# Molecular characterization of methicillin resistant *Staphylococcus aureus* isolated from hospitals environments and patients in Northern Palestine

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**ABSTRACT**

**BACKGROUND:** *Staphylococcus aureus* (*S. aureus*) is considered one of the most common pathogen to humans. Infections caused by this microorganism can be acquired through both hospital and community settings. This study was carried out to investigate molecular characterization of MRSA strains isolated from the patients and their environment in two hospitals (Rafidia hospital and Thabet hospital) in Northern Palestine, and to determine the clonal identity between these strains and their possible contribution to healthcare-associated infections (HAIs).

**METHODS:** Two hundred sixty five swabbed samples were collected from these hospitals, *S. aureus* was isolated, antibiotic resistant genes were Panton–Valentin leukocidin (PVL) gene were detected and *SCCmec* and *spA* were typed by PCR and/or sequencing.

**RESULTS:** The prevalence of MRSA among *S. aureus* isolates was 29% and 8.2% in Rafidia hospital and Thabet hospital, respectively. All strains resistant to oxacilllin disk were carried *mecA* gene. Majority of strains (84.6%) carried *SCCmec* type II (n = 11), type IVa and non-typeable were also detected. In addition, PVL was detected in 2 (14.3%) clinical strains. ERIC PCR patterns revealed that 2 strains recovered from patient bed and nasal swab isolated from Thabet Hospital were nontypeable, *spA* typing showed that they belonged to type t386 and have identical DNA sequences. Other 2 clinical isolates were spa typed, one belonged to clone t044, while the other is new clone not exist in database.

**CONCLUSIONS:** Results may give evidence that environmental contamination possibly contributing to (HAIs.

**Keyword:** *S. aureus*, Healthcare-associated infection, Environmental contamination, MRSA. Palestine.

**INTRODUCTION**

*Staphylococcus aureus* (*S. aureus*) is considered one of the most common pathogen to humans and animals. Infections caused by this microorganism can be acquired through both hospital and community settings and it is still one of the most common causes of hospital-acquired infections. *S. aureus* is the most common cause of surgical wound infections and pneumonia, and the second most common cause of bacteraemia. Other infections caused by this pathogen include endocarditis, septicaemia, osteomyelitis, meningitis, skin infections, gastroenteritis, and toxic shock syndrome [1,2].

Multidrug-resistant strains of *S. aureus*, particularly MRSA, represent a major clinical and epidemiological issue in hospitals among patients and hospital staff [3]. It was also found that contamination of the inanimate environment with MRSA occurred when either infected or colonized individuals were present in hospital rooms [4,5]. In hospitals many items were reported previously might serve as a source for the transmission of MRSA [2,4,6-8].

It was shown by molecular methods that identical or closely related strains were isolated from the patients and their environment, suggesting possible environmental contamination contributing to endemic MRSA[9,10]. The aim of this study was to investigate molecular characterization of MRSA strains isolated from the patients and their environments in these hospitals, and to determine the clonal identity between these strains and their possible contribution to Healthcare-associated Infections. This study has not been investigated previously in Palestine. The results of the present study might be valuable to both health professionals and the scientific community, and may aid the current understanding the epidemiology of MRSA.

**MATERIALS AND METHODS**

*Samples collection and bacterial identification*

The samples in this study were collected from two hospitals in Northern Palestine between October and November 2013. These hospitals are Rafidia Hospital in Nablus City and Thabet Hospital in Tulkarm City, with a total number of beds 215 and 114, respectively, for the year 2013. A total of 196 samples have been collected from different environmental surfaces (n=100 from Rafidia Hospital and n=96 from Thabet Hospital). Other 69 samples have been also collected from patients' nares or obtained from labs of these hospitals (n=34 from Rafidia Hospital and n=35 samples from Thabet Hospital). These samples were cultured and identified in Department of Biology and Biotechnology, Science College, An-Najah National University, Nablus-Palestine. Different hospital environmental surfaces included were incubators of new born, breathing masks, bed side cabinets, floors, door knobs, bed rails, faucets and others. These surfaces were sampled with a saline moistured sterile cotton swab, immediately transferred into 5 mL Tryptone Soy Broth (TSB) and incubated for 18-24 h at 37ºC. Thereafter, samples were subcultured on Mannitol salt agar (MSA). Yellow colonies (3-4 colonies) were then subcultured on nutrient agar (NA) and furthermore they were identified by Gram stain, catalase test and Coagulase test. Samples from nares patients were obtained by swabbing nares and processed as mentioned above.

