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Prevalence of the Panton-Valentine Leukocidin Gene in Clinical Isolates of *Staphylococcus aureus* Isolated From Hospitals the Ilam Province of Iran

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Abstract

Background: Panton-Valentine leukocidin (*luk-pv*) is a cytotoxin that causes leukocyte destruction and tissue necrosis.

Objectives: The aim of this study was to determine the prevalence of the *pv1*, *femA*, and *mec*A genes in *staphylococcus aureus* isolates from clinical specimens in hospitals in Ilam, Iran.

Materials and Methods: One hundred and sixty *Staphylococcus aureus* isolates were collected from hospitals in Ilam, Iran, and phenotypic and genotypic examinations for methicillin-resistant *S. aureus* (MRSA) isolates were carried out. The antibiotic profiles of these isolates, and the minimal inhibitory concentration (MIC) of MRSA isolates was determined using the agar dilution method with vancomycin, cefoxitin, and oxacillin. All isolates were examined using polymerase chain reaction (PCR) primers for the *fem*A, *mec*A, and Panton Valentine leukocidin (*luk-pv*) genes.

Results: The results showed 91 isolates (56.88%) were coagulase-positive, and 69 isolates (43.12%) were coagulase-negative *Staphylococcus aureus* (CNSA). Out of 91 (56.88%) coagulase-positive staphylococci, 32 isolates (35.16%) were resistant to cefoxitin, and 30 isolates (32.96%) were resistant to oxacillin, using disc diffusion method. PCR revealed the presence of the *fem*A gene (510 bp band) in all coagulase-positive isolates (100%), and the *mec*A gene (513 bp band) was detected in 32 isolates (35.16%); out of 32 MRSA isolates, 13 isolates (40.62%) were positive for presence of the *luk-pv* gene (433 bp band).

Conclusions: The cefoxitin disk diffusion method showed the best results when compared to oxacillin disk, similar to results from detecting the *mec*A gene in PCR as a golden test. Studies on MRSA that carry the *luk-pv* gene should continue to provide significant insight into the prevalence and epidemiology of these important resistant pathogens. Also, the rate of pvl gene-producing isolates showed to be relatively high, and it should be detected in all staphylococcal infections.

Keywords: Methicillin Resistance, *Staphylococcus aureus*, Paton-Valentine, Ilam

1. Background

Staphylococcus aureus, frequently a member of the normal skin flora and the nasal cavity, often causes abscesses; infections of wounds to the skin, and soft tissue; osteomyelitis, endocarditis; pneumonia; and so on. It may also cause staphylococcal scalded skin syndrome, a severe disease in infants, or toxic shock syndrome (1). The virulence of *Staphylococcus aureus* depends on a variety of components, such as capsule and protein A, the clumping factor and coagulase, hemolysins, enterotoxins, toxic-shock syndrome [TSS] toxins, exfoliatins, and Panton-Valentine leukocidin [PVL] (2, 3). Panton Valentine leukocidin (PVL) is a cytotoxin, one of the β-pore forming toxins; its presence is associated with the increased virulence of certain strains of *S. aureus*. It is present in the majority of community-associated methicillin0resistant *S. aureus* (CA-MRSA) and is the cause of necrotic flesh eating lesions, an aggressive condition that often kills patients within 72 hours (4). PVL creates pores in the membranes of infected cells. PVL is produced from the genetic material of a bacteriophage that infects *S. aureus*, making it more virulent (5, 6). Epidemiological and clinical data provide compelling evidence that the high virulence potential of community-acquired MRSA is associated with genes like *lukF-PV* and *lukS-PV* (PVL), but direct evidence that PVL plays a role in pathogenesis has been limited (7). The presence of PVL in *S. aureus* appears to be associated with increased disease severity, ranging from cutaneous infections requiring surgical drainage to severe chronic osteomyelitis and severe necrotizing pneumonia, which could be fatal (8). The production of PVL is related to furuncles, cutaneous abscesses, and severe necrotic skin infections in school children and in certain communities (9).

2. Objectives

This study was conducted to examine Panton-valentine toxin production and methicillin resistance in clinical isolates of *Staphylococcus aureus* in Ilam Hospitals using the disc diffusion method and molecular method by PCR.

3. Materials and Methods

3.1. Samples Collection

A total of 160 non-duplicated *S. aureus* isolates used in this study were randomly collected from in-patient and outpatient populations of two government hospitals from Ilam, Iran between September 2012 and October 2013. Then, all isolates were identified as *S. aureus* using conventional microbiological tests. Pure stock cultures of all isolates were stored frozen at -76°C in Skim Milk broth, containing 10% glycerol (10).

