

The Association of *Panton-Valentine leukocidin* and *mecA* Genes in Methicillin-Resistant *Staphylococcus aureus* Isolates From Patients Referred to Educational Hospitals in Ahvaz, Iran

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Received: July 13, 2014; Revised: September 5, 2014; Accepted: September 27, 2014

Background: *Staphylococcus aureus*, an important human pathogen is one of the main causative agents of nosocomial infection. Virulence genes play a major role in the pathogenicity of this agent and its infections. Methicillin-Resistant *Staphylococcus aureus* (MRSA) isolates are major challenge among infectious agents that can cause severe infections and mortality. Methicillin-resistant *S. aureus* produces a unique type of Penicillin Binding Protein 2a (PBP2a) that has low affinity for β -lactam antibiotics. Most of the MRSA bacterial strains can also produce a leukotoxin as Panton-Valentine Leukocidin (PVL) that increases the virulence of MRSA strains and can cause severe necrotic pneumonia. The presence of *pvl* gene is a genetic marker for the MRSA populations.

Objectives: The aim of this study was to explore the association of *pvl* and *mecA* genes in clinical isolates of MRSA.

Materials and Methods: Fifty MRSA isolates were collected from 200 clinical samples from three different educational hospitals in Ahvaz, Iran, and identified by biochemical tests including catalase, oxidase, tube coagulase, mannitol fermentation, and sensitivity to furazolidone, resistance to bacitracin, PYR test and Voges-Proskauer test. Their resistance to methicillin was evaluated using the disc diffusion method. DNA was extracted by boiling and then the presence of *pvl* and *mecA* genes was investigated by the polymerase chain reaction method using specific primers.

Results: The results revealed that *mecA* and *pvl* genes were positive for 15 (30%) and 3 (6%) of the isolates, respectively. None of *mecA* positive isolates was positive for *pvl* gene.

Conclusions: It can be concluded from these results that fortunately the prevalence of *pvl* gene is low in MRSA isolates in this region and there is no association between the presence of *pvl* and *mecA* genes in these isolates.

Keywords: MRSA; Iran; Virulence Factor; Methicillin Resistance; Panton Valentine Leukocidin

1. Background

Among the etiologic agents of infectious disease, *Staphylococcus aureus*, especially Methicillin-Resistant *Staphylococcus aureus* (MRSA) strains are the main agents of human infections (1). This pathogen can cause nosocomial infection in such a manner that it is the most common pathogen isolated from patients in hospitals that can cause wide range of infections from skin and soft tissue infections till toxic shock syndrome and life threatening syndromes. During the end of 1990s, increasing of *S. aureus* infections was led to isolation of MRSA (2-5). Nearly, all *S. aureus* isolates produce various toxins and enzymes that are important in the pathogenicity of this bacterium (6, 7). With the emergence of resistant strains at 1961 that had multidrug resistance, the treatment of *S. aureus* infections had been paid more attention. The MRSA stains are widely distributed around the world that shows their resistance to both penicillins and cephalosporins (8, 9).

The MRSA produces a unique type of Penicillin Binding Protein 2a (PBP2a) that has low affinity for β -lactam antibiotics (10, 11). Most of MRSA strains can also produce a leukotoxin as Panton-Valentine Leukocidin (PVL) that increases their virulence and can cause severe necrotic pneumonia (8, 12). Pathogenicity of *S. aureus* results from the surface cell wall structures and different exoproteins that PVL is one of the most important of them for overcoming host immunity and disease progress (13). Pore forming toxins perforate membranes of host cells, mainly the plasma membrane and also intracellular organelle membranes. They directly kill target cells, in order to intracellular delivery of other bacterial or external factors, releasing nutrients or escaping from phagosomal space. Panton-valentine leukocidin is a member of pore forming toxins that targets host leukocytes. The PVL-producing strains are commonly involved in the skin and soft tissue infections as well as

able to cause life threatening infections like pneumonia (7, 8, 12). Two open reading frames are responsible for coding PVL, i.e. lukS-pv and lukF-pv. The presence of *pvl* gene is a genetic marker for the MRSA populations (14). Recently, reports about infections caused by *pvl*-positive strains have been increased that necessitate investigations about the prevalence of this virulent marker among MRSA strains in hospital and also community acquired infections.