*Oxacillin disk diffusion test*

Oxacilllin (1 μg) antibiotic disks (Oxoid) were used to detect MRSA. Zones of inhibition was determined in accordance with procedures of the Clinical and Laboratory Standards Institute [11]. According to Oxacilllin, *S. aureus* isolates were considered susceptible if inhibition zones were ≥13 mm after incubation on 4% NaCl Meuller Hinton Agar (MHA) at 35ºC for 24 hours.

*DNA extraction*

*S. aureus* genome was prepared according to method described previously [12]. Briefly, cells were scraped off an overnight NA plate with a sterile loop, washed twice with 1 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), then the pellet was resuspended in 0.5 ml of sterile distilled $H_2O$, and boiled for 10-15 min. The cells then were incubated on ice for 10 min. The debris pelleted, DNA concentration was determined using spectrophotometer and the samples stored at -20ºC until use for further DNA analysis.

## PCR assay for PVL detection

The primer sequences used for the PVL gene detection were described previously [13]. The PCR reaction mix with a final volume of 25 μL, was performed with 12.5 μL of PCR premix (ReadyMix[TM] Taq PCR Reaction Mix with MgCl$_2$, Sigma), 0.4 μM of each primer and 2 μL of DNA template. The amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf) according to the following thermal conditions: denaturation at 94 ºC for 30 s, annealing at 50 ºC for 30 s, extension at 72 ºC for 1 min (30 cycles), with a final extension step at 72ºC for 2 min. The PCR products were detected by electrophoresis through 1.5% agarose gels to determine the size (433 bp) of amplified fragment.

## PCR assay for SCCmec typing

 The primer sets used for *SCCmec* typing were described previously [14] . Each PCR reaction mix (25 μL) was performed using 12.5 μL of PCR premix (ReadyMix[TM] Taq PCR Reaction Mix with MgCl$_2$, Sigma), 0.2 μM of each primer, and 2 μL DNA template. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf) according to the following thermal conditions: initial denaturation for 4 min at 94ºC was followed by 35 cycles of denaturation at 94ºC for 30 s, annealing at 50ºC for 30 s and extension at 72ºC for 2 min, with a final extension step at 72ºC for

4 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel after ethidium bromide staining (0.5 μg/ml). The *SCCmec* type was determined on the basis of the expected fragments size, for the subtypes I, II, III, IVa, IVb, IVc, IVd, IVh and V would be 613, 287, 243, 776, 1000, 677, 1242, 663 and 325 bp, respectively.

*ERIC PCR assay*

Enterobacterial repetitive intergenic consensus (ERIC) PCR was performed using Primer ERIC1 and Primer ERIC2 which described previously [15]. Each PCR reaction mix (25 μL) was performed using 12.5 μL of PCR premix (ReadyMix$^{TM}$ Taq PCR Reaction Mix with MgCl$_2$, Sigma), 1 μM of each primer, 3 μL DNA template, concentration of dNTPs was modified to 400 μM , MgCl$_2$ to 3 mM and Taq DNA polymerase to 2 U. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf) according to the following thermal conditions: initial denaturation for 2 min at 94ºC was followed by 30 cycles of denaturation at 94ºC for 1 min, annealing at 25ºC for 2 min and extension at 72ºC for 3 min, with a final extension step at 72ºC for 5 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel. A binary matrix of band presence or absence was analyzed by the unweighted pair group method for arithmetic averages (UPGMA), using SPSS Statistics version 20 (IBM).

