%)	MSSA –ve (%)	Total +ve (%)
SEA	162 (88.5)	21 (11.5)	59 (88)	8 (12)	221 (88.4)
SEB	111 (60.6)	72 (39.4)	49 (73.1)	18 (26.9)	160 (64)
SEC	176(96.2)	7 (3.8)	64 (95.5)	3 (4.5)	240 (96)
SED	5 (2.7)	178 (97.3)	0 (0)	67 (100)	5 (2)
SEE	43 (23.5)	140 (76.5)	12 (17.9)	55 (82.1)	55 (22)
SEG	145 (79.2)	38 (20.8)	56 (83.6)	11 (16.4)	201 (80.4)
SEh	128 (70)	55 (30)	48 (71.6)	19 (28.4)	176 (70.4)
SEi	101 (55.2)	82 (44.8)	38 (56.7)	29 (43.3)	139 (55.6)
SEj	85 (46.4)	98 (53.6)	22 (32.8)	45 (67.2)	107 (42.8)
SELM	162 (88.5)	21 (11.5)	61 (91)	3 (9)	223 (89.2)
SELn	139 (76)	44 (24)	53 (79)	14 (21)	192 (76.8)
SElo	179 (97.8)	4 (2.2)	67 (100)	0 (0)	246 (98.4)
TST	54 (29.5)	129 (70.5)	22 (32.8)	45 (67.2)	76 (30.4)
ETA	77 (42)	106 (58)	36 (53.7)	31 (46.3)	113 (45.2
ETB	0 (0)	183 (100)	0 (0)	67 (100)	0 (0)
PVl	183 (100)	0 (0)	66 (98.5)	1 (1.5)	249 (99.6)

Genetic Diversity of S. aureus Isolates (Cluster Analysis)

Dendrograms were constructed on the basis of similarity index among *S. aureus* using the RFLP patterns obtained after restriction with both *Alu*I and *Cfo*I enzymes to study genetic relatedness among the isolates.

For *Alu*I restriction enzyme, the 0.17 similarity cut-off value gave 11 major clusters (A-K genotypes and 88 patterns), while for *Cfo*I restriction enzyme, a 0.30 similarity cut-off value gave 16 major clusters (A-O genotypes, and 56 patterns).

The dendrograms revealed that *S. aureus* isolates showed different distributions of genotypes depending on the restriction enzyme. When PCR products were cut with *Alu*I, half of the isolates clustered in genotype A (116 isolates) followed by genotype B (37 isolates) and genotype F (17 isolates), while the remaining isolates were distributed as following; 14 isolates in genotype D, 10 isolates in genotype E, 9 isolates for both genotypes C and I, 8 isolates in genotype H, 7 isolates in genotype G, 5 isolates in genotype J and 1 isolate in genotype K. In addition, 80% of the isolates belonging to cluster A (92 isolates) were MRSA. There was not a distinct cluster formed from isolates from any of the two hospitals. The majority of clusters contained mixed geographical isolates with exception of a few of the clusters where the majority of isolates were from one hospital.

The pattern distribution was changed upon digestion with *Cfo*I enzyme where more than half of the isolates belonged to three genotypes D, C and F with 64, 45 and 25 isolates in each genotype respectively. The remaining isolates were distributed as follows; 23 isolates in genotype H, 17 isolates in genotype B, 16 isolates in genotype I, 15 isolates in genotype A, 6 isolates in genotype G, 4 isolates in both genotype E and M, 2 isolates in genotypes N and K and one isolate for genotypes O and P.

Discussion

MRSA is one of the most commonly isolated pathogen associated with general and nosocomial infections worldwide including Jordan [5,24]. The incidence of MRSA varies by location from one institution to another, and from Country to another. This study shows that the prevalence of MRSA is high totaling about 73.2% of the total clinical tested isolates.

Various studies from different European countries showed high prevalence of MRSA. For instance, in 2010 MRSA accounted for 25-50% of *S. aureus* specimens in southern Europe and UK [25]. Similarly, in Portugal, the MRSA prevalence reached 52.2% in 2010 [26]. In contrast to these results, in Skane county of southern Sweden, the reported cases of MRSA were less than in the southern Europe Counties, however, the incidence of MRSA infection was rising as the number of cases increased from 31 in year 2000 to 315 cases in year 2010 [25]. In USA, prevalence of MRSA approached 60% of the *S. aureus* isolates in 2003 [27]. In India, the proportion of CA-MRSA in children <5 years was 73.7% [28]. The high prevalence of MRSA in different parts of the world could be due to different protocols in using antibiotics in these parts of the world.

