Identification of inducible clindamycin resistance in *Staphylococcus aureus* methicillin resistance from clinical isolates by d-zone test

Saadat S¹, Solhjoo K²*, Kazemi A², Mardaneh J³

Received: 05/08/2013 Revised: 08/18/2013 Accepted: 10/05/2013

1. Young Researchers Club, Islamic Azad University, Jahrom Branch, Jahrom, Iran
2. Dept. of Microbiology, School of Medicine, Jahrom University of Medical Sciences, Jahrom, Iran
3. Professor Alborzi Clinical Microbiology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

Journal of Jahrom University of Medical Sciences, Vol. 11, No. 4, Winter 2014

**Abstract**

**Introduction:**
The increasing incidence of infections caused by strains of *Staphylococcus aureus*, especially methicillin-resistant (MRSA), has led to the increased use of effective antibiotics such as clindamycin and erythromycin for treatment of systemic and local infections caused by this organism. However, concern over the possibility of emergence of clindamycin resistance during therapy has discouraged some clinicians from prescribing it. The aim of this study was to identify the induced clindamycin resistance in clinical isolates of methicillin-resistant *Staphylococcus aureus*.

**Materials and Methods:**
In this cross-sectional study, 100 *S.aureus* strains isolated from clinical specimens were collected from laboratories in Shiraz (Shahid Faghihi, Nemazi and MRI) hospitals. Re-identification of the isolates was performed by conventional microbiological and biochemical tests. Methicillin resistant strains were selected by disc diffusion method, and inducible clindamycin resistance in these strains was identified using D-zone Test.

**Results:**
The result of susceptibility testing showed that out of 100 *Staphylococcus aureus* samples 44 isolates (44%) were resistant to methicillin. Forty-six percent of the isolates were resistant to erythromycin and 51% to clindamycin.

**Conclusion:**
The results showed that performing D-zone test in clinical laboratories for identification of induced clindamycin resistance, reporting the results to the physician for prescribing these two antibiotics for treatment of infections caused by inducible clindamycin resistant strains, and replacing the appropriate treatment regimen are essential measures to be taken.

**Keywords:** Staphylococcus aureus, Clindamycin, Meticillin, Drug Resistance

**Introduction**
*Staphylococcus aureus* is a major cause of nosocomial infections (1-2). Macrolide-Lincosamide- Streptogramin B (MLSB) antibiotics are currently being widely used for the treatment of Staphylococcal infections (3). This group inhibits protein synthesis by binding to the 50s ribosomal subunit (4-5). Clindamycin is a drug classified under this group and is the preferred drug for treating certain skin and soft tissue infections and serious staphylococcal infections (6-7).
Clindamycin is an oral drug that can penetrate the skin and its structures better, does not require renal balance and accumulates in abscesses; therefore, it has a positive effect on treatments; unlike in β-lactam drugs, high microbial populations cannot inhibit clindamycin (3, 8-9).

Lincosamide resistance mechanisms are developed in three ways: (1) Reducing permeability or drug rejection (efflux), which causes resistance to macrolides, azalides and streptomycin of group B, but does not affect clindamycin (9-11); (2) Inactivating lincosamides through lincosamide transferase gene coded by the gene lnuA (1,11); (3) Changing drug target site as the most common resistance mechanism. Methylase is responsible for changing drug target site. This enzyme causes the methylation of ribosomal 23Sr RNA and prevents the drug from binding to the target site. The said enzyme is coded by erythromycin resistance methylase (erm) gene. There are four erm genes, namely, ermA, ermB, ermC and ermF, with ermA and ermC being the most common. Since all three drug classes have a mutual target site, bacteria become resistant to all antibiotics of this class; this type of resistance is called MLSB phenotype. This type of resistance exists in two forms; inducible, i.e. MLSB resistance (iMLSB) and constitutive, i.e. constitutive MLSB resistance (cMLS). In constitutive resistances, active mRNA is continuously produced even in the absence of an inducer substance as it does not require an inducer substance. In addition, isolates are resistant to clindamycin and erythromycin. In inducible resistances, inactive mRNA is activated when methylase enzyme is produced in the presence of an inducer such as erythromycin. In this case, resistance to erythromycin leads to the production of a D-shaped zone of inhibition around the clindamycin disk (10, 12).

Since inducible resistance to clindamycin cannot be detected by common laboratory methods, strains resistant to erythromycin may be mistaken for those resistant to clindamycin and might thus never get prescribed (10,13). Induction tests can help distinguish Staphylococcus aureus with inducible resistance from those with constitutive resistance. This test is carried out by the diffusion disk method. Erythromycin disk is placed at a 15-26 mm distance of the erythromycin disk. After the incubation, the presence of a D-shaped zone of inhibition around the clindamycin disk with its flat edge on the side of erythromycin disk indicates inducible resistance. Given that in this test, the inhibition zone is D-shaped, it is called a D-test. The method’s sensitivity has been confirmed with a 100% certainty compared to PCR (8,14). The present study purports to detect and determine the rate of inducible clindamycin resistance in staphylococcus aureus clinical isolates.

