Original article

Prevalence of methicillin resistant *Staphylococcus* species isolated from burn patients in a burn center, Ahvaz, Iran

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Abstract

Introduction and objective: Today methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase negative *Staphylococci* (MRCNS) are frequent causes of nosocomial infection. Extensive burn injuries, extended hospitalization and inappropriate antibiotic therapy have been identified as risk factors for MRSA and MRCNS carriage and infection. The aim of this study was to assess the prevalence of methicillin resistance among clinical isolates of *Staphylococci* taken from burn patients using four separated methods and also determination of susceptibility pattern to amikacin, ciprofloxacin, vancomycin, carbenicillin and gentamicin.

Materials and methods: A total of 185 clinical staphylococcal isolates from wound and blood specimens were evaluated for susceptibility to oxacillin using oxacillin and cefoxitin disk diffusion method, agar screening containing 6 microgram oxacillin /ml, oxacillin E test and polymerase chain reaction for predicting *mecA* gene.

Results: The results showed that 27.8% of wound and blood specimens were infected by *Staphylococci* and among these 60% were identified as methicillin resistant. We found no significant differences between the results of PCR assay and conventional disk diffusion method by oxacillin and cefoxitin disk, however the results of cefoxitin disk was more significant than oxacillin and gave better results. Both of the sensitivity and specificity value were similar (99%, 100%) for E test and agar screen test. Furthermore in E test for detection of minimum inhibitory concentration (MIC), more than 93% of MRSA and 15% of MRCNS isolates had MIC value more than 256µg/ml. We also determined a significant difference pattern between methicillin resistant and methicillin susceptible *Staphylococci* to five antimicrobial agents.

Conclusion: In conclusion, our results showed that the prevalence of methicillin resistant *Staphylococci* in our center was very high and cefoxitin disk test is reliable alternation for

detection of methicillin resistant *Staphylococci*.

Keywords: *Staphylococcus aureus*, *Coagulase-negative Staphylococci*, *mecA*, Cefoxitin, Oxacillin, Methicillin resistance

Introduction

Staphylococcal infection in hospitalized patients has been a major concern for well over a century. It is known that *Staphylococcus aureus* is the predominant bacteria responsible for burn and surgical wound infection [1] and it is perhaps the single most common cause of healthcare-associated infection throughout the world [2]. Coagulase-negative *Staphylococci* (CNS) belong to a group of opportunistic pathogens since they are found as normal inhabitants of the skin and mucus membranes in different parts of the body. CNS are a leading cause of nosocomial infections, especially in neonates, immunocompromised individuals and burn patients. CNS strains have become a serious problem as they express methicillin resistance, which involves all β-lactam antibiotics and leads to a significant limitation in therapeutic options [3].

Methicillin-resistant *Staphylococci* strains are those strains that have acquired the ability to grow in the presence of methylpenicillins and derivatives, including methicillin, oxacillin, and nafcillin. The *mecA* gene is inducible and encodes polypeptide with high molecular weight (78-kD PBP2a) [4]. It occurs in both methicillin resistance *Staphylococcus aureus* (MRSA) and methicillin-resistant coagulase-negative *Staphylococci* (MRCNS) and is highly conserved [5].

Hospital-associated MRSA isolates often show multiple resistances to other commonly used antimicrobial agents, including aminoglycosides, erythromycin, clindamycin, co-trimoxazole and tetracycline while community-associated MRSA isolates are often resistant only to β-lactam agents and erythromycin [6]. MRSA colonization rates in burn patients have been reported up to 39% and hence MRSA outbreaks in burn units are not uncommon [7].

Data show approximately 75% of hospital strains of CNS that are resistant to methicillin [8]. Patients with extensive burn injuries are especially susceptible to infection with MRSA due to loss of the skin barrier and reduced immunological capacity [9]. Numerous studies have shown that the incidence of MRSA throughout Iran is rising, however, regionally the rates differ dramatically [10-12]. Our previous study, conducted in 2005 using disk diffusion method, showed that the incidence of MRSA isolates from burn patients at Taleghani burn hospital was 58% [10]. Currently, a number of standardized methods have been recommended by Clinical and Laboratory Standard Institute (CLSI) for detection of MRSA, including broth and agar dilution, disk diffusion and agar screen methods [13]. But the usefulness of polymerase chain reaction (PCR) assay for the *mecA* gene as "gold standard" is well established [14].

The purposes of our study were (i) to estimate the prevalence of MRSA and MRCNS among clinical isolates of *Staphylococcus spp*. using oxacillin and cefoxitin disk test, agar screening test and PCR; (ii) to compare PCR detection of *mecA* gene with some standard techniques available in our center; (iii) to determine oxacillin MIC and susceptibility pattern to various antibiotics.

