Malaysian J Pathol 2017; 39(2) : 115 – 122

ORIGINAL ARTICLE

Frequency and molecular epidemiology of Panton-Valentine leukocidin gene in *Staphylococcus aureus* colonising HIV-infected patients

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Abstract

Background: HIV-infected patients pose a high risk of contracting skin and soft tissue infections caused by *Staphylococcus aureus*. Those who are colonized with methicillin-resistant *S. aureus* (MRSA) that carry Panton-Valentine leukocidin (PVL) are predisposed to severe infections that could lead to necrotic skin infections. However the association of *S. aureus* specifically methicillin sensitive *S. aureus* carrying PVL gene in HIV patients has not been widely reported. Here, we study the prevalence and the molecular epidemiology of PVL-producing *S. aureus* in HIV-infected patients. *Methods:* Swabs from four body sites of 129 HIV-infected patients were cultured for *S. aureus* and identified by standard microbiological procedures. The isolates were subjected to antimicrobial susceptibility testing by disk diffusion against penicillin, erythromycin, clindamycin, and cotrimoxazole. PCR was used to detect the PVL gene and genetic relationship between the isolates was determined by using pulse field gel electrophoresis. *Results:* A total of 51 isolates of *S. aureus* were obtained from 40 (31%) of the patients. The majority (43.1%) of the isolates were obtained from the anterior nares. Thirteen (25.5%) of all the isolates were resistant to more than one category of antibiotics, with one isolate identified as MRSA. Thirty-eight (74.5%) isolates (including the MRSA isolate) carried PVL gene where the majority (44.7%) of these isolates were from the anterior nares. A dendogram revealed that the isolates were genetically diverse with 37 distinct pulsotypes clustered in 11 groups. *Conclusion*: *S. aureus* obtained from multiple sites of the HIV patients were genetically diverse without any clonality observed.

Keywords: *Staphylococcus aureus*, MRSA, Panton-Valentine leukocidin, HIV-infected patients

INTRODUCTION

Staphylococcus aureus is one of the important normal flora on human skin. Despite it being a commensal, it can become a pathogen causing a wide spectrum of clinical infections ranging from benign superficial skin infections to invasive infections such as endocarditis, osteomyelitis, and necrotising pneumonitis.[1] Although the anterior nare is the most frequent carriage site for this bacterium,[2] other parts of the body have also been reported to be colonized by *S. aureus.*[3]

Staphylococcus aureus ranks among the most common causes of bacterial infections in HIV-infected patients.[4] Patients with HIV who are being colonised by methicillin-resistant *S. aureus* (MRSA) have been identified to be at increased risk of developing skin and soft tissue infections (SSTI) compared to the general population.[5,6] Skin and soft tissue infections include boils, carbuncles, furuncles and skin abscesses. *S. aureus* carriage is an important predisposing factor for HIV-infected patients to exacerbate the clinical conditions since 80% of clinical infections are due to endogenous strains.[7,8]

S. aureus which causes invasive infections is associated with many virulence factors. Panton-Valentine leukocidin (PVL) is one of the virulence factors that are capable of causing lysis of leukocytes and tissue necrosis.[9,10] PVL gene encodes for *S. aureus* toxin to create pores on host cell membrane. Being immunocompromised, HIV-infected patients pose a high risk of

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contracting *S. aureus* infections, especially strains which possess PVL genes.

The epidemiology of nasal colonisation with *S. aureus* in community-acquired infections has been well studied. However, the molecular characteristics of the *S. aureus* isolated from HIV-infected patients are still lacking. In this study, we determined the frequency of *S. aureus* colonisation on multiple body sites and distribution of PVL genes in HIV-infected patients, and the phylogenetic relationship amongst the isolates.

MATERIALS AND METHODS

Collection of samples and culture

A total of 516 samples were taken from 129 HIV-confirmed patients attending the Infectious Diseases Outpatient Clinic at Sungai Buloh Hospital, Selangor, Malaysia. The sample size was calculated by using the Krejcie and Morgan formula. After obtaining a written consent from the patients, swabs were collected at four body sites i.e., anterior nares, throats, skin and axilla and from any skin lesions which were present in the patient. Subsequently, the swabs were cultured on mannitol salt agar (Oxoid, UK) and incubated at 37°C for 48 hours. Suspected *S. aureus* colonies were streaked on blood agar for purity and incubated for further 24 hours at 37°C. Gram stain, catalase test, rapid *S. aureus*-specific agglutination test (Staphaurex Plus, Remel, UK) and VITEK® 2 (BioMerieux, France) were performed to confirm their identity.

