



Identification of Pantone–Valentine leukocidin virulence gene in methicillin–resistant *Staphylococcus aureus* isolated from clinical specimens of burn patients in Zare Hospitals of Sari, Iran

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ABSTRACT

Objective: To identify the pathogenic gene of Pantone-Valentine leukocidin in methicillin-resistant *Staphylococcus aureus* strain isolated from clinical specimens of burn patients in Shaheed Zare Burn Hospital.

Methods: A total of 104 samples of *Staphylococcus* were collected and 78 aureus samples were isolated from Zare Hospital patients from November 2016 to July 2017. All isolates were identified using a standard biochemical and laboratory methodology in accordance with CLSI principles, and disk agar diffusion antibiogram were performed to identify methicillin resistant colonies. Then the presence of the Pantone-Valentine leukocidin gene was tested by PCR.

Results: Of the methicillin-resistant *Staphylococcus aureus* samples, 80% were negative for the Pantone-Valentine leukocidin gene, and only 20% of the samples had Pantone-Valentine leukocidin gene. Male and female patients showed no significant difference in the positivity rate of Pantone-Valentine leukocidin gene (16.12% vs. 33.33%) ($P=0.25$). Besides, there was no significant difference in bacterial resistance or susceptibility to antibiotics between samples with or without the Pantone-Valentine leukocidin gene.

Conclusions: In recent years, increased resistance has been a serious threat. The resistance or susceptibility of *Staphylococcus aureus* strains to different antibiotics is different in different geographical locations.

KEYWORDS: Methicillin; Resistance; Pantone-Valentine leukocidin; PCR; *Staphylococcus aureus*

1. Introduction

Staphylococcus aureus (*S. aureus*) is a human pathogen which is implicated in nosocomial infections. Because of the complexity of various virulence factors, it becomes a serious threat to human. Among infectious organisms in burn patients, *S. aureus*, particularly methicillin-resistant *S. aureus* (MRSA), is a major challenge with the potential to cause significant morbidity and mortality[1,2]. For the first time, MRSA was reported in the 1960s as soon as the recognition of the penicillinase-resistant β -lactam antibiotic methicillin[3]. The burn lesion is commonly exposed to bacterial colonization and infection because of the deficiency of

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How to cite this article: Ranjkesh A, Salari Z, Nejadi M, Behboudi E, Ramezani A, Zandi M, et al. Identification of Pantone-Valentine leukocidin virulence gene in methicillin-resistant *Staphylococcus aureus* isolated from clinical specimens of burn patients in Zare Hospitals of Sari, Iran. J Acute Dis 2020; 9(2): 121-125.

Article history: Received 21 July 2019; Revision 4 March 2020; Accepted 16 March 2020; Available online 11 May 2020

the normal skin physical barrier and the additional decrease in cell-mediated immunity[3]. Also, the ability of *Staphylococci* to survive in polymorphonuclear leukocytes is an extra problem. Furthermore, polymorphonuclear leukocytes bactericidal activity is weak in burned patients, which allows bacteria to survive for a longer time[4].

S. aureus has several virulence factors in both cell-associated and secreted kinds which enhance cellular adhesion, bacterial proliferation, invasion and evasion of immune responses. These virulence factors include Pantan-Valentine leukocidin (PVL), toxic shock syndrome toxin 1 (TSST-1), staphylococcal enterotoxins (SEs) and hemolysins[5]. PVL is a toxin which is commonly involved in the skin and soft tissue infections, necrotizing pneumonia, diffuse cellulitis and osteomyelitis[6]. Some of the toxins like TSST-1 and SEs are superantigens. Interestingly, more than 20 superantigens have been discovered in *S. aureus* strains, and at least 80% of the main strains have at least one. Bacterial toxins SEs are involved in staphylococcal food poisoning, whereas toxic shock syndrome and staphylococcal scalded skin syndrome are mediated by TSST-1 and exfoliative toxins[7].