Materials and methods

Location and sampling procedures

Taleghani burn hospital is a 160 bed university affiliated referral hospital, serving a population of about six million in south west part of Iran. This hospital is the only referral center in Khuzestan province and three neighbor provinces. Approximately 1200 patients with all stages of burns are admitted annually. From August 2006 to October 2007, all the burn wound biopsies and blood cultures were tested. During this period, 501 biopsy and 102 blood samples from 603 patients were cultured for *Staphylococci.* All *Staphylococci* isolates were identified using conventional methods in the microbiology laboratory [15].

Microbiological procedures

Disk diffusion testing was performed according to the Kirby-Bauer method, as described in the guidelines of the CLSI with a 1µg oxacillin and Mueller-Hinton agar (Merck, Germany) [13]. The CLSI recommends the direct colony suspension method for testing *Staphylococci* for potential methicillin or oxacillin resistance. The plates were incubated in ambient air at 35°C, and inhibition zones around the disk were measured after 24h. Inhibition zones with ≤17mm diameters for CNS and ≤10mm for *S. aureus* were considered as resistant. Any discernible growth within the zone of inhibition was indicative of methicillin resistance. In addition, all isolates were screened by the disk diffusion method for resistance to cefoxitin according to CLSI guidelines to ensure that they were methicillin resistant.

Based on CLSI recommendation, a zone of ≤19mm for *S. aureus* and ≤14mm for CNS were reported as oxacillin resistant. There is no intermediate category with the cefoxitin disk diffusion test [13]. Furthermore, to evaluate the susceptibility pattern of methicillin resistant *Staphylococci*, we used five antimicrobial agents including gentamicin (10µg), carbenicillin (100µg), ciprofloxacin (5µg), vancomycin (30µg) and amikacin (30µ). All methodological variants were assessed using the same inoculum which was standardized to 0.5 McFarland turbidity. Two standard strains were processed in parallel as controls for the disk diffusion test: *Enterococcus faecalis* ATCC 29212 and *S. aureus* ATCC 25923.

The E test gradient technology is based on a combination of the concepts of dilution and diffusion principles for susceptibility testing. E test strips for oxacillin were provided by AB BIODISK (Solna, Sweden). MICs were performed according to the manufacturer's instructions. E test strips were placed on Mueller-Hinton agar plates containing 2% NaCl, which enhance the growth of microcolonies and the expression of the resistance.

Inoculum suspensions were adjusted to the turbidity of 0.5 McFarland standard and the plates were then incubated at 35°C for a full 24h. After the period of incubation, the E test MIC results were read where the edge of the inhibition ellipse intersects the MIC scale on the strip. According to the manufacturer's instructions and CLSI, MIC breakpoints for defining interpretative MRSA and MRCNS were 4µg/ml and 0.5µg/ml respectively [16]. In addition, the oxacillin-salt agar screening plate procedure may be used in order to detect and confirm the presence of MRSA. This test was performed as directed in CLSI guidelines [13]. For each isolate, 1µl or a swab of 0.5 McFarland suspensions was streaked on a Mueller-Hinton agar plate supplemented with 4% NaCl and 6mg of oxacillin per ml. The plates were then incubated in ambient air at 35°C for 24h. Any growth on the plate was recorded as indicating oxacillin resistance.

Multiplex PCR

All staphylococcus isolates were evaluated by detection of the *mecA* gene by PCR amplification. PCR was performed with six complementary primers: mecA1(5'-AAT CTT TGT CGG TAC ACG ATA TTC TTC ACG-3'); mecA2(5'-CGT AAT GAG ATT TCA GTA GAT AAT ACA ACA-3'); S.aureus1(5'-ATC AAA AAG TTG GGG AAC CTT TTC A-3'); *S. aureus*2 (5'-CAA AAG AGC GTG GAG AAA AGT ATC A-3'); *S. epidermidis*1(5'-AAC AGG TGA ATT ATT AGC ACT TGT AAG-3'); *S. epidermidis*2 (5'-ATT GCT GTT AAT ATT TTT TGA GTT GAA-3'). In this study we used two different multiplex PCR assays that include both species- specific primer pairs for internal control (*S. aureus and S. epidermidis*) and a primer pair for the specific detection of *mecA.* Amplification with these primers was predicted to generate a108bp, 124bp and 174bp DNA fragment that could be resolved by electrophoresis in 2% (w/v) agarose gels stained with ethidium bromide (Fig. 1).

