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Molecular Study of Panton-Valentine Leukocidin Genes among *Staphylococcus aureus* Clinical Isolates in Khartoum State, Sudan

Najem Aldin M. Osman[1,*], Intisar E. Alrayah[2], Yassir Mahgoub Mohamed[3], Ali M El-Eragi[4], Muataz M. Eldirdery[3], Mohamed Ahmed Salih[5]

[1]Department of Biotechnology, Faculty of Science and Technology, Omdurman Islamic University, Sudan
[2]College of Applied Medical Science, Shaqra University, KSA
[3]Department of Microbiology, Tropical Medicine Research Institute, National Center for Research, Sudan
[4]Department of Microbiology, Faculty of Medicine, King Khalid University, KSA
[5]Department of Biotechnology, Biotechnology Park, Africa City of Technology, Sudan
*Corresponding author: najemosman@hotmail.com

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Abstract *Staphylococcus aureus* strains carrying Panton- Valentine Leukocidin genes (PVL) are an emerging threat worldwide, causing variety of infections even in healthy individuals. Intensive efforts through the last years have been carried out towards the detection and analysis of PVL genes. The prevalence and characterization of such genes has not been done in Sudan. In this study we investigated the prevalence and the molecular characteristics of PVL genes among *S. aureus* clinical isolates, comparing their PVL allelic variant with that of PVL positive strains from different countries. Standard microbiological procedures were used for the identification of isolates, polymerase chain reaction for determination of PVL genes and standard sequencing for mapping of *lukS/F*-PV genes. In Silico tools were used for sequence analysis. Among 210 *S. aureus* isolates, PVL genes were detected in 122 (58%). Sequence analysis for *lukS/F*-PV genes from 12 representative isolates detected a new point mutation in *lukS* PV region. Collectively, our findings showed a high frequency of PVL genes among *S. aureus* isolates and revealed a novel nonsynonymous mutation. Phylogenetic analysis revealed that Sudanese isolates were closely related to each other.

Keywords: *PVL, S. aureus, Sudan*

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1. Introduction

The *Staphylococci aureus* is a bacterium of major human pathogen. It has been reported as the cause of spectrum of infections in hospitals and throughout the communities [1].

Pathogenicity of *S. aureus* is related to a number of virulence factors that allow it to adhere to surface, invade or avoid the immune system and cause harmful toxic effects to the host. These factors include cell surface, components (e.g. protein A, fibrinogen-binding protein, collagen-binding protein and clumping factors) and exoprotiens (e.g. enterotoxin, exofoliatin, toxic shock syndrome toxin and Panton-Valentine leukocidin (PVL) [1]. PVL is a pore-forming cytotoxin that targets human and rabbit mononuclear and polymorphonuclear cells (PMNs) [2]. When injected intradermally into rabbit, it induces severe inflammatory lesions, leading to capillary dilation, chemotaxis, and polymorphonuclear karyorrhexis, and skin necrosis [3]. Studies have shown that PVL is encoded by two co-transcribed genes, *lukS*-PV and *lukF*-PV (*lukS/F*-PV) and the toxic effect results from the synergistic action of the two separate exoprotiens [4].

Recently there has been much interest in PVL due to its involvement in severe disease among children and young adults with no known exposure to health care establishment [1]. The association of PVL gene with community acquired pneumonia was first noted by Lina *et al*., (1999). PVL was also significantly associated with strains causing invasive skin infections such as furunculosis (93%) and coetaneous abscess (50%), compared with superficial folliculitis (0%). PVL was not observed in strains associated with infective endocarditis, urinary tract infections, toxic shock syndrome, or mediastinitis, although only few strains were tested [5]. Diep *et al*., (2004) reported a similar association of PVL and skin and soft-tissue infections caused by methicillin resistant *Staphylococcus aureus* (MRSA) isolated from inpatients and outpatients from San Francisco General Hospital and inmates in county jails [6].

