Citation: Rania O. Abdulraheem. Molecular Detection of Community- associated and Hospital acquired Methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin gene isolated from Wound Infection Sudanese Patients. African Journal of Medical Sciences, 2016, 1(12) ajmsc.info

Molecular Detection of Community- associated and Hospital acquired Methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin gene isolated from Wound Infection Sudanese Patients

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Abstract

Background: Panton-Valentine leukocidin (PVL) genes encode a potent toxin in staphylococcal wound infections. These infections have increased over the past years in Sudan and other developing countries.

Objective: To detect community-associated and hospital acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin gene isolated from Wound Infection Sudanese Patients.

Materials and methods: This study is a qualitative, descriptive cross sectional study performed in Khartoum (Sudan) to detect PVL genes using molecular techniques among *Staphylococcus aureus* (*S.aureus*) isolates. The study was conducted during the period from March to August (2012). 90 cultures of *S. aureus* were investigated. These cultures were identified according to the standard bacteriological procedures. The strains were isolated from community-acquired wound infections and from hospital-acquired wound infections. *S. aureus* strains isolated were also tested for methicillin sensitivity using the Kirby-Bauer disc diffusion technique. Genomic DNA was extracted from the staphylococcal cultures by a standard molecular procedure and was used as the template for amplification by using primers specific for *luk*S-PV and *luk*F-PV which form the operon for the PVL gene locus.

Results: *S. aureus* was confirmed in 83 (92.2%) out of the 90 isolates collected. 38 (45.8%) *S.aureus* strains were isolated from community-acquired wound infections; and 45 (54.2%) strains from hospital-acquired wound infections. The total methicillin-resistant *S.aureus* (MRSA) strains isolated were 43 (51.8%) strains. 8 (18.6%) MRSA strains were isolated from community-acquired wound infections; and 35 (81.4%) were isolated from hospital-acquired wound infections. PVL gene was detected in 18 (21.7%) of the 83 confirmed *S.aureus* isolates. Among community-acquired *S.aureus* isolates PVL gene was detected in 13 (72.2%) strains; and among hospital-acquired *S.aureus* isolates PVL gene was detected in 5 (27.8%) strains. PVL gene was detected in 6 (33.3%) MRSA strains; and it was detected in 12 (66.7%) methicillin-sensitive *S.aureus* (MSSA) strains.

Conclusion: The molecular technique is reproducible and reliable for detection of the PVL gene in *Staphylococcus aureus* strains isolated from wound infection cases.

Key words: PVL gene, Molecular technique, Methicillin-resistant *Staphylococcus aureus*.

Introduction

Staphylococcus aureus (*S. aureus)* is a facultative anaerobic Gram-positive bacterium. It is estimated that 20% of the human population are long-term carriers of *S. aureus*[1].
The reason *S. aureus* is a serious pathogen is its ability to have a combination of bacterial immuno-evasive strategies. One of these strategies is the production of the carotenoid pigment (staphyloxanthin), which is responsible for the characteristic golden color of *S. aurous* colonies. This pigment acts as a virulence factor, primarily by being a bacterial antioxidant which helps the microbe to evade the reactive oxygen species which the host immune-system uses to kill pathogens [(2,3)]. *S. aurous* is one of the most common causes of nosocomial infections, and it often causes postsurgical wound infections. It has played a role in a number of outbreaks of fatal bacterial infections; and may increase the expression of the staphylococcal protein A[4].
Panton-Valentine leukocidin (PVL) gene is a cytotoxin belonging to the β-pore-forming toxins. The presence of PVL gene is associated with increased virulence of certain strains of *Staphylococcus aureus*. PVL gene creates pores in the membranes of infected cells [5].
PVL gene exotoxin constitutes essential components of the virulence mechanisms of *S. aureus*. Nearly all *S. aureus* strains secrete lethal factors that convert host tissues into nutrients required for bacterial growth[6].
The PVL gene factor is encoded in the prophage 'Φ-PVL' which is a virus integrated into the *S. aureus* bacterial chromosome. Its genes secrete two proteins: *Luk*S-PV and *Luk*F-PV. These two proteins act together as subunits, assembling in the membranes of the host defense cells, particularly the monocytes, and macrophages. The subunits fit together and form a ring with a central pore through which cell contents leak and acts as a superantigen [(7, 8)].
Thus *S. aureus* presents a serious challenge to medicine due to its highly virulent toxin genes. This study shall provide an insight into these traits.

