

Survey of *ermA*, *ermB*, *ermC* and *mecA* genes among *Staphylococcus aureus* isolates isolated from patients admitted to hospitals in Tehran, Iran by PCR and sequencing.

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Abstract

Staphylococcus aureus is one of the gram positive bacteria that has created many problems in treatment. The antibiotic resistance is an important problem and debatable topic in the world. In recent years, because of overuse of antibiotics and transition of resistance genes, frequency of resistant staphylococcal infections, are increasing. Clindamycin inductive resistance causes failure in treatment. The aim of current study is detection of clindamycin inductive resistance *S. aureus* isolates among patients admitted to Tehran hospitals by multiplex PCR. A total of 80 isolates of *S. aureus* were collected from hospitalized patients in Tehran. The antibiotic susceptibility tests were applied by MIC and disk diffusion methods. The identification of clindamycin inductive resistance isolates was performed by D-zone test. To detection of *ermA*, *ermB*, *ermC* and *mecA* genes, multiplex PCR was administrated. In current experiment, among 80 isolates, resistance rate to erythromycin and clindamycin were 70% and 45% respectively. By D-zone test, 15 samples were positive. The frequency of *ermA*, *ermB* and *ermC* genes in *S. aureus* isolates were 5%, 7.5% and 10% respectively. The results of this study demonstrated that the antibiotic resistance is a main problem in patient's treatment. By identification of resistant isolates and apply appropriate treatment, can be somewhat prevent from outspread of resistant isolates.

Keywords: Methicillin resistance *Staphylococcus aureus* (MRSA), Clindamycin inductive resistance, D-zone test.

Accepted on January 2, 2019

Introduction

Staphylococcus aureus species are known as human pathogens which cause skin and soft tissue infections, acute septicemia, pneumonia and toxic shock syndrome [1,2]. These organisms are resistant to most of drugs and constant against most of disinfectants agents [3]. Nowadays, antibiotic resistance of *S. aureus* is a major problem in society [4]. Different types of antibiotic resistance mechanisms have been found in *S. aureus* that beta-lactamase producing is the most common which associated gene with enzyme production is located on the plasmid. Resistance to nafcillin by *mecA* gene which is located on chromosome, resistance to vancomycin (vancomycin and nafcillin resistance genes exist in these strains) and plasmid resistance to tetracycline, erythromycin and aminoglycosides are the types of resistance mechanisms [5,6]. Resistance to nafcillin, methicillin and oxacillin is independent from beta-lactamase producing [7,8]. The methicillin resistant *S. aureus* (MRSA) is a problem in development of hospital infections. A study has shown that 38.6% of *S. aureus* which isolated from hospitalized patients in Shariati hospital and Tehran Children's Medical Center are MRSA isolates [9]. MRSA are resistant to certain types of oxacillin antibiotics (nafcillin, methicillin, oxacillin and cloxacillin) and all of the beta lactam antibiotics

such as penicillin, amoxicillin and cephalosporin's [10]. The *Staphylococcus* which are resistant to erythromycin show cross-resistance with macrolides (spiramycin, azithromycin and clarithromycin), lincosamides (clindamycin and lincomycin) and type B streptogramin. Increasing of MLS (Macrolide-Lincosamide-Streptogramin) resistant strains in clinical specimens, indicates increasing of clindamycin utilization [11]. Erythromycin and clindamycin are different classes of antibiotics which bind to 50S ribosomal subunit of bacteria and inhibit protein synthesis. In *Staphylococcus* resistance to these antibiotics is create by methylation of target site on ribosome that mostly related to methylase gene *erm* (rRNA) [12]. The *ermA*, *ermB* and *ermC* are types of *erm* gene which discovered in *Staphylococcus* [12,13]. The *ermA* as a chromosomal gene is the most common and *ermB* which is located on plasmid have not found in primates. The frequency of these plasmids (85-98%) increases by use of erythromycin and clindamycin. 95% of acne causing *S. aureus* isolates which are under influence of clindamycin have *ermC* gene [4]. Also, resistance to macrolides can occur through efflux which is dependent to *mrsA* gene, the other resistance mechanism is inactivation of lincosamides by chemical alterations that related to *inuA* gene that rarely occurred [12,13]. The routine antibiotic susceptibility tests for *S. aureus* with inducible