2. Objectives

The aim of the present study was to discover the association of *pvl* and *mecA* in MRSA strains isolated from hospitalized patients in three educational hospitals of Ahvaz City, Iran.

3. Materials and Methods

3.1. Sampling

Fifty *S. aureus* isolates were collected from patients in three educational hospitals of Ahvaz, including Imam Khomeini, Golestan and Taleghni hospitals. These samples were obtained from 200 samples collected from different sections of the above-mentioned hospitals including Outpatient Department (OPD), skin, men surgery, ophthalmology, nephrology, pediatric, women, orthopedic, ear, nose and throat (ENT), neonates and internal medicine. Samples were collected from skin lesions, blood cultures, burns, intravenous catheters, wound drainages, abscesses, tracheal secretions, synovial fluids, ocular secretions and urines. These isolates were identified based on standard morphological and biochemical tests including catalase, oxidase, tube coagulase, mannitol fermentation, sensitivity to furazolidon, resistance to bacitracin, PYR test and Voges-Proskauer (15).

3.2. Determining Antibiotic Susceptibility

In order to determine methicillin resistance of isolates Muller-Hinton Agar (MHA, Merck, Germany) screening test was used according to CLSI (clinical and laboratory standards institute) (16). Briefly, a 0.5 McFarland suspension was prepared from pure culture of isolates and then cultured on MHA containing oxacillin (6 µg/mL). For this purpose using a sterile swab the bacterium was inoculated as a dot with a 10 - 15 mm diameter and a strike culture was prepared on the other part of plate and incubated at 35°C. The results were studied after 24 and 48 hours of incubation. Growth of only one colony on this culture medium revealed methicillin resistance of that isolate. *Staphylococcus aureus* ATCC 29213 as the negative control

and sensitive to methicillin and *S. aureus* ATCC 33591 as the positive control and resistant to methicillin were used in all experiments. Furthermore, the susceptibility of isolates to oxacillin was surveyed using the standard Kirby-Bauer disc diffusion method (17).

3.3. DNA Extraction

The isolates were cultured in Trypticase Soy Broth (TSB, Merck, Germany) for 18 h at 37°C. Then, 1.5 mL of culture was centrifuged (12000 rpm, 10 minutes) and the precipitate was dissolved in one mL deionized water and boiled for 20 minutes. After centrifugation (5000 rpm, 5 minutes) the supernatant was slowly mixed with phenol-chloroform-isoamyl alcohol (1: 24: 25) at 1: 1 ratio and the aqueous phase was harvested by centrifugation at 13000 rpm, 30 seconds. Cold isopropanol was added to aqueous phase (60.100, v/v) and stored overnight at -22°C. The DNA was then precipitated and washed subsequently with absolute and 70% ethanol. Finally, DNA was air-dried at 37°C and dissolved in 200 µL sterile deionized water and stored at -22°C till experiments (18).

3.4. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was performed in a final 25 µL volume reaction containing PCR buffer (10x), Magnesium Chloride (MgCl₂) (2 mM), Deoxynucleotide Triphosphates (dNTPs) (0.2 mM), forward and reverse primers (10 pmol/µL), template DNA (1 µL), Taq DNA polymerase (1.5 u) and deionized water. The PCR method was performed in thermal cycler (Bio-Rad, USA) according to the following program: initial denaturation (94°C, 2 minutes), 35 cycles each composed of initial denaturation (94°C, 30 seconds), primer annealing (55°C, 45 seconds) and extension (72°C, 75 seconds) and a final extension (72°C, 4 minutes) (19). Positive control for *mecA* and *pvl* genes and negative control (distilled water) were also regarded in each series of PCR reaction. The PCR product was subjected to electrophoresis in 1% agarose gel containing DNA safe stain and documented using gel documentation (UVI TEC, Cambridge, UK).

