A comparative characterization of nasal and clinical isolates of
Staphylococcus aureus from west of Iran

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ABSTRACT

Background and Objectives: Recently, the rise of methicillin-resistant Staphylococcus aureus (MRSA) isolated from hospital healthcare workers (HCWs) and various infectious samples has become one of the main concerns in hospital settings. Therefore, epidemiological studies are necessary to monitor antibiotic resistance patterns in each region and to study the pathogenesis of this strain to control infections.

Materials and Methods: In this cross-sectional study, a total of 100 S. aureus isolates, including 50 isolates obtained from the anterior nares of healthcare workers, as well as 50 other isolates cultured from the various clinical specimens from the referral hospitals in Khorramabad (West of Iran) were tested. All isolates were examined to determine antibiotic resistance pattern, and the presence of staphylococcal enterotoxin A (sea), staphylococcal enterotoxin B (seb) and meca genes.

Results: The meca gene was found among 36% (18/50) of the clinical S. aureus isolates (CSIs) and 14% (7/50) of nasal S. aureus isolates (NSIs), with statistically significant difference (X2 = 6.53; p = 0.011). The difference between the frequency rate of sea gene among MRSA strains isolated from clinical specimens (46.6%, 7/15) was significant compared to strains isolated from nostrils (14.3%, 1/7) (X2 = 3.85; p = 0.049).

Conclusion: The frequency of meca, sea, and seb genes among the clinical samples was more than strains isolated from the nostrils of healthcare personnel.

Keywords: MecA; Methicillin resistance; Staphylococcal enterotoxins; Staphylococcus aureus

INTRODUCTION

Staphylococcus aureus is a pathogen and opportunistic bacterium which can colonizes the skin and nasal cavity of many people, causes local and even systemic diseases such as bacteremia, osteomyelitis, and pneumonia (1, 2). There has been a dramatic increase in the occurrence of S. aureus infections, especially methicillin-resistant S. aureus (MRSA), which is resistant to several classes of antibiotics, as well as disinfectants (3-5).

The meca gene is mainly responsible for the methicillin resistance phenotype among MRSA isolates (6). Reviews have shown that the mean frequency of MRSA in Iran has been reported at 43%, and Lorestan province is ranked amongst the provinces with the high prevalence rates (30-70%) of MRSA (6). Nostrils are the primary ecological niche for MRSA and the association between nasal carriage of MRSA and infectious diseases has been proven in recent years (7, 8). The colonization of MRSA strains in the nares of hospital health care workers (HCW) is considered

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to be the main source of transmission of these resistant clones to patients (9).

Previous studies in Iran revealed the prevalence of S. aureus and MRSA strains among medical personnel was 22.7 and 32.8%, respectively (10). Approximately 15 to 80% of isolated staphylococci from different resources are enterotoxigenic (11). Up to now, 21 staphylococcal enterotoxins (SEs) or staphylococcal enterotoxin-like (SEI) proteins have been identified (12, 13), the most prevalent being staphylococcal enterotoxin A (SEA) and B (SEB) (14). The SEs and SEI proteins share common characteristics: they are small molecules with similar three-dimensional structures, heat-stable and have super antigenic properties, which are implicated in food poisoning (11, 15). The most SEs and SEI genes are located on mobile genetic elements such as plasmids, which could be horizontally transferred between staphylococcal strains. While serologic methods are not sufficient to identify the different types of these toxins, molecular techniques such as PCR and real-time PCR have been developed to detect SE and SEI genes (11, 16).

Regarding the importance of mecA and enterotoxin genes in the pathogenesis of S. aureus, data about strains (nasal and clinical) isolated from Lorestan province hospitals has not been documented. This study aimed to determine the frequency of staphylococcal enterotoxin A (sea), staphylococcal enterotoxin B (seb) and the mecA genes among S. aureus isolated from the anterior nares of healthcare workers (HCWs), as well as various clinical specimens.