The aim of the current study was to evaluate the prevalence of genes encoding leukocidins in *S. aureus* strains with resistance and sensitivity to methicillin isolated from burn patients in Shaheed Zare Hospital, Sari, Iran.

2. Patients and methods

The present study was approved by the Ethic Committee Faculty of Medicine, Sari Branch, Islamic Azad University, Sari, Iran with the number 208 305 139 520 25. In addition, the consent form was obtained from all patients.

From November 2016 to July 2017, 1283 isolates were taken from burned patients in Shahid Zare Burn Hospital, Sari, Iran. The blood culture 675 (53.36%) and swab samples from burned lesions of the patients 608 (48.06%) were collected as soon as clinical symptoms of infection appeared and then were transferred to the laboratory (Figure 1). Standard biochemical tests such as catalase, coagulase, mannitol salt agar, disk agar diffusion (DAD), antibiogram, and DNA test were used to detect *S. aureus* strains[8]. Totally 104 *staphylococci* and 78 *aureus* were detected. Then suspensions inoculate in sterile, molar Hinton agar medium. After a few minutes the antibiotic discs were laid with 22 mm distance and 16 mm distance from plat walls. Plates then were incubated in 37 °C for 24 h and then the diameter of the halo of Lacking growth for all antibiotics was measured and methicillin-resistant strains were selected for polymerase chain reaction (PCR) to detect genes of Leukocidin Pantone valentine. DNA was extracted from the bacterial colony by a commercial DNA isolation kit (Pouya gene Azma kit)[9]. And PCR was used to detect the *pvl* gene representing MRSA strains. The primers were as following: F-ATCATTAGGTAATGTCTGCACATGATCC A and R-GCATCAASTGTATTGGATAGCCAAAAGC that can amplify a DNA fragment with size 433 bp[10]. We used *S. aureus* strain ACTC49775 as a positive control. Eppendorf thermocycler

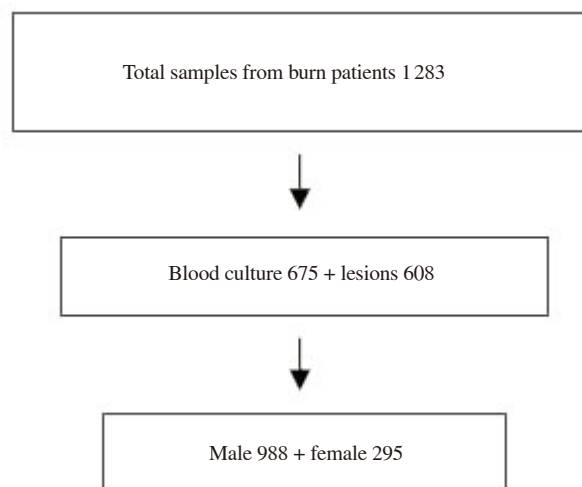


Figure 1. The study flow chart.

was used for DNA amplification (Roche Co., Germany). DNA amplification was performed in a final volume of 25 µL containing 12 µL of MgCl₂, 0.2 mM of dNTP mix, 1 µL of each primer, 1 U of Taq polymerase, 5 µL PCR buffer and 5 µL of template DNA.

The amplification was performed with an initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 61 °C for 30 s, and extension at 72 °C for 60 s with a final extension at 72 °C for 5 min. The PCR products were separated on 1.5% (w/v) agarose gel with 0.5 mg/mL of loading dye and were analyzed by gel electrophoresis.

The data were analyzed using the Statistical Package for the Social Sciences 22 (SPSS Inc., Chicago, IL, USA). Qualitative variables were assayed using the *Chi-square* test. The significance level of the tests was set at $\alpha=0.05$.