Oxacillin and cefoxitin resistance are generally used as indicators for methicillin resistance. In this study about (85.8%) and (84.7%) of MRSA isolates were resistant to oxacillin and cefoxitin respectively. However, these results contrasted results reported by Anand *et al.* [29] who reported near complete agreement between results of disc diffusion and PCR methods in identification of MRSA.

This could be due to the fact that the susceptibility to cefoxitin has increased in MRSA strains that could be defective in PBP2a [30].

When the isolates were tested for antibiotic resistance against 16 commonly used antibiotics, various susceptibility patterns and obvious multidrug resistance profiles (resistant to ≥ 3 antibiotics) appeared in MRSA isolates compared to MSSA isolates. The high prevalence of cross resistance to antimicrobial agents could be related to the dissemination of transposons with insertion sequences in the 50-kb *mec* region gene [12]. Fluit *et al.* [31] found that 87% of MRSA isolates were multidrug resistant while only 3% of MRSA isolates were resistant to β-lactam antibiotics, all the isolates were susceptible to vancomycin. When the isolates in this study were tested for sensitivity to vancomycin and teicoplanin, none of the isolates showed any resistance irrespective of whether they were MRSA or MSSA isolates. In addition to full susceptibility to teicoplanin and vancomycin, MSSA isolates were completely susceptible to gentamicin, nitrofurantoin, and cefoxitin. When the MRSA isolates were tested against penicillin, as expected, most of them were resistant to penicillin (94.5%) due to the production of penicillinase which destroys the beta-lactam ring of penicillin [32]. Similar results were obtained by Rajaduraipandi *et al*. [33] who reported that all their MRSA isolates were resistant to penicillin and the majority of them were resistant to ampicillin. When the isolates were tested for resistance to levofloxacin, an antibiotic that is not extensively used in Jordan, only 22.8 % of the isolates were resistant. This appears

contrasting to high resistance rates of *S. aureus* isolates to levofloxacin reported from a Japanese study in which 80–95% resistance was reported [34]. The high resistance profile for this particular antibiotic probably reflects the excessive use of this class of antibiotics in Japan while in North America levofloxacin resistance is about 35%, a percentage closer to what was found in this study possibly indicating the alternative use of other antibiotics with treatment [35].

Most of the isolates were sensitive to linezolid, a member of oxazolidinones antibiotic class, which may be due to its low use as treatment in combating *S. aureus* infections as 91.4 % susceptibility was detected. Similar results were reported from Poland and UK of full susceptibility [36,37]. Gentamicin resistance which is extensively used in treatment of *Staphylococcus* infection is on the rise. For instance, Moyo *et al.* [38] reported a 27.2% resistance to gentamycin in *S. aureus.*

However, in this study only about 34% of isolates were resistant to gentamicin, contrasting results reported by Pulimood *et al.* [39], who reported that 85.5 % of MRSA isolates were resistant to gentamicin in India and results reported by Qureshi [40] who reported a gentamicin resistance of 97.8% in Pakistan. Moreover, in this study we observed that 5% and 35% of MRSA were resistant to rifampicin and sulfamethoxazole (SXT), respectively. These findings are contradictory to those of Shittu *et al.* [41], who reported that 73.8% and 85.2% of MRSA were resistant to these two antibiotics, a result possibly indicting the different approaches in using these antibiotics in clinical settings.

Table 1 shows average number of toxin genes per site of isolates. It is noticed that there is no major differences among the different samples sources although the average number of toxin genes is somewhat high ranging from about 8 to 10 genes per sample source.

These results appeared similar somewhat to results reported by Ferry *et al.* [42] who showed that 84% of the *S. aureus* clinical isolates harbored at least one toxin gene and results by Morandi *et al.* [43] who reported that 79% of *S. aureus* from dairy products harbored at least one toxin gene. Further, Demir *et al*. [44] reported that 65.8% of the clinical isolates exhibited at least one toxin gene. However, these results appeared to be completely different from results reported by Peles *et al.* [45] who reported that only 15% of *S. aureus* isolated from milk samples in Hungary harbored at least one toxin gene indicating the low prevalence of toxin genes in non-pathogenic *S. aureus* isolated from milk samples. However, the results appeared different from those reported by Naffa *et al.* [23] who showed that only 23% of the clinical isolates exhibited at least one toxin gene. The exceptionally high prevalence of toxin genes among the tested isolates in this study could be due to the nature of the tertiary hospitals in which most of the patients are referred from primary health care establishments. It is worth mentioning that in this study, none of the isolates contained the *etb* toxin gene. These results appeared similar to other reported studies [46-48] in which the researchers reported very low numbers of isolates exhibiting this toxin gene. Demir *et al.* [44] also reported only 9.2% of the tested clinical isolates harbored this toxin gene.

