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Frequency of *pvl* gene in methicillin resistant *Staphylococcus aureus* Isolates collected from Northwest Iran

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ABSTRACT

Staphylococcus aureus infections, particularly infections caused by methicillin-resistant *S. aureus* (MRSA) strains, are emerging as a major public health problem. The aim of the present study was to determine the prevalence of methicillin-resistant *staphylococcus aureus* (MRSA) by phenotypic and genotypic methods in clinical specimens and detection of the Panton – Valentine leukocidin (PVL) gene in the MRSA strains. In an 11-month study, 710 clinical specimens were collected from patients attending to several teaching hospitals of Urmia city, Northwest Iran. The isolates were examined by conventional culture method for detecting *S. aureus* strains and further confirmation with standard biochemical tests, including catalase, coagulase and DNase. MRSA isolates phenotypically were screened by disk diffusion method. Then DNA was extracted from our MRSA isolates and *mecA* gene amplified by PCR. Finally, pvl genes were identified among MRSA isolates which were positive for *mecA* gene. Among test isolates, 114 isolates (16%) were confirmed as *S. aureus*, from which 48 (42.1%) were recorded as MRSA. *pvl* gene was detected in 13 (27%) MRSA isolates. Our study showed that the prevalence of PVL-positive MRSA isolates, justify further detailed inspection to prevent possible future endemics in the studied hospitals and likewise other hospitals in the region.

KEY WORDS: MRSA, STAPHYLOCOCCUS AUREUS, *PVL GENE*, MEC A GENE

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INTRODUCTION

Staphylococcus aureus is a Gram-positive opportunistic bacterium of great clinical significance, expressing diverse virulence factors that facilitate its adherence, colonization, intercellular interaction, immune system evasion, and tissue damage. Moreover, this microorganism has developed resistance to β-lactam antibiotics by β-lactamase expression or the presence of penicillin binding protein 2a (PBP2a) directly related with methicillin-resistant *S. aureus* (MRSA) strains.The *mec* A gene codifies for PBP2a and is a structural part of a mobile genetic element named Staphylococcal cassette chromosome *mec* (SCC*mec*), which can insert itself into a specific region of its central genome. The different types of SCC*mec* are classified according to the combination of the *mec* complex (*mecA* gene and its regulators) and the cassette chromosomal recombinase (*ccr*), which codifies for enzymes responsible for SCC*mec* mobility. *Mec* A gene is not present in methicillin-sensitive *S. aureus* (MSSA) strains and the presence of this gene is regarded as a criterion of resistance (Merlino, Watson et al. 2002 Llarrull, Fisher et al. 2009 Chambers and DeLeo 2009 Jensen and Lyon 2009, Borbón-Esquer, Villaseñor-Sierra et al. 2014).

In 1961 the first MRSA strain was identified in the United Kingdom. Especially in the past two decades the prevalence of these strains have increased in the world (El-Din, El-Shafey et al. 2003). These strains are usually associated with hospital-acquired infections (HA-MRSA) that shows resistance to many antibiotics, including β-lactams, semi-synthetic penicillins, cephalosporins and carbapenems. The prevalence of HA-MRSA is variable in different parts of the world, ranging from 4.1% in Panama up to 59% in Korea (Klevens, Edwards et al. 2006, Klein, Smith et al. 2007, Alvarez, Ramirez et al. 2008).

Genotyping of MRSA strains has been employed in epidemiological studies to determine its prevalence, dissemination, risk factors, and association with antimicrobial resistance (Kondo, Ito et al. 2007). Production of the Panton – Valentine leukocidin (PVL) in MRSA strains is considered to be associated with disease severity (Lina, Piémont et al. 1999). PVL is a Staphylococcal leukocidin which only attacks macrophages and polymorphonuclears, and has two components “S (33kDa)” and “F (34kDa)” which is controlled by *luk S-PV* and *luk F-PV* genes, respectively (Narita, Kaneko et al. 2001). With respect to the presence of certain chromosomal cassettes, the majority of the published studies have shown an association of SCC*mec* type II strains with hospital infections, whereas type IVa strains, with or without the presence of the *pvl* gene, have been associated with community-acquired infections (Borbón-Esquer, Villaseñor-Sierra et al. 2014).The aim of the present study was to determine the prevalence of *pvl* gene in MRSA isolates by genotypic methods in clinical specimens.