*SpA gene amplification*

The polymorphic X region of the spa gene was amplified using primers spa-1113f and spa-1514r as described previously [16] . Each PCR reaction mix (25 μL) was performed using 12.5 μL of PCR premix (ReadyMix$^{TM}$ Taq PCR Reaction Mix with MgCl$_2$, Sigma), 0.2 μM of each primer, and 2 μL DNA template. DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf) according to the following thermal conditions: initial denaturation (4 min at 94°C), followed by 35

cycles of denaturation at 94°C for 40 s, annealing 55°C for 40 s, and extension 72°C for 90 s, with a final extension 72°C for 5 min. Amplified PCR products were purified from gel by the MinElute PCR purification kit (Qiagen, Hilden, Germany) and sequenced by dideoxy chain termination method using ABI PRISM sequencer, model 3130 (Hitachi Ltd, Tokyo, Japan), Bethlehem University, Bethlehem, Palestine. The Kreiswirth spa nomenclature obtained by the *spA* typing tool (http://fortinbras.us/cgi-bin/spatyper/spaTyper.pl) was adopted for the present study. Sequence information was further submitted for accession number in primary bioinformatics web servers.

### *Multiplex PCR For antibiotic resistance genes detection*

The PCR primers used to detect 9 antibiotic resistant genes in a multiplex PCR approach were described previously [17]. Multiplex PCR amplification was carried out in a 25µl volume contained approximately 3µl of template DNA, 1X PCR buffer, 3 U Taq DNA polymerase, 0.2 µM of each of the 18 primers, 4 mM $MgCl_2$. The DNA amplification was carried out using the thermal cycler (Mastercycler personal, Eppendorf), after initial denaturation step at 94ºC for 2 min, followed by 30 cycles of initial denaturation at 94ºC for 30 s, 55 ºC for 30 s and 72ºC for 30 s, with a final extension step at 72 ºC for 4 min. Amplification Products (15 µl) were analyzed on a 2.0% agarose gel.

### *Statistical analysis*

Statistical analysis was done using Minitab software, version 15 by applying Chi square dependency Test to check if the prevalence of *S. aureus* is depending on site or not in the two hospitals. A *P* value of $< 0.05$ was considered to be dependent.

## RESULTS

Our results showed that, 71 (53%) and 79 (60.3%) samples were mannitol fermenter from Rafidia hospital and Thabet hospital, respectively. Incidence rate of *S. aureus* in

Rafidia hospital and Thabet hospital was 23.1% and 37.4%, respectively. The number of MRSA among *S. aureus* in both hospitals was 13 strains, Rafidia hospital 29% (n=9) and Thabet hospital 8.2% (n=4). Data about number of samples, incidence mannitol fermenter isolates, *S. aureus*, MRSA are presented in Table I. Statistical analysis showed that the prevalence of *S. aureus* is depending on the site, in the two hospitals using Chi Square Test and the *P*-value was 0.011.

Results showed that these 13 strains were resistant to oxacilllin using Disk diffusion method as well as all showed *mecA* gene by PCR technique. These strains carried different antibiotic resistance genes including *aacA-aphD* (Gentamicin), *erm* (*C*) (Erythromycin and clindamycin), *tetK* (Tetracyclin). Data are presented in Figure 1. *SCCmec* typing identified that the majority (84.6%) of isolates carried *SCCmec* type II (n = 11), which is traditionally associated with Hospital Acquired-MRSA (HA-MRSA). Only one (7.7%) strain No. 64T harbored *SCCmec* type IVa, which is traditionally associated with Community Acquired-MRSA (CA-MRSA), as well as one (7.7%) strain 3HPR was nontypeable. Panton–Valentin leukocidin (PVL) was detected in 2 (14.3%) samples (3HPR and 4HPR) isolated from patients; PVL that was detected in 4HPR sample was associated with *SCCmec* type II, while that was detected in 3HPR *SCCmec* was nontypeable. PVL genes of these positive samples were sequenced. Data about MRSA strains and representative of MRSA strains with *SCCmec* types and PVL are shown in Figure 1 and 2.