Table 6 shows a summary of the number of isolates harboring the total number of toxins tested. Almost all the isolates but 1 contained genes for *pvl*, while *selo, sec, selm*, and *sea* genes were found in 246, 240, 225 and 221 isolates respectively (Table 6). Similar results were reported by Ramdani-Bouguessa *et al.* [49] who reported that *pvl* was the most detected toxin gene (72%) of the tested clinical isolates. Similar results were also reported from a Tunisian study [50] where 79% of the community acquired MRSA contained the *pvl* gene while 51% of the health care associated MRSA infections contained the *pvl* gene. However, as with distribution of antibiotic resistance, toxin distribution varied between studies from different areas of the world. For instance, Zhao *et al.* [51] reported the prevalence of *pvl* presence in 41.5% of the MSSA strains and only 2% of the MRSA strains. These differences appeared to be due to different lineages of *S. aureus* where each geographical region appeared to have different lineages. With regard to association of the average number of toxin genes and the source of the sample, there was no apparent difference in the average number of toxin genes between the different sources of samples even irrespective of whether the isolate is MRSA or MSSA.

The present study shows that RFLP analysis revealed 88 distinct restriction patterns when coagulase gene was digested with *Alu*I in addition to 6 isolates that were not digested with the enzyme. In contrast, only 56 distinct patterns were recognized when the *Cfo*I enzyme was used in addition to 10 isolates that were not digested by the enzyme. The numbers of patterns appeared to be different from previous studies [20] who reported only 10 patterns among 85 *S. aureus* isolates. Similarly, Karahan and Cetinkaya [52] reported only 23 RFLP patterns among 200 *S. aureus* isolates. The difference in the pattern numbers observed among S. *aureus* isolates analyzed in the above mentioned studies and the pattern numbers among *S. aureus* isolates in this

study appears large, highlighting the high genetic variation among the isolates in our study. This could be explained in part by the larger number of isolates analyzed in this study as opposed to the low number analyzed in the Hooky *et al.* [20] study. RFLP clusters upon the construction of a dendrogram in this study revealed that the analyzed isolates were genetically diverse and comprised a heterogeneous population with 11 clusters at 0.17 similarity level for *Alu*I enzyme. However, the isolates yielded 16 clusters at 30% similarity level after digestion with *Cfo*I enzyme in which cluster D contains the majority of the isolates (64).

The similarity cut off was calculated based on the similarity matrix and suggests that all of the isolates shared only 17 % and 30% of their RFLP fragments following digestion with *Alu*I and *Cfo*I respectively. The low similarity indicates that there is a high level of DNA polymorphism among these isolates. Among the *Alu*I clusters, genotype A appeared to be predominant with 115 isolates, most of which are MRSA. The presence of *mec*A gene is not genotype specific since it was distributed all over the genotypes.

Similar to our study, Hookey *et al.* [20] studied the coagulase gene RFLP patterns of 85 *S. aureus* isolates comprising major epidemic strains of MRSA in United Kingdom and reported only 7 phylogenetic groups for the isolates. Most epidemic strains of MRSA were clustered in groups D and G. Moreover, different clustering patterns and genotypes were obtained when the dendrogram were constructed for MRSA isolates as a group compared to those for MSSA isolates as a group. For instance, following *Alu*I digestion, 61 MRSA isolates were clustered in genotype C rather than genotype A. Similar changes in the cluster arrangement were observed when a dendrogram was constructed for MRSA isolates restricted with *Cfo* I where the majority of isolates (50) were clustered in genotype A rather than genotype D.

In the present study restriction with *Alu*I enzyme yielded more distinct patterns than patterns of *CfoI*, and this is expected since the *Alu*I enzyme has more restriction sites than of *Cfo*I enzyme [19]. The sharing between RFLP fragments of *Alu*I enzyme (17%) is less than the *Cfo*I enzyme (30%) suggesting that the discrimination power of the *Alu*I enzyme provides higher resolution than the *Cfo*I enzyme and therefore the use of *Alu*I enzyme has higher discriminatory power than the use of the *Cfo*I enzyme. Nevertheless, *Cfo*I enzyme restriction yielded higher number of clusters than *Alu*I restriction. Moreover, there were no apparent differences observed among isolates collected from Jordan University Hospital or the King Abdullah University Hospital in terms of RFLP patterns or antibiotic resistance profiles.

Conclusions

MRSA isolates exhibited higher antibiotic resistance profiles than the MSSA isolates. The average number of toxin genes per isolates was similar for both MRSA and MSSA. However, the overall high number of toxin genes present in the strains may indicate the invasiveness nature of the isolates as all of the isolates where obtained from clinical samples. The RFLP of the coagulase gene shows that the samples collected from only two major hospitals are heterogenic but there was no apparent genetic separation between the samples based on the location of collection.

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