Antimicrobial susceptibility testing

The *S. aureus* colonies were initially suspended in alkaline peptone water to a turbidity of 0.5 McFarland Standards, prior to determination of their susceptibility to antibiotics by disk diffusion method. The antibiotics tested were penicillin (P10), cefoxitin (FOX30), erythromycin (E15), clindamycin (CC2) and cotrimoxazole (SXT). All the antibiotic disks were purchased from Oxoid, UK. The breakpoints interpretation was based on Clinical Laboratory Standards Institute.[11] Those colonies demonstrating resistant pattern, was further subjected for confirmatory of their antibiotic susceptibility using minimal inhibition concentration (MIC) method by VITEK®2 AST–GP71 Test Kit. The MIC of ⩽4 mg/L to cefoxitin was regarded as MRSA.

Detection of PVL genes

All of the *S. aureus* isolated were screened for PVL gene by PCR. Chromosomal DNA of the isolates was extracted from the colonies by using an extraction kit according to the manufacturer's instruction (Qiagen, Germany). The extracted DNA was used as the template in PCR for detection of the PVL gene. The primers' *(lukS-PV* and *lukF-PV*) sequence, constituents of the PCR mix and the amplification conditions were according to a method that was previously described.[9] DNA of a reference strain *S. aureus* (ATCC 49775) was used as positive control and concurrently run in the amplification reaction. An amplicon of 433-bp size following agarose electrophoresis was indicative of the presence of PVL gene in the isolates.

Pulse-field gel electrophoresis (PFGE)

Prior to performing PFGE, *S. aureus* colonies were cultured in brain heart infusion broth and incubated overnight at 37°C. Genomic DNA for PFGE was prepared according to a standard protocol.[12] Briefly, a slice of the DNA-agarose plug was digested with 20 U/ml of *SmaI* for 30 minutes at 25°C and the DNA fragments were separated via electrophoresis performed in 1% agarose (Seakem Gold, Switzerland) on CHEF DR III (Biorad, US) at ramped pulsed times of 5 to 40 sec for 19 hours at 200 V. Interpretation of the DNA banding patterns was according to a published method.[12] An image of the dye-stained gel was captured electronically and dice coefficient of similarity was calculated to compare the macro restriction patterns.[13] Clustering was based on the unweighted pair-group average method (UPGMA) and was performed using BioNumerics 4.0 software.[14]

RESULTS

A total of 516 swabs (i.e., 4 swabs per person) were collected from the 129 patients. Forty patients (31.0%) were positive for *S. aureus* which yielded 51 isolates (Table 1). The largest number of isolates were obtained from the anterior nares (43.1%), followed by the throat (29.5%), skin (21.6%) and axilla (5.9%). The majority (75%) of these patients had *S. aureus* strains isolated from a single site of the body, while 25% of the patient had *S. aureus* on more than two sites on their bodies. Fourteen (35%) patients had *S. aureus* exclusively in their anterior nares, nine (22.5%) patients with *S. aureus* only in their throats, five (12.5%) patients harboured the organism on their skin, and two (5%) had *S. aureus* on their axilla.

Antimicrobial susceptibility test on the isolates showed that majority (84.3%) of the

TABLE 1: Profile of 51 *S. aureus* isolated from 129 HIV-infected patients attending a tertiary hospital