4. Results

From a total of 50 samples, 27 samples (54%) were belonged to Imam Khomeini Hospital, 16 samples (32%) from Golestan Hospital and 7 samples (14%) from Taleghani Hospital. According to the patient gender, 26 samples (52%) were isolated from men while 24 samples (48%) were from women. The number of samples from different clinical specimens and hospital sections are presented in Tables 2 and 3, respectively.

Table 1. Specific Primers for Amplification of *mecA* and *pvl* Genes of *Staphylococcus aureus*

Primer	Sequence	Base
<i>MecA1</i> ^a	5' GTAGAAATACT GAACGT CCGATAA 3'	24
<i>MecA2</i>	5' CCAATCCACATTGTTTCGGTCTAA 3'	25
<i>Luk-PV1</i>	5' ATCATTAGGTAATAATGCTCTGGACATGATCCA 3'	31
<i>Luk-PV2</i>	5' GCATCAAGTGTATTGGATAGCAAA AGC 3'	27

^a Reference (19).

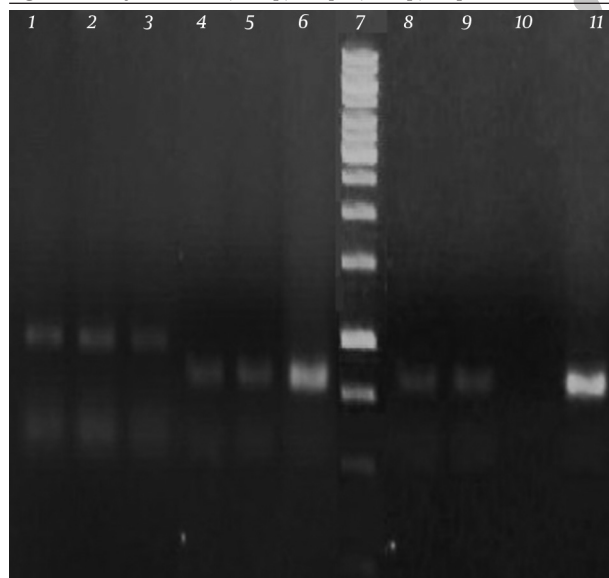
Table 2. The Source of *Staphylococcus aureus* Isolates in Clinical Samples

Clinical Sample	Number of Isolates
Skin lesions	11
Blood cultures	9
Burns	6
Urines	3
Abscesses	3
Tracheal Secretions	3
Synovial Fluids	2
Ocular Secretions	3
Intravenous Catheters	6
Wound Drainages	4

Table 3. Distribution of Samples in Different Hospital Wards ^a

Ward	Number of Isolates
OPD	13
Skin	13
Men surgery	1
Ophthalmology	3
Nephrology	8
Pediatric	3
Women	3
Orthopedic	2
ENT	2
Neonates	1
Internal Medicine	1

^a Abbreviations: OPD, Outpatient Department; ENT, Ear, Nose and Throat.

Figure 1. Analysis of *mecA* (314 bp) and *pvl* (433 bp) Amplification

1: Positive Control for *pvl*; 2 and 3: Positive *pvl* Clinical Isolates; 4, 5, 6, 8 and 9: Positive *mecA* in Clinical Isolates; 7:100 bp Molecular Weight Marker; 10: Negative Control (Distilled Water) and 11: Positive Control for *mecA*.

The results of antibiotic susceptibility tests revealed that all isolates were resistant to methicillin. Following PCR reaction with *mecA* and *pvl* specific primers, 314 bp and 433 bp PCR products were produced for *mecA* and *pvl* genes, respectively (Figure 1).