3.2. Laboratory Methods

Specimens were diagnosed by Gram's stain and were cultured on 10% sheep blood agar and MacConkey's agar. Staphylococcal isolates were identified by Gram's staining, catalase production, and haemolysis on blood agar, the oxidative-fermentative test, and the production of bound and free coagulase, mannitol fermentation and 7.5 percent NaCl tolerance, and heat labile DNase. Tube coagulase production is considered the "gold standard" for identifying *S. aureus* (10).

3.3. Antimicrobial Susceptibility Test

A disk diffusion test of Penicillin (10 U), vancomycin (30 μg), Ampicillin (10 μg), Gentamicin (10 μg), Erythromycin (15 μg), Clindamycin (2 μg), Amikacin (30 μg), ciprofloxacin (5 μg), Tetracycline (30 μg), and Co-trimoxazole (25 μg) (Mast, Merseyside, United Kingdom) was carried out using the Kirby-Bauer method according to CLSI Guidelines 2011. Mueller-Hinton agar plates with 2% NaCl were overlaid with the inoculums (turbidity equivalent to that of a 0.5 McFarland Standard) of the *S. aureus* clinical isolates. Zone diameters were measured at 24 (48 hours for vancomycin) following CLSI criteria (11). The minimal inhibitory concentration (MIC) was determined only for MRSA isolates by the agar dilution method. Briefly, gradient plates of Mueller-Hinton agar (Hi-media, India) were prepared with vancomycin, cefoxitin, and oxacillin (0.5 - 256 mg/L, Sigma- Aldrich). The 0.5 McFarland equivalent inoculum was prepared using an 18 - 24 hours-old culture spotted onto gradient plates. Plates were incubated overnight at 35°C for 24 hours before assessing the visible growth, as recommended by the national committee for clinical laboratory standards (11). MSSA (ATCC 6538) and MRSA (ATCC 33591) were included as control strains for disk diffusion (12).

3.4. Detection of Methicillin-Resistant Isolates

Detection and confirmation of methicillin-resistant *S. aureus* isolates was carried out using the disk diffusion method, and the following disks were used: oxacillin (1 μg) and cefoxitin (30 μg). After incubating the plates for 24 hours at 37°C, the antimicrobial activity was evaluated by determining the diameter of the inhibition zone, as recommended by the CLSI (9, 10). Then, results were compared to each other.

3.5. DNA Extraction

Total genomic DNA was extracted using phenol-chloroform isoamyl alcohol (Merck, Darmstadt, Germany). The specimens were pelleted, followed by adding 250 μL buffer I and buffer II containing RNase (Cinna Gen, Tehran, Iran). A 550 μL volume of phenol was added to the aliquots before being centrifuged at 10,000 g for 5 minutes. The supernatant clear phase was then collected into a new eppendorf tube, and the latter stages were repeated twice, in order to wash cell debris. A 0.1 volume of sodium acetate 0.1 M was then added to the tubes and washed twice using ethanol 100 and 80%, respectively. The tubes were then centrifuged at 12,000 g for 15 minutes. The pellet was finally dried and re-suspended in a 30 μL Tris-EDTA buffer (13). DNA extracts were stored at -20°C until required for PCR screening (13).

3.6. Detection of femA, mecA, and luk-pv Genes by PCR

In all coagulase-positive isolates, the resistant genes were detected by PCR. They included genes for *fem*A, *mec*A, and Panton-valentine (*luk-pv*) producing toxin (14). The PCR primers used to detect the resistant genes are listed in Table 1 (1, 13). The PCR mixture was prepared in a final volume of 25 mL. The amplification mixture consisted of a 2.5 mL template DNA, 2 mL primers, 2 mL of a 10-fold concentrate PCR buffer, 2 mL dNTP, 0.5 mM $MgCl_2$, 15 mL D.W. and 1 U of Taq DNA polymerase (CinnaGen, Tehran, Iran). A thermocycler (Master Cycler Gradient; Eppendorf, Hamburg, Germany) was programmed for the detection of *fem*A genes with the following program: initial denaturation at 94°C for 3 minutes was followed by 30 cycles of amplification with 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds, except for the final cycle, which had an extension step of 4 minutes (14). The PCR products were submitted to electrophoresis on 1.5% agarose gel (MBI Fermentas) containing 0.4 mL of ethidium bromide and visualized by using UV trans illumination and photographed (BioDoc- Analyse; Biometra, Goettingen, Germany). The PCR program for the detection of the *mec*A gene was as follows: initial denaturation at 94°C for 3 minutes was followed by 30 cycles of amplification with 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, except for the final cycle, which had an extension

step of 4 minutes (13). The PCR program for the detection of the *luk-pv* gene was as follows: initial denaturation step of 2 minutes at 94°C; 30 cycles of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C; and a final elongation at 72°C for 7 minutes (1).