3. Results

These samples were isolated from 988 (77.5%) male and 295 (22.5%) female patients. The mean age was (35.17±17.73) years old (range 1 to 62 years), and the mean of total burns was (35.12±21.28)% (range 8%-99%). A total of 503 samples were positive for bacterial infection, including 399 (79.32%) infected with Gram-negative bacteria and 104 (20.68%) with Gram-positive staphylococci. Of the total 503 specimens, 399 specimens belonged to the Gram-negative bacterium, including 20 (3.98%) *Escherichia coli*, 30 (5.96%) *Serratia*, 175 (34.79%) *Acinetobacter*, 40 (7.95%) *Enterobacter*, 30 (5.96%) *Proteus*, 6 (1.19%) *Hafnia*, 5 (0.99%) *Klebsiella* and 48 (9.54%) *Pseudomonas*. A total of 78 (15.51%) of the tested isolates were identified as *S. aureus*. Of these 78 *S. aureus* samples, 40 (51.28%) were resistance and 38 (48.72%) were sensitive to methicillin. Totally 80% of *S. aureus* positive samples were PVL negative and only 20% were PVL positive. Male and female patients showed no significant difference in positive positivity of the *pvl* gene (16.12% vs. 33.33%) ($P=0.25$). Of the 40 resistance samples, all *S. aureus* samples were resistant to methicillin and oxacillin antibiotics, followed by antibiotic vancomycin (97.50%).

Table 1. Sensitivity and resistance to different antibiotics between male and female patients.

Antibiotics	Resistant	Sensitive	P-value
Cefazolin			
Male	74.19%	25.81%	0.024
Female	33.33%	66.67%	
Cephalexin			
Male	74.19%	25.81%	0.024
Female	33.33%	66.67%	
Ampicillin			
Male	96.77%	3.23%	0.008
Female	66.67%	33.33%	
Tetracycline			
Male	80.64%	19.36%	0.560
Female	88.88%	11.12%	
Streptomycin			
Male	80.64%	19.36%	0.560
Female	88.88%	11.12%	
Vancomycin			
Male	0	100.00%	0.060
Female	11.11%	88.89%	
Ciprofloxacin			
Male	80.00%	20.00%	0.300
Female	62.50%	37.50%	
Oxacillin			
Male	100.00%	0	0
Female	100.00%	0	
Methicillin			
Male	100.00%	0	0
Female	100.00%	0	

The resistance rates of ampicillin, tetracycline, and streptomycin were 90.00%, 82.50%, and 82.50% (Figure 2). Male and female patients showed significant differences in resistance rate and susceptibility rate to cefazolin ($P=0.024$), cephalexin ($P=0.024$) and tetracycline ($P=0.008$). A total of 74.19% of the bacterial samples from male patients were resistance to cefazolin and cephalexin antibiotics, 35.0% of female patients were resistance to cefazolin and cephalexin

antibiotics, with significant difference ($P=0.024$). Male and female patients also showed significant differences in resistance rate of ampicillin antibiotics (96.77% vs. 66.6%) ($P=0.008$) (Table 1). Besides, there was no significant difference in bacterial resistance or susceptibility to antibiotics between samples with or without the *pvl* gene (Table 2).

Table 2. Resistance and susceptibility rate of samples with or without the *pvl* gene [n(%)].

Antibiotic	PVL positive	PVL negative	P-value
Cefazolin			
Resistant	4 (10.0)	22 (55.0)	0.32
Sensitive	4 (10.0)	10 (25.0)	
Cephalexin			
Resistant	4 (10.0)	22 (55.0)	0.32
Sensitive	4 (10.0)	10 (25.0)	
Ampicillin			
Resistant	6 (15.0)	30 (75.0)	0.11
Sensitive	2 (5.0)	2 (5.0)	
Tetracycline			
Resistant	7 (17.5)	26 (65.0)	0.67
Sensitive	1 (2.5)	6 (15.0)	
Streptomycin			
Resistant	7 (17.5)	26 (65.0)	0.67
Sensitive	1 (2.5)	6 (15.0)	
Vancomycin			
Resistant	0	1 (2.5)	0.61
Sensitive	8 (20.0)	31 (77.5)	
Ciprofloxacin			
Resistant	6 (15.0)	23 (57.5)	0.92
Sensitive	2 (5.0)	7 (17.5)	
Oxacillin			
Resistant	8 (20.0)	32 (80.0)	0
Sensitive	0	0	
Methicillin			
Resistant	8 (20.0)	32 (80.0)	0
Sensitive	0	0	

PVL: Panton-Valentine leukocidin.