Table 4. Results of *mecA* and *pvl* Screening in 50 Methicillin-Resistant *Staphylococcus aureus* Isolates

Isolate	<i>mecA</i>	<i>pvl</i>
1	-	-
2	-	-
3	-	-
4	-	-
5	-	-
6	-	-
7	-	-
8	+	-
9	+	-
10	-	-
11	-	-
12	-	+
13	+	-
14	-	-
15	-	-
16	-	-
17	-	-
18	+	-
19	-	-
20	-	-
21	+	-
22	-	-
23	-	-
24	+	-
25	-	-
26	-	+
27	-	-
28	+	-
29	-	-
30	+	-
31	-	-
32	-	-
33	+	-
34	-	-
35	-	-
36	-	-
37	-	-
38	-	-
39	-	-
40	-	-
41	-	-
42	-	+
43	-	-
44	-	-
45	+	-
46	+	-
47	+	-
48	+	-
49	+	-
50	+	-

Table 4 shows the total number of MRSA isolates and their *mecA* and *pvl* markers. Based on the obtained results, 30% (15 samples) of MRSA strains were positive for *mecA* gene while 6% (3 samples) had *pvl* gene. None of the samples had simultaneously both virulence markers.

5. Discussion

Staphylococcus aureus is one of the most common pathogens that have been isolated from patients in hospitals, especially MRSA strains are among important nosocomial infections for humans (1). Having knowledge about the prevalence of MRSA and their virulence factors is useful for treatment and control of community and hospital acquired *S. aureus* infections. Following the emergence of MRSA strains, molecular studies showed that some of these isolates carry *pvl* gene (20). The *pvl* positive strains lead to infections with different clinical appearance even in immunocompromised patients lead to necrotic pneumonia, which its mortality can be as much as 75% (8). Therefore, frequent monitoring of this pathogen, its antibiotic susceptibility and determining their virulence factors is of great importance in control and treatment of infections.

According to the results of this study, only 30% of isolates identified as MRSA were positive for *mecA* gene. This explains that it maybe *mecA* positive MRSA isolates have different mechanism for methicillin resistance than *mecA*. These results are in agreements with other reports such as reported by Bagdonas et al. in Lithuania (23.4%) (21), Turnidge et al. in Australia (24%) (22), Li et al. in China (12%) (23) and also in different parts of Iran, e.g. Fatholahzadeh et al. in Tehran (36%) (24) and by Japoni et al. in Shiraz (43%) (25). However, the frequency of *mecA* positive isolates in this study is more than the results of Bagdonas et al. Turnidge et al. and Li et al. while is less than the results obtained by Fatholahzadeh et al. (2009) and Japoni et al. This finding is a hopeful result that the frequency of *mecA* in Ahvaz is yet less than other studied areas of Iran. The occurrence of *pvl* gene in previous studies has been reported from 2% - 35% (26, 27). In comparison the obtained results for *pvl* gene in the present study is near to the lower limit obtained from similar studies. No association between *mecA* and *pvl* genes was found in our study. It is possible that *mecA* and *pvl* genes but not both of them be present on the chromosome of clinical isolates of *S. aureus*.

The obtained results in the present study are in agreement with the finding of Okon et al. (27) and Terry Alli et al. (9) that reported no *pvl* positive strain is there among tested MRSA strains. Okon et al. (27) have suggested it is maybe that resistance determinants be carried on other mobile elements, such as plasmids, transposons, and phages; so, their elimination from bacterial cell would result in the absence of *mecA* gene and consequently no association with *pvl* gene. Affolabi et al. (28) also reported no significant association between *pvl* and *mecA* in *S. aureus* isolates from Cotonou. However, the *pvl* gene has been reported in some MRSA in other studies (29, 30).

According to the results of this study, PCR assay for MRSA gene can be useful for definite diagnosis of MRSA strains. Identification of *mecA* positive strains can be used as a guide for separating infected patients from

others in hospital environment in order to prevent gene transfer among clinical strains and also distribution of virulent factors.

Acknowledgements

The authors wish to thank the research vice chancellor of Shahid Chamran University in Ahvaz City, Iran, for providing research grant (Grant No. 874095).

Authors' Contributions

Hossein Motamedi: Study design, laboratory experiment, and manuscript preparation. Seyyed Soheil Rahmat Abadi: Laboratory experiments. Seyyed Mojtaba Moosavian: Sample providing. Maryam Torabi: Sample providing.

Funding/Support

This study was funded and supported by Shahid Chamran University of Ahvaz, Iran.

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