3.7. Statistical Analysis

All data analyses were performed using SPSS software version 11.5 for Windows. By using the w2-test and Fisher's exact test, P values < 0.05 were considered statistically significant.

4. Results

4.1. Samples Collection

The clinical specimens were collected from blood (31%), wounds (20%), urine (21%), catheters (7%), sputum (12%), other sources (9%).

4.2. Diagnosis of Isolates Using Microbiological Methods

Out of 160 isolates of Staphylococci collected. The results showed 91 isolates (56.88%) were coagulase-positive, and 69 isolates (43.12%) were coagulase-negative for *staphylococcus aureus* (CNSA).

4.3. Antibiogram Profile of Isolates

Table 2 show the results of antibiotic resistance testing of the MRSA and CNSA isolates with the ten antibiotics studied. The patterns of MICs of oxacillin, cefoxitin, and vancomycin on the MRSA isolates were determined with concentrations varying from 4 μg/ml for vancomycin to 64 - 128 μg/mL for oxacillin and 32 μg/mL for cefoxitin.

4.4. Detection of Methicillin-Resistant Isolates

Out of 91 (56.88%) coagulase-positive staphylococci, 32 isolates (35.16%) were resistant to cefoxitin, and 30 isolates (32.96%) were resistant to oxacillin, using disc diffusion method.

4.5. Detection of femA, mecA, and luk-pv Genes by PCR

PCR revealed the presence of the *fem*A gene (510 bp band) in 91 isolates (100%); those were coagulase-positive, and in all isolates, those were resistant to cefoxitin when using the disk diffusion method (Figures 1 - 3). The *mec*A gene (513bp band) was detected in 32 isolates (35.16%); those were resistant to cefoxitin. Out of 32 MRSA isolates, 13 isolates (40.62%) were positive for the presence of the *luk-pv* gene (433 bp band).

Table 1 The Primer Sequencing for the Detection of *fem*A and *luk-pv1* Genes (1)

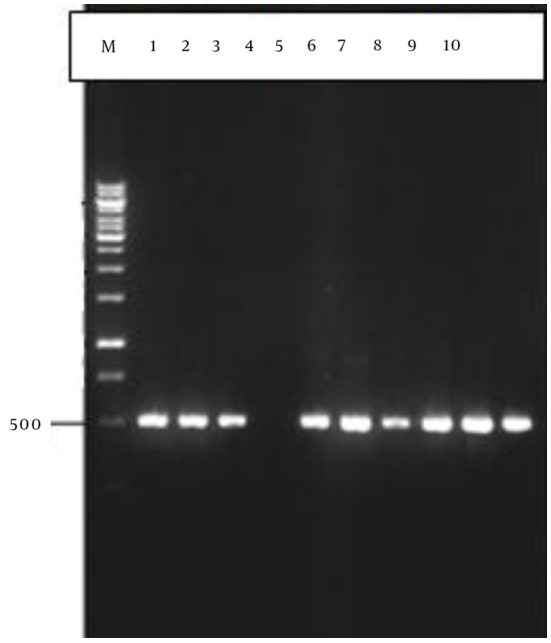
Primer	Primer Sequence	Size of Product, bp
Luk-PV1		433
F	5′- ATCATTAGGTAAAATGTCTGGACATGATCCA-3´	
R	5′- GCATCAAGTGTATTGGATAGCAAAAGC-3′	
***Fem*A**		510
F	5'- AAAAAAGCACATAACAAGCG-3'	
R	5'- GATAAAGAAGAAACGAGCAG-3'	
***mec*A**		513
F	5´- AAAATCGATGGTAAAGGTTGGC-3´	
R	5'- AGTTCTGCAGTACCGGATTTGC-3´	

Table 2. Antibiotic-Resistant Pattern of MRSA Isolates[a]