Patient ID / Isolate no.	Site	Resistant to antibiotic	PVL gene	PFGE profile
6	N	PEN	+	III
	T	PEN	-	III
7	N	None	+	V
8	N	PEN	+	VII
9	N	PEN, SXT	+	III
10	N	PEN	-	nd
14	N	PEN	+	V
16	N	PEN, ERY	+	V
	S	PEN, ERY, CC	-	V
	T	PEN, ERY	+	V
17	N	PEN	+	V
18	N	PEN, ERY, SXT	+	I
	S	PEN, ERY, SXT	+	I
20	N	None	-	nd
	S	None	+	nd
22	A	PEN	+	XI
25	T	PEN	+	IX
26	N	PEN	+	V
30	T	PEN	+	nd
32	S	PEN, FOX, ERY, CC, SXT	+	VI
	T	PEN,ERY,SXT	+	III
33	N	PEN	+	V
	T	None	+	III
36	S	PEN	+	IX
42	T	PEN	+	nd
43	S	PEN, ERY	-	nd
47	S	PEN	+	III
48	N	None	-	V
50	A	PEN	+	nd
	S	PEN	+	III
51	N	PEN	-	IX
	S	PEN	+	nd
52	S	PEN	-	III
55	N	PEN, ERY, SXT	-	I
	T	PEN	-	IV
61	S	PEN	+	III
63	T	PEN, SXT	+	V
68	N	None	+	III
77	T	None	+	IX

Patient ID / Isolate no.	Site	Resistant to antibiotic	PVL gene	PFGE profile
78	N	PEN	+	X
79	A	PEN	+	IX
86	T	None	+	VIII
87	N	PEN, SXT	+	VI
91	N	PEN, ERY, SXT	+	I
	T	PEN, ERY, SXT	+	I
94	N	PEN	+	III
99	T	PEN	+	III
105	T	PEN	-	V
115	T	PEN	-	V
129	N	PEN	+	III
130	N	PEN	-	II

Site: N - anterior nares, T - throat, S - skin, A - axilla; Antibiotic: PEN - penicillin, FOX - cefoxitin, ERY - erythromycin, CC - clindamycin, SXT - cotrimoxazole, None - susceptible to all antibiotics; PVL: (+) - present, (-) - absent; PFGE: nd - not done, I-XI - groups

isolates were resistant to penicillin (Table 2). Resistance to erythromycin ranked second (21.6%), followed by cotrimoxazole (17.6%), clindamycin (3.9%) and one isolate (2.0%) was resistant to cefoxitin. The cefoxitin-resistant isolate (strain 32S) represented the only MRSA strain isolated, while the other 50 isolates were methicillin-sensitive *S. aureus* (MSSA). The presence of only one MRSA isolate suggests that only 0.8% of this cohort of patients carried MRSA while 91.2% were MSSA carriers. Eight isolates from seven patients were found to be susceptible to all the tested antibiotics. Another eight isolates were resistant to three or more than three categories of antibiotics. Interestingly, seven isolates (16N, 16S, 16T, 32S, 32T, 55N and 55T) had different antimicrobial susceptibility profiles, even though they originated from the same patient. The summary of the results obtained from this work is shown in Table 2.

Detection of amplicons of 433-bp in size by PCR, showed that PVL genes were present in 38 isolates (including the MRSA isolate), indicative of 74.5% of the isolated *S. aureus* carried PVL genes. These 38 PVL-positive isolates were obtained from 32 patients, signifying 24.8% of the patients carry PVL genes. *S. aureus* isolated from the anterior nares (44.7%), showed the largest number of PVL-positives, followed by the throat (27.5%), skin (23.5%) and axilla (5.9%). It was also noted that although *S. aureus* (6N, 6T, 16N, 16S and 16T) was isolated from multiple sites of two patients, not all of the isolates contained PVL gene. Isolates 6N, 16N and 16T harboured PVL gene whereas 6T and 16S did not. And also, isolate 32S (MRSA) that was obtained from the skin contained PVL gene, but isolate 32T which was also from the same patient did not bear the PVL gene.

TABLE 2: Antibiogram pattern of 51 *Staphylococcus aureus* isolates obtained from HIV-infected patients

Antibiotic		Susceptible	Intermediate	Resistant
Penicillin	P10	8 (15.7%)	0	43 (84.3%)
Cefoxitin	FOX30	50 (98%)	0	1 (2%)
Erythromycin	E15	29 (56.8%)	11 (21.6%)	11 (21.6%)
Clindamycin	CC2	35 (68.6%)	14 (27.5%)	2 (3.9%)
Cotrimoxazole	SXT	41 (80.4%)	0	10 (17.6%)