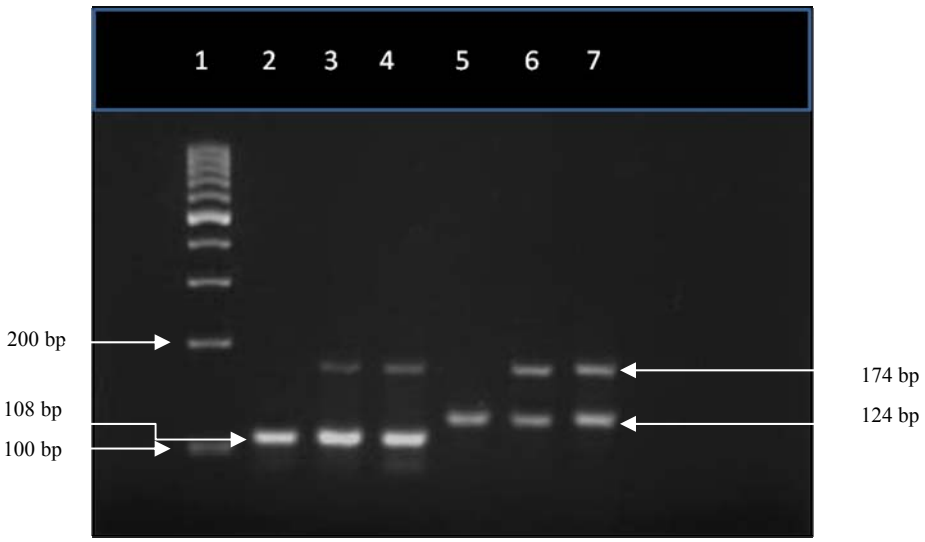

Fig. 1: Detection of mecA gene by PCR. Line 1: 100bp marker, Line 2: *S. aureus mecA*$^-$ (negative control), Line3: *S. aureus mecA*$^+$ (positive control), Line 4: Sample test (*S. aureus* with *mecA* gene), Line 5: *Coagulase negative Staphylococci mecA*$^-$ (negative control), *Line 6: Coagulase negative Staphylococci mecA*$^+$ (positive control), Line 7: Sample test (*Coagulase negative Staphylococci* with *mecA* gene)

The PCR protocols were optimized to 40 cycles consisting of 1min at 90°C, 1min at either 50 or 61°C for annealing and 90 s at 72°C, followed by a final 10min extension at 72°C. Each reaction mixture of 25µl volume consisted of 17.85 of double sterile water, 2.5µl of 10× polymerase buffer (100 mmol l^{-1} Tris-HCl pH 9.0, 500mmol l^{-1} KCl, 4mmol l^{-1} $MgCl_2$), 400µmol l^{-1} dNTPs, 50pmol of each primer and 2.5 units of Taq polymerase with 2.5µl staphylococcal DNA (about 10ng) as template [17]. For template preparation, an overnight culture was extracted with Fermentase nucleic acid purification kit (www.fermentas.com). Positive and negative controls were included using *S. aureus* ATCC 25923 and 43300 (methicillin-susceptible and methicillin-resistant) [18].

The percentages of sensitivity and specificity were calculated according to Mulder Sensitivity that represent the number of *mecA*-positive strains detected as

resistant by phenotypic methods divided by the total number of *mecA* positive strains (either susceptible or resistant). Specificity was calculated through dividing the number of *mecA*-deficient strains classified as susceptible according to phenotypic criteria by the total number of *mecA*-negative samples [19].

Results

Prevalence of methicillin resistant Staphylococci

From August 2006 to October 2007, a total of 185 *Staphylococcus spp.* were isolated in 501 biopsies and 102 blood specimens taken from 603 patients. From 185 isolates, 97 *S. aureus* and 88 coagulase-negative *Staphylococci* (84 *S. epidermidis*) were reported. The frequency of *Staphylococci* in wound and blood specimens were 161 of 501 (32.1%) and 24 of 102 (23.5%) respectively. From 185 *Staphylococci* isolates, 112 isolates were methicillin resistant. MRSA and MRCNS in *Staphylococci* isolates were 61% and 60% respectively.

Comparison of PCR detection of mecA gene and conventional susceptibility testing for the detection of methicillin resistant Staphylococci

The presence of *mecA* gene using PCR was considered as the reference or gold standard method for calculating the Sensitivity and specificity of the other tests in this study. Results from the multiplex PCR assay were correlated very well with those from conventional disk diffusion susceptibility tests. Sensitivity in cefoxitin disk diffusion test was slightly better than oxacillin disk. There were two false negatives for oxacillin (1.7%), two CNS were *mecA*$^+$ but susceptible to oxacillin disk. The cefoxitin disk detected methicillin resistance correctly in all isolates compared to the presence of *mecA* gene.

Totally based on oxacillin and cefoxitin disk diffusion, there was no significant difference between conventional susceptibility testing and PCR for predicting methicillin resistance *Staphylococci* ($p<0.05$). In E test and agar screen with 6µg/ml oxacillin, we found the same value of sensitivity and specificity (99%, 100%). These values were less than cefoxitin but more than oxacillin. The overall results obtained with the different techniques are shown in Table 1.

Table 1: Sensitivity and specificity of different diagnostic methods based on PCR amplification of the mecA gene as reference method

Reference method	Parameter	Different diagnostic methods			
		Etest	Oxacillin disk	Cefoxitin disk	Agar screening
PCR of *mecA*	Sensitivity	99 %	98 %	100 %	100 %
	Specificity	100 %	100 %	100 %	100 %

Determination of MIC value and Antibiotic susceptibility pattern

We also evaluated the susceptibility pattern between methicillin resistance and methicillin susceptible *Staphylococci* using different antibiotic disks. It is important to emphasize that we have determined a significant difference between them. The highest resistance percentage belonged to ciprofloxacin (81.2%) and then amikacin (81%), carbenicillin (64.6%) and gentamicin (64.3%) (Table 2). Our results showed that the MICs value in 93% of the MRSA isolates were over 256µg/ml. Similarly in MRCNS, 15% of isolates were more than 256µg/ml and 83% in a range between 0.6 and 6µg/ml.

Table 2: Percentage of resistant pattern among methicillin resistant and methicillin susceptible *Staphylococci* isolates

Isolates	Antimicrobial agents				
	Amikacin	Ciprofloxacin	Vancomycin	Carbenicillin	Gentamicin
Methicillin resistant	81%	81.2%	2%	64.6%	64.3%
Methicillin susceptible	25%	68.2%	0%	30.3%	30.6%

Discussion

Approximately 32% (89.4 million persons) and 0.8% (2.3 million persons) of the U.S. population are colonized with *S. aureus* and MRSA respectively [20]. This study revealed that 27.8 % of burn wound and blood specimens were infected by *Staphylococci* and among them about 60% were identified as methicillin resistant. The prevalence of methicillin resistance among *Staphylococci* isolated from burn patients at our hospital has not been determined accurately to date. Our results are close to other studies in Iran and some countries. In three separated studies in Iran, which reported by Japoni *et al.* [11], Mehdinejad *et al*. [12] and Ekrami *et al.* [10], the average prevalence of methicillin resistance *Staphylococci* in burn patients was between 60%-80%.

In a study in Korea, Song *et al.* [21] reported that the incidence of MRSA was 98% within a burn center. This is markedly higher than those reported from other countries [21]. In another study in the United States the rate of MRSA in a burn center was 33% [22]. These variations are reflected by several studies from different continents [23]. The variations are due to differences of local conditions, such as climate or microbial prevalence, but others are likely to be caused by differing prevention protocols, topical and systemic treatment of burn wounds, sampling regimens as well as study lengths [24].

It is well established that methicillin resistant *Staphylococci* colonization could be the result of an exogenous vector, a nosocomial infection and/or endogenous vector. The colonized or infected patient and in the case of burns, older patients and those with large burns are at a higher risk of colonisation. The spread in hospitals occur mainly through hands of healthcare workers and medical equipment, such as hydrotherapy showers [23,24].

Increases in prevalence methicillin-resistant *Staphylococci* is a fact and some investigators have confirmed it. Guggenheim *et al.* [24] reported this increase from 3% in 1986-1997 to 16% in 1998–2001 and 13% in 2002-2005, and results for methicillin-resistant CNS show an even greater increase [25]. This high value is presumably related to the inappropriate use of antimicrobial drugs and insufficient infection control measures in Iranian hospitals. Our results showed 1.7% false negative causes by oxacillin disk diffusion method (heteroresistance to methicillin) and without any false results for cefoxitin.

Compared to other studies, we employed a fairly large number of isolates and this is beneficial for accurate statistical interpretation. Similar to other studies we found a higher sensitivity and specificity values for the cefoxitin disk test in comparison with the oxacillin disk method [26,27]. Based on our results and also CLSI recommendation, we preferred cefoxitin

disk over the oxacillin disk for predicting methicillin resistance in both MRSA and MRCNS. Even though, almost the same sensitivity and specificity were observed for both E test and agar screening tests, we believe the agar screening with 6mg/L oxacillin is easier, more reliable and a cheaper method. The overall results suggest the cefoxitin disk test is a reliable alternative for the *mecA* gene detection and can be useful for those labs which do not have PCR facilities.

Conclusion

Our results showed the prevalence of methicillin resistant *Staphylococci* in our center was very high and cefoxitin disk test is an alternative reliable for detection of methicillin resistant *Staphylococci*. Finally, this paper emphasizes the need for preventive measures and choosing effective antimicrobials against MRSA and MRCNS infection in the burn unit.

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