In this study, ERIC PCR profile revealed that 11 of MRSA had 11 different patterns. Other 2 strains (48BT from patient bed) and (99AT from nasal swab) isolated from Thabet Hospital were nontypeable (showed no bands under conditions of ERIC PCR). All MRSA strains were differentiated into four discriminatory clusters at a 50% similarity level, namely, C1 to C6. ERIC PCR profile of these MRSA strains is

presented in Figure 3. *SpA* gene typing of these 2 strains showed that these were belonged to type t386 and have identical sequence of DNA and clustered together in phylogenetic tree (Figure 4) . Other 2 clinical isolates (4HPR, 3HPR), were investigated by *spA* typing, indicating that these strains were clonally non related. Strain 4HPR was belonged to clones t044, while strain 3HPR is new clone not exist in database. The nucleotide sequences reported in this study were further registered at the GenBank database under the accession numbers (KJ544514- KJ544518 and KJ930035).

## DISCUSSION

Results of this research showed that 3.6% (7/196) of environmental surfaces in hospitals in Northern Palestine were contaminated with MRSA. The proportion of hospital surfaces contaminated with MRSA have varied considerably in published reports, ranging from 0.5% to 44% of surfaces in patients rooms on regular hospitals wards and from few percent to 64% of surfaces in burn units with MRSA patients [4,10,18,19]. Rates of environmental contamination vary on the basis of the site of infection in source patients. Environmental contamination may contribute to transmission of healthcare associated pathogens when healthcare workers or patients come into direct contact with contaminated surfaces [4,5]. Transmission pathogens depend on several factors such as viability of pathogens on these environmental surfaces, frequency of contact between patients, healthcare workers and contaminated surfaces, relative humidity, ambient temperature, surface substrate and dose of transmitted pathogens [20,21].

In spite of changes in the epidemiology of MRSA strains, it is clear that some strains are found geographically restricted while others are found worldwide; these strains showed significant variation in the ability to cause outbreaks [22]. Molecular epidemiologic analysis of the isolates from the environment and the patients is much more informative than simple isolation of microbes from environmental surfaces or other items in hospital [23]. *SCCmec* types (I–III) usually were detected in hospital settings or from patients with a history of hospitalization. Results of this study showed that majority of isolates carried SCCmec type II independent on the source of sample. These results were in contrast to reports published recently from Palestine [12,24], which showed that *SCCmec* type III were detected from clinical strains. Our results were consistent with these reports

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that *SCCmec* type I was not detected while both types II and IV were presented. *SCCmec* type II detected from patients nasal swabs this may give indication that these strains were obtained from hospital environmental surfaces. Results of this study were in contrast to other report published recently [25], which showed that 95.5% and 4.5% of MRSA from Palestine, Jordan and Iraq were belonged to *SCCmec* type III and V, respectively.

ERIC PCR profile revealed that 2 strains (48BT from patient bed) and (99AT from nasal swab) isolated from Thabet Hospital were nontypeable. Also *spA* gene typing confirmed that these 2 strains had the same type (t386) and also identical sequences.  This may considered as evidence that environmental contamination with MRSA possibly contributing to healthcare-associated infections (HAI). These results were in agreement with previous reports [9,10], which showed that identical or closely related isolates were recovered from the patients and their environment, possibly contributing to endemic MRSA. Our study showed that clone t386,  t044 and one did not exist in database were detected in Palestine. These results were in contrast to previous study which showed that only 2 clones circulate in Palestine [25]. The epidemiology of MRSA can differ between hospitals, even if these hospitals are in the same region. Assessment of epidemiological situation for the establishment of adequate infection control programmes, and to monitor MRSA strains evolution is considered an important issue for each hospital[22]. These data confirms the tendency of *SCCmec* types IV and V which are traditionally associated with CA-MRSA to spread in hospital settings as mentioned previously [24,26,27]. Type IVa was isolated from bed side cabinet from Thabet Hospital.