MATERIAL AND METHODS

BACTERIAL ISOLATES

A total of 710 different clinical specimens, including urine, wound, blood, broncho-alveolar lavage, skin and soft tissue, cerebrospinal fluid and body fluids were studied for isolation of *S. aureus* during January-December 2015 from patients admitted to several hospitals in Urmia city, West Azerbaijan, Iran. Initially, all isolates were identified using standard microbiological and laboratory methods, including growth on blood agar and type of hemolysis, Gram stain, catalase test, growth on mannitol salt agar, slide and tube coagulase tests, and DNase test (Forbes et al. 2007). Later, all *S. aureus* isolates were stored in nutrient broth supplemented with 15% glycerol at -20°C until use.

PHENOTYPIC DETECTION OF MRSA ISOLATES

Resistance to methicillin was determined by Kirby – Bauer disk diffusion method on Muller Hinton agar (MHA) using cefoxitin disk (Hi Media, India) as described in the guidelines of Clinical and Laboratory Standards Institute (CLSI) documents . As recommended in the CLSI guidelines, direct colony suspension method was used for testing of *S. aureus* isolates for potential methicillin and or oxacillin resistance. The plates were incubated at 35°C for 18–24 hours aerobically, and growth inhibition zones around the disk was measured. Inhibition zone diameters ≤21 mm considered as resistant. Any visible growth within the zone of inhibition was also considered as methicillin resistant.

DNA EXTRACTION

DNA extraction was performed according to Sadeghi *et al.* (Sadeghi and Mansouri 2014). Briefly, for making a starter culture, a single colony of *S. aureus* was inoculated on nutrient agar. Three or four colonies of overnight growing bacteria from the starter culture were suspended in 450 µl of TE (Tris-EDTA) buffer (10 mM Tris, 1 mM EDTA, pH 8). Cell lysis was obtained by treatment with 5 µl of proteinase K (20 mg/mL) for 20 min at 50 °C followed by addition of 60 µl of 10% SDS for 10 min at 68 °C. In the next step, 80 µl of cetyltrimethylammonium bromide (CTAB)/NaCl and 100 µl of 5 M NaCl were added and incubated at 65 °C for 10 min. Then, chloroform/isoamyl alchohol (700 µl) was added and centrifuged at 11000 × g for 8 min. Supernatant was

transferred to another tube and the DNA was precipitated with isopropanol, washed with 70% ethanol, dried, and dissolved in 100 µl of deionized water.

MOLECULAR DETECTION OF MEC A GENE

All *S. aureus* isolates were evaluated by PCR amplification for detection of *mecA* gene by using *mecA P4 (5'-TCCAGATTACAACTTCACCAGG-3') and mecA P7 (5'- CCACTTCATATC TTGTAACG-3')* primers (McClure, Conly et al. 2006). PCR performed in a 25µl volume containing 10 pmol of each primers, 200 µM dNTP (Roche, Germany), 2.5 µl (50 mM MgCl2), 0.5 µl Taq polymerase (2.5 u) (Roche, Germany), 5 µl PCR buffer 10x (Roche,Germany) and 5 µl DNA-template. *S.aureus* ATCC 33591 and ATCC 25923 were used as positive and negative controls, respectively. The following PCR condition was used: 94°C (4 min),30 cycles with 94°C (45 s) ,56°C (45 s), 72°C (1 min) and finally 72°C (7 min) ; 4°C hold.

MOLECULAR DETECTION OF PVL GENE

PCR for detection of *pvl* gene was carried out by using primers as below:

pvl-1:5'ATCATTAGGTAAAATGTCTGGACATGATCCA–3' and *pvl*-2: 5' GCATCAAGTGTATTGGATAGCAAAAGC –3' (McClure, Conly et al. 2006). PCR performed in a 50 µl containing 20 pmol of each primer, 5 µl of 10x buffers, 1.5 µl of dNTP (10pmol), 3 µl of $MgCl_2$ and 32.5 µl of distilled water and 4 µl of the template DNA. DNA denatured for 5 minutes at 95 °C following with 35 cycles of denaturing performed for 30 S at 92°C, with annealing at 55 °C for 30 S, extension at 72 °C for 45 S and finally, 10 minutes of the final extension performed at 72 °C.

AGAROSE GEL ELECTROPHORESIS FOR DETECTION OF PCR PRODUCTS

PCR products were visualized following electrophoresis in 1.7% agarose gels run at 70 V with ethidium bromide staining (Sigma, USA). *pvl* gene and *mecA* gene positive isolates yielded an amplification product of shining band in 433 and 162 base pair, respectively, with the standard positive control under UV trans-illuminator (UVP, USA) (SANTOS, TEIXEIRA et al. 1999).

