Original Article

Staphylococcus aureus carrying *luk*S/F Panton-Valentine Leukocidin (PVL) toxin genes in hospitals of Lahore city

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Abstract

Introduction: Panton Valentine-Leukocidin (PVL) toxin is secreted by *Staphylococcus aureus* and is mostly associated with skin and soft tissue infections (SSTI). This study aims to find out the prevalence of *luk*S/F-PV gene, which encode PVL toxin from strains of SSTI, burn wounds and nasal colonizers of out-patients and to measure the antimicrobial susceptibility of *S. aureus* isolates.

Methodology: This is an analytical observational cross-section study and was conducted from July 2014 to June 2015 at four tertiary care hospitals and PCSIR Laboratories Complex, Lahore, Pakistan. A total of 376 random clinical swabs were collected from SSTI (n = 179), nasal nares (n = 134) and burn wounds (n = 63) from out-patients' departments (OPD). The specimens were cultured on nutrient and mannitol salt agar (MSA) and the organism was identified by catalase, coagulase, and DNase tests. Antimicrobial susceptibility, methicillin, inducible clindamycin, and high-level mupirocin (HLMR) resistance were determined as per CLSI guidelines. Molecular identification of *mec*A and *luk*S/F-PV genes was performed by PCR.

Results: We isolated 127 S. *aureus*, where 41 (32.3%) were MRSA and 86 (67.7%) were MSSA. All MRSA carried *mec*A gene whereas *luk*S/F-PV gene was found in 21 MRSA and 31 MSSA strains. Overall, a high antimicrobial resistance was found against MRSA and *luk*S/F-PV positive MSSA. Inducible clindamycin and high-level mupirocin resistance (HLMR) was 23.6% and 19.5% respectively.

Conclusions: A high rate of PVL toxin gene was detected among *S. aureus* strains and a high prevalence of antimicrobial resistant strains was observed.

Key words: *luk*S/F-PV; CA-MRSA; skin and soft tissue infections; SSTI; Panton-Valentine Leukocidin toxin; PVL.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes multiple types of infections with high rates of morbidity and mortality [1]. For the last two decades, this organism caused serious threats to public health, infection control, and economics [2]. The reason is its capability to cause a variety of diseases such as pneumonia, bacteremia, endocarditis, urinary tract infections (UTI's), food poisoning and prosthetic device infections [3]. In addition to these, it can also cause a wide range of skin and soft tissue infections (SSTI) such as mild impetigo, fatal necrotizing fasciitis. According to the estimates, MRSA is responsible for about 90% SSTI's [4] that predominately include 50% to 70% abscesses and 25% to 75% cases of cellulitis [5]. A number of virulence factors present in *S. aureus* are involved in evasion of the immune system and impose

pathogenicity [1]. However, a major contribution to its pathogenicity is due to an exotoxin called Panton-Valentine Leukocidin (PVL) that destroys human mononuclear and polymorph nuclear cells [6]. It has been found to be involved in pneumonia [6] and infections related to the skin as its association with SSTI has been strongly reported previously [7].

Less than 5% of the strains of *S. aureus* encompass PVL, however, a high frequency has been reported in the isolates that cause necrotic skin lesion and severe necrotizing pneumonia [8]. *S. aureus* is a commensal organism and colonizes in the nose of healthy persons asymptomatically which is a major risk factor for multiple types of infection spread [1]. Infections associated with PVL positive strains of *S. aureus* in the past were thought to be only present at community level and were restricted only to community people with no

history of hospitalization or admission to a health care facility, however this phenomenon has now greatly changed and these organisms are also prevailing in hospital environments [9].

Since the emergence of MRSA there is a gradual increase in the rate of resistance and efficiency of treatment has been diminished. In addition to β-lactams which lost their effectiveness decades ago against this organism, some non-beta lactam drugs such as clindamycin, mupirocin, and vancomycin have also been challenged by these decisive bacteria leaving behind a narrow chance of effective antibiotic choice [10]. Therefore, the imperative aspects of this study are to measure antimicrobial resistance and prevalence of *luk*S/F-PV genes in our locality. To the best of our knowledge, this is the first study to report the prevalence of *luk*S/F-PV genes from Pakistan.

Methodology
Study design and location
It was a cross-sectional study conducted from July 2014 to June 2015. Samples were collected from patients of four tertiary care hospitals of Lahore city namely Jinnah Hospital, Services Hospital, Mayo Hospital and Lahore General Hospital (LGH). The research was carried out at Food and Biotechnology Research Center (FBRC), Pakistan Council of Scientific and Industrial Research (PCSIR) Laboratories Complex, Lahore. Before starting the study, approval was taken from the ethical review committee of the Institute of molecular biology and biotechnology (IMBB), The University of Lahore and PCSIR Laboratories Complex, Lahore. A written consent was also obtained from each patient under study.