Panton–Valentin leukocidin (PVL) was detected in 2 strains isolated from patients; 4HPR strain was associated with *SCCmec* type II, while the other strain (3HPR) was

nontypeable *SCCmec*. Few MRSA isolates (14.3%) carried the PVL-toxin gene, this could be explained by the fact that these MRSA isolates were originally hospital acquired. PVL gene detection indicates that these strains might produce a potent toxin, which is responsible for unusual virulence forming pores in leukocytes [28]. PVL-producing strains can cause complicated chronic deep-skin infections or necrotizing pneumonia with an extremely high mortality, even in young and previously healthy patients [29-31]. Diagnostic procedures are needed that allow the detection of PVL-positive strains, as well as their assignment to clonal groups, in order to monitor epidemiological developments.

In this study, bacterial samples were isolated only from the environmental surfaces, patients' nares or obtained from labs of hospitals, samples were not isolated from nares or hands of medical personnels. We concluded that special attention to infection control policies, work practices, and cleaning techniques are necessary to reduce the risk potential of MRSA transmission in hospital staff and patients. Our results may give evidence that MRSA environmental contamination possibly contributing to HAI and further investigations are needed

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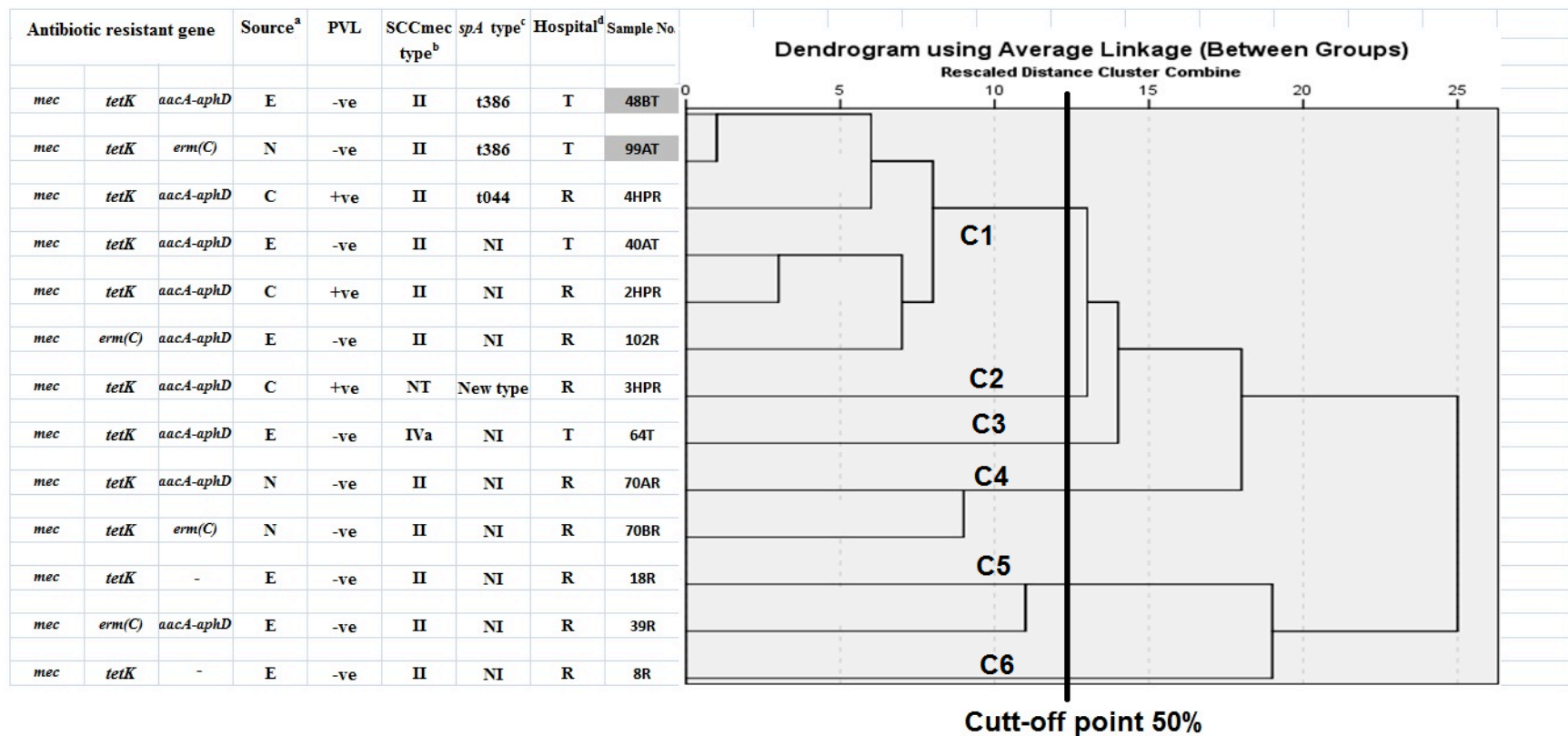
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**Table I.** Number of mannitol fermenter isolates, *S. aureus*, MRSA isolates recovered from Rafidia Hospital and Thabet Hospital.

