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Prevalence of Methicillin-Resistant *Staphylococcus aureus* Carrying Panton-Valentine Leukocidin Gene in Cutaneous Infections in the City of Isfahan

Solmaz Ohadian Moghadam [1], Seyed Asghar Havaei [2], Mohammad Reza Pourmand [1*]*

[1] *Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, IR Iran*
[2] *Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, IR Iran*

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ABSTRACT

Background: Methicillin-Resistant *Staphylococcus aureus* (MRSA) is a major cause of Nosocomial and community infections that are becoming increasingly difficult to combat, because of emerging resistance to all classes of antibiotics. Moreover Panton-Valentine leukocidin (PVL) is an important virulence factor in *S. aureus* and causes white blood cell destruction, necrosis and accelerated apoptosis. The aim of this study was to determine the frequency of PVL-positive MRSA in cutaneous infections, for epidemiological purposes and also to determine antibiotic resistance of the isolates.

Methods: Collectively, 56 isolates of *S. aureus* were obtained from Isfahan University of Medical sciences affiliated hospitals and confirmed with biochemical tests (coagulase, mannitol fermentation, and DNase). Then polymerase chain reaction (PCR) was used to detect *pvl* gene. Coagulase gene was used as internal control. The antibiotic susceptibility of all isolates to methicillin was determined using disk diffusion method.

Results: Out of 56 isolates 14.3% were PVL positive that 37.5% were from abscess and 62.5% were from wound. Among all of these isolates 67.8% were MRSA and also 75% of PVL-positive isolates were MRSA.

Conclusion*:* The prevalence of PVL positive MRSA in cutaneous isolates is high. Future works are necessary for a more complete understanding of distribution of these virulent isolates in nasal carriers to decrease the risk of infections.

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∗ *Corresponding Authors:* Seyyed Asghar Havaei, PhD., Department of Microbiology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, IR Iran. *Tel:* +98 913 3133539, *E-mail:* havaei@med.mui.ac.ir and Mohammad Reza Pourmand, PhD., Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, IR Iran. *Tel/Fax:* +98 21 88954910, *E-mail:* pourmand@gmail.com

Introduction

Staphylococcus aureus is a very strong pathogen in developing infections which are acquired from hospitals and communities and cause a broad spectrum of diseases from mild skin infections up to the very severely invasive ones. Pathogenicity depends on its numerous virulence factors (1). One of the most important virulence factors of this bacterium is the leukocidins which are toxins with 2 separate synergic components (2). As Gladstone van Heyningen discussed, it attacks man's and rabbit's mononuclear and polymorphonuclear cells (3). Panton-Valentine leukocidin was, for the first time, identified by Panton and Valentine from supernatant of *S. aureus* V_8 which was isolated from a patient with chronic furunculosis (4). The toxins biological activity was studied by Woodin *et al.* (5) Panton-Valentine leukocidin includes S and F proteins, components both necessary for its function and none is operative by itself (6). These dimeric molecules are connected to each other and assemble in human's polymorphonuclear cells membrane to form an octameric structure and open Ca^{+2} channels (7). This virulence factor induces tissue necrosis, leukocytosis and apoptosis acceleration (8). Another feature of *S. aureus* is its resistance against different groups of antibiotics especially methicillin. It is thus very hard to fight against (9, 10). For years, *S. aureus* strains, resistant to methicillin (MRSA) have developed and produced the dangerous toxin of Panton-Valentine leukocidin which causes severely complicated cutaneous infections and necrotizing pneumonia with a high rate of mortality. Their strains have also the potential of community epidemiological propagation (11). Due to the importance of these strains, numerous studies have been conducted on their prevalence in hospitals and communities of Florida (12), Germany (13), France (14), Latvia (15), Minnesota (16), Austria (17), Iran (18), Switzerland (19) USA (20). Results of a study by Havaei *et al*. showed high prevalence of *pvl* gene among cutaneous isolates.

Regarding the very importance of these strains, we studied the prevalence of these isolates in a hospital using the PCR assay method.

Methods

Bacterial Isolates

The study started in 2008 for a 6 month period. In this research, cutaneous samples (abscess and wound) from patients who were admitted to Alzahra hospital affiliated to the Isfahan University of Medical Sciences were taken to the microbiology lab of Isfahan Medical Faculty to approve the diagnosis of *S. aureus*. A subculture was initially performed on blood agar. Catalase, coagulase, mannitol fermentations and DNase tests were then applied. Totally, 96 samples were tested, among them 56 isolates were confirmed as S. *aureus* (25 abscess and 318 wound).