**Materials and methods**

In the present cross-sectional study, 100 clinical isolates were collected from a number of hospitals in Shiraz (Shahid Faghihi, Namazi and MRI) over a six month period from March to August of 2012. These specimens were isolated from a total of 44 blood samples, 18 urine samples, 11 skin samples and 10 ulcer samples, 9 sputum samples, 4 nasal samples and 4 pharynx samples. In order to determine of the identity of microorganisms, gram staining and catalase testing, coagulase, mannitol fermentation and DNase were used. Staphylococcus aureus strains verified by the microbiological and biochemical methods were once again verified by the molecular PCR method for the nuc gene using the following specific primers made by Cinnagen Company. Length of the fragment produced by the primers’ activity was 279bp (15).

Forward primer: 5’-GCGATTGATGGTGATACGGTT-3’
Reverse primer: 5’-AGCCAAGCCTTGACGAACTAAAGC-3
After 7 minutes of initial denaturation at 94°C, 30 cycles of PCR were performed including 30 seconds of denaturation at 94°C, 60 seconds of primer binding at 50°C and 75 seconds of elongation at 72°C; eventually, a 5-minute elongation of the intended fragment at 72°C ended the process. PCR products were examined on a 1.2% agarose gel electrophoresis and a UV transilluminator.

In the next stage, these bacteria were examined by oxacillin susceptibility test and based on CLSI guidelines. Subsequently, in order to evaluate inducible clindamycin resistance by the diffusion disk method and based on CLSI standards, erythromycin (15μg) and clindamycin disks (2μg) (MAST Company, UK) were used (16). For this purpose, a 0.05 McFarland suspension yielded from a 24-hour bacteria culture was taken and cultured on Mueller Hinton agar medium using a sterile swab. The erythromycin and clindamycin disks were then placed on the culture medium at 15 mm distance of each other. Once the disks were incubated for 18 hours at 35°C, various phenotypes of inducible clindamycin resistance were examined and recorded (17).

**Results**

In the present study, 100 Staphylococcus aureus isolates isolated from clinical isolates were examined with regard to the presence of the nuc gene once microbiological and biochemical tests were conducted. All strains possessed this gene and were verified with a 100% certainty (Figure 1). Among the tested Staphylococcus aureus strains, 44 isolates (44%) were meticillin-resistant, 56 (56%) were methicillin-susceptible and 10 were clindamycin inducible resistant (Fig. 2).

![Figure 1: Nuc gene PCR product electrophoresis](image1)

![Figure 2: D-zone test](image2)
An induction test performed on 100 isolates led to the detection of D-zone phenotype in 10 isolates. These isolates had a clear inhibition zone around the clindamycin disk with a flat edge adjoining the erythromycin disk (Fig. 1). The D+ phenotype was observed in 2 isolates. In this phenotype, the zone of inhibition had a flat surface adjoining the erythromycin disk, but small colonies existed within the area expanding from the edge of the inhibition zone to the clindamycin disk. Among the 10 isolates with inducible clindamycin resistance, 8 (80%) were resistant to methicillin and 2 (20%) were susceptible to methicillin (Table 1). Six isolates (6%) were resistant to erythromycin and susceptible to clindamycin, but the produced inhibition zone was not flat (negative phenotype). In 2 isolates (2%) with a hazy D-zone phenotype (HD phenotype), growth was detected around two disks. In other words, in addition to the weak and integrated growth around the disk, a flat edge also existed, adjoining the erythromycin disk. HD phenotype indicates clindamycin resistance, but not induction. Thirty-six isolates (36%) showed constitutive resistance to erythromycin and clindamycin, that is to say, they revealed growth around both disks without the haze (R phenotype). In 46 isolates (46%), susceptibility to both antibiotics and development of a large inhibition zone was observed (S phenotype). The results of the diffusion disk test showed that 46% and 51% of the isolates were resistant to erythromycin and clindamycin respectively (Table 2). In addition, 2 isolates were susceptible to clindamycin but resistant to erythromycin.