For the PFGE work, only 43 isolates were examined because eight isolates were lost during subculturing and maintenance. PFGE of the *SmaI* digested chromosomal DNA subtyped the 43 strains into 37 reproducible and distinct PFGE profiles (pulsotypes). The pulsotypes consisted of 14-17 DNA fragments that ranged from 20 to 600 kb in size. A dendogram based on the matrix of F-values was then constructed (Fig. 1). Based on 75% similarity, 11 groups were observed. In general, there was no major group that clustered strains from common sites of colonisation. It was also observed that six isolates (32S, 32T, 33N, 33T, 55N and 55T) obtained from multiple sites of three patients, were separated into different groups. On the other hand, four patients had strains (6T, 6N, 16N, 16S, 16T, 18N, 18S, 91N, 91T) that were genetically similar and grouped together. The single MRSA strain (32S) was significantly different and thus not placed in any groups. No clonality amongst the isolates was observed.

DISCUSSION

The anterior nare has been well-recognised as the predominant site of *S. aureus* colonization.[2,15] The relatively absence of human defense in the vestibule of the nose where the skin is continuous with the nasal mucous membrane, allows the

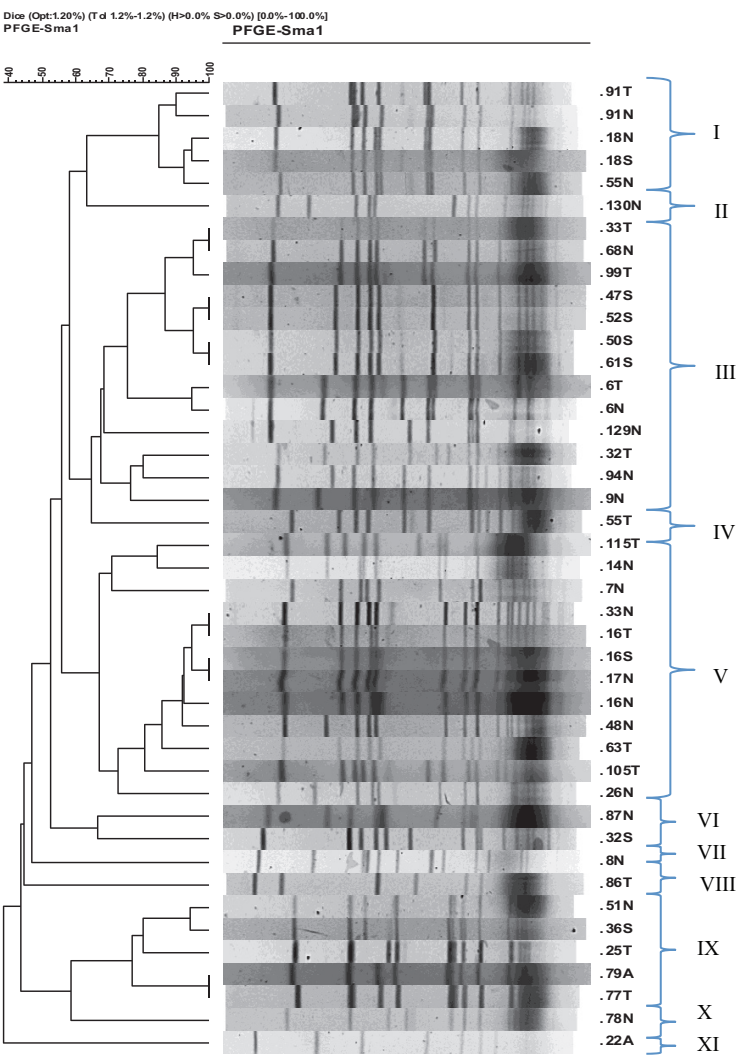

FIG. 1: Phylogenetic relationship of 43 *Staphyloccocus aureus* isolates obtained from multiple sites of HIV-infected patients. Clustering based on unweighted pair-group method (UPGMA) at 75% similarity, 37 pulsotypes obtained were grouped into 11 groups (I-XI)

bacterium to establish successfully in the anterior nares. As in the earlier studies,[2,15] we also found that the anterior nares are the most common site for colonisation of *S. aureus*, compared to other sites of the body. Although culturing of swabs from anterior nares alone has been shown to detect the organism in most *S. aureus* carriers, an additional culture of throat swabs could increase the detection.[16-18] This is confirmed by our isolation of *S. aureus* in the throat of nine patients whose anterior nares failed to detect *S. aureus*. Although our results showed that the anterior nares were the most predominant site for detection of *S. aureus* compared to other sites of the body, surveillance from the anterior nares alone can miss colonising *S. aureus*. Hence, the importance of sampling *S. aureus* from other anatomic sites, especially the throat, would increase the sensitivity of screening of *S. aureus* colonisers.[16,19]

