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Inducible clindamycin resistance in clinical isolates of *Staphylococcus aureus* due to *erm* genes, Iran

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ABSTRACT

Background and Objectives: Resistance to macrolide can be mediated by *erm* and *msrA* genes in *Staphylococcus aureus*. There are the evidences that show *erm* genes may be causative agent of inducible or constitutive resistance. The aim of this study was to investigate the incidence of inducible clindamycin resistance and determine the most frequency of *erm* and *msr*A genes among *S. aureus* isolates.

Materials and Methods: In this study a total of 124 non duplicated clinical isolates of *S. aureus* were tested with disk diffusion method. All isolates were tested by PCR for *mec*A, *erm*A, *erm*B, *erm*C and *msr*A genes.

Results: According to PCR results, 48.4% had *mec*A gene and 51.6% were *mec*A negative. By phenotypic D-test method, 32.3% revealed inducible resistance and recorded as D and D^+. Sensitive and constitutive phenotypes were found in 54.8% and 12.9% of isolates respectively. Inducible clindamycin resistance was more prevalent in MRSA (29%) than MSSA isolates (2.4%). Among studied *erm* genes, the most frequency genes were *erm*A and *erm*C with 41.1% and 17.7% respectively. Three isolates of them had D phenotype, while the PCR results of *erm* genes were negative. All isolates were negative for *erm*B or *msr*A genes.

Conclusion: Since *S. aureus* isolates with inducible resistance may mutate and change to constitutive resistance, to prevent treatment failure, we suggest that inducible resistance test be performed on erythromycin resistant/clindamycin sensitive isolates.

Keywords: D- test, Inducible clindamycin resistance, *Staphylococcus aureus*

INTRODUCTION

In the past decade *Staphylococcus aureus* especially methicillin-resistant strains were known as the most important pathogens that were frequently isolated, and caused serious and life threatening clinical infections such as nosocomial and community-acquired infections (1-3). Vancomycin and teicoplanin are commonly used to treat the infections with methicillin-resistant *S. aureus* (MRSA)(1), however, recently isolation of *S. aureus* with decrease susceptibility or resistance to glycopeptides (4) caused encourage of physicians to prescribe of other alternative treatments such as Macrolide - Lincosamide - Streptogramin (MLS) (5).

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Macrolide (erythromycin), lincosamide (clindamycin), and streptogramin (quinupristin-dalfopristin) antimicrobial agents (collectively MLS_B agents) have been used to treat staphylococcal infections (6).

However, among MLS because of pharmacokinetics properties such as good oral absorption and excellent tissue penetration, clindamycin is the most used antibiotic, but excessive use of MLS in the treatment of infections, has been led to increase of resistance to these antibiotics (5). Resistance to MLS antibiotics among staphylococci can be occurred by various mechanisms, including: I- an active efflux pump encoded by *msrA* gene (cause resistance to macrolids and type B streptogramins, and not to clindamycin) (6), II- Enzymatic inactivation of antibiotic (7) and III- ribosomal target modification that is the major mechanism of resistance (8) and affects macrolides, lincosamides, and type B streptogramins (MLS_B resistance)(6, 9). In staphylococci, the four genes, *ermA*, *ermB*, *ermC* and *ermF,* are frequently involved in resistance to MLS (10). The expression of MLS_B resistance can be inducible or constitutive and is not related to the type of the *erm* genes (8). *S. aureus* isolates with constitutive resistance in vitro, demonstrate resistance to both erythromycin and clindamycin whereas *S. aureus* isolates that harbor inducible resistance are resistant to erythromycin but appear susceptible to clindamycin ($iMLS_B$) (11). Although, after contact to clindamycin in vivo, they may mutate and produce constitutive resistance that becoming resistant to all MLS antibiotics (12) and may cause treatment failure (13-14). In addition, isolates with *msrA*-mediated efflux pump also have the same phenotype and are resistant to erythromycin and sensitive to clindamycin, however they cannot produce constitutive resistance during treatment (14).

Lack of identity of inducible clindamycin resistance leads to false laboratory reports and could lead to clinical failure when clindamycin is used therapeutically and cause treatment problems (6, 15). On the other hand, labeling of staphylococci as clindamycin resistant, while they are only resistance to erythromycin, could stop prescription of clindamycin, in cases that infections have occurred by truly clindamycin-susceptible staphylococcal isolates (6, 16). A simple laboratory test (as titled D-zone test) can differentiate between staphylococci that have inducible *erm* genes-mediated resistance and those which have efflux pump-mediated resistance (14).

The aim of present study was to determine the incidence of inducible clindamycin resistance and investigate the prevalence of *ermA*, *ermB*, *ermC*, and *msrA* genes among the clinical isolates of *S. aureus*.