Materials and methods

This was a qualitative, descriptive, cross sectional study performed in Khartoum (Sudan) to detect PVL gene carried by *S.aureus* isolates using PCR technique. The study was conducted during the period from March to August 2012. The Mann-Whitney U statistical test and the receiver-operator-characteristic curve analyses were performed with SPSS software, version 16.0 (SPSS Inc., Chicago, USA). They were used to identify differences in PVL genes between groups of samples that were PVL positive (PVL^+) and PVL negative (PVL^-). The level of statistical significance was set at a p - value of <0.05.
S.aureus isolates were kindly provided by the Ministry of Health Laboratory Department (Khartoum State). 90 cultures of *S. aureus* were studied. These cultures were identified

according to colonial morphology, Gram stain, catalase, coagulase, mannitol fermentation, and DNAse tests. From these 90 cultures, 83 isolates were fully confirmed as *S. aureus.* 38 *S.aureus* strains were isolated from community-acquired wound infections; and 45 strains from hospital-acquired wound infections; covering both males and females. *S. aureus* strains isolated were also tested for methicillin sensitivity using the Kirby-Bauer disc diffusion technique. The confirmed isolates were sub-cultured onto agar slopes and incubated overnight at 37° C prior to molecular testing of PVL gene.

Genomic DNA was extracted from the staphylococcal cultures by a standard procedure and was used as the template for amplification by using primers specific for *luk*S-PV and *luk*F-PV, which form the operon for the PVL gene locus. DNA was extracted using phenol chloroform extraction technique (chemical method). Positive and negative controls were used with each run of PCR assay. A commercial DNA extraction kit (Cuagen, USA) was used according to the manufacturer's instructions.

100 ml of nutrient broth was prepared and autoclaved. A loopful of *S.aureus* was inoculated in the broth and incubated overnight at 37°C. The culture broth was centrifuged at 12,000 rpm for 15 min at 4°C. The resulting pellet was washed with an equal volume of 0.85 % saline, vortexed, and centrifuged at 12000 rpm for 15 min in 4°C. The pellet was suspended in 0.35 ml of SPES buffer, mixed well, and incubated at room temperature for 1 to 2 minutes. An equal volume (400 ml) of phenol was added, vortexed properly for1 minute and centrifuged at 12000 rpm at room temperature for 5 minutes. The aqueous phase was transferred to a fresh tube and an equal volume of absolute ethanol was added, and incubated at -20°C for 30 min. for cold precipitation of DNA. This was then centrifuged at 12000 rpm for 15 minutes at 4° C. The supernatant was discarded and the pellets were washed in 70% ethanol. The pellets were allowed to air dry at room temperature for 15 minutes, then dissolved in a minimal amount of TE buffer for complete solubilization, and stored at - 4°C.

The *luk*F-PV and *luk*S-PV genes, coding for the PVL gene toxin, were detected by PCR, as described by Lina *et al* [9]. The reaction was performed was according to QIAGEN Hot StarTaq DNA Polymerase (QIAGEN Inc., Valencia, CA). The primer sequences used were as follows:

Forward primer: ATCATTAGGTAAAATGTCTGGACATGATCCA.

Reverse primer: GCATCAASTG TATTGGATAGCAAAAGC.

PCR amplification was carried out in a total volume of 50 µL in sterile PCR tube, containing 25 µL 2xtaq, 1µL mgcl2, 1µL each primer (Working primer 10%), 17µl H_2O, and 5 µL template DNA. Thermo-cycling conditions were Gene-Amp 9600 thermocycler (Applied Biosystems). Amplification PCR program started with an initial denaturation at 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 40 sec, extension at 72°C for 1 minute; and final extension at 72°C for 5 minutes. The amplified products (5 µl) were separated by electrophoresis in 1.5% agarose gel and visualized by staining with ethidium bromide using UV gel documentation system. A 433-bp PCR product was amplified with the above mecA-gene specific primers (Fig. 1)

Results

S. aureus was confirmed in 83 (92.2%) out of the 90 isolates collected. 38 (45.8%) *S.aureus* strains were isolated from community-acquired wound infections; and 45 (54.2%) strains from

