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RESEARCH ARTICLE

Antimicrobial susceptibility, virulence factors and biofilm formation among *Staphylococcus aureus* isolates from hospital infections in Kerman, Iran

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ABSTRACT

Objective: The aims of present study were to determine the antimicrobial susceptibility, virulence factors and biofilm formation among MRSA hospital isolates of *Staphylococcus aureus*.

Methods: Thirty non-repetitive strains of *S. aureus* isolated from three hospitals in Kerman, Iran. Antimicrobial susceptibility was determined by disk diffusion breakpoints method according to CLSI guideline. The minimum inhibitory concentration (MIC) of vancomycin and methicillin were measured by the broth microdilution and E-test procedures. Virulence factors (protease, DNase, lecithinase, capsule and hemolysis) associated with the above isolates was studied. Biofilm was quantified by microtiter technique.

Results: In total, 14 (46.7%) *S. aureus* were isolated from lower respiratory tract, six (20.0%) from urinary tract and remaining 10 (33.3%) were recovered from wounds, blood and orthopedic patients. All of the isolates were susceptible to tigecycline, eight (26.7%) were found to be resistant to methicillin (MRSA) and 4 (13.3%) showed reduced susceptibility to vancomycin. No any vancomycin resistant isolate was detected ($p \leq 0.05$). MIC results showed that four of the isolates (13.3%) exhibited MIC 4 µg/mL to vancomycin while, five (16.6%) demonstrated MIC 32 µg/mL to methicillin. The isolates were also resistant to amoxicillin/clavulanic acid, tetracycline and tobramycin. It was found that, six (75 %) of MRSA strains produced lecithinase, seven (96.7%) demonstrated protease and DNase activities as compared to MSSA isolates. Biofilm analysis revealed that twenty (66.7%) isolates formed strong, seven (23.3%) formed moderate and three (10.0%) had weak biofilm.

Conclusion: From the results, it can be concluded with emergence of few isolates of vancomycin intermediate, treatment options available for these infections are limited; therefore, monitoring, and management of infections due to MRSA with reduced susceptibility to vancomycin, must be done in order to control spread of these strains in the hospital environment.

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Key words: *Staphylococcus aureus*, methicillin resistance, MIC, virulence factor, biofilm

İran Kerman'da hastane kökenli *Staphylococcus aureus* suşlarında antimikrobiyal duyarlılık, virulans faktörleri ve biyofilm yapımı

ÖZET

Amaç: Bu çalışma hastane kaynaklı *Staphylococcus aureus* suşlarının antimicrobial duyarlılık, virülans faktör ve biyofilm oluşturma özelliklerini belirlemeyi hedeflemektedir.

Metotlar: İran, Kerman'da 30 adet, tekrarlamayan *S. aureus* suşu hastanelerden izole edildi. Antimicrobial duyarlılıklar disk diffüzyon metoduyla CLSI rehberinin sınırdeğerlerine göre çalışıldı. Vankomisin ve metisilin için minimum inhibitor konsantrasyonu (MİK) sıvı mikrodilüsyon ve E-test prosedürleri ile belirlendi. Bu suşlarla birlikte olan virülans faktörleri (proteaz, DNaz, lesitinaz, kapsül ve hemoliz) çalışıldı. Biyofilm değerlendirmesi mikrotitre tekniği ile miktarı belirleme şeklinde yapıldı.

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Bulgular: Toplam olarak 14 (% 46, 7) *S. aureus* suşu alt solunum yollarından, altı suş (% 20,0) üriner sistemden ve kalan 10 suş (% 33,3) yaralar, kan ve ortopedi hastalarından izole edildi. Bütün suşlar tigesikline duyarlıydı, sekiz suş (% 26,7) metisiline dirençli (MRSA) bulundu ve dört suş (% 13,3) vankomisine azalmış duyarlılık gösterdi. Suşların hiç biri vankomisine dirençli değildi ($p \leq 0.05$). MİK sonuçları; vankomisin için suşlardan dördünde (% 13,3) MİK 4 µg/mL olarak bulunurken beşinde (% 16,6) metisilin için MİK 32 µg/mL olarak bulundu. Suşlar aynı zamanda amoksisilin/klavulanik asit, tekrasiklin ve tobramisine dirençli idiler. MSSA suşları ile karşılaştırıldığında MRSA suşlarından 6'si (% 75,0) lesitinaz, yedisi (% 96,7) proteaz ve DNaz aktivitesi gösterdi. Yirmi suş (% 66,7) kuvvetli, yedi suş (% 23,3) orta ve üç suş (% 10,0) zayıf biyofilm yapmaktaydı.