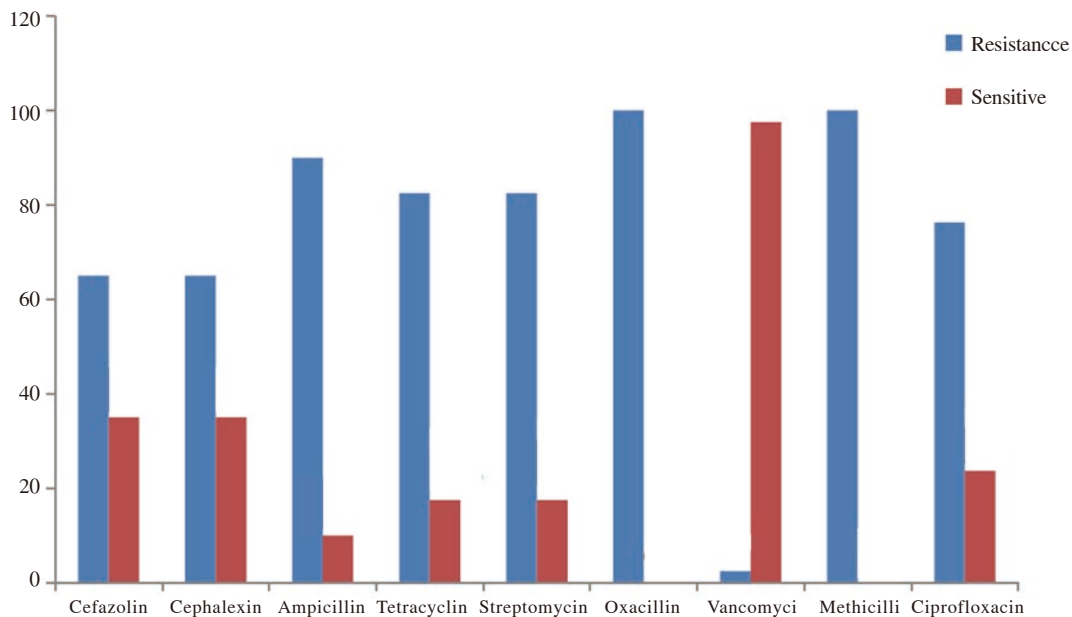


Figure 1. Bacterial sensitivity and resistance to different antibiotics (%).

4. Discussion

Today, antibiotic resistance becomes one of the major problems in medicine, and widespread antibiotic abuse plays a key role in the emergence of resistant bacteria. *S. aureus* as the second nosocomial pathogen is part of the natural flora of the skin and nasal ducts. This bacterium is resistant to a wide range of antibiotics. Recent studies have shown that *S. aureus* strains PVL has a high virulence and is responsible for severe infections such as bone marrow, joint, and necrotizing pneumonia.