| Samples Category | Rafidia Hospital | Thabet Hospital | Total |
|---|---|---|---|
| Environmental | 100 | 96 | 196 |
| Clinical and nasal swab | 34 | 35 | 69 |
| Mannitol fermentor isolates | 71 (53%) | 79 (60.3%) | 150 |
| Coagulase +ve (*S. aureus*) | 31/134 (23.1%) | 49/131 (37.4%) | 80/265 (31.3%) |
| Environmental | 22/100 (22%) | 36/96 (37.5%) | 58/196 (29.6%) |
| Clinical and nasal swab | 9/34 (26.5%) | 13/35 (37.1%) | 22/69 (31.9%) |
| MRSA | 9/31 (29.0%) | 4/49 (8.2%) | 13/80 (16.3%) |
| Environmental | 4/22 (18.2) | 3/36 (8.3%) | 7/58 (12.07%) |
| Clinical and nasal swab | 5/9 (55.6%) | 1/13 (7.7) | 6/22 (27.3%) |

| Antibiotic resistant gene | | | Source[a] | PVL | SCCmec type[b] | spA type[c] | Hospital[d] | Sample No. |
|---|---|---|---|---|---|---|---|---|
| mec | tetK | aacA-aphD | E | -ve | II | t386 | T | 48BT |
| mec | tetK | erm(C) | N | -ve | II | t386 | T | 99AT |
| mec | tetK | aacA-aphD | C | +ve | II | t044 | R | 4HPR |
| mec | tetK | aacA-aphD | E | -ve | II | NI | T | 40AT |
| mec | tetK | aacA-aphD | C | +ve | II | NI | R | 2HPR |
| mec | erm(C) | aacA-aphD | E | -ve | II | NI | R | 102R |
| mec | tetK | aacA-aphD | C | +ve | NT | New type | R | 3HPR |
| mec | tetK | aacA-aphD | E | -ve | IVa | NI | T | 64T |
| mec | tetK | aacA-aphD | N | -ve | II | NI | R | 70AR |
| mec | tetK | erm(C) | N | -ve | II | NI | R | 70BR |
| mec | tetK | - | E | -ve | II | NI | R | 18R |
| mec | erm(C) | aacA-aphD | E | -ve | II | NI | R | 39R |
| mec | tetK | - | E | -ve | II | NI | R | 8R |



Dendrogram using Average Linkage (Between Groups)
Rescaled Distance Cluster Combine