Sonuç: Bu sonuçlardan bu enfeksiyonlarda tedavi seçeneklerinin sınırlı olduğu anlaşılmaktadır. Bundan dolayı vankomisine duyarlılığı azalmış MRSA suşlarının etken olduğu enfeksiyonların izlenmesi ve yönetimi bu suşların hastane ortamına yayılmasının kontrolüyle yapılmalıdır.

Anahtar Kelimeler: *Staphylococcus aureus*, metisilin direnci, MİK, virülans faktörleri, biyofilm

INTRODUCTION

Methicillin resistant *S. aureus* (MRSA) has been the most commonly recognized hospital pathogen around the world and it is often isolated widely from ICU, surgery, internal, orthopedic and burn units of the hospitals. Similarly, MRSA accounts for 10% to 40% cases of health care associated - pneumonia (HCAP), hospital- acquired pneumonia (HAP) and ventilator- associated pneumonia (VAP) in tertiary care hospitals.[1,2] It has been illustrated that resistant to methicillin among the *S. aureus* isolates was 39.5%, while, resistance to other antibiotics tested were found to be 26.3%.[3] Furthermore, some medical centers reported more than half of *S. aureus* isolates were MRSA.[4,5] It is estimated that MRSA infections caused 19,000 deaths per year in the United States hospitals alone, with an associated 3-4 billion US dollars per annual healthcare costs.[4,5]

Hands of medical personals is one of the main factor contribute in the transfer of MRSA strains in hospital unites. Similarly, contaminated hospital equipments reported to be important cause of transmission of MRSA in ICU and hemodialysis. With the emergence of multidrug resistant (MDR) MRSA strains in the hospitals and lack of other effective alternatives drug, vancomycin was used for treatment of MRSA.[6] However, in-vitro susceptibility of MRSA to vancomycin is no longer universal because of emergence of vancomycin intermediate and resistant strains of *S. aureus* that resulted in treatment failure.[7] Recent data suggests high rates of clinical and bacteriologic blood stream infections (BSIs), when vancomycin was used to treat MRSA strains with an MIC to vancomycin of 2 µg/mL or greater. The first clinical isolate of vancomycin intermediate *S. aureus* (VISA) was identified in 1997, and these strains have now been reported worldwide.[8,9] To date, VISA strains (vancomycin MIC 4-8 µg/ml) are characterized by a resistance mechanism that is usually associated with vancomycin exposure. A study published in 2005 found that nearly one percent of all hospital inpatient were associated with *S. aureus* infections that many were resistant to methicillin and vancomycin.[10]

Epidemiology of MRSA in Iran showed that among 235 isolates were evaluated by conventional antibiotic susceptibility tests and PCR, 112 strains were MRSA (47.5%).[11] Similarly, a cross sectional study carried out at university teaching hospital, serving as the referral center for patients from north west region of Iran, showed six VISA *S. aureus* strains (VISA) with MIC=4 µg/mL, five of them reported as MRSA.[12]

The proportion of patients whose death was attributable to methicillin resistant virulent MRSA was significantly higher than that for methicillin sensitive *S. aureus* (MSSA).[13] In critically ill patients, after accurate adjustment for disease severity and acute illness, it was found MRSA bacteremia to have a higher attributable mortality than MSSA bacteremia which was mainly due to increases in virulence factors.[14] There are several reports on relation between biofilm and antibiotic resistance made biofilm based infections rarely resolved.[15] In this regard, *S. aureus* is among leading nosocomial pathogens capable of producing severe biofilm related infections such as colonization on central venous catheters, lower respiratory tract infections (due to contaminated ventilators) and osteomyelitis.[5,7] Biofilm formation is also important, because this mode of growth is associated with the chronic nature of the subsequent infections, and colonizing bacteria can resist against phagocytosis and evade the body's defense system.[16]

The purpose of present study was to investigate antimicrobial susceptibility, virulence factors and biofilm formation among *S. aureus* strains isolated from hospital infections in Kerman, Iran.