resistance, demonstrate the resistance to erythromycin and susceptibility to clindamycin. Thus, clindamycin therapy may be given for *erm* and *mrsA* mutants, which ultimately leads to failure in therapy [12]. On the other side, clindamycin is one of the most commonly used antibiotics, especially for treatment of skin and soft tissue infections caused by *S. aureus* and is a supersede drug in patients with allergies to penicillin [14]. Induced resistance to clindamycin in *S. aureus* is common which is not detected by routine tests and according to CLSI (Clinical and Laboratory Standards Institute) guidelines, D-zone test is the best method to identify of clindamycin induction resistance [4,15]. For detection of inducible clindamycin resistance, the standard test is D-zone test. In routine antimicrobial susceptibility tests, those which are clindamycin inducible resistance, emerge as erythromycin resistant and clindamycin susceptible [16]. Resistance rate to erythromycin class is so fast, so these medications should not be used alone for the treatment of chronic infections [17]. This test as a simple and reliable test with high sensitivity and specificity, detects clindamycin inductive resistance in clinical laboratories [2]. Placing erythromycin and clindamycin disks at a distance of 15-20 mm from each other and formation of a flat region close to erythromycin disk demonstrates clindamycin inductive resistance which indicates the presence of *erm* gene [17]. Therefore, providing a useful solution to use the appropriate treatment regimen by determination of the antibiotic resistance pattern in the target population is necessary. The D-zone test can be an acceptable method to identification of clindamycin inductive resistance of *S. aureus* isolates. The aim of current study is detection of inducible clindamycin resistance *S. aureus* isolates among patients admitted to Tehran hospitals by multiplex PCR. Also due to cheapness, being simple and importance of inductive resistance detection in effective treatment of *S. aureus*, is recommended.

Methods and Materials

In this descriptive study, by referring to the clinical laboratories of the hospitals affiliated to the Shahid Beheshti University of Medical Sciences, the *Staphylococcus* isolates were collected and transmitted to the Microbiology Lab in Shahid Beheshti University of Medical Sciences. To confirm *S. aureus* isolates, gram stain, β -hemolysis on blood agar, growth on mannitol salt agar, catalase, coagulase and DNase tests were applied [18]. According to CLSI guidelines (2015), susceptibility of the isolates to erythromycin, clindamycin, oxacillin, trimethoprim, sulfamethoxazole and tetracycline (Mast Co. UK) with disk diffusion method on Mueller Hinton agar (Merck Co. Germany) was performed. *S. aureus* ATCC25923 was used as a quality control [19]. Determination of MIC (Minimum Inhibitory Concentration) for vancomycin was performed based on CLSI guidelines. *S. aureus* suspension in BHI (Brain Heart Infusion) broth with 0.5 McFarland concentration was prepared and inoculated on Mueller Hinton agar (Merck Co. Germany). The clindamycin 2 μ g and erythromycin 15 μ g disks, were placed at a distance of 12 mm from each other and after incubation for 18-24 h in 35°C, preventive zone diameter was measured. D-shaped zone of clindamycin from the side of