Until recently genes coding for PVL were infrequently encountered, being noted in <5% of *S. aureus* isolates worldwide [7]. However a very high proportion of newly emerging community associated-MRSA (CA-MRSA) strains carrying PVL genes with rate 77% to 100% was reported in various studies [8]. In an epidemiologic study from Minnesota, PVL was present in 77% of all CA-MRSA and in only 4% of hospital associated-MRSA (HA-MRSA) [9]. In the future, screening for the PVL virulence factor in *S. aureus* may become a routine laboratory procedure [10].

Molecular characteristics of PVL positive *S. aureus* have been reported worldwide, in US [11], in Europe [12], in Japan [13]. Although characterization of some Egyptian strains has been studied by Enany *et al*., (2010) but information from Africa is still limited. O'Hara *et al*., (2008) detected twelve single nucleotide polymorphisms (SNPs) among *lukS-PV* and *lukf-PV* genes. The majority of which are synonymous. A non synonymous mutation at position 527, however, serves as the basis of the H and R isoforms [11,14,15]. The PVL nucleotide sequence is highly conserved and one genetic polymorphism in *lukS-PV* results in a non synonymous amino acid change in *LukS-PV* [11].

From the different cited literature review *S. aureus* strains carrying PVL genes which is a potential virulence factor, are involved in severe human infections, most worrying however, is the increasing number of reports of PVL positive strains of *S. aureus*. This study is focused to determine the frequency of PVL genes among *S. aureus* isolates from Khartoum state, to analyze the molecular characteristics of the isolates through partial sequencing of *lukS-PV* and *lukf-PV* genes and to compare their allelic variations with that of PVL positive from different countries.

2. Materials and Methods

This study was carried out mainly in Khartoum state; during years 2012 to 2014. Specimens were collected from Khartoum major hospitals, including Khartoum teaching hospitals, Alribat teaching hospital, and the Military hospital. Study subjects are patients suffering from any *S. aureus* suspected infections including throat infection, otitis, wound infection, pneumonia, septicemia, UTI, skin abscess, osteomyelitis, throat infection, and enteritis.

2.1. Bacterial identification

All the procedures were carried out in Tropical Medicine Research Institute, Department of Microbiology. The isolation and identification of bacterial isolates was done according to standard biochemical tests [16] and [17]. 210 *S. aureus* isolates were recovered from different sites of infections. *S. aureus* ATCC 25923 was used as control strain in all procedures.

2.2. Molecular Detection of PVL Genes

2.2.1. DNA Extraction

DNA extraction was done using DNeasy kit 69504 (supplied by QIAGEN). Pretreatment of bacterial cells was done according to the manufacturer instructions.

2.2.2. PCR Amplification

The specific primers used in this study were synthesized and purchased from Metabion International-Germany. A single PCR assay targeting *Staphylococcus aureus* species specific *lukS/F-PV* gene (a determinant of leukotoxin) was performed, the primers used was *luk-PV*-1, (5- ATCATTAGGTAAAATGTCTGGACATGATCCA-3) and *luk-PV*-2,(5-GCATCAACTGTATTGGATAGCAAAAGC-3) [5]. The amplifications were performed using Maxime PCR PreMix Kit i-Taq 20 µl (INTRON Biotechnology, South Korea). Amplification was performed as described previously [5].

The PCR products (433bp) were analyzed on a 1.5% agarose gel. The gel electrophoresis was performed at 80V for 45 min. and the analysis was done by using an automated gel photo documentation system see Figure 1-A.

2.2.3. Sequencing of PVL Gene

Partial standard sequencing for coding sequence of *lukS/F-PV* genes was performed by Macrogen Company (Seoul, South Korea). The PCR products of the PVL gene were prepared with concentration not less than 50 ng /µl and sent to the company for mapping.