Antibiotic	Pen	Van	Amp	Gn	E	Da	Ak	Cip	Te	Ts
MRSA	98 (85.96)	0	100 (87.71)	55 (48.25)	62 (54.38)	37 (32.45)	24 (21.05)	48 (42.10)	59 (51.75)	48 (2.10)
CNSA	86 (100)	0	86 (100)	64 (74.41)	68 (79.06)	46 (53.48)	31 (36.04)	40 (46.51)	66 (76.74)	54 (62.79)

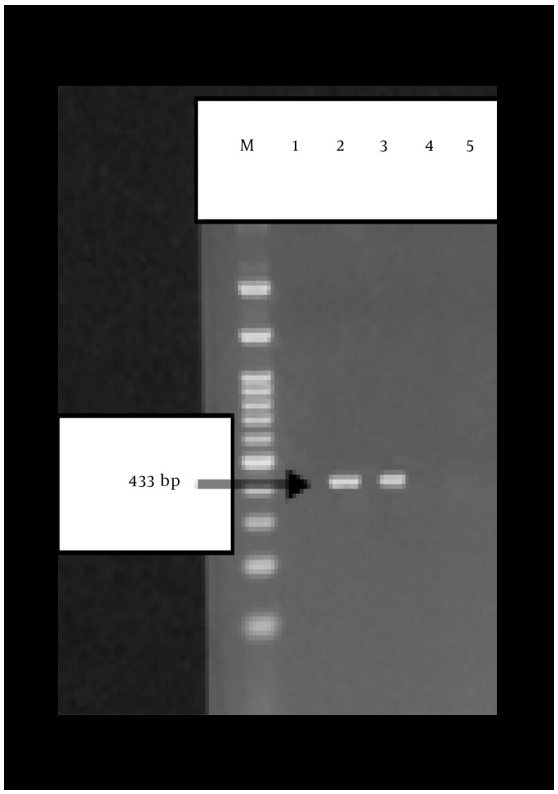
Abberivations: Ak, Amikacin; Amp, Ampicillin; Cip, Ciprofloxacin; Da, Clindamycin; E, Erythromycin; Gn, Gentamicin; Pen, Penicillin; Te, Tetracycline; Ts, Co-trimoxazole; Van, Vancomycin.
[a]Data are presented as No. (%).

Figure 1. Agarose Gel Electrophoresis for the Detection of the *fem*A Gene (510 bp) in all Coagulase-Positive *Staphylococcus aureus* Strains by PCR



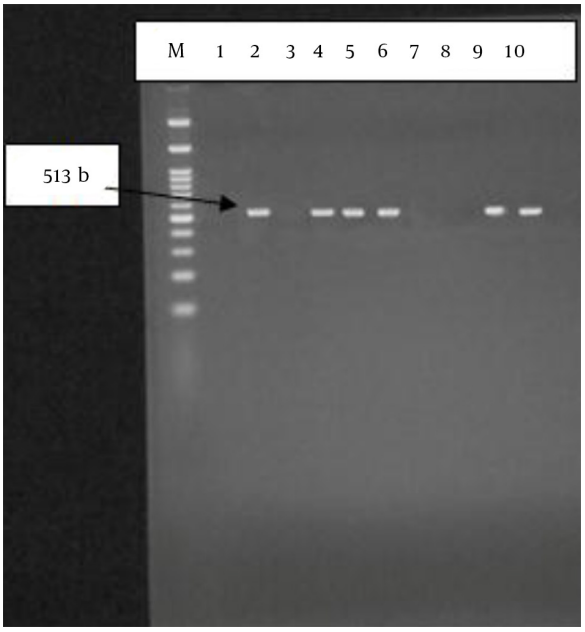
Lanes 2, 3, 5, 6, 7, 8, 9, 10, positive strains; lane 4, negative strain (MSSA *S. aureus* ATCC 6538); lane 1, positive control (MRSA *S. aureus* ATCC 33591); lane M, molecular weight marker (100 bp).

Figure 2. Agarose Gel Electrophoresis for the Detection of the *luk-pv* Gene (433 bp) in *Staphylococcus aureus* Strains by PCR



Lane M, molecular weight marker (100 bp); Lane 1, negative control; lanes 2, 3, positive strains to *luk-pv* genes; lanes 4, 5, negative strains.

Figure 3. Agarose Gel Electrophoresis for the Detection of the *mec*A Gene (413 bp) in *Staphylococcus aureus* Strains by PCR



Lanes 4, 5, 6, 9, 10, positive strains; lanes 3, 7 and 8, negative strains; lane 2, positive control (MRSA *S. aureus* ATCC 33591); lane 1, negative control (MSSA *S. aureus* ATCC 6538); lane 19, water; lane M: molecular weight marker (100 bp).

5. Discussion

Methicillin-resistant *S. aureus* produces a low affinity penicillin-binding protein (PBP2/ or PBP2a), in addition to usual PBP (15). Available data show that the structural genes of this PBP (*mec*A) are present in resistant strains, but not in susceptible ones (15). The *mec*A genes and its associated elements are located in the chromosome (16). There are two resistant mechanisms: oxacillin inactivation, which occurs with the high-level production of β-lactamase, and modified resistance, called MOD-SA, which results from the production of modified PBPs with altered resistance to oxacillin (9). In the present study, 160 *S. aureus* strains were collected from in-patient and outpatients of two government hospitals in Ilam, Iran. They were tested for MRSA and Panton-Valentine leukocidin toxin-producing *S. aureus* species. In the present study, the percentage of MRSA isolated from all patients was only 35.9%. Similar results were reported by Mohajeri et al. (2013) in Kermanshah, in western Iran, with a percentage of MRSA of 36% (17). Our results are in agreement with data collected by Salimnia and Brown (2005) among staphylococcus isolates in outpatient and inpatients at the Detroit medical center (DMC) and from Outreach specimens (18), and Malathi et al. in 2009, who found a percentage of 36.4% MRSA isolated from patients (19). The occurrence of MRSA results in this study was lower than some results in Iran and other countries. In Iran, a 56% prevalence of MRSA infection was reported by Japoni et al. in 2004, reporting from the Nemazi hospital in Shi-

raz (16). The prevalence of a 75% MRSA infection was reported by Izadi et al. in Tehran in 2012 (20). In the United States, a Texas hospital reported a 47% prevalence of MRSA infection in 2003 (21). In this study, the rate of Panton-Valentine leukocidin toxin-producing staphylococci was evaluated at 40.62% among MRSA isolates. Our data is in agreement with a survey conducted by Mariem et al. in two Tunisian university hospitals (22), and in agreement with a study by Al-Talib et al. conducted in Malaysia (23). Furthermore, the prevalence of MRSA-producing *Luk-pv* toxins observed here was lower than that reported in Belgium by Kaur et al. In their survey, the prevalence of MRSA isolates producing *Luk-pv* was reported at 85.1% (1). In Canada, in 2000, Mehrotra et al. reported on the prevalence of MRSA isolates producing *Luk-PV* toxin at a rate of 100% (24).

In our study, the oxacillin disk diffusion and cefoxitin disc diffusion methods were compared with PCR. The cefoxitin disk diffusion method showed 100% sensitivity and was superior to the oxacillin disk diffusion method (93.18% sensitivity) regarding the detection of oxacillin-resistant *S. aureus* using the PCR method for detecting the *mec*A gene. A survey conducted by Velasco et al. (25) showed that the cefoxitin disk diffusion method, recently recommended by the NCCLS and CLSI as a screening test, was the best method, showing 100% sensitivity and 98% specificity. Recent studies evaluating cefoxitin disks for the detection of MRSA also obtained good results, with a sensitivity of about 100% and specificity of 99% (26). Cauwelier et al. 2004, reported resistance to methicillin in 155 clinical MRSA isolates using different methods, including oxacillin and cefoxitin disks, latex agglutination, and an agar screening test. The cefoxitin disk diffusion method presented 100% sensitivity and 99% specificity, whereas the sensitivity of the oxacillin disk diffusion method fell to 97.1% (27). According to our data, compared to the gold standard (*mec*A gene detection), the cefoxitin disk diffusion method is preferable to the oxacillin disk diffusion method for the detection of MRSA. Furthermore, our data showed that the rate of *pvl* production among coagulase-positive *S. aureus* was 40%. In a survey conducted by Lina et al. a total of 172 strains were tested for the *pvl* gene, and they reported that 55% of isolates had this gene (28). Kaur et al. in 2012, reported that, from 70 species, 85.1% of *S. aureus* isolates produced the *pvl* gene (1). Al-Talib et al. in 2009, collected a total of 230 staphilococci species and *pvl* genes in any isolates no detected (23).

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Footnotes

Authors' Contribution: Study concept and design: Arman Rostamzad; drafting of manuscript: Arman Rostamzad; critical revision of the manuscript for important intellectual content: Arman Rostamzad and Nabi Rostamneia.

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