C1
C2
C3
C4
C5
C6

Cutt-off point 50%

**Figure 1:** Dendrogram of MRSA isolates based on the UPGMA method derived from analysis of the ERIC-PCR-profiles. Antibiotic resistant gene profile, source of samples, PVL, SCC*mec* type, and hospitals are also shown. Shaded cells showed strains have the sample profile using ERIC PCR.
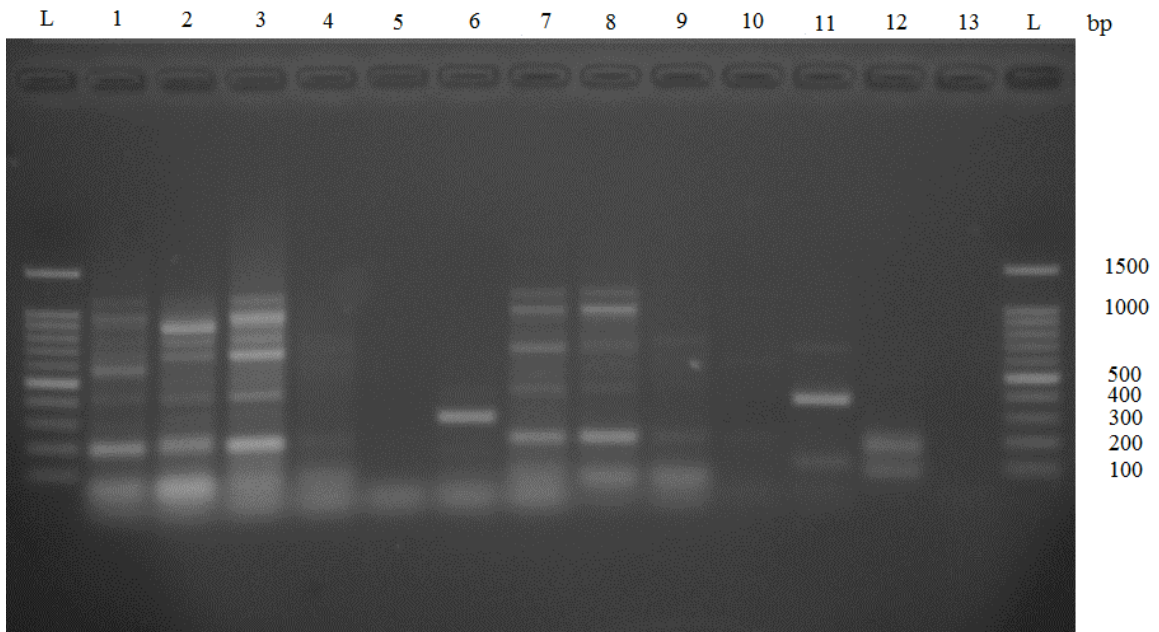
C1-C6: ERIC-PCR clusters

[a] E: Environment; N: Narse; C: Clinical.

[b] NT: non typeable
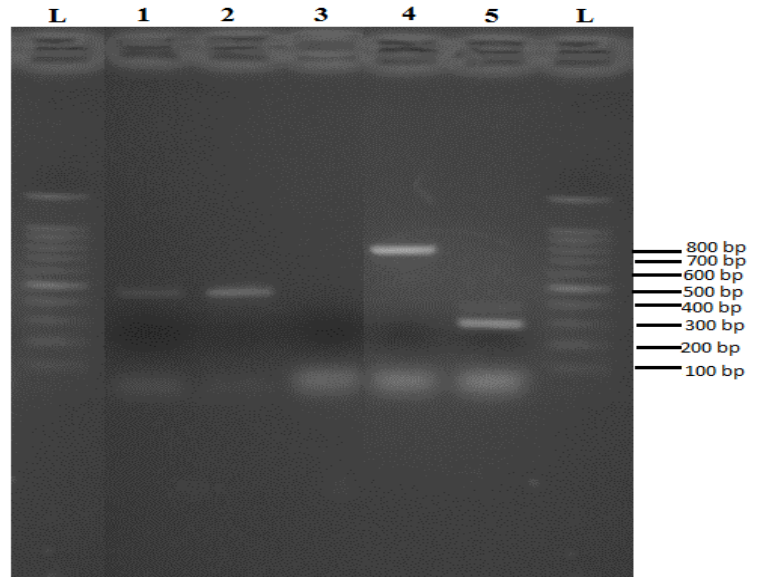
**Figure 2.** Representative samples showing detection of Panton-Valentin leukocidin (PVL) a
SCC*mec* types in MRSA isolates by PCR. Lanes: L, 100 bp DNA ladder; 1 and 2, PVL posit
strains; 3 negative control; 4, *SCCmec* type IVa and 5, type II.