METHODS

Patient's sources

Thirty non- repeated clinical isolates of *S. aureus* were recovered from three university hospitals (Afzalipoor, Shahid-Bahonar and Shafa with 250 and 170-bed, respectively) in the city of Kerman, southeast of Iran. Demographic information about the patients' sex, age, type of samples (blood, wound, nasal, and urine), hospital units (ICU, surgical, orthopedic and emergency) and length of hospitalization were obtained from hospital medical records. Several clinical manifestations such as fever, chill, tachypnea, tachycardia, chest pain and peripheral emboli such as Osler' nodes and subungual hemorrhages were also considered in this investigation.

Sample collection

Duplicate samples were collected from infected sites by an expert laboratory technician, inoculated in to 5 mL sterile Stuart Transport medium (Merck, Darmstadt, Germany) and transferred to Department of Microbiology laboratory Kerman University of Medical Sciences within 24 h of collections. A loopful of the bacterial suspensions were diluted (10^{-2}) with 5 mL sterile normal saline (0.9%) and inoculated primarily on to sterile Tryptic Soy Agar (BioMérieux, Marcy l'Etoile, France), Baird-Parker agar (Merck, Darmstadt, Germany) supplemented with egg yolk-tellurite emulsion and on blood agar plates. After incubation at 37°C for 24 h, bacterial identification were performed by standard microbiological and biochemical tests as described previously.[17] The individual colonies of isolates were preserved in sterile Eppendrof tubes containing 1 mL sterile nutrient broth (Merck, Darmstadt, Germany) and 40% glycerol at -70°C for further analyses.

Antimicrobial susceptibility testing

Antimicrobial susceptibility of all thirty *S. aureus* isolates was tested by the Kirby-Bauer agar disk diffusion breakpoint method according to Clinical Laboratory Standards Institute (CLSI 2012) guide lines.[18] Oxoid antibiotic disks were obtained from Hi-media, India and used in the following concentrations (in µg mL-1): methicillin (MET) [10 µg], tetracycline (TE) [30 µg], amikacin (AN) [30 µg], tigecycline (TGC) [15 µg], gentamicin (GM) [10 µg], ciprofloxacin (CIP) [5 µg], ceftazidime (CAZ) [30 µg], vancomycin (VAN) [30 µg], erythromycin (E) [15 µg], chloramphenicol (C) [30 µg], tobramycin (TM) [10 µg], amoxicillin + clavulanic acid (AMC) [30 µg]. The CFU/mL of inoculums was adjusted to 1×10^8, 100 µL were spread all over Muller-Hinton agar. Susceptibility to tigecycline was classified based on the EUCAST criteria (www.eucast.com); susceptible, MICs of ≤0.5 µg/mL or zone around disk ≤17 mm.

MRSA screening

Preliminarily MRSA Screening test was performed in accordance to the CLSI guidelines (2012) using cefoxitin agar. Bacterial suspension (5 mL) of 0.5 McFarland (1×10^8 CFU/mL) was prepared from each *S. aureus* isolate then a swab was dipped and streaked on the surface of a Muller-Hinton agar (MHA) (Hi-media, India) supplemented with 6 µg/mL cefoxitin and 4% NaCl. After incubation for 24 h at 35°C, if any growth was detected, the isolate was considered MRSA. Similarly, a loopful of bacterial samples was inoculated on nutrient agar plates supplemented with 4 µg/mL oxacillin and incubated for 24 h at 35 ºC. MIC vancomycin and methicillin (oxacillin monohydrate sodium salt) were determined by the broth microdilution method using flat bottomed 96-well polystyrene microtiter plate. Serial twofold dilutions in triplicate sets were done with concentrations ranging from 0.5 to 32 µg/mL respectively as recommended by CLSI.[18] To each well bacterial suspension at concentration of 1×10^6 CFU/mL was added and incubated for 24 h at 35°C. In addition, MIC vancomycin for each isolate was measured by E strip test (E-test) (Hi-Media, India) according to manufacturers' instructions and CLSI guidelines. The MIC <4 µg/mL was considered susceptible, MIC 4-8 µg/mL was considered intermediate and MIC ≥32 µg/mL considered resistant to vancomycin. The MIC was measured twice to confirm the existence of VISA. Simultaneously, a laboratory isolate of *S. aureus* sensitive to above antibiotics were used as negative control.