STATICAL ANALYSIS

Data were analyzed using SPSS statistical software (version 15, SPSS, Chicago, USA), Chi-square exact test was used to test for significant association between categorical variables. *P-value* less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Distribution of methicillin resistant S. aureus:

Out of 710 clinical specimens, 114 isolates (16%) were confirmed as *S. aureus,* of which 48 (42.1%) were recorded as methicillin resistant, mostly isolated from urine, wound discharge and blood (Table1).

Table 1: Distribution of methicillin-resistant *Staphylococcus aureus* (MRSA) isolates according to clinical specimens.

Specimens	No. of *S. aureus* isolates (%)	MRSA (%)
Urine	33(28.94)	16(14.03)
Wound	29(25.43)	15(13.15)
Blood	14(12.28)	8(7.01)
Broncho-alveolar lavage	11(9.64)	3(2.63)
Skin and soft tissue	8(7.01)	1(0.87)
Cerebrospinal fluid	7(6.14)	3(2.63)
Body fluids	5(4.38)	1(0.87)
Other specimens	7(6.14)	1(0.87)
Total	114(100)	48(42.1)

Phenotypic detection of methicillin resistant S. aureus and molecular detection of mecA gene:

The presence of *mecA* gene using PCR considered as the gold standard method for calculating the specificity and sensitivity of the other tests in this study. Results from conventional disk diffusion susceptibility tests correlated very well with those from the PCR assay. The cefoxitin disk detected MRSA isolates correctly in all cases compared to the presence of *mecA* gene by PCR (42.1%) (Figure 1). Entirely based on cefoxitin disc results there was no substantial differences between conventional susceptibility testing and PCR for calculating methicillin resistant *S. aureus* (p>0.05). The overall results obtained with different techniques are shown in Table 2.

Detection of MRSA isolates carrying pvl gene:

Among MRSA isolates, 13 (27%) contained *pvl* genes and the remaining isolates were recorded as *pvl* negative (Figure 2).

S. aureus is one of the most common infectious agents which has become a frequent cause of nosocomial infection. This bacterium is simply gained and comprises potential to become resistant to many common in-use antibiotics and the prevalence of resistant strains posing serious therapeutic and infection control problems within the hospital environment (Khosravi, Hoveizavi et al. 2012). The infections caused by MRSA are important and can even cause mortality, so increasing antibiotic

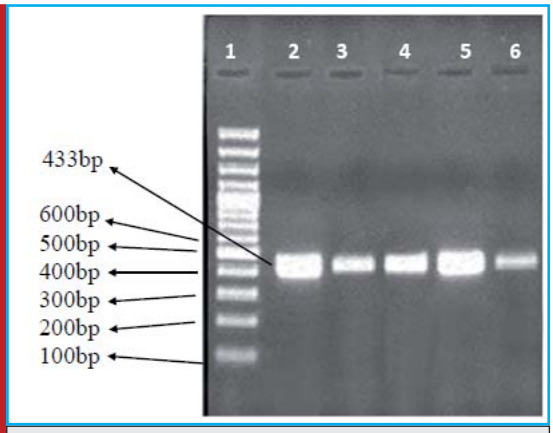

FIGURE 1. **PCR products for the *pvl* gene.** Lane 1: 100 bp molecular weight marker. Lane 2: *Staphylococcus aureus pvl* gene-positive control. lanes 3-6: *pvl positive Staphylococcus aureus* isolates (433bp).

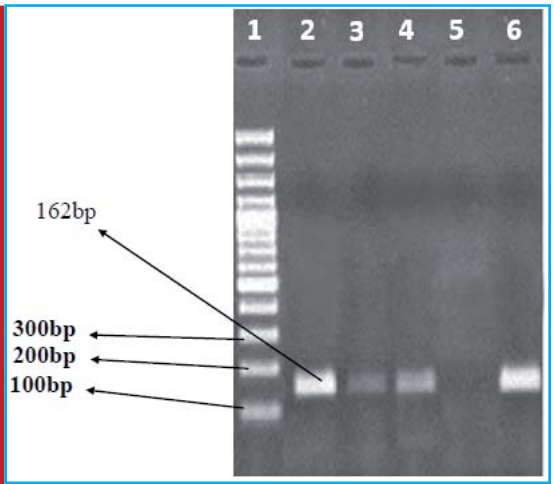

FIGURE 2. **PCR products for the *mecA* gene.** Lane 1: 100 bp molecular weight marker. Lane2: Positive control (MRSA). Lanes 3, 4 and 6: *mecA* positive *Staphylococcus aureus* isolates (162bp). Lane 5: Negative control.

resistance is a concern and should be monitored (Merlino, Watson et al. 2002).