Genomic DNA Extraction

DNA was isolated using Bioneer kit as recommended by the manufacturer; with the modification that 25 λ/ml lysostaphin was added

to bacterial suspension. Finally the cell lysate was used as the template DNA for PCR.

PCR Assay

All the isolates were tested for the presence of *pvl* genes using the PCR test in which standard strain NCTC 13300 as positive control, distilled water as negative control and *coa* gene as internal control were used. DNA was amplified on an Eppendorf thermocycler with the final volume of 50 µlit containing 5 µlit of 10x buffers, 3 µlit of $MgCl_2$ and, 1.5 µlit of dNTP (10PMol), 20pMol of each primers (lukS-F/pv-1 and lukS-F/pv-2), 32.5 µlit of distilled water was added and 4 µlit of the DNA preparation was taken. Isolates were denatured for 5 minutes at 95°C following with 35 cycles of denaturing was performed for 30 S at 92°C, with annealing at 55°C for 30 S and extension at 72°C for 45 S. Finally, 10 minutes of final extension was performed at 72°C. PCR products were analyzed by electrophoresis through a 1.5% agarose gel.

Primers used for *coa* gene as internal control, with relevant product of 900 bp, were as follow:

CoA 1-5' CGA GAC CAA GAT TCA ATA AC3'

CoA 2-5'AAA GAA AAC CAC TCA CAT CAC A3'

Also primers used for lukS/F-PV, with relevant product of 433 bp, were as follow:

Luk PV-15'ATCATTAGGTAAAATGTCTGCACATGATCCA3'

Luk PV-25'GCATCAASTGTATTGGATAGCCAAAAGC3'

Antimicrobial Susceptibility Testing

Susceptibility to antibiotics was determined by agar disc diffusion method using, Muller Hinton agar medium and clindamycin, rifampicin, tobramycin, ceftriaxone, ciprofloxacin chloramphenicol, oxacillin, gentamicin, tetracyclin, cotrimoxazole, erythromycin and vancomycin MAST disc and all were incubated at 37°C over night except for oxacillin that we were used Mueller Hinton agar medium containing 2% NaCl and the plates were incubated at 35°C over night *(Table 1)*.

Statistical Analysis

Statistical analysis was conducted using SPSS (version 12.0) for the analysis of relation between carrying *pvl* gene and the patient's age and sex. The Chi-square test was done for the determination of statistical significance. Differences at 0.95 confidence level ($p < 0.05$) were considered significant.

Results

In this study 56 isolates of *S. aureus* were collected and analyzed from Alzahra hospital affiliated to the Isfahan University of Medical Sciences.

Results showed that 14.3% of these isolates carry Panton-Valentine Leukocidin (PVL) gene among which 44.64% of the isolates were abscess and 55.3% were wound. Among all of these isolates 67% were MRSA and also 75% of PVL-positive isolates were MRSA.

Data obtained from *Chi-square test* (χ^2test) showed that the presence of leukocidin gene is not significantly different in male and female patients ($p = 0.4$) *(Table 2)*.

Table 1. Frequency of antibiotic resistance

Type of antibiotic Isolates	Number of resistant isolates In MRSAs N 38 (%)	Number of resistant isolates N 56 (%)
Clindamycin	20(35.7)	20(52.6)
Rifampicin	8(14.3)	8(21)
Tobramycin	27(48.2)	27(71)
Ceftriaxone	27(48.2)	27(71)
Ciprofloxacin	25(44.5)	25(65.8)
Chloramphenicol	2(3.6)	2(5.3)
Cotrimoxazole	22(39.3)	22(57.9)
Oxacillin	28(50)	28(73.7)
Gentamicin	25(44.6)	25(65.8)
Tetracycline	32(57.1)	32(84.2)
Erythromycin	35(62.5)	35(92.1)
Vancomycin	0(0)	0(0)

The average age of patients whom the isolates were taken from, was between 39.2 and according to the T-student test there is no significant relation between the age of patients and the presence of Panton-Valentine leukocidin gene ($p = 0.85$) *(Table 3).*

Discussion

In this research, 56 *S. aureus* isolates were collected from a hospital in Isfahan. PCR tests showed that 14.3% of the isolates carry *pvl* genes, the prevalence of which is much higher than that reported from European countries (1). The prevalence of PVL-positive MRSA was 10.7% and among PVL-positive isolates 75% were MRSA (6MRSA out of 8 PVL-positive). Though the prevalence of this gene has been reported to be 35% amongst *S. aureus* isolates (21), these differences in the rate of prevalence are possibly due to different geographical areas and the type of assay used to diagnose the gene.

Other researchers who detected *pvl* in *S. aureus* using Immunodiffusion agar in a hospital in France, reported that PVL-producing *S. aureus* were responsible mostly for necrotizing skin infections such as furuncle and abscess (22-25). It is noted in various reports that a patient with abscess or recurrent furuncle should be primarily suspected of PVL related *S. aureus* infection (26).

This is especially true in high risk groups such as athletics with close encounter. In the present study, *Luks/f-pvl* gene was detected using PCR and analyzed by electrophoresis on 1.5% gel agar.

Also noted in various studies the fact is that *S. aureus* carrying *pvl* genes are the cause of epidemic infections that referred to as "super adapted *S. aureus* isolates" (27). In contrary to some studies which associate *S. aureus* carrying *pvl* genes to MRSA and especially community associated methicillin resistant *S. aureus* (CA-MRSA) (28, 29) in this study the prevalence of such isolates were almost high in MSSA isolates. MRSA carrying these genes were hospital-acquired methicillin resistant *S. aureus* (HA-MRSA). It is worthy to mention that the prevalence of this gene was the

same in male and female and that the average infected age was 37.

Table 2. Frequency of PVL gene according to patient's sex

PVL gene	Male N (%)	Female N (%)	Sum N (%)
Positive	5(13.1)	3(16.8)	8(14.3)
Negative	33(86.8)	15(83.3)	48(85.7)
Sum	38(100)	18(100)	56(100)

$p = 0.4$

Table 3. Comparison of SD and average of patients' age according to presence of PVL gene

PVL gene	Age average	*SD factor	P
Positive	38.9	14.7	0.85
Negative	39.3	14.4	

*SD: Standard Deviation

This result is similar to results from other research (29, 30). These findings are in agreement with results from Wannet *et al.* in Holland (31). Generally, the results of this research show the high prevalence of *S. aureus* carrying *pvl* gene in the hospital under study. These isolates were multi resistant *(Table 1)*. As it is also reported in frequent studies, MRSAs are bacteria resistant to a series of antibiotics in addition to methicillin (32).

In a study conducted by Fey and his colleagues in 2003 (33), it was reported that among the CA-MRSA samples tested, 81% were resistant to penicillin and oxacillin. Resistance to erythromycin, clindamycin and ciprofloxacin were 13%, 6 % and 6 % respectively. None of the isolates were multi-resistant. (As to be resistant to more than 3 non-beta lactam antibiotics). Although in this study, among the HA-MSRA samples 87.5% of the isolates were multi-resistant (33).

In the late 1990, the first isolates positive for PVL-MRSA, was observed (34). And these strains have become globally distributed in the recent year (35).

In one study in Algeria, on the MRSA strains, PVL was the most common toxin producing gene identified. Among these positive PVL-MRSA strains, 97.2% were resistant to kanamycin, 73% to tetracycline, 25% to erythromycin, 11.3% to clindamycin, 7% to gentamicin, 2.3% to chloramphenicol and 2.3 % of the strains were resistant to rifampicin (36).

In our study among the 56 isolates, 67.8% were MRSA, all of which were HA-MRSA. Among these HA-MRSA isolates 52.6%, were resistant to clindamycin. 21% to rifampicin, 71% to tobramycin, 71% to ceftriaxone, 65.8% to ciprofloxacin, 5.3% to chloramphenicol, 73.7% to oxacillin, 57.9% to cotrimoxazole, 65.8% to gentamicin, 84.3% were to tetracycline and 92.1% were resistant to erythromycin. In these isolates, multi resistant isolates were present. And also no vancomycin resistant isolate was found *(Table 1).*

Since PVL virulence factor is carried by a bacteriophage and is also transferable to other *S. aureus* (9), the risk of epidemic infection with such isolates is high in hospitals.

Physicians should thus take suitable strategies to prognose such isolates and assign quick and suitable treatments. It is therefore very important to identify and decolonize the carriers because infections by these isolates are very invasive and even lethal and their epidemics will impose irremediable outcomes.

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