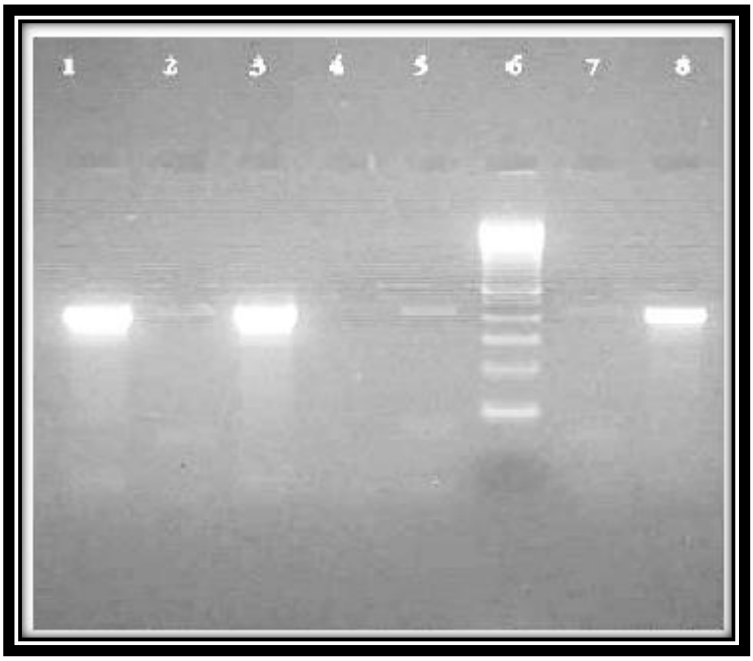

Fig. (1): PCR detection of PVL gene

Legend: 1 = Positive control 2 = Negative control 3 = PVL positive 4 = PVL negative 5 = PVL negative 6 = Molecular weight marker 7 = PVL negative 8 = PVL positive

hospital-acquired wound infections. Among community-acquired wound infection patients, 26 (68.4%) *S.aureus* strains were isolated from males and 12 (31.6%) were isolated from females (Table 1).

Table (I): *Staph. aureus* isolates according to gender and type of wound infections

Gender	Community-acquired wound infections	Hospital-acquired wound infections	Total
Males	26 (68.4 %)	32 (71.1 %)	58 (69.9 %)
Females	12 (31.6 %)	13 (28.9 %)	25 (30.1 %)
Total	38 (100 %)	45 (100 %)	83 (100 %)

On the other hand, among hospital-acquired patients, 32 (71.1%) *S.aureus* strains were isolated from males and 13 (28.9%) *S.aureus* strains from females (p = 0.79). The total methicillin-resistant *S.aureus* (MRSA) strains isolated were 43 (51.8%) strains. 8 (18.6%) MRSA strains were isolated from community-acquired wound infections; and 35 (81.4%) were isolated from hospital-acquired wound infections (Table 2).

Table (2): MRSA isolation and PVL gene detection according to type of wound infections

Type of wound infections	MRSA	PVL gene
Community-acquired	8 (18.6 %)	13 (72.2 %)
Hospital-acquired	35 (81.4 %)	5 (27.8 %)
Total	43 (100 %)	18 (100 %)

Also 32 (74.4%) MRSA strains were isolated from males in the community-acquired group; and 11 (25.6%) were isolated from females (p = 0.35).
By PCR technique, PVL gene all *S.aureus* isolates belonged to *mec* type and carried the *luk*F-PV and *luk*S-PV genes. PVL gene was detected in 18 (21.7%) of the 83 confirmed *S.aureus* isolates. Among community-acquired *S.aureus* isolates PVL gene was detected in 13 (72.2%) strains; and among hospital-acquired *S.aureus* isolates PVL gene was detected in 5 (27.8%) strains (p = 0.01).
As shown in Table (3), methicillin sensitivity, PVL gene was detected in 6 (33.3%) MRSA strains; and it was detected in 12 (66.7%) methicillin-sensitive *S.aureus* (MSSA) strains (p = 0.07). As per gender differences, PVL gene was detected in 13 (72.2%) males and 5 (27.8%) females from whom *S.aureus* strains were isolated (p = 0.8)

Table (3): PVL gene detection according to methicillin sensitivity and gender

PVL gene detection	MRSA	MSSA	Males	Females
Positive	6 (33.3 %)	12 (66.7 %)	13 (72.2 %)	5 (27.8 %)
Negative	37 (56.9 %)	28 (43.1 %)	45 (69.2 %)	20 (30.8 %)