In the present study, high rate of resistance against methicillin and oxacillin (100%), ampicillin (90%), tetracycline (65%), streptomycin (82.5%), ciprofloxacin (76.3%), ceftazolin (65%) and cephalexin (65%) were observed in the isolated community *S. aureus* strains. In a study by Karmakar *et al.*, resistance of *S. aureus* isolates to ampicillin (91%), nalidixic acid (91%), chloramphenicol (36%), streptomycin (27%), kanamycin (55%), cefoxitin (68%), novobiocin (64%), and erythromycin (82%) was found[11]. This report confirms that *S. aureus* is resistant to a wide range of antibiotics. In a study, Ruu *et al.* studied 100 samples with *S. aureus* using the real time PCR method and showed that the *pvl* gene was negative in 51 cases (58% PVL negatives methicillin-sensitive *S. aureus* (MSSA) and 42% negative PVL MRSA) and 49 cases positive (46% positive PVLs MSSA and 54% PVL positive MRSA)[12]. These results were not matched to our research findings. In our research 8 cases (20%) carried positive PVL strains (100% MRSA) and 32 cases (80%) were negative PVL strains (100% MRSA). In another study in the UK, the results revealed that less than 2% of *S. aureus* strains carry the *pvl* gene and this gene can be transported both in the MRSA and MSSA strains.

Worldwide emergence of MRSA is a serious concern to public health and a challenge to clinicians. Numerous factors are involved in drug resistance and the virulence of *S. aureus*. In the late 1990s first PVL positive MRSA was identified and these strains have been disseminated worldwide recently[13]. There are several studies to assess the role of PVL in enhancing the virulence of *S. aureus* strains and their pathogenicity. PVL increases the pathogenicity of *S. aureus* via necrosis, facilitates apoptosis and reduces immune system polymorphonuclear and mononuclear cells, and is responsible for mortality and morbidity[14]. PVL is commonly recognized as a marker for colonized MRSA, necessary for deep dermal infections and soft tissues[15,16]. It is considered that the global measure of PVL among MRSA isolates is different. Reports have been demonstrated that a lower prevalence of PVL (5% in France, 4.9% in the UK, 8.1% in Saudi Arabia, and 14.3% in Bangladesh)[14,17-19], which reflect the noteworthy difference in the prevalence of PVL in various geographical areas and populations. Kaur *et al.*[20] from India have revealed 62.85% prevalence of PVL in MRSA and MSSA (MRSA: 85.1% and MSSA: 48.8%), higher prevalence of PVL in MRSA compared to current study findings. Also, Johnsson *et al.*[21] have reported *pvl* gene in only 1 isolate (1%) from 65 cases suffered *S. aureus* bacteremia, in 2 (2.19%) isolates from 91 cases suffered cutaneous infections, and

in 4 (7.27%) isolates from 55 patients suffered respiratory tract infections. Rostamzad *et al.*[22] demonstrated that from 32 MRSA isolates, 13 (40.62%) isolates were positive for *pvl* gene among hospitals isolates. PVL in children (<14 years of age) was more prevalent in comparison to adult patients. Parallel reports were acquired in another study from India[23]. Epidemiological studies offered that high pathogenicity of community-acquired MRSA is correlated with the *pvl* gene but direct evidence of this correlation is still retaining to be discovered[24]. In *S. aureus*, through PVL expression of toxins and other factors can be evaluated in *in vivo*[25]. Furthermore, expression of the *pvl* gene can be facilitated by sub-MIC values of β -lactam antibiotics[26]. The health education is weak in the studied community and they usually use β -lactam antibiotics to treat any infections; probably this is the disturbing factor of high expression of the *pvl* in *in vitro*. Consequently, detecting of *pvl* gene in isolated *S. aureus* colonies indicates the emergence of pathogenic *S. aureus* in population in the studied area.

The present study reveals the increased rate of multi-drug resistant *S. aureus* infections in public. The prevalence of the *pvl* gene among the MRSA isolates in this study was low. The presence of PVL in multi-drug resistant bacteria such as MRSA can be a lethal and challenging condition for clinicians.

Conflict of interest statement

The authors report no conflict of interest.

Funding project

This article was financially supported by Sari Branch, Islamic Azad University with grant number 410.

Authors' contribution

M.O. and S.M.G. design of the work; A.R., A.R., and M.M. data collection, S.A., H.K, and M.P. data analysis, Z.S. interpretation, M.M.E. and A.R. drafting the article, S.M.G. critical revision of the article, M.O. final approval of the version.

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