Identification of the virulence factors

All MRSA isolates were screened for protease, DNase, coagulase, lecithinase and hemolytic activity on blood agar and compared with MSSA as described previously.[19,20] Similarly, presence of capsule was detected according the method described by Welch et al.[21]

Primary attachment assay

The initial attachment to biologic surfaces is an important step in biofilm formation, as well as in the subsequent pathogenesis of *S. aureus* biofilm-associated infections. Therefore, we tested the capability of the MRSA strains to attach to a polystyrene surface. Briefly, overnight *S. aureus* cultures were

adjusted to an optical density (OD600nm) of 0.5 (1×10^8 CFU/mL) and diluted to 10^3 CFU/mL. Aliquots (100 µL) of the suspension were inoculated on microtiter plate wells. After 30 min of incubation at 37°C, the plate was gently rinsed three times with sterile phosphate-buffered saline (PBS) (pH= 7.2) and then covered with sterile 150 µL of Tryptic Soy Broth (TSB). Primary attachment was expressed as the mean percentage of CFU (± SD) remaining on the microtiter plate compared to the initial inoculum.

Biofilm formation assay under static condition

The biofilm formed by the above isolates was quantified by microtiter method as described previously with some modification.[22] Briefly, one loopful from each isolated colony was inoculated into a sterile TSB medium (2 mL) containing glucose (1% W/V) to optimize biofilm production. The optical density (OD650nm) was then adjusted to 0.13 to reach 0.5 McFarland standard (1.5×10^8 CFU/mL) followed by further dilution of prepared bacterial suspension to reach ~10^6 CFU/mL and addition of 100 µL of each prepared inoculums to 96-well polystyrene flat bottom tissue culture microplate. Similarly, 100 µL of the TSB medium without any bacterium (negative control) was added to the related wells, microtiter plate was then incubated at 37°C under static condition. One set was kept uninoculated as negative control. After 24 h incubation at 37°C, non-adherent cell suspensions were slowly and aseptically aspirated, washed and replaced with 10 µl of sterile phosphate buffered solution (pH= 7.2) to remove any remaining suspended cells. In order to fix the biofilm, 150 µL of methanol (Merck, Darmstadt, Germany) was added to each well and kept at room temperature (25°C) for 20 min. The methanol was then removed and replaced with 200 µL of crystal violet solution (1% W/V). The wells containing biofilm matrix washed slowly with sterile deionized water and kept at room temperature till dried. Thereafter, 200 µL of glacial acetic acid (33% V/V) was added to each well and the optical density (OD) of each well was measured at OD570nm by using Synergy 2 multi-mode microplate reader (BioTek, USA). The isolates were classified into strong, moderate, weak and no biofilm based on attachment to microplate and formula given by Stepanovic et al.[23]

RESULTS

Of the thirty non-repeat strains of *S. aureus* that were included in the study, fourteen (46.7%) were obtained from pulmonary infections, six (20%) from UTI, five (16%) were from wound infections and abscesses, three (10%) from blood and two (6.7%) were obtained from orthopedic patients. The highest number of the samples collected from ICU (29%) and lowest numbers of *S. aureus* were detected from newborne (3%). Twenty-three (76.7%) of the patients were male and seven (23.3%) were female. We found that, incidence of infection was much higher in the patient ≥50 years old as compared to other age groups and have highest rate of hospitalization (12 days) (p≤0.05).

Table 1. Antibiotic susceptibility of *S. aureus* isolated from patients hospitalized in different hospital units in Kerman, Iran

Antibiotics	Resistance No (%)	Intermediate No (%)	Sensitive No (%)
Methicillin	8 (26.7)	0 (0)	22 (73.3)
Tetracycline	20 (66.6)	8 (26.6)	2 (6.6)
Amikacin	7 (23.3)	3 (10)	20 (66.6)
Tigecycline	0 (0)	0 (0)	30 (100)
Gentamicin	10 (33.3)	2 (6.6)	18 (60)
Ciprofloxacin	10 (33.3)	2 (6.6)	18 (60)
Ceftazidime	12 (40)	11 (36.6)	7 (23.3)
Vancomycin	0 (0)	4 (13.3)	26 (86.6)
Erythromycin	11 (36.6)	5 (16.6)	14 (46.6)
Chloramphenicol	0 (0)	0 (0)	30 (100)
Tobramycin	10 (33.3)	0 (0)	20 (66.6)
AmoxiClav	26 (86.6)	0 (0)	4 (13.3)
Total	n=30 (100%)		

The antibiotic susceptibility of *S. aureus* isolates is illustrated in Table 1. All of the isolates were sensitive to tigecycline (MRSA and MSSA) whereas, 26 (86.6%) were sensitive and remaining four (13.3%) were intermediate to vancomycin (P≤0.05). No any VRSA isolate was detected in our study. Most important observation was in case of methicillin. It was found that (n= 22, 73.3%) of the *S. aureus* population was sensitive to methicillin and (n= 8, 26.7%) remaining was resistant to this antibiotic. Colonies on nutrient agar containing 4 µg/mL oxacillin monohydrate sodium salt appeared after 24 h incubation at 35°C. Twelve (40%) isolates were resistant to ceftazidime, (n= 26, 86.6%) resistant to amoxicillin + clavulanic acid, (n= 10, 33%) were resistant to tobramycin and (n= 20, 66%) were resistant to tetracycline. The rate of resistance to ceftazidime was relatively lower than other antibi-

otics used against MRSA. The MIC results of the vancomycin and methicillin is presented in Table 2. It was found that only four *S. aureus* isolates exhibited MIC 4 µg/mL, while, 13 isolates demonstrated MIC 2 µg/mL to vancomycin. No vancomycin resistant isolate was detected. The results were further confirmed by E-test. Similarly, the results of MIC to methicillin revealed that, 10% (n= 3) of the isolates had MIC 32 µg/mL and 33% (n= 10) showed MIC 4 µg/mL to methicillin respectively.

Table 2. MIC of vancomycin and methicillin against 30 hospital isolates of *S. aureus*

Antibiotic concentration (µg/mL)	0.25	0.5	1	2	4	8	16	32
Vancomycin, N (%)	1 (3.3)	2 (6.6)	10 (33.3)	13 (43.3)	4 (13.3)	0 (0)	0 (0)	0 (0)
Methicillin, N (%)	0 (0)	1 (3.3)	4 (13.7)	3 (10)	10 (33)	4 (13.3)	5 (16.6)	3 (10)

Furthermore, in-vitro screening *S. aureus* for virulence factors showed that, 96.7% of the MRSA isolates could produce detectable extracellular protease; while, in 25% of the MSSA isolates this enzyme was detected (p≤0.05) as shown in Table 3. In case of lecithinase enzyme, 75% of MRSA population showed enzyme activity, whereas, in case of MSSA, 12.5% showed the enzyme activity. Results of capsule staining revealed that 62.5% of the MRSA isolates had capsular polysaccharide; this was 37.5% for MSSA (Table 3).

Table 3. Frequency (%) of the virulence factors among methicillin resistant (MRSA), methicillin sensitive (MSSA) isolates of *S. aureus* isolated in this study

Virulence factor	MRSA, n= 8 (%)	MSSA, n=8 (%)
Coagulase	7 (96.7)	6 (75.0)
Protease	7 (96.7)	2 (25.0)
Lecithinase	6 (75.0)	1 (12.5)
DNase	7 (96.7)	4 (50.0)
β-hemolysis	7 (96.7)	2 (25.0)
Capsule	5 (62.5)	3 (37.5)

Primary attachment assays revealed no significant initial binding differences between those isolates with strong biofilm and moderate or weak biofilm forming *S. aureus* to polystyrene surface. Quantification of the biofilm formation among thirty isolates of *S. aureus* revealed that, Twenty (66.6%) isolates formed strong, seven (23.3%) moderate and three (10.0%) had weak attachment to microplate and biofilm. In addition, all the MRSA isolates were found to form biofilm while, MSSA isolates were also formed moderate and weak biofilm.