2.3. Bioinformatics Analysis

The identity of the nucleotide sequence of the isolated strains was detected by comparing them with the published sequences using online public basic local alignment search tool (Blast n) (http://www.ncbi.nlm.nih.gov/blast), [18]. The Sequences chromatogram was viewed by FinchTV program version 1.4.0 (http//www.geospiza.com/product/finchtv.shtml). Also it was used to compare the nucleotides sequences of the isolates each the forward and reverse sequences. Multiple sequence alignment was performed by BioEdit software version 7.0.9.1, [19] through ClustalW multiple alignment to compare our isolates sequence with the control sequence (ATCC 49775, GeneBank accession NO AB006796) and with other highly similar published sequences from different selected countries including: Egypt (FJ821791), USA (EF571829), China (AB678712), UK (EF571788) and India (JF778651). In GeneMarkS version 4.25 (http://exon.gatech.edu/genemark/genemarks.cgi), the genes sequences were translated into amino acid sequence [20]. The phylogenetic and molecular evolutionary analyses were conducted using the online soft ware Phylogeny.fr. [21] MEGA6 software (version 0.06), was used for confirmation [22]. Mutation analysis was done online by project hope software (http://www.cmbi.ru.nl/hope/report/2064?10).

3. Results

From the 210 pure *S. aureus* isolates, PVL positive isolates were 122 (58.1%). Twelve representative isolates were selected for PVL sequencing. Eight isolates were from community origin including 12, 90, 120, 146, 154, 160, 171, and 198, while the other four isolates (49, 116, 129 and 172) were from hospital origin. The isolates were from different sites of infection see Table 1.

Table 1. Characteristics of PVL positive representative isolates

Isolate number	Site of infection	Origin	PVL
12	Paronychia	Community	+
49	Skin abscess	Hospital	+
90	Wound	Community	+
116	Osteomyelitis	Hospital	+
12O	Wound	Community	+
129	Osteomyelitis	Hospital	+
146	Skin abscess	Community	+
154	Skin abscess	Community	+
160	Throat infection	Community	+
171	Ear infection	Community	+
172	Wound	Hospital	+
198	Pneumonia	Community	+

3.1. In Silico Analysis

Homology analysis through BLAST tool showed 99% identity of our sequences comparing with above mentioned sequence obtained from Gene- Bank. Multiple sequence alignment revealed two types of mutations (substitution) among all isolates. The mutations had occurred in *lukS-PV* region, one of them is at position 663 (GTG / GTT) detected in seven isolates when compared with the control, the isolates were 12, 49, 90, 116, 129, 154, and 160. This mutation has been reported recently as synonymous polymorphism. A single nucleotide polymorphism at position 610 (AAT / GAT) was detected in all sequenced isolates as shown in Figure 1- B, C.

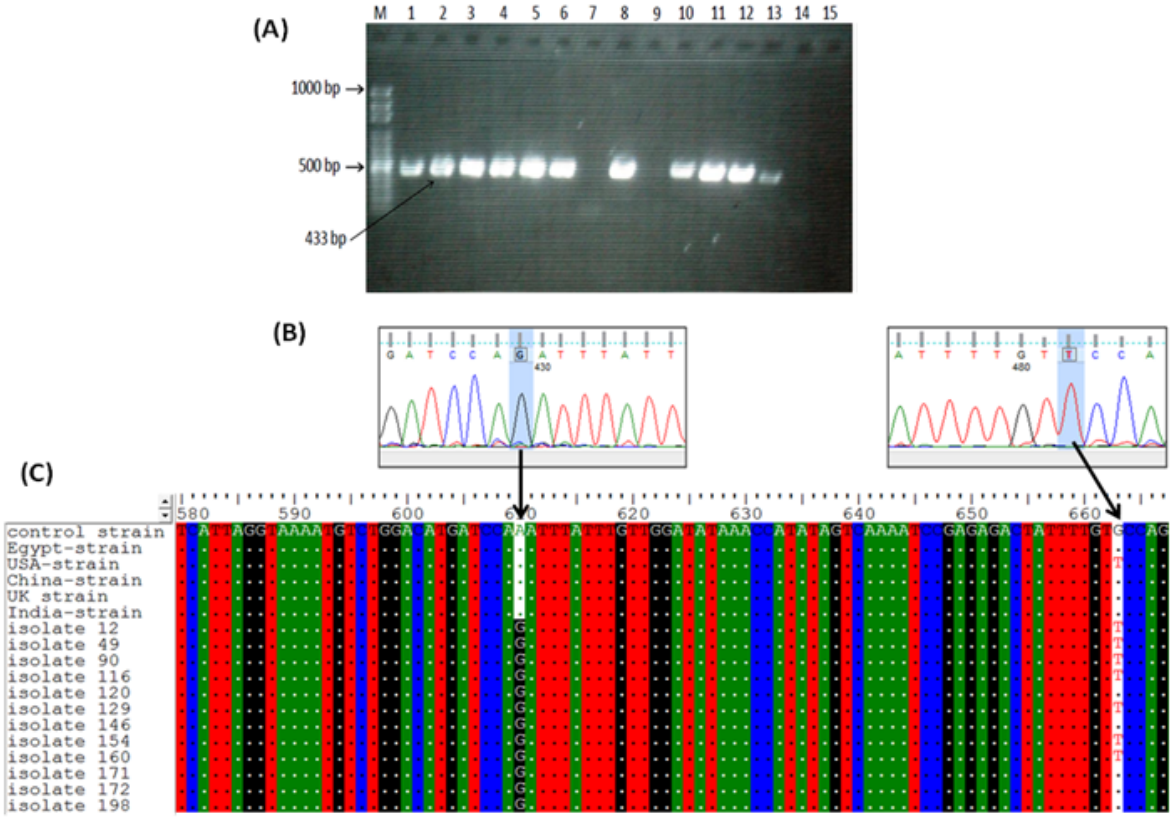

Figure 1. A. Molecular detection of PVL virulence gene by PCR analyzed in 1.5% agarose gel electrophoresis M: 100 bp molecular ladder, lane 1: positive control, lane 15: negative control, lane 2, 3, 4, 5, 6, 8, 11, 12, and 13 typical PVL positive isolates, lane: 7, 9, 14 PVL negative isolates. B. *lukS-PV* gene sequence chromatogram as showed by FinchTV software, left: site of new detected mutation, right: synonymous nucleotide polymorphism. C. BioEdit multiple sequence alignment of the twelve isolates and other selected strains from data base

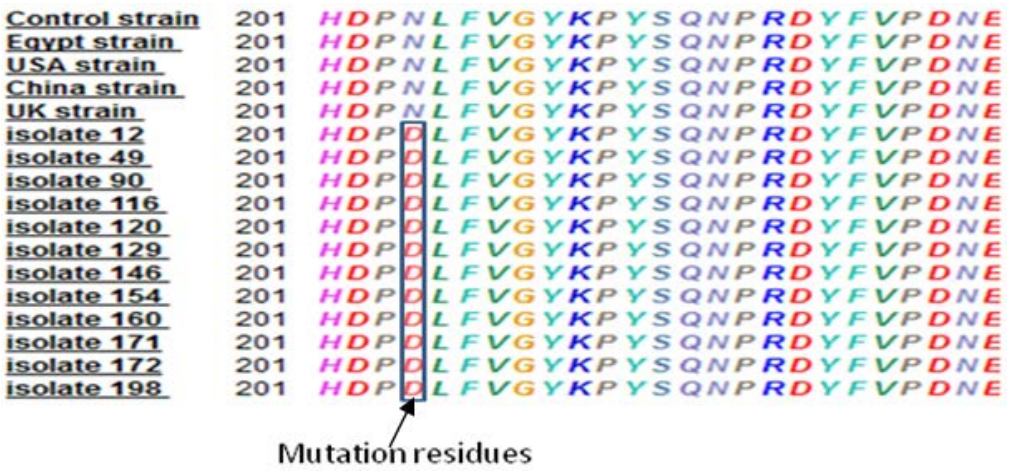

Figure 2. Amino acids multiple sequence alignment of *lukS-PV* gene showed the mutation residues (Aspartic acid) at position 204 for the twelve Sudanese strains. The alignment was performed using ClustalW sequence alignment

3.2. Amino Acids Sequence and Alignment

The result of translation and multiple amino acids sequences alignment revealed that the change at position 663 (GTG / GTT) gives the same type of amino acid which is valine, while the change at position 610 (AAT / GAT) gives another different amino acid (Asparagine → Aspartic acid) at position 204 of amino acid sequence (Figure 2).