erythromycin disk was carefully investigated. Extraction of DNA was performed by GeNet Kit (Cat. No. K-3000). To investigate the quality of extracted DNA, in terms of quantity all samples were checked by WPA Bio-wave II Nano spectrophotometer at 260/280 wavelength. Multiplex PCR was performed for *ermA*, *ermB* and *ermC* genes by master mix 2X (Sina Colon Co. Cat. No. PR901638) which included the following instances: 0.4 mmol of each dNTP, 3 mmol MgCl₂, 0.08 U/ μ l Taq-polymerase. The primer sequences for *ermA*, *ermB* and *ermC* genes were as follows respectively: *ermA* F: AGCGGTAAACCCCTCTGA, *ermA* R: TTCGCAAATCCCTTCTCAAC with, *ermB* F: CCGTTTACGAAATTGGAACA GGTAAGGGC, *ermB* R: GAATCGAGACTTGAGTGTGC and *ermC* F: AATTGGCTCAGGAAAAGGGC, *ermC* R: AAATCGTCAATCCCCGCATG. The PCR product size for *ermA*, *ermB* and *ermC* genes was 190, 360 and 564 bp respectively. The primer sequences were checked by Gene Bank (Blast) and finally the PCR program were as follows: Initial denaturation at 94°C for 5 min for one cycle, secondary denaturation at 94°C for 45 s, the annealing at 58°C for 45 s, the extension at 72°C for 45 s for 36 cycles and final extension at 72°C for 5 min for one cycle. The PCR products were analysed by electrophoresis on 1.5% agarose gel in TBE (Tris-Borate-EDTA), then the gel was stained by ethidium bromide (10.5 μ g/ml) afterward the results were checked by Gel Doc in the wavelength of 280 nm.

Results

In this descriptive study, from October 2015 to November 2016, samples collection was performed from patients referred to Tehran hospitals. After identification by biochemical and microbial tests, 80 isolates were approved as *S. aureus*. 75% of samples belong to males and 25% of them referred to females. 45 of isolates were collected from urine, 10 isolates from wound, 5 isolates from tracheal, 4 isolates from sputum, 10 isolates from blood and 6 isolates from other clinical specimens were collected (Table 1). Among 80 isolates of *S. aureus* the resistance rate to erythromycin was 70%, to clindamycin was 45%, to tetracycline was 50%, to cotrimoxazole was 35% and 90% were resistant to oxacillin (Figure 1). By MIC and disk diffusion results, all of the isolates were susceptible to vancomycin (Figure 2). The D-zone test results demonstrated 15 isolates were positive phenotypically (Figure 3). Among these isolates 55 of them were MRSA (including *mecA* gene by PCR) and 45 isolates were methicillin susceptible *S. aureus* (MSSA) which confirmed by cefoxitin disk diffusion agar. The *ermA*, *ermB* and *ermC* genes were detected in 8 (10%), 6 (7.5%) and 4 (5%) of isolates respectively. Also, 2 isolates had all three genes simultaneously.

Table 1. Frequency of collected samples.

Origin of samples	Number of samples
Urine	45

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Wound	10
Blood	10
Tracheal	5
Sputum	4
Other clinical specimens	6
Total	80

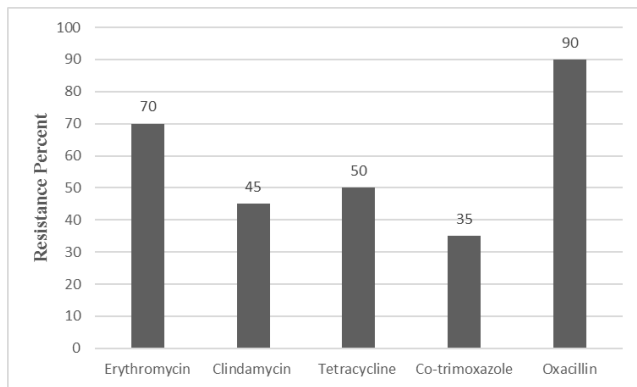


Figure 1. Comparison of the antibiotic resistance rate.

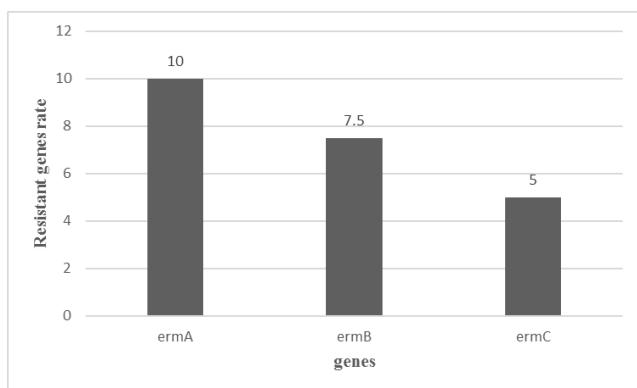


Figure 2. Frequency of resistance genes.