The prevalence of *S. aureus* colonisation varies according to the population studied. In the general population, the *S. aureus* colonisation is usually between 25-50%.[15] In Malaysia, the prevalence of MRSA in patients attending a hospital in Kuala Lumpur was reported to be 44%, with the highest number isolated from soft tissue infections.[20] Similarly in Nigeria, 42% of their patients and healthcare workers were MRSA carriers,[21] but much lower prevalence (4.3%) of MRSA in patients in Indonesian hospitals was reported.[22.] In HIV-infected patients, the incidence of MRSA colonisation is expected to be higher than non-HIV infected patients because of factors such as low CD4 T-cell count, high HIV viral load and frequent exposure to antibiotics.[23] A previous report estimated that the colonisation of *S. aureus* in HIV-outpatient was between the range of 0 to17%.[24] In our study, the rate of MRSA carriers is very low (0.8%), indicating that the frequency of MRSA amongst this cohort of HIV-infected patients is rare. Similarly in other countries, low prevalence of 1% and 3% was also reported in Spain and Singapore, respectively,[25,26] but a much higher prevalence of 12.8% was recorded in HIV-infected patients who attended a Referral Centre in Iran.[27]

In this study, we found a high percentage (24.8%) of the patients carry PVL-positive MSSA. In Nigeria and New Zealand, the carriage rate of PVL gene in MSSA was 40.3% and 37%, respectively.[28,29] Indonesia reported a low carriage rate of 1.5% of PVL genes in MSSA[22]. Unfortunately in our study, we are not able to make any comparison between the rate of MSSA and MRSA since we obtained only one MRSA isolate. While many previous reports have focused on infections caused by PVL-positive MRSA in HIV-infected patients, reports on severe infections caused by PVL-positive MSSA are limited.

It has been shown that HIV-infected patients have a higher risk of developing SSTI caused by *S. aureus* than non-HIV infected patients.[30,31] For the treatment of SSTI in HIV-infected patients, oral administration of cotrimoxazole and clindamycin are usually recommended. Our results show that prevalence of *S. aureus* resistant to clindamycin is relatively low (3.9%) compared to cotrimoxazole (17.6%), thus suggesting that clindamycin is preferable to cotrimoxazole. We also observed that five patients harboured MSSA strains that were not only resistant to cotrimoxazole but also positive for PVL. It is highly possible that these five patients have a high risk of developing SSTI because SSTIs in HIV-infected patients have been associated with co-occurrence of cotrimoxazole resistance and PVL-positive *S. aureus*.[32]

Molecular typing of isolates have paramount importance in showing clonality and genotypic patterns of *S. aureus* infections. Although the turnaround time for PFGE is slower and time-consuming than PCR, it has been accepted as the 'gold standard' for molecular typing of *S. aureus,* particularly for molecular typing of MRSA.[33] In general, the dendogram obtained revealed that the *S. aureus* colonising HIV-infected patients were genetically diverse and did not originate from one common source. This observation supports other reports that *S. aureus,* in particular, was genetically diverse.[33,34] This study also shows that *S. aureus* isolates are non-clonal as they were randomly distributed and there was no clustering of the strains of common sites. A patient may have *S. aureus* isolates obtained from multiple sites but the isolates may be genetically different. Multiple body sites are usually seen in older or in MRSA-infected patients.[35]

In conclusion, sampling from different sites of the body increased the yield of detection of *S. aureus* colonisation. Although the most predominant site of colonisation is the anterior nares; swabbing the anterior nares alone would have missed 31.7% of the carriers. In general, the rate of the PVL carrying gene in *S. aureus* among the HIV patients are within the rate that has been described in other studies. However, due to the limitation of our study, we are not able to compare this rate from non HIV-infected patients.

The relatively high prevalence of PVL-positive MSSA would be a matter of concern. As the *S. aureus* on HIV-infected patients is genetically diverse, the impact of colonisation with mixed strains of *S. aureus* on the development of clinical disease is still unknown, and thus warrants further study.

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