**Figure 3.** DNA fingerprints generated by ERIC PCR analysis of 13 MRSA isolates recovered from clinical and environmental surfaces on 1.5% agarose gel. Lanes numbered with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 are referring to samples 8R, 18R, 39R, 40AT, 48BT, 64T, 70AR, 70BR, 102R, 2HPR, 3HPR, 4HPR and 99AT, respectively.
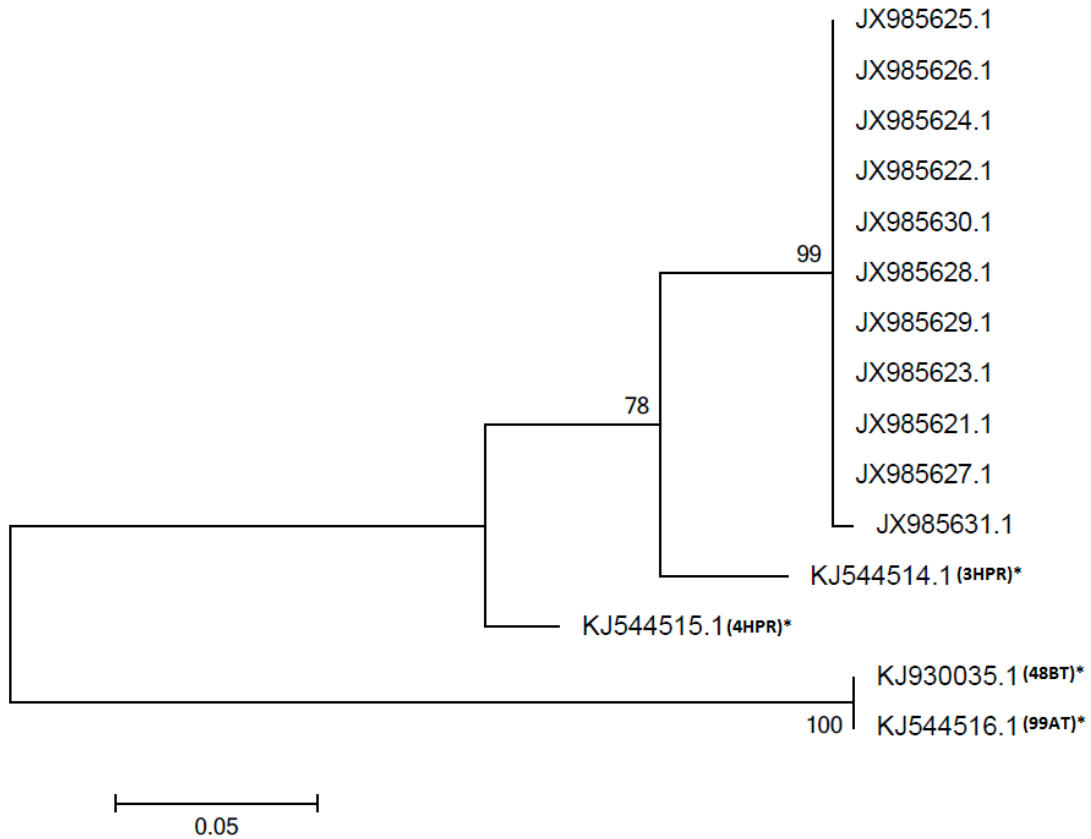
**Figure 4.** Phylogenetic analysis by Maximum Likelihood method based on the partial *spA* gene sequence. The *spA* gene nucleotide sequences reported in this study were denoted by asterisk. Reference sequences were reported from Palestine, Jordan and Iraq used for phylogenetic analysis [25]. The tree was bootstrapped with 1000 replicate, and the genetic distance corresponding is shown by the bar. Evolutionary analyses were conducted in MEGA6.