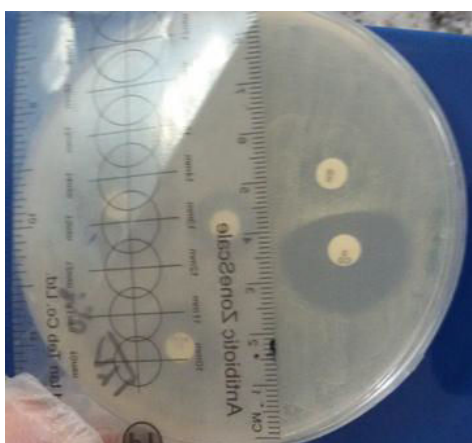


Figure 3. D-zone test on *Staphylococcus aureus* isolates.

Discussion

According to the results of other studies in recent years, *S. aureus* is one of the most common gram positive bacteria which is involved in nosocomial infections. Treatment of infections caused by *S. aureus* is difficult and causes mortality in hospitalized patients. Due to ability of *S. aureus* isolates to acquire resistance and overuse of antibiotics, resistance rate is increasing in this bacteria. In current study, 70% of isolates were resistant to erythromycin, 45% to clindamycin, 50% to tetracycline, 35% to co-trimoxazole and 90% to oxacillin and D-zone test of 15 isolates were positive. According to study by Sasirekha et al. in India, 153 isolates of *S. aureus* were collected and antibiotic susceptibility tests for cefoxitin, oxacillin and D-test to determination of clindamycin inductive resistance were performed. The results showed that 42 (45.27%) of isolates were resistant to methicillin, 43 of isolates were susceptible to clindamycin and 63 (17.41%) of isolates were resistant to erythromycin which 14 of them (15.9%) showed inductive resistance, 29 (95.18%) of isolates had real sensitivity to clindamycin and 20 (13%) of isolates showed intrinsic resistance to clindamycin [20]. The rate of resistance in this study was less than current experiment that reason of this difference can be sample size, the type of antibiotic used in that area or difference in year of sampling. As regards the rate of antibiotic resistance is increasing every year, in a study by Ansari et al. in Nepal, 15718 clinical specimens from blood, urine, sputum and pus were collected, the antibiotic susceptibility tests and identification of resistant isolates to methicillin by cefoxitin, oxacillin disks and identity of inductive resistance to clindamycin by D-zone test, showed 306 of isolates were *S. aureus* and all of the isolates were susceptible to vancomycin and teicoplanin. Resistance to methicillin by cefoxitin disk in 43.1% and resistance to oxacillin in 39.2% of isolates were observed which 100 of isolates were resistant to erythromycin and inductive resistance of clindamycin was reported in 38 (12.4%) of isolates [21]. The rate of resistance to erythromycin was more than current study that it depends on antibiotic prescribing in those regions, but the rate of resistance to clindamycin were consist with current study. In other study by Chaturvedi et al. in India in, 6468 different clinical specimens were collected and resistance to mupirocin by E-test and agar dilution methods and inductive resistance to clindamycin by D-test were determined. The results of this study showed that 82 of isolates were resistant to methicillin and 15 (18.3%) of isolates were resistant to mupirocin that 8 isolates (53.3%) had high level resistance and 7 isolates (46.7%) had low level resistance and 4 isolates among resistant isolates to methicillin and mupirocin had inductive resistance to clindamycin [22]. Also in other study by Juayang et al. in Philippines, 94 infections caused by *S. aureus* were detected during 2010-2012 that 38 (40.6%) had methicillin resistance and 37 (39.4%) showed clindamycin inductive resistance. The most samples (71.05%) were wound and abscess specimens with methicillin resistance, while in blood specimens 5.3% was reported. The results of antibiotic susceptibility tests showed that all of the isolates (100%) to linezolid, 95% to tetracycline and 0% to penicillin G were