Discussion

S. aureus had become a significant cause of hospital-acquired infection in many parts of the world. *Staphylococcus aureus* colonizes approximately 30% of the general population. The organism is mainly transmitted between persons by close contact. The worldwide spread of PVL gene-positive *S. aureus* had been documented[10].
PVL gene is one of many toxins associated with *S. aureus* infection. It was described as a key virulence factor, allowing *S. aureus* to target and kill specifically the neutrophil white blood cells[11].
The high prevalence of PVL gene among *S. aureus* wound infections observed worldwide is due to the spread of a single clone that can be identified on the basis of genotyping characteristics[12].
However, reports of prevalence rates of PVL gene among *S. aureus* infections in developing countries, to the best of our knowledge, are lacking. In recent studies performed in the Mediterranean region, the Balkans (Serbia) and the Middle East, there was a high prevalence of PVL gene-positive *S. aureus* infections[13].

The PCR assay is specific, simple and reliable in detection of *S. aureus* PVL genes. It could be performed in less than 2 hours following full isolation and identification of *S. aureus* in pure agar culture.

As shown in Table (1), among community-acquired patients, 26 (68.4%) *S.aureus* strains were isolated from males and 12 (31.6%) *S.aureus* strains from females. Also among hospital-acquired patients, 32 (71.1%) *S.aureus* strains were isolated from males and 13 (28.9%) *S.aureus* strains from females. Statistically this relationship was not significant (p = 0.79).

In wound infections, PVL gene had been associated more frequently leading to direct invasion and tissue destruction. In UK, 390 *S. aureus* clinical isolates were screened for PVL genes and 20.8% of soft tissue specimens were found to contain the genes for PVL gene[14].

In our study PVL gene was detected in 21.7% (18/83)) of wound infection cases; giving almost an identical similarity (Table 2).

Cedric Badiou and his colleagues studied the detection of *Staphylococcus aureus* PVL gene in soft tissue clinical specimens. They recovered 185 *S. aureus* isolates from patients with such infections. They analyzed these specimens for the presence of the PVL locus gene by PCR. 72 (39%) were positive for the PVL locus gene[15].

Other workers found that 50-93% of *S. aureus* strains responsible for cutaneous abscess and cellulites formation and 85% of *S. aureus* strains responsible for community-acquired infection had harbored the genes encoding for PVL as compared with none of those causing nosocomial infection[4].

Cynthia and her co-workers studied the community-associated methicillin-resistant *S. aureus* isolates and hospital-associated infections. They identified 352 patients who had hospital-associated-MRSA cultures. The PVL gene *mec* type IV phenotype was identified in 128 (36%) of these 352 patients[16].

In the present study and among hospital-acquired *S.aureus* isolates PVL gene was detected in 5 (27.8%) strains (Table 2).

Vandenesch and his co-workers showed that the MRSA PVL^+ strains belong predominantly to community-acquired MRSA isolates. They examined whether $mecA^+$ strains produced more PVL than $mecA^-$ strains. They found no difference in the PVL concentrations between $mecA^+$ and $mecA^-$ strains. They concluded that it is unlikely that *mecA* has an impact on PVL production[17].

In our study the *luk*F-PV and *luk*S-PV genes, coding for the PVL gene toxin, were detected by PCR using mecA-gene specific primers. PVL gene was detected in 6 (33.3%) of MRSA strains and in 12 (66.7%) of MSSA strains (Table 3).

In Hong Kong Ho and his colleagues studied the molecular epidemiology and household transmission of community-associated methicillin-resistant *S. aureus.* They detected community-associated methicillin-resistant *S. aureus* in 6 (13%) of 46 household contacts. They performed PCR for detection of the genes encoding PVL, and observed that 23 were PVL positive[18]. PVL gene is a cytotoxin that causes leukocyte destruction and tissue necrosis. Although there is agreement that PVL gene strains are epidemiologically linked to severe infections and necrosis, conflict remains over whether PVL gene is itself pathogenic[19].

From this study, it may be recommended that future research should be carried out to investigate measures aiming at decolonizing carriers, interrupting person-to-person transmission, and preventing potentially lethal outbreaks. PVL gene infection control measures

need to focus on good hygiene, avoiding sharing personal items and environmental cleaning Conclusion: The molecular technique is reproducible and reliable for detection of the PVL gene in *Staphylococcus aureus* strains isolated from wound infection cases.

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