Table 1: The results of phenotypes obtained from isolates susceptible (MSSA) and resistant (MRSA) to Methicillin

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>MRSA</th>
<th>MSSA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>D+ phenotype</td>
<td>2 (100%)</td>
<td>0 (0%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>D phenotype</td>
<td>6 (80%)</td>
<td>2 (20%)</td>
<td>8 (8%)</td>
</tr>
<tr>
<td>Neg phenotype</td>
<td>2 (33.3%)</td>
<td>4 (66.7%)</td>
<td>6 (6%)</td>
</tr>
<tr>
<td>HD phenotype</td>
<td>1 (50%)</td>
<td>1 (50%)</td>
<td>2 (2%)</td>
</tr>
<tr>
<td>R phenotype</td>
<td>30 (83.3%)</td>
<td>6 (16.7%)</td>
<td>36 (36%)</td>
</tr>
<tr>
<td>S phenotype</td>
<td>3 (6.5%)</td>
<td>43 (93.5%)</td>
<td>46 (46%)</td>
</tr>
<tr>
<td>Total</td>
<td>44 (44%)</td>
<td>56 (56%)</td>
<td>100 (100%)</td>
</tr>
</tbody>
</table>

Table 2: Frequency of resistance of isolates to erythromycin and clindamycin based on diffusion disk test

<table>
<thead>
<tr>
<th>Drug Resistance Rate</th>
<th>Susceptible</th>
<th>Semi-susceptible</th>
<th>Resistant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythromycin</td>
<td>52 (52%)</td>
<td>2 (2%)</td>
<td>46 (46%)</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>47 (47%)</td>
<td>2 (2%)</td>
<td>51 (51%)</td>
</tr>
</tbody>
</table>

Discussion
Clindamycin of MLSB antibiotic class is taken into consideration when treating Staphylococcus aureus infections. Give that vancomycin is used for the treatment of methicillin-resistant strains, finding a replacement for this antibiotic is crucial. As for clinical laboratories, the phenotypic detection of inducible clindamycin is essential to the treatment of patients. Inducible clindamycin resistance for strains of Staphylococcus aureus is reported to be between 7% and 94%. The variance found between geographical regions, even from one hospital to another, could be a result of different antibiotic prescriptions. (12).

In the study conducted by Fiebelkorn et al., from among the 114 Staphylococcus aureus isolates resistant to erythromycin,
33 were also recorded as inducible clindamycin resistant (3). Shoja et al. reported the inducible clindamycin resistance of the isolates to be 9.75%, 3.38% of which were susceptible to methicillin (11). Steward et al. examined 128 Staphylococcus aureus isolates by the diffusion disk method and found 38 to have inducible resistance to clindamycin disk (18). In a survey conducted by Nafisi et al. in Shahrekord, inducible resistance to clindamycin was reported to be 9% in methicillin-resistant strains and 2.3% in methicillin-susceptible strains. They reported the rate of inducible resistance in MRSA strains to be ten times more than in MSSA strains (19). Mohajeri et al. reported the frequency of erythromycin and clindamycin resistant isolates to be 41.5% and 23.3% respectively, and the rate of inducible resistance to clindamycin to be 10%. This rate was 19.5% in MRSA isolates and 3.9% in MSSA isolates (20). Naderinasab et al. examined 128 isolates and perceived the D-zone phenotype in 6 of them (1 Staphylococcus aureus isolate and 5 coagulase negative Staphylococcus isolates) (21). Abdollahi et al. reported the frequency rate of inducible resistance in erythromycin resistant Staphylococcus aureus strains to be 9.38% in Sanandaj; Saderi et al. reported the same rate to be 6.4% in Tehran, which is less than the rate found in the present study (22-23). In their study conducted in Mashhad, Seifi et al. examined 212 Staphylococcus aureus isolates isolated from the patients and reported the erythromycin and clindamycin resistance in MRSA strains to be 88.6% and 52.3%; meanwhile, out of the 52.3% clindamycin resistant strains, 20.5% had the inducible type (24). Comparison of results of the present study to other studies shows that, except rare cases, the frequency rate of inducible clindamycin resistant strains is approximately 10%. The variance in results obtained from the present study compared to those of particular other studies can be attributed to certain factors such as difference in antibiotic resistance patterns of strains in different geographic regions, quality of antibiotic disks used in different studies, accuracy of the tests and reading of the results. In the present study, the two isolates susceptible to clindamycin were also resistant to erythromycin. This state could be a result of the inactivation of clindamycin due to the effect of lincosamide nucleotide transferase, which solely inactivates lincosamides. The results of the present study showed that the rate of inducible resistance in Staphylococcus aureus is rather high, particularly in methicillin-resistant isolates. This point reveals how common this type of inducible resistance is, and that, upon conducting the D-zone test, clindamycin may be selected as a suitable drug for treatment. Therefore, given the incapability of conventional antibiogram methods to detect this type of resistance, it is advised that the D-zone test be used for a careful assessment of susceptibility testing in Staphylococcus aureus isolates.

**Conclusion**

The results of the present study uncovered the soaring rise of the inducible resistance of clindamycin antibiotic; avoiding the arbitrary and indiscriminate use of this antibiotic and its prescribing solely based on antibiotic allergy test results can prevent the resistance from developing. On another note, clinical laboratories should conduct the D-zone test and report its results to the physicians in order for them to be able to choose the appropriate treatment regimen.

**Conflict of interests**

The authors declare no conflicts of interest in this study.
References: