Distribution of Genes Encoding Resistance to Macrolides Among Staphylococci Isolated From the Nasal Cavity of Hospital Employees in Khorramabad, Iran

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Abstract

Background: Epidemiological data on antibiotic susceptibility of Staphylococcus strains isolated from nasal carriers in each region can be helpful to select appropriate drugs to eradicate carriage states, control nosocomial infections and also treat patients.

Objectives: The current study aimed to investigate the antibiotic resistance profile and the molecular prevalence of the ermA, ermB, ermC and msrA genes among Staphylococcus strains isolated from the anterior nares of hospital employees.

Patients and Methods: In this cross-sectional study, a total of 100 Staphylococcus isolates, 51 Staphylococcus aureus, 49 coagulase-negative staphylococci (CoNS) were isolated from the anterior nares of hospital employees in Khorramabad, Iran. Susceptibility pattern to macrolide antibiotics were determined using the disk diffusion method. The polymerase chain reaction (PCR) assay was applied to determine the major erythromycin-resistant genes (ermA, ermB, ermC and msrA).

Results: Fifty-three (53%) isolates were simultaneously resistant to erythromycin, azithromycin and clarithromycin (cross-resistance); while 8 (8%) isolates had variable macrolide susceptibility pattern. Among the S. aureus isolates, the difference in prevalence of resistance to erythromycin between males and females was significant (P = 0.011). The frequency of ermA, ermB, ermC, and msrA genes were 3%, 5%, 33% and 20%, respectively. It was also found that out of 53 isolates resistant to erythromycin, 44 (83%) Isolates (eight S. aureus and thirty-six CoNS strains) carried at least one of the four tested genes. Eight (8%) isolates had intermediate phenotype to erythromycin, in which 4 (50%) isolates carried ermB or ermC genes. In addition, out of 39 erythromycin-susceptible isolates, 3 (7.7%) isolates were positive for ermB or ermC genes.

Conclusions: No entire association was found between genotype and phenotype methods to detect macrolides-resistant isolates. In addition, distribution of genetically erythromycin-resistant isolates is geographically different among staphylococci. It is recommended removing S. aureus from nasal carriers by proved approaches such as local or systemic administration of effective antibiotics or bacterial interference.

Keywords: Coagulase-Negative Staphylococci, Erythromycin, Nasal, PCR, erm, Staphylococcus aureus

1. Background

Staphylococcus aureus and coagulase-negative staphylococci (CoNS) are well known to most clinicians as a cause of nosocomial and community-associated infections worldwide (1, 2). Staphylococci are able to develop resistance to many routinely used antibiotics to which they were susceptible first (1). Moreover, this condition has become more complicated because of various factors such as horizontal acquisition of foreign genetic elements and ability to establish persistent infections (e.g. biofilm formation) in the host. Nowadays, due to wide spread use of indwelling and prosthetic medical devices, CoNS have gained much attention as considerable agents involved in nosocomial infections (3). A major part of these infections are caused by the transmission of the bacteria from hospital employees as reservoir to patients. In particular, this situation can lead to serious infections in immunocompromised patients who are hospitalized with underlying diseases (4-8). Macrolides including erythromycin are the antibiotics used against Gram-positive and some Gram-negative bacteria. Three mechanisms in Gram-positive bacteria that result in resistance to erythromycin are as follows: (I) modification in the ribosomal target site, mediated by the methyltransferases encoded by erm (erythromycin resistance methylase) genes, (II) efflux pump encoded by msrA/B (macrolide specific resistance genes) and (III) ereA/B (erythromycin esterases) genes (9-12). Among these mechanisms, the erm encoded methy-
lases are the major factor of resistance to macrolides. Among many reported and sequenced *erm* genes, three major genes of *ermA, ermB* and *ermC* are present in staphylococci (12, 13). Due to the development of resistant strains, epidemiological data on antibiotic susceptibility of *Staphylococcus* strains isolated from nasal carriers in each region can be helpful to select appropriate drugs to eradicate carriage states, control the nosocomial infections and also treat the patients (14, 15).

2. Objectives

The current study aimed to investigate the antibiotic susceptibility profile and the prevalence of the *ermA, ermB, ermC* and *msrA* genes among staphylococcal strains isolated from the nares of hospital personnel in Khorramabad, Iran.