Collection of samples
A total of 376 non-repetitive clinical specimens were collected aseptically from infection sites of patients who reported at outpatient department (OPD) and hospitalized patients at dermatology and burn wards of the hospitals. Nasal swabs of a number of patients who reported at OPD of dermatology were also taken. A transportable cotton swab in Amies medium (Transwab M40, Corsham, UK) was used to collect specimens from SSTI (n = 179), nasal nares (n = 134) and burn wounds (n = 63). Clinical samples were transported to the microbiology laboratory at PCSIR laboratories complex within three hours of collection. Male to female ratio was found to be 2:1.

Bacterial identification
Specimens were streaked on nutrient agar and mannitol salt agar (Oxoid, Basingstoke, UK) and incubated at 37^0 C for 18 – 20 hours aerobically. Gram-positive cocci were identified on the basis of their colony morphology and shape under the microscope after Gram staining and were further confirmed by recommended biochemical tests of catalase, free and bound coagulase and DNase for *S. aureus* [11].

Identification of MRSA strains
MRSA strains were isolated phenotypically by the use of cefoxitin (30 μg) disks, a zone of inhibition against this disk was noted as recommended by CLSI guidelines 2014 [12].

Antimicrobial susceptibility testing
Antimicrobial susceptibility of the isolates was done by the Kirby-Bauer disk diffusion method using Mueller Hinton agar (Oxoid, Basingstoke, UK) media. Plates were incubated at 37 °C for 24 hours aerobically. Implanted antibiotics were penicillin (30μg), cefoxitin (30μg), erythromycin (15μg), clindamycin (2μg),

Table 1. Percentage of antibiotic resistance pattern.

Antibiotics	MRSA (%) n = 41	*luk*S/F-PV positive MSSA (%) n = 31
Penicillin (30μg)	100%	100%
Cefoxitin (30μg)	100%	0%
Erythromycin (15μg)	70.7%	12.9%
Clindamycin (2μg)	56%	9.6%
Trimethoprim/sulfamethoxazole (1.25/23.75μg)	63.4%	25.8%
Chloramphenicol (30μg)	31.7%	3.2%
Ciprofloxacin (5μg)	97.5%	6.4%
Tetracycline (30μg)	97.5%	90.3%
Fusidic acid (10μg)	58.5%	3.2%
Mupirocin (200μg)	19.5%	0%
Vancomycin (30μg)	0%	0%
Linezolid (30μg)	0%	0%

trimethoprim / sulfamethoxazole (1.25/23.75 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), tetracycline (30 μg), fusidic acid (10 μg), mupirocin (200 μg), vancomycin (30 μg) and linezolid (30μg) as shown in Table 1. Interpretation of the susceptibility was done as per CLSI recommendations [12].

Inducible clindamycin resistance

Inducible clindamycin resistance was checked by the D-zone test, which was performed by placing erythromycin (15μg) and clindamycin (2μg) discs at a distance of 15-20 mm apart. Formation of D-type zone against clindamycin was considered as inducible clindamycin-resistant strain (Figure 1) [13].

High-level mupirocin resistance (HLMR)

High-level mupirocin resistance (HLMR) was measured by placing the mupirocin (200μg) disc according to recommendations of CLSI [12]. For quality control and interpretation of results, we used American Type Culture Collection (ATCC) strain 25923 (MSSA) and ATCC strain 33951 (MRSA) during all process where required.

PCR amplification of mecA and lukS/F genes

DNA was extracted using a wide range commercial DNA isolation kit (ONE-4-ALL Genomic DNA Mini-Preps Kit, Canada Inc). For the detection of *mec*A gene, we used forward primer: 5′-GTAGAAATGACTGAACGTCCGATAA-3′ and reverse primer: 5′-CCAATTCCACATTGTTTCGGTCTAA-3 having accession number X52593.1 [14] and *Luk*S/F gene was identified by using already published primers which were forward primer: 5′-ATCATTAGGTAAAATGTCTGGACATGATCCA-3′ and reverse primer: 5′-GCATCAACTGTATTGGATAGCAAAAGC-3′ having accession number X72700.1 [6]. For PCR conditions in thermocycler (MyCycler BioRad Lab.

Figure 1. Characteristic of inducible clindamycin resistance D-test.

D shaped zone of inhibition due to the inducible clindamycin resistance.

Inc., Hercules, CA, USA) were the following: initial denaturation at 95ºC for 4 minutes; 30 cycles of denaturation of DNA at 95ºC for 30 seconds, annealing at 58°C for 40 seconds, primary extension at 72°C for 1 minute and final extension at 72° C for 7 minutes. PCR products were observed on 1% agarose gel with 3-5μL ethidium bromide (10mg/mL, Invitrogen, Loughborough, UK) staining using Gel Doc instrument under UV light.

Results

We isolated a total of 127 S. *aureus* (33.77%) from 376 specimens, where 41 (32.3%) were MRSA and 86 (67.7%) were MSSA isolates as shown in Table 2. All MRSA strains contained *mec*A gene while *luk*S/F-PV gene was found in 21 MRSA and in 31 MSSA strains. Majority of the MRSA (n = 15) and MSSA (n = 49) were isolated from SSTI (n = 64) followed by 17 MRSA and 03 MSSA from burn wounds and 09 and 34 isolates of MRSA and MSSA respectively were recovered from nasal nares swabs. Types of SSTI's with no. of isolates

Table 2. Prevalence of MRSA, MSSA and *luk*S/F-PV gene from different clinical samples.

Total number of isolates (n = 127)		MRSA (n)	MSSA (n)	*luk*S/F-PV +ve
Skin and Soft Tissue Infections (SSTI) (n = 64)	Abscesses (n = 15)	03	12	09
	Folliculitis (n = 07)	01	06	04
	Impetigo (n = 10)	02	08	05
	Carbuncle (n = 14)	02	12	05
	Furuncle (n = 12)	05	07	08
	Surgical wounds (n = 04)	01	03	01
	Hyper IgE syndrome (n = 02)	01	01	01
Burn wounds (n = 20)		17	03	03
Anterior nares swabs (n = 43)		09	34	17
Total = 127		**41 (32.3%)**	**86 (67.7%)**	**52 (40.9%)**

and gene carriage is shown in Table 2. PVL gene *LukS*/F prevalence was detected by PCR as bands of its product on gel electrophoresis are shown in Figure 2. The overall prevalence of *LukS*/F was found to be 40.9% (Table 2) and graphical representation of all isolates with gene carriage is shown in Figure 3.

Gender and Age

Male to female ratio was 2:1 and 44% of the patients belonged to 17 to 30 years of age group.

Antimicrobial Resistance

Overall, MRSA were about 98% resistant to tetracycline and ciprofloxacin, 58% to fusidic acid, 63% to trimethoprim/sulfamethoxazole whereas most effective drugs were vancomycin and linezolid with no resistant isolate. On the other way, *luk*S/F-PV positive MSSA were 100% resistant to penicillin, 90% to tetracycline, 25.9% to trimethoprim/sulfamethoxazole and zero resistance was observed against vancomycin and linezolid (Table 1). Inducible clindamycin resistance was found in 30 (23.6%) isolates out of which 9 were MRSA and 21 were MSSA (Figure 1); all of them were isolated from nasal swabs and SSTI specimens. Mupirocin, which is used for de-colonization of commensal bacteria showed resistance in 8 isolates all from burn patients. This was high-level mupirocin resistance (HLMR) and was not detected in OPD patients. Hospitalized patients of burn center were prescribed mupirocin for de-colonization of nasal *S. aureus*.

Discussion

MRSA is a growing worldwide concern and is strongly associated with many complex diseases, including skin and soft tissue infections (SSTI). The present study demonstrates high prevalence of *mec*A gene (n = 41) and *lukS/F* gene (n = 52) where it was found in 21 MRSA and 31 MSSA isolates both from hospitalized and OPD patients which shows that strains harboring this gene may also be inside hospitals or health care setups which is an alarming situation. The burden of health care associated infections (HAI) is also increasing and CA-MRSA strains which were previously restricted among community patients are causing infections in hospital environments which is of great concern [9] because the presence of PVL toxin is more dangerous for the immune system, since it destroys leukocytes and evades immune defenses [1].

Toxin genes *lukS/F*-PV were predominated among SSTI isolates where abscesses and furuncle were the leading infections harboring PVL toxin genes, which

Figure 2. Agarose gel electrophoresis analysis of PCR amplicon of *mec*A gene and *luk*S/F gene.
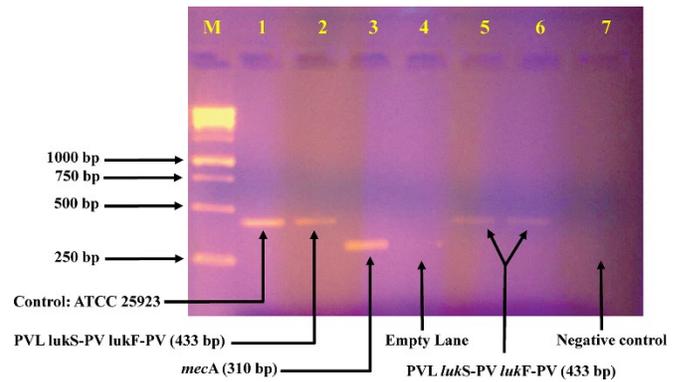

Lane M shows molecular marker; Lane 1: positive control ATCC (25923); Lane 2,5,6 shows *luk*S/F-PV (433bp) and lane 3 shows *mec*A (310bp); Lane 7: negative control DNA free water.

reinforces the importance of culturing bacteria from infectious sites before the start of antibiotic therapy. Similar studies conducted in various parts of the world [15-17] confirm the ubiquitous presence of *lukS/F* genes. The ratio of MRSA strains from hospitalized and out-patient department (OPD) was 85% and 24% respectively which nearly matched with a similar study from Kenya [18].

Inducible clindamycin resistance was observed in 23.6% isolates however one study showed a little high percentage of 30.2% [19]. Macrolide-Lincosamide-Streptogramin B (MLS$_B$) antibiotics if prescribed in place of beta-lactam drugs in SSTI treatment, inducible clindamycin test must be performed. High-level mupirocin resistance (HLMR) was observed in burn samples only, it coincides with studies in Iran where it was 25% [20] and 28 % [21] from burn units. Mupirocin

Figure 3. Prevalence of PVL positive and negative isolates.
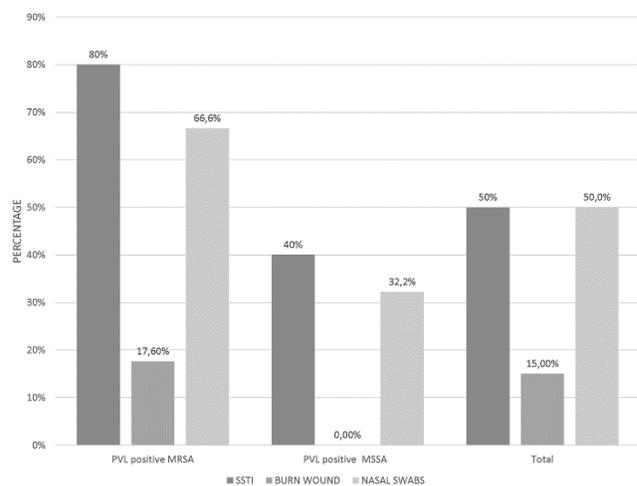
Percentage of PVL genes carried by MSSA and MRSA strains.

is used as a decolonizing agent, it is widely used in burn patients to lower the risk of MRSA colonization, however, its inappropriate use may lead to HLMR which is usually found among multidrug-resistant bacteria [22].

We isolated 32.3% MRSA strains. The rise of MRSA strains is of great concern for the whole world including Pakistan where previous studies showed 30% to 45% [23-26] and even higher (60%) by Perveen *et al*. [27] and 50% to 70% by Kaleem *et al*. [28]. In this alarming situation effective measures are necessary to mitigate the MRSA iceberg where it is linked to inappropriate infection control measures, contaminated intravenous catheters, surfaces (door handles, sink, floor, ventilator and incubator) and hand colonization which are major causes of the spread of strains [29]. Another major factor to acquire resistance is the irrational and broad spectrum use of antibiotics which is not according to WHO guidelines [30].

Limitations to this study are that we could not record the case history of patient infection and antibiotic use nor we knew whether the infection was recurrent or the first time. All the data obtained represented major hospitals and the number of males to female patient ratio was high and samples were taken topically from the infectious site.

Conclusion

There is a progressive increase in strains carrying PVL toxin and resistance against non-beta lactam drugs. These pathogens (CA-MRSA) are now making their way into hospitals. The clinical microbiology laboratories should consider routine testing and reporting of inducible clindamycin resistance in *S. aureus* to prevent the clinical treatment failure. A decolonizing agent such as mupirocin should also be used appropriately. The national multicenter survey should also be carried out to have a fair idea of resistance pattern and implement a proper antibiotic policy to combat this serious threat. Patient education should also be carried in order to stop the spread of SSTI strains to other household members.

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