DISCUSSION

During the past four decades, an MRSA strain has evolved from a controllable nuisance into a serious public health concern. Many MRSA infections occur in hospitals and healthcare facilities. There are several reports on distribution and frequency of MRSA infections in Iranian hospitals, Fatholahzadeh et al.,[24] isolated 99 MRSA from hospital patients in Tehran during a 15 month period. It was found that all MRSA isolates were susceptible to vancomycin (MIC90 ≤ 2 µg/mL). In the other study, results showed that the rate of MRSA had risen up to 43% in Nemazi hospital (Shiraz, Iran) during 4 years.[25] There is a little information available on antimicrobial susceptibility, virulence factors and biofilm formation among MRSA hospital isolates of *S. aureus* in south east (Kerman), Iran.[26]

The present study was conducted to address these issues that pose serious health care concern in our region. We found that, significant number of patients infected with *S. aureus* were from ICU and aged above 50 years old. This is since MRSA isolates have high incidence rate among elderly especially in ICU and can form biofilm on hospital instruments like ventilator and cause ventilator associated pneumoniae and catheter related spesis.[20] It is well known that, the clinical syndrome associated with invasive MRSA disease included bacteremia, pneumonia, cellulitis, osteomyelitis, endocarditis, and septic shock many of which facilitate by biofilm. Fortunately, the rate of MRSA among *S. aureus* population was low in spite of increasing resistance to methicillin around the world, and the isolates remain susceptible to other antibiotics as well. The important observation in our study was sensitivity of all MRSA strains to tigecycline.

The current definition for VISA is an *S. aureus* isolate with a vancomycin broth and E-test MIC of 4 to 8 µg/mL. MICs equally or greater than 32 µg/mL are characterized as resistant.[18]

A recent investigation on vancomycin intermediate *S. aureus* in a medical center in Taiwan showed that 4.2% of VISA isolates were associated with a higher mortality rate (60%) than VSSA.[27] Golan et al.,[28] reported a statistically significant increase in vancomycin MICs over a 4 year period (2002-2005) at their institution. Similarly, several authors have addressed the question of whether MRSA is more virulent than methicillin sensitive *S. aureus* (MSSA). Soriano and colleagues performed a retrospective case control study of 908 (225 MRSA) episodes of bacteremia and matched 163 pairs and suggested that MRSA was not an independent factor for mortality.[29] Chen et al.,[30] studied the susceptibility MRSA to nine antimicrobial agents, it was found that, among these MRSA isolates, 99.7% were susceptible to vancomycin; however, 30.0% (n= 517) exhibited MICs of 2 mg/L and six (0.3%) were intermediately susceptible (VISA, MICs of 4µg/ml) to vancomycin.[30]

Many virulence factors are associated with MRSA that enable them to cause disease.[8] In our study, the MRSA strains were produced more lecithinase and protease DNase, coagulase and had more hemolysis activity on blood agar as compared to MSSA isolates which may influenced the pathogenicity of these strains and severity of infections. We also found that our MRSA isolates formed strong and moderate biofilm, making them more resistant to antimicrobial agents.

However, we could not clearly establish relation between biofilm production and antibiotic resistance, since some MSSA strains produced strong biofilm, but were sensitive to antibiotics tested. In a study carried by Rezaei et al.,[31] it was found that twenty six (8.8%) of all *S. aureus* isolates were recognized as MRSA. All the MRSA isolates have the ability of biofilm formation which 15.4%, 19.2% and 65.4% of them were strong, medium and weak biofilm producer respectively.

Based on above data, we suggest that strict control and surveillance must be undertaken to prevent spread of MRSA strains in the hospital environments and units.

Further research is needed to understand the molecular genetics of MRSA isolates.

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