susceptible [23], that the rate of resistance to clindamycin was somewhat similar to the current study. Iraj et al. in Hamedan, Iran, 520 nasal swabs from children under the age of 12 were collected and antibiotic susceptibility tests by disk diffusion (for oxacillin, erythromycin, clindamycin, cefazolin, vancomycin and D-test) showed that 118 (22.3%) of children had *S. aureus* and just one isolate showed methicillin resistance. Also 5% of these isolates acquired from community, 6.3% of them acquired from hospital and had clindamycin inductive resistance [24] that the rate of resistance was less than present study. The reason of this different can be attributed to the surveyed community, because resistance rate is lower in kids. In a study by Fatemeh et al. in Isfahan, Iran, 585 isolates of *S. aureus* were collected from 3 clinical centers in Tehran (2005-2006), antibiotic susceptibility tests by disk diffusion for 13 antibiotics, MIC test for methicillin (according to broth micro dilution and PCR) to detection of *mecA* gene was performed, which in total 321 (54.7%) isolates identified as *S. aureus* and were resistant to kanamycin (88%), cefotaxime (65%), methicillin (66%), oxacillin (88%), ampicillin (100%), erythromycin (41%), clindamycin (38%), sulfamethoxazole-trimethoprim (41%), vancomycin (0%), chloramphenicol (40%), ciprofloxacin (93%), gentamycin (20%) and tetracycline (64%). All of the isolates were resistant to methicillin and 63% of isolates with interstitial resistance had *mecA* gene [25]. The resistance rate to erythromycin and clindamycin in this study was less than current study which is related to the time of sampling that by passing the time, due to acquire of resistance elements to antibiotic, resistance of isolates has increased. Also in Razavi et al. experiment in Karaj, Iran, 130 isolates of *S. aureus* were collected from blood, tracheal aspirate, urine and wound specimens and antibiotic susceptibility test was performed by disk diffusion method also presence of *mecA* gene was detected by molecular tests. They showed that 100 of isolates were *S. aureus* and 56% of them had *mecA* gene which indicates the high prevalence of methicillin resistance in Iran, also 45.4% of methicillin resistant isolates at least were resistant to three classes of antibiotic and just 4 isolates among 130 isolates were susceptible to all of the tested antibiotics and all of the *S. aureus* isolates were susceptible to vancomycin and resistant to penicillin, 58% to methicillin, 44% to erythromycin, 60% to gentamycin, 56% to ciprofloxacin, 42% to ceftriaxone, 16% to rifampin and 53% to amikacin [26]. Sample type, sampling time, type of the disk which used and sample size are instances that cause alterations in antibiotics percentage. In current experiment among 80 isolates *ermA*, *ermB* and *ermC* genes were detected in 10%, 7.5% and 5% of isolates respectively. In other study by Ghanbari et al. in Isfahan, they were found that 4 isolates had *ermC*, 2 isolates had *ermB* and 1 isolate had *ermA* [26], that current study was similar to this experiment. Also in a study by Mousavian et al. in Ahwaz, 41.1% of isolates had *ermA*, 17.7% had *ermC* and none of the isolates had *ermB* gene [28,29]. The prevalence of some of these genes in this study was much more than current experiment which the reason of these differences can be the region and time of sampling.

Conclusion

The prevalence of antibiotic resistance in current study has been of concern, therefore control of infections, preventing from dissemination of resistant bacteria, need to have accurate management in drug prescription and identification of resistant isolates. Also due to cheapness, being simple and importance of inductive resistance detection in effective treatment of *S. aureus*, it's recommended that this test be performed in laboratory routinely that will prevent from reporting of resistant isolates instead of susceptible isolates.

Acknowledgment

This study was funded by Research Department of the School of Medicine, Shahid Beheshti University of Medical Sciences (grant No 6049).

Ethical Statement

The present study was acknowledged by the Ethics Committee of Shahid Beheshti University of Medical Sciences with reference number IR.SBMU.MSP.REC.1396.378.

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