MATERIALS AND METHODS

Bacterial isolates. During this cross-sectional study, a total sample size of 100 S. aureus strains originated from nasal and clinical sources were investigated. Fifty of the tested strains were included from our previous study (July 2011 to January 2012) on nasal S. aureus isolates (NSIs) obtained from anterior nares samples of HCWs employed in the four referral hospitals in Khorramabad (West of Iran) (17). The remaining fifty strains were clinical S. aureus isolates (CSIs) collected from culturing of various clinical specimens (October 2015 to February 2016). The isolates were identified by the following methods: Gram-staining and positive reactions for catalase, coagulase, DNase, and mannitol fermentation on mannitol salt agar (yellow colonies) (18). The isolates which were identified as S. aureus, were stored at -70°C in TSB medium containing 15% glycerol for further investigations. This study was approved by the Ethical Committee of Lorestan University of Medical Sciences, Khorramabad, Iran. (IR.LUMS.REC.1397.060).

Antimicrobial susceptibility testing. To determine the antibiotic resistance profile of S. aureus isolates, the disc diffusion method was used, according to CLSI guidelines (19) by applying the following antibiotic disks (Rosco, Denmark): penicillin G (10U), cefoxitin (30 μg), tetracycline (30 μg), rifampin (5 μg), trimethoprim / sulfamethoxazole (23.75 / 1.25), erythromycin (15 μg) clindamycin (2 μg) and ciprofloxacin (5 μg).

PCR amplification of mecA, sea, and seb genes. DNA of all S. aureus isolates was purified using an AccuPrep® DNA Extraction Kit (Bioneer, Korea) according to the manufacturer’s protocol and with some modifications. First, a fresh culture of strains was prepared in Luria Bertani broth (LB; Merck, Germany). Then, 1 ml of each bacterial suspension was centrifuged for 5 minutes at 3000 rpm. The supernatant was discarded, and 10 μl of Lysostaphin enzyme solution (100 μg/ml in sterile deionized water; Sigma) was added to the harvested bacteria (17). After incubation for 1 hour at 37°C, DNA extraction was continued according to the kit instruction. Specific primers designed by Mehrotra et al. (Table 1) were used to detect the sea, seb and mecA genes. PCR for the amplification of the sea and seb genes was performed as a multiplex assay as follows: 2.5 μl PCR buffer (10x), 200ng DNA as a template, 1.2 unit of Taq DNA Polymerase, 20pml of each primer, 0.4mM dNTP, 1.5mM MgCl2, and distilled water was added up to 25 μl final volume. The mecA gene was amplified in a sep-

Table 1. Primers used for the amplification of staphylococcal enterotoxin A (sea), B (seb) and mecA genes.

| Gene | Primer sequence (5’ to 3’) | Product size (bp) |
|------|-----------------------------|-------------------|
| sea  | F: GGTTATGCAATGTCGGGTGGG  | 102               |
|      | R: CGGCACATTATTCTCTCCTCGG  |                   |
| seb  | F: GTATGGTGTTGTAACTGAGC    | 164               |
|      | R: CCAAAATAGTGGACGGTTAAGG  |                   |
| mecA | F: ACTGCTATCCACCCTCAAC     | 163               |
|      | R: CTGGTAGAGTGTGTAATCTGG   |                   |
arate reaction, including all of the above mentioned conditions, except for 20pmol of mecA primers, 0.2 mM dNTP and 1 unit of Taq Polymerase. The PCR program was set up as mentioned in Table 2 in the Thermocycler (Bio-Rad, My cycler, US) (20). The amplified DNA fragments were visualized in 1.5% agarose gel electrophoresis. A Staphylococcus aureus strain carrying both sea and seb genes and another containing the mecA gene were prepared as positive controls by Dr. Jalil Fallah Mehrabadi (Malek Ashtar University, Tehran).

Table 2. Temperature program to replicate seb, sea and mecA genes.

| Step           | Temperature | Time |
|----------------|-------------|------|
| Initial denaturation | 94°C        | 5 min |
| Denaturation     | 94°C        | 1 min |
| Annealing       | 57°C        | 1 min |
| Extension       | 72°C        | 1 min |
|                 | 35 Cycle    |      |
| Final extension | 72°C        | 8 min |

Statistical analysis. Descriptive data were analyzed by SPSS software version 19. The Chi-Square (X²) test was used to compare the resistances and also the frequency of enterotoxins among CSIs and NSIs. P values of <0.05 were considered statistically significant.

RESULTS

Microbial isolates and antibiotic susceptibility. A total of 100 S. aureus isolates, including 50 CSIs, as well as 50 NSIs were investigated. All isolates (100%) showed resistance to penicillin. Resistance rate to cefoxitin (MRSA), erythromycin, rifampin, clindamycin, trimethoprim/sulfamethoxazole (Co-trimoxazole), tetracycline, and ciprofloxacin were found 22%, 22%, 7%, 22%, 14%, 30% and 15%, respectively. Also, the intermediate susceptibility phenotypes were observed for erythromycin (7%), clindamycin (6%), ciprofloxacin (5%), tetracycline (3%), cefoxitin, and trimethoprim/sulfamethoxazole (1%). The pattern of microbial susceptibility of isolates, based on the site of sampling is listed in Table 3.

Frequency of enterotoxin A and B using multiplex PCR. PCR results revealed that among the 100 isolates evaluated for the presence of enterotoxin A and B genes, 36 (36%) carried at least one of the enterotoxin genes. The frequency of sea and seb genes were 35% and 8% among all samples, respectively. Twenty-eight isolates carried only the sea gene, one isolate contained the seb gene, and 7 isolates simultaneously harbored both genes (Fig. 1). The prevalence of the enterotoxin genes based on the origin of samples (CSIs or NSIs), is illustrated in Fig. 2. The difference in the relative frequency of enterotoxin genes among CSIs and NSIs was not statistically significant (χ² = 1.286, p = 0.733).

Frequency of mecA gene and enterotoxins among MRSA isolates. All 100 isolates were assessed to detect the mecA gene as a source of resistance to methicillin. The mecA gene was positive in 25% (25/100) of total isolates, in which 36% (18/50) CSIs and 14% (7/50) NSIs harbored this gene (Fig. 3). This difference was statistically significant (χ² = 6.53; p = 0.011). 22 isolates with the cefoxitin-resistant phenotype (MRSA) were positive for the mecA gene in the PCR test. Interestingly, 3 clinical isolates in the disk diffusion method were susceptible to cefoxitin, while carrying the mecA gene.

Of the 22 MRSA isolates, 8 isolates were positive for at least one of the tested enterotoxins genes. The prevalence of sea and seb genes among MRSA isolates was 36.3% (8/22) and 9% (2/22), respectively. However, out of the 78 isolates with susceptible phenotype for cefoxitin (MSSA), 27 (34.6%) isolates were positive for at least one of the enterotoxin genes, with a frequency of 34.6% and 69.7% for sea and seb genes, respectively. The occurrence of mecA and enterotoxin genes, based on the cefoxitin susceptibility and the origin of isolates is shown in Table 4. While the difference between the frequency rate of sea gene among MRSA strains isolated from clinical specimens (46.6%, 7/15) was significant compared to strains isolated from nostril (14.3%, 1/7) (χ² = 3.85; p = 0.049), the difference in a total number of sea and seb genes among CSIs and NSIs was not statistically significant (χ² = 2.163; p = 0.141).

DISCUSSION

Although the overall difference of antimicrobial resistance pattern between strains isolated from clinical samples and nares of hospital employees was not
Table 3. Antibiotic susceptibility pattern of clinical isolated S. aureus (CSIs) and nasal isolated S. aureus (NSIs).

| Antibiotic | CSIS n=50 (%) | NSIS n=50 (%) | 22= MRSA Resistant No. (%) |
|------------|---------------|---------------|----------------------------|
|            | Resistant     | Intermediate | Susceptible                | CSIs n=15 | NSIs n=7 |                     |
| Cotrimoxazole | (18) 9       | 0             | (82) 41                    | (10) 5     | (2) 1     | (88) 44            | (6.46) 7     | (1.57) 4 |
| Rifampin    | (8) 4         | 0             | (92) 46                    | (6) 3      | 0         | (94) 47           | (6.26) 4     | (8.42) 3 |
| Cefoxitin   | (30) 15       | (2) 1         | (68) 34                    | (14) 7     | 0         | (86) 43           | (100) 15    | (100) 7  |
| Erythromycin| (30) 15       | (2) 1         | (68) 34                    | (14) 7     | (12) 6    | (74) 37           | (80) 12     | (1.57) 4 |
| Ciprofloxacin| (18) 9       | (4) 2         | (78) 39                    | (12) 6     | (6) 3     | (82) 41           | (3.53) 8    | (1.57) 4 |
| Tetracycline | (38) 19       | (2) 1         | (60) 30                    | (22) 11    | (4) 2     | (74) 37           | (6.86) 13   | (8.42) 3 |
| Clindamycin | (28) 14       | 0             | (72) 36                    | (16) 8     | (12) 6    | (72) 36           | (80) 12     | (4.71) 5 |
| Penicillin G | (100) 50      | 0             | 0                          | (100) 50   | 0         | 0                 | (100) 15    | (100) 7  |

Fig. 1. Agarose gel electrophoresis of multiplex PCR products for enterotoxin A and B genes (sea, seb). Lane 1, a strain negative for sea and seb genes; Lane 2, positive control strain containing both the enterotoxin genes; Lanes 3-5, isolates contain sea and seb genes, simultaneously; Lane 6, sea gene positive strain (102 bp); Lane 7, seb gene positive strain (164 bp); lane M, 100 bp DNA size marker.

Fig. 2. The frequency of enterotoxin A (sea) and B (seb) genes among 100 isolates of Staphylococcus aureus. A, Frequency of the sea and seb genes among 50 CSIs; B, Frequency of the sea and seb genes among 50 NSIs ($x^2 = 1.286$, $p = 0.733$).

Fig. 3. Agarose gel electrophoresis of PCR products generated from mecA gene amplification. Lanes 1-10, mecA gene positive isolates; Lanes 13-15 mecA gene negative isolates; Lanes 11 and 12, positive control strains; Lane M, 100 bp DNA size marker.

statistically significant, our results revealed that the prevalence of antibiotic resistance was higher among CSIs. Resistance to cefoxitin (MRSA) was statistically significant between both group samples ($p = 0.047$). In the other words, the frequency of MRSA isolates from CSIs was approximately 2 times that of NSIs. It is obvious that due to the increased use of an-
tobiotics in hospital settings, the selective pressure on hospital-acquired infectious agents such as *S. aureus* is higher than the acquired infectious agents from the community (21). Methicillin-resistant staphylococci have been reported in the UK since 1961 (22), and today its spread in community and health care settings is considered a global concern and is associated with significant mortality and morbidity. These resistant strains have been isolated from various CSIs as well as from NSIs (23-25). Based on the CLSI recommendations, the disk diffusion method using cefoxitin disk (30 mg) has a higher sensitivity to detect MRSA phenotypes (19, 26). The results of our study showed that 14% and 36% of *S. aureus* isolated from NSIs and CSIs were methicillin-resistant (MRSA), respectively.

In the Middle East, nasal carriers for MRSA have been reported to be between 0%-13.2% (23). Mamishi et al. in Tehran reported that the prevalence of MRSA isolated from NSIs and CSIs were 14.9% and 48%, respectively (27). Also, in the study by Wongboot et al. 47% (70/149) of the CSIs and 3% (1/37) of the NSIs were MRSA(28). According to the CLSI guidelines, the criterion for resistance to methicillin is a MIC greater than 4 mg/l for oxacillin or the existence of the *mecA* gene (29). Therefore, in the current study, all isolates were evaluated for the presence of the *mecA* gene by the PCR method. The results of the frequency for the *mecA* gene in various studies such as Rajabiani et al. in Tehran (36.3%), and Hoseini Alfatemii et al. in Shiraz (42.3%) were in agreement with our results (36% of the CSIs and 14% of the NSIs) (30, 31). The occurrence of the *mecA* gene among CSIs compared with NSIs was statistically significant. Selective pressure from the widespread use of β-lactams in hospital systems, as well as compliance with hospital-acquired infectious disease control programs by staff, can explain such discrepancy. MRSA phenotypes are mainly created by the acquiring of the *mecA* gene, and in fewer cases by PBP4 and the *ica* gene (32). In this study, 3 clinical isolates were susceptible to cefoxitin (MSSA) but were *mecA* carriers. In previous studies, similar results have been reported for the discrepancy between phenotype and genotype of MRSA strains (33, 34). For the characterizing of such isolates, Hososaka et al. classified oxacillin susceptible and *mecA*-positive phenotypes (OS-MRSA) as new types of MRSA, which have distinct characteristics from MRSA acquired from the community or hospital (29).

Our PCR results revealed that 30%, 0%, and 6% of the CSIs and 26%, 2% and, 8% NSIs were carriers for the *sea*, *seb*, and *sea*+*seb*, respectively. These results are consistent with those of the study by, Wongboot et al. (2013), which stated that of 149 isolates of *Staphylococcus aureus* obtained from the clinical samples, 45%, 11%, and 5% were carriers of the *sea*, *seb*, and *sea*+*seb* genes, respectively. Besides, it is reported that *S. aureus* isolated from nares, carried 38%, 8%, and 5%; the above genes, respectively (28). Despite our results, Peck et al. (2006), found the most common enterotoxin gene in *S. aureus* taken from blood samples was *seg* gene (41/70, 58.6%), while for nasal isolates was *sei* (67/95, 70.5%) (35). The results of our study demonstrated that 36.3% and 34.6% of isolates of MRSA and MSSA harbored at least one of the tested enterotoxin genes, respectively; but this difference wasn’t significant. These results are not consistent with those of the study by Wongboot et al. (2013), in which the prevalence of enterotoxin genes

| Source of isolate | No. of isolates tested by disk diffusion with CFX | PCR for *mecA* | PCR for enterotoxin genes |  |
|------------------|-------------------------------|-----------------|--------------------------|  |
|                  | No. of positive | No. of negative | *sea* | *seb* | *sea*+*seb* |
| Clinical n=50    | 15 resistant (MRSA) | 15 | 0 | 6 | 0 | 1 |
|                  | 35 susceptible | 3 | 32 | 9 | 0 | 2 |
| Nasal n=50       | 7 resistant (MRSA) | 7 | 0 | 0 | 0 | 1 |
|                  | 43 susceptible | 0 | 43 | 13 | 1 | 3 |
| Total            | 100 | 25 | 75 | 28 | 1 | 7 |

1CFX, cefoxitin (30 µg)
2*sea* gene was more frequent in clinical than nasal strains ($X^2 = 3.85; p= 0.049$)
3$X^2 = 2.163; p= 0.141$
among MRSA was higher than MSSA (28). It should be noted that the differences between toxin profiles among various samples and studies could be due to the existence of genes of staphylococcal enterotoxins on various genetic elements, such as bacteriophage, plasmid, and pathogenic islands. It is evident that the difference in the genetic content could affect the prevalence of these genes. On the other hand, the distribution of the genes of enterotoxin A and B varies in different genetic clones. Cha et al. proved that the main genetic clones containing the sea gene were ST59, ST30, ST1 (35, 36).

Therefore, it is not to be expected that our tested isolates would have different clones in terms of genetic background, which requires more complementary experiments. In addition, Staphylococcus aureus toxins profile could be completely variable based on the geographical location (the spread of specific clones), origins of sample (clinical, nasal), and even the site of isolation (blood, ulcer, etc.). As far as the authors know, this is the first study to evaluate the frequency of the mecA gene and enterotoxins of S. aureus in two different clinical and nasal populations in Khorraramabad city. However, the limited sample size, lack of additional examinations such as the minimum inhibitory concentration (MIC) for oxacillin, the molecular typing, and the determination of the clonality of isolates were the limitations of this study.

In summary, the results of this study proved that the frequency of the mecA gene in clinical specimens is more common than those isolates from nares of clinical workers in selected hospitals in Khorraramabad, and the gene is found in about one-third of the samples. It is assumed, selective pressure of antibiotics on clinical strains significantly increases the frequency of genetic exchanges between bacteria in hospital settings. It is obvious, in addition to transferring of resistance-encoding genes, some of the virulence-associated genes may also be transmitted. Therefore, the screening of hospital staff for S. aureus carriage, training of patients and medical staff, adherence to the principles of hospital infections controlling, and hygienic conditions in contact with physicians or tools among personnel and patients, particularly the careful observance of food hygiene principles in cooking and handling can help to prevent and reduce the S. aureus infections and intoxications. One of the limitations in the current experiment was the small sample size. Therefore studies should be carried out using more samples to confirm the results.

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