Approximately 89.4 million persons (32%) and 2.3 million persons (0.8%) of the US population are colonized with *S.aureus* and MRSA, respectively (Kuehnert, Hill et al. 2005). The rate of MRSA in all community-associated *S.aureus* infections ranges from 2.5% to 39% in Asian countries (Vandenesch, Naimi et al. 2003). In our study, 42.1% of isolates were recorded as MRSA, while other studies conducted in Iran have shown MRSA prevalence is 55% in Tehran (Saderi, Habibi et al. 2008), 37% in Tabriz (NIKBAKHT, Nahaei et al. 2007) and 50% in Hamedan (Zamani, Sadeghian et al. 2007).

Such strains can be spread by close contact with an infected person, touching contaminated surfaces and stuffs, unhealthy and crowded living conditions and poor personal hygiene. In addition, MRSA is difficult to treat due to multi-drug resistance and cause confusion in the usual sensitivity tests to detect the resistance due to non-uniform expression of them. Therefore, the detection of *mec*A and *pvl* genes represents a quick and more specific method for early identification of CA-MRSA isolates (McClure, Conly et al. 2006). Also, a combination of *mec*A and *pvl* genes is capable to produce super adaptable *S. aureus* strains (22-25). Most of CA-MRSA strains have the virulence factor, PVL, which is not often found in HA-MRSA or MSSA strains. But a low occurrence of MSSA was reported, and has been led to necrotizing pneumonia and death (Vandenesch, Naimi et al. 2003).

In recent years, an impressive worldwide spread of PVL-positive CA-MRSA clones have been observed (Francis, Doherty et al. 2005). Out of the MRSA isolates in our study, 27% carried PVL-encoding genes. Previous studies in Iran have reported the prevalence to be 19% in the capital of Iran; Tehran (Lari, Pourmand et al. 2011), 7.23% in the Southwest; Ahvaz (Khosravi, Hoveizavi et al. 2012), and 5.47% in the South; Shiraz (Alfatemi, Motamedifar et al. 2014). In contrast to the present study, some previous reports have described an extremely high prevalence of *pvl* genes in MRSA. In Western Nepal, Tunisian and Texas, the prevalence of PVL-positive MRSA isolates was 56.8%, 79%, 94.9% respectively (Bocchini, Hulten et al. 2006, Mariem, Ito et al. 2013, Bhatta, Cavaco et al. 2016). Some investigations revealed a low prevalence of PVL genes in MRSA. In Turkey, UK and Austria PVL-positive MRSA occurrence were detected 1.3%, 1.6%, 3.7%, respectively (Holmes, Ganner et al. 2005, Krziwanek, Luger et al. 2007, Kilic, Guclu et al. 2008). These findings may reflect the difference in the prevalence of this gene in different geographic regions and also kind of assay used for detecting the genes.

Since there is a strong evidence of involvement of *pvl* gene in pathogenesis of *S. aureus* strains, so diagnosis and treatment of infections caused by *S. aureus* strains harboring these genes is very important. Detec-

TABLE 2: Sensitivity and specificity of diagnostic methods in identification of methicillin-resistant *S. aureus*.

Test	No. of MRSA identified (%)	Sensitivity	Specificity
Cefoxitin disk	48(40.67%)	100%	100
mecA	48(40.67%)	100%	100

tion of PVL is commonly carried out by using molecular techniques (Khosravi, Hoveizavi et al. 2012). According to the results of this study, the phenotypic methods with cefoxitin susceptibility testing and PCR assay for MRSA gene can be useful for definite diagnosis of MRSA strains. Our study was limited because *SCCmec* typing was not performed.

CONCLUSION

In conclusion, the prevalence of PVL-positive MRSA isolates, found to be 27% in this study, justify further detailed inspection to prevent possible future endemics in the studied hospitals and likewise other hospitals in the region. Moreover, if these strains spread to parts of hospital, including pediatrics, intensive care and cardiac intensive care unit could be life threating. Therefore, identification of these strains and treatment of relative infections is important in prevention of colonization and spread.

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