3.3. Phylogenetic Analysis

The construction of phylogenetic tree revealed that all strains have closely related sequence. Our isolates appeared in two groups, the first one containing isolates 146, 171, 120, 172, and 198 were so closed to the India and control strain, and the second group containing isolates 12, 49, 90, 116, 129, 154, and 160 were so closed to the USA strain, see Figure 3.

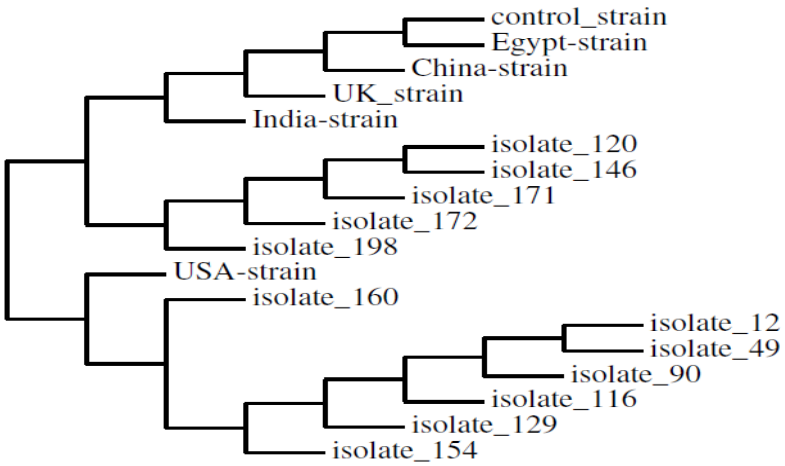

Figure 3. Phylogenetic tree of the mutant Sudan *LukS PV* gene and other PVL genes obtained from database

4. Discussion

PVL is an important virulence determinant of *S. aureus*, the general view of this study showed an increase in PVL positive *S. aureus* isolates. To our knowledge, there is no published data about the prevalence and allelic variants of such genes in Sudan. Our study revealed that 58.1% of *S. aureus* isolates were PVL positive. The prevalence of PVL in Algiers hospital was 72% [23], while Lina *et., al* found that it was 37.2%. This variation in frequency may be due to the nature of origin of isolates, because PVL genes are more frequently among community strains. [1,5,24,25].

Concerning the sequence, it is almost certain that PVL genes are well conserved at nucleotide level even when isolates have different genetic backgrounds [26]. All our isolates have similar nucleotide sequence except for position 663 in which seven isolates have T while the other five have G. this point mutation has been reported previously [11,14,27], and it results in no amino acid change. Interestingly, our study showed a new point mutation at position 610 (A/G) of *lukS-PV* gene sequence in all isolates from different origins (community and hospital) when compared with the control and even with the selected strains from different countries (Figure 3- B, C). This mutation results in non synonymous change in amino acids sequence at conserved region of *lukS-PV* gene protein sequence (Asn → Asp). The residue in which the mutation occurred is part of an interpro domain named "Leukocidin/porin" (IPR016183). The mutated residue is located on the surface of a domain with unknown function. The residue was not found to be in contact with other domains of which the function is known. However, contact with other molecules or domains is still possible and might be affected by this mutation. The mutant amino acid has different charge which may affect the contact between the mutant residues and neighboring residues.

The phylogenetic analysis presented here revealed that the PVL genes are highly conserved, our isolates appear in two groups, the first group is so closed to US strain and the second one is so closed to Indian and UK strains, but they are closely related to each other.

In conclusion, this study provides information about *S. aureus* PVL genes prevalence and sequence and it showed a novel mutant *lukS-PV* gene in Sudan. Further studies are required to investigate the functional implications of such mutation in the protein structure and function.

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Competing Interests

The authors have no competing interests

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