3. Patients and Methods

3.1. Isolation and Identification of Nasal Staphylococci

During this cross-sectional study, a total of 340 volunteers from hospital staff (males and females), employed in various wards of four referral and state hospitals (Shohaday-e-ashayer, Asalian, Shahid Madani and Taemin) in Khorramabad city (west of Iran) were included and screened for nasal carriage of *S. aureus* from July 2011 to January 2012. The subjects with a history of any antibiotic consumption for at least ten days at the time of sampling were excluded. The sample size was estimated via formula to calculate the ratio with an accuracy of 0.05 (d = 0.05).

\[ n = \frac{z^2 \times P(1-P)}{d^2} \] (1)

To collect samples, the convenience sampling method was used. Three hospitals under study were general (400, 350 and 150 beds) and one hospital was specialized (100 beds). Finally 100 adults of both genders (40 males and 60 females) were enrolled. Information about variables including gender, work experience and the ward which hospital staff worked was collected by a questionnaire. The samples were collected from both anterior nares of the subjects by rotating a sterile cotton wool swab. The swabs were placed and transported in 3 mL tryptic soy broth (Merck, Germany) and incubated at 37°C. After overnight incubation, a loop full of inoculated broth medium was subcultured on 10% sheep blood agar, mannitol salt agar and nutrient agar (Merck, Germany) (16). Presumptive staphylococcal colonies (growth on mannitol salt agar, Gram-positive and catalase-positive cocci) were tested further for the production of coagulase and DNase enzymes according to the standard bacteriological procedures (17). Isolates with positive reactions (coagulase-positive and DNase-positive) were identified as *S. aureus*; other staphylococcal species with negative test results designated as CoNS. Duplicate isolates from the same subject were excluded.

3.2. Bacterial Susceptibility Testing

Susceptibility of *Staphylococcus* isolates to antibiotics (Mast, Merseyside, United Kingdom) including erythromycin (15 µg), clarithromycin (15 µg), azithromycin (15 µg), cefoxitin (30 µg), penicillin G (10 U), vancomycin (30 µg), tetracycline (30 µg), clindamycin (2 µg), trimethoprim/sulfamethoxazole (1.25/23.75 µg) and rifampin (5 µg) was determined by the Kirby-Bauer disk diffusion method on muller-hinton agar, according to the procedures described by the clinical and laboratory standards institute (CLSI) (18).

3.3. DNA Extraction

Total DNA (consisting of the chromosomal and the plasmid) of all the isolates was extracted and collected using AccuPrep® Genomic DNA extraction kit (Bioneer, Korea) with some modifications. Briefly, a 1 ml of Luria-Bertani broth (LB) bacterial colony of each strain was suspended in 1 mL of lysogeny broth (LB) medium, and incubated at 37°C for 18 hours. The bacterial cells were harvested by centrifugation at 7,500 g for 10 minutes and resuspended in 200 µL of phosphate-buffered saline (PBS) and mixed well. To ensure adequate cell breakage, 10 µL lysostaphin (100 µg/ml in sterile deionized water; Sigma) was added to the bacterial mixture and incubated at 37°C for 30 minutes (19). Then, 10 µL of proteinase K (20 mg/mL) was added to the sample and after incubating at 56°C for 30 minutes, the process was continued according to the manufacturer’s instructions. Finally, DNA was extracted and preserved at -20°C.

3.4. Primers and PCR

The four primer pairs used to amplify the *ermA, ermB, ermC* and *msrA* genes are listed in Table 1 (12). In the current study, multiplex polymerase chain reaction (PCR) amplification was performed for *ermA, ermC* and *msrA* genes as previously described by Zmantar et al. (12) in a 25 µL reaction mixture consisting of: 1X PCR reaction buffer, 2.5 mM MgCl₂, 0.2 mM dNTP, 25 pmol of each of the three primer pairs, 1.2 U of Taq DNA polymerase (Fermentas, Lithuania) and 200 ng DNA template. For *ermB* gene amplification, the *ermB* primers (25 pmol) were added in a separate PCR reaction under the same above-mentioned conditions. Amplification was carried out using a gradient thermal cycler (Bio-Rad, My cycler, US) as the following program: five
minutes at 94°C, 30 cycles of amplification, consisting one minute at 95°C, 0.5 minute at 55°C and two minutes at 72°C, ending with 10 minutes at 72°C (final extension). To verify amplification, 5 µL of PCR products were subjected to agarose gel electrophoresis (1.5% agarose, 1X TAE, 100V). Ethidium bromide-stained DNA fragments were then visualized on a UV transilluminator at 300 nm in comparison with a 50-bp molecular size standard ladder (Fermentas, Lithuania).

To verify the authenticity of the amplicons, PCR products of three positive samples containing one of the genes of ermB, ermC or msrA as representative were subjected to sequencing (Macrogen, Korea). The resulting sequences were aligned with corresponding sequences in the GenBank database using at the national center for biotechnology information (NCBI) BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi? program = blastn and PAGE-TYPE = blast search and LINK-LOC = blasthome). To minimize the random errors, all of the devices were calibrated before use. All observations were carried out by one microbiologist.

3.5. Statistical Analysis

Descriptive data were presented as frequencies and percentages via SPSS software version 16. Chi-square test and T-test were used to determine any significant differences between prevalence of the tested genes among S. aureus and CoNS strains. P values less than 0.05 were considered statistically significant.

3.6. Ethical Issues

The study was approved by ethical committee of Lorestan University of Medical Sciences, Khorramabad, Iran (No. 200/49017). All nasal samples were taken from hospital employees volunteered for the study. Informed consent was obtained from all precipitants before sampling. Privacy and confidentiality of personal information were saved and protected.

4. Results

4.1. Staphylococcal Isolates and its Susceptibility Patterns

A total of 51 S. aureus strains were isolated from anterior nares of the 340 screened subjects (i.e. the nasal carriage rate for S. aureus was 15%). Of the 51 S. aureus-carriers, 22 (43.1%) were males and 29 (56.9%) were females. On the other hand, 49 randomly selected CoNS strains, isolated from nares of non-S. aureus carriers [18 males (36.7%), 31 females (63.3%)] were also included and tested in the study. There was no statistical difference between the two groups (S. aureus nasal carriers and CoNS) in terms of gender (P = 0.72). Average work experience for CoNS obtained subjects was 6.4 ± 0.89 years, and within S. aureus-nasal carriers were 4.8 ± 0.68 years. Based on the T-test result, difference in the mean of work experience between the two groups was not statistically significant (P = 0.22). The resistance profile for all isolates to macrolides as well as other tested antibiotics is listed in Table 2. The majority of isolates expressed resistance to penicillin (96%). No resistance to vancomycin was noted. Twenty-nine (29%) isolates were resistant to cefoxitin (eight S. aureus and twenty-one CoNS). Fifty-three (53%) isolates were simultaneously resistant to erythromycin, azithromycin and clarithromycin (cross-resistance); while 8 (8%) isolates had various macrolide susceptibility patterns. For example, two isolates were intermediate to erythromycin, while they were resistant to azithromycin and clarithromycin. In addition, two isolates were found susceptible to erythromycin, while they were resistant to clarithromycin. The frequency of resistance to all tested antibiotics between CoNS and S. aureus isolates were statistically significant (P < 0.001). Distribution of susceptibility phenotypes to erythromycin among the isolates of S. aureus and CoNS according to the gender is shown in Table 3. Statistical analyses showed that among the S. aureus isolates difference in prevalence of resistance to erythromycin was significant between males and females (P = 0.011); while such difference was not significant within CoNS isolates (P = 0.771).

Table 1. Oligonucleotide Sequences Used for Erythromycin-Resistant Genes Amplification

| Resistance Gene | Primers Sequences (5’ → 3’) | Product Size, bp |
|-----------------|------------------------------|-----------------|
| ermA            | Forward TAICTTACGTGAGAGGAT    | 139             |
|                | Reverse CTACACTTGGCTTAGGAAA   |                 |
| ermB            | Forward CITACACTTGGCTTAGGATT  | 142             |
|                | Reverse GTTACTCTTGGCTTAGGAAA  |                 |
| ermC            | Forward CTGTGTGATGGATAITTCC   | 190             |
|                | Reverse ATCTTATAGCAACCCGTATTC|                 |
| msrA            | Forward TCCACTATGACAAACATC    | 163             |
|                | Reverse AAATCGTCCTTATGGTGT    |                 |

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Table 2. Prevalence of Resistance to the Tested Antibiotics Among the Isolates of Staphylococcus aureus and CoNS Using the Disk Diffusion Method 4, 10

| Antibiotics | S. aureus, n = 51 | CoNS, n = 49 | Total, n = 100 |
|-------------|------------------|-------------|--------------|
| Penicillin  | 49 (96.1)        | 47 (96.1)   | 96 (96)      |
| Clarithromycin | 13 (25.5)      | 44 (89.8)   | 57 (57)      |
| Azithromycin | 11 (21.6)        | 44 (88.8)   | 55 (55)      |
| Erythromycin | 10 (19.6)        | 43 (87.7)   | 53 (53)      |
| Tetracycline | 12 (23.5)        | 47 (96)     | 59 (59)      |
| Clindamycin | 9 (17.6)         | 37 (75.5)   | 46 (46)      |
| TS          | 5 (9.8)          | 38 (77.5)   | 43 (43)      |
| Cefoxitin   | 8 (15.7)         | 21 (42.8)   | 29 (29)      |
| Kanamycin   | 7 (13.7)         | 18 (36.7)   | 25 (25)      |
| Rifampin    | 3 (5.9)          | 4 (8.2)     | 7 (7)        |

Abbreviations: CoNS, coagulase-negative staphylococci; TS, Trimethoprim/sulfamethoxazole.

*pValue according to the Chi-square test < 0.001.

4 Values expressed as No. (%).

4.2. Distribution of Macrolides Resistance Genes Using PCR

All of the isolates were screened for the presence of the four genes ermA, ermA, ermC and msrA as the main causative agents of resistance to macrolides. As predicted, the positive isolates for the ermA, ermA, ermC and msrA genes showed 139, 142, 190 and 163 bp bands, respectively (Figure 1). The frequency rate of ermA, ermA, ermC and msrA genes were 3%, 5%, 33% and 20%, respectively; separately illustrated for S. aureus and CoNS isolates in Figure 2. It was found that among the 53 isolates resistant to erythromycin, 44 (83%) isolates (eight S. aureus and thirty-six CoNS) carried at least one of the four tested genes. Of these erythromycin-resistant isolates, nine (approximately 17%) isolates (one S. aureus and eight CoNS) harbored genes of ermC and msrA, and one (1.8%) CoNS isolate also harbored ermA and ermC genes simultaneously. However, combination of three or four genes was not found among the tested isolates (Table 4). Eight (8%) isolates had intermediate phenotype to erythromycin, in which 4 (50%) isolates carried ermA or ermC genes (Table 4). In addition, it was found that out of 39 erythromycin-susceptible isolates, 36 (92.3%) isolates did not harbor any of the four antibiotic resistant genes, but the remaining isolates (three isolates) were positive for ermA or ermC genes, while they were still susceptible to erythromycin (Table 4). Interestingly, of the 29 methicillin-resistant staphylococci, 22 (75.9%) isolates were resistant to erythromycin and 19 (65.5%) isolates harbored at least one of the tested genes.

4.3. Nucleotide Sequence Accession Numbers

Nucleotide sequences were submitted to GenBank under the accession numbers AB937975, AB937976 and AB937977 for the ermA, ermC and msrA genes, respectively.

5. Discussion

Staphylococcus aureus carrier status can lead to nosocomial infections in hospitalized patients (6, 8). On the other hand, CoNS are leading cause agents of opportunistic and device-related infections (3). In the current study, nasal carriage rate for S. aureus was 15%. In several studies performed in various regions of the world, this carriage status in healthcare workers have been reported ranging from 18.6% to 50% (20). Moreover, in Iran, the frequency of nasal carriage among hospital employees is reported approximately 12.7% - 45% (21). Unfortunately, the results of disk diffusion testing showed that all the evaluated isolates were multidrug-resistant. So that, 29% of strains were methicillin-resistant (Table 2). It is a serious threat when a methicillin-resistant strain is transferred and spread among hospitalized patients (therapeutic failure) (22). It was observed that the frequency of resistance among CoNS isolates was significantly higher than those among S. aureus isolates; and this finding was also attributable to resistance to erythromycin and cefoxitin (Table 2). While the majority of the erythromycin-resistant strains had cross-resistance to other macrolides; however eight strains exhibited heterogeneous susceptibility pattern toward macrolide antibiotics (Table 2). It is presumed that this discrepancy is due to alterations in the chemical structure of various generations of macrolides. The association between resistance to methicillin and the presence of tested genes was significant (P = 0.031). The simple explanation for this phenomenon is carrying and transmission of the various adjacent resistance genes on genetic elements such as plasmids. Several studies concerning the distribution of erm genes and efflux pumps are conducted using southern hybridization and PCR techniques (13, 23, 24). Despite previous studies in which have primarily focused on clinical strains of staphylococci (1, 11, 23-28), the current study presents the first report on the occurrence of main genes implicated in macrolides-resistance in Staphylococcus strains obtained from nares of hospital employees. The obtained PCR results demonstrated that the most prevalent gene in both S. aureus and CoNS strains was ermC (33%). Also, the frequency of this gene (ermC) in erythromycin-resistant S. aureus and CoNS were 13.7% and 53%, respectively. These results are consistent with those of the study by Cetin et al. (25) performed with CoNS in Turkey in which a frequency of 30% for ermC was yielded.
Martineau et al. (24) reported an occurrence of 66% for *S. epidermidis* strains. Fluit et al. (9) found that the *ermC* gene is located on a small plasmid; therefore, horizontal transmission of this gene from resistant strains to susceptible strains is unavoidable. Regarding *ermA*, some studies showed that this gene, as the most prevalent gene among clinical strains of *S. aureus*, has a frequency of 21% to 67% (1, 24-27). Similar findings were also published in Denmark, where *ermA* was responsible for 80.6% resistance to erythromycin among 428 tested clinical *S. aureus* strains from 1959 to 1988 (23). In contrast, the current study PCR results demonstrated that *ermA* was not observed in the tested *S. aureus* strains. It was also found that *ermB* gene was carried in a minority of *S. aureus* (7.8%) and CoNS (2%) strains; while previous studies revealed that this gene was primarily originated from animal strains and generally its frequency was low (24-27). However, Zmantar et al. in two different studies (12, 28) reported that *ermB* was the most common gene when they evaluated 81 *S. aureus* strains isolated from human auricular. Finally, *msrA* gene, encoding an ATP-dependent efflux pump (24), was more prevalent in CoNS (41.8%) than *S. aureus* strains (20%). These observations are in line with the findings of Lina et al. (26) who reported the prevalence of *msrA* gene as 14.6% and 2.1% among CoNS and *S. aureus* strains, respectively. Similar data were also obtained (41%) from a study conducted by Zmantar et al. (28) when 77 clinical CoNS strains were investigated. Some staphylococcal strains in the current study showed discrepancies between the genotype and phenotype. So that, three strains (two *S. aureus* and one CoNS) harbored *ermB* or *ermC* gene, while they were susceptible to erythromycin. Zmantar et al. (28) and Coutinho et al. (1) also hypothesized that this phenomenon may be caused by down-regulation or mutation in promoter region of *erm* genes. On the other hand, Martineau et al. (24) demonstrated that such strains carrying the resistant genes would exhibit phenotypical resistance, upon sub-culturing in media with increasing gradients of the corresponding antibiotic. Moreover, nine *Staphylococcus* isolates were resistant to erythromycin, but did not carry any of the tested erythromycin-resistant genes. Authors presume that other variants of *erm* genes or efflux pump (*msrB*) involve in this subject, which were not evaluated in the study (1, 24, 29, 30). Although the current study is...
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Figure 1. Gel Electrophoresis of DNA Fragments generated by PCR of \textit{erm} and \textit{msrA} genes in Erythromycin-Resistant Staphylococci Isolated From the Nares of Hospital Employees in Khorramabad, Iran

A, Multiplex PCR amplification of \textit{ermA}, \textit{ermC} and \textit{msrA} genes; source of DNA for lanes: 1, negative control strain of erythromycin-sensitive \textit{Staphylococcus aureus} ATCC 25923; lanes 2, 4 and 7, three different \textit{S. aureus} isolates (\textit{ermC}); lane 3, a coagulase-negative staphylococci (CoNS) isolate (\textit{ermA}); lanes 5 and 6, two different CoNS isolates contain \textit{ermC} and \textit{msrA} genes simultaneously; lane 8, a CoNS isolate contains \textit{msrA} gene. B, PCR products resulted from amplification of \textit{ermB} gene; lanes 1 and 2, two different \textit{S. aureus} isolates contain \textit{ermB} gene; lane 3, \textit{S. aureus} ATCC 29213 (negative control); lanes M, DNA molecular size marker (50-bp ladder; Fermentas, Lithonia); the electrophoresis was run on 1.5% agarose gel, stained with ethidium bromide.

the first report on the occurrence of genetic determinants responsible for macrolide resistance among \textit{Staphylococcus} strains originated from hospital employees; however, lack of further in vitro investigations, such as determining the minimum inhibitory concentration (MIC) for erythromycin, detection of other genes involved in macrolide
resistance and limited sample size, were the weakness of the current study. In conclusion, no entire association was found between genotype and phenotype methods to detect macrolides resistance. In addition, distribution of genetically erythromycin-resistant isolates is geographically different among staphylococci. It is recommend controlling S. aureus in nasal carriers by proved approaches such as local or systemic administration of effective antibiotics or bacterial interference. In addition, to prevent CoNS-associated nosocomial and indwelling medical device infections, it is recommended that healthcare workers wash hands and avoid touching their noses during the patient examination, use sterile masks and gloves and finally, insert prosthetic and catheter devices aseptically.

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Footnotes

Authors' Contribution: The concept, design, study supervision, PCR set up, manuscript preparation and critical revision of the manuscript: Gholam Reza Goudarzi; sample collection, culture and performance of PCR: Farzad Tahmasbi; statistical analysis of data: Khatereh Anbari; sample collection in women's hospital: Masoumeh Ghafarzadeh; all authors have read and approved the final manuscript.

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