MATERIALS AND METHODS

Isolation and identification of bacteria. During of one year period 124 clinical isolates of *S. aureus* were collected from three teaching hospitals affiliated to Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. The bacteria which were consecutively isolated from patients in various wards and different specimens such as: catheter, blood, wound, discharge, abscess, burn, and so on, were transported to Microbiology Laboratory in School of Medicine and were confirmed by standard microbiology tests including: Gram staining, catalase, slide and tube coagulase, mannitol fermentation and production of DNase enzyme (17).

Antibiotic susceptibility testing. Antimicrobial susceptibility of the isolates was determined by using Kirby-Bauer disk diffusion method according to Clinical and Laboratory Standard Institute (CLSI) guidelines. Briefly a 0.5 McFarland suspension of bacteria were prepared and inoculated on Mueller-Hinton's agar plates (Merck, Germany). The tested antimicrobial agents were penicillin (10U), oxacillin (1µg), cefoxitin (30µg), gentamicin (10µg), Trimetoprim-sulfametoxazol (1.25/23.75µg), azithromycin (15µg), imipenem (10µg), meropenem (10µg), ciprofloxacin (5µg) and rifampin (5µg). The minimal inhibitory concentrations (MICs) of vancomycin were determined by E-Test (Bio Mérieux) according to CLSI guidelines. *S. aureus* ATCC 25923 and *S. aureus* ATCC 29213 were included as standard strains and quality control for disk diffusion and MIC tests; respectively (CLSI, 2007).

Disk approximation test with erythromycin and clindamycin (D-Zone test). Inducible clindamycin resistance, was determined using disk approximation test with erythromycin and clindamycin (D-zone test) as recommended by CLSI (CLSI, 2007). Briefly, 0.5 McFarland suspensions were prepared with organisms from an overnight growth and then inoculated and spread over the surface on Mueller-Hinton's agar plates (Merck, Germany). One erythromycin disk (15 µg) and one clindamycin disk (2 µg) (MAST, Group Ltd, Merseyside, UK) were

placed on the inoculated plates in a distance of 15 mm from each other. Plates were incubated at 35°C and read after 18 h. Inducible clindamycin resistance was confirmed by forming of a flattening shape of the clindamycin inhibition zone (D shape) around the erythromycin disk which indicated erythromycin had induced clindamycin resistance. Furthermore, the staphylococcal isolates were grouped to different phenotypes according to a study as previously described (14). These phenotypes were: S phenotype (sensitive to both erythromycin and clindamycin), R phenotype (constitutive resistance and were resistant to both erythromycin and clindamycin), D phenotype (resistant to erythromycin and clindamycin zone like D) and D^+ (resistant to erythromycin and D shape zone for clindamycin with small colonies growing within the D zone)(14).

DNA extraction. DNA was extracted from *S. aureus* isolates by boiling method (2). Bacteria were inoculated on Mueller-Hinton's agar plate overnight at 37°C. After this time, one to five colonies were suspended in 100 µl of TE buffer (10 mMTris, 1 mM EDTA, pH 7.8) and boiled for 10 minutes at 100°C. After centrifugation, bacterial suspensions at 9000 × g for 30second at 4°C, the supernatant was collected and used as DNA template for PCR reaction (2).

Identification of *mecA* gene. For identification of *mecA* gene in methicillin-resistant isolates, which were screened by resistance to oxacillin and/or cefoxitin disks, polymerase chain reaction (PCR) was performed using by primer pair *mecA* F 5'-GTAGAAATGACTGAACGTCCGATGA-3' and *mecA* R 5'- CCAATTCCACATTGTTTCGGTCTAA -3', that amplified a 310-bp Product (Tiwari and Sen, 2006). PCR condition in a Mastercycler (Eppendorf, Germany) were as follows: 94°C for 4 min, 35 cycle of 94°C for 1 min, 62°C for 1 min, 72°C for 45-s and final extension 5 min in 72°C. PCR products were electrophoresed on a 1% agarose gel and visualized under ultraviolet illumination. *S. aureus* ATCC 29213 and *S. aureus* ATCC 33591 were used as *mecA* negative and *mecA* positive control strains respectively.

Amplification of *erm* and *msrA* determinants by PCR. DNA amplification was performed using specific primers for detection of *erm* and *msr*A genes. Oligonucleotide primers used for PCR were as follows: *ermA*/F:5'–TATCTTATCGTTGAGAAGGGATT–3', *ermA*/R: 5'– CTACACTTGGCTTAGGATGAAA–3' which amplified a 139 bp, *ermB*/F: 5' –CTATCTGATTGTTGAAGAAGGATT–3', *ermB*/R: 5' –TTTACTCTTGGTTTAGGATGAAA–3' which amplified a 142 bp, *ermC*/F:5'–CTTGTTGATCACGATAATTTCC –3', *ermC*/R 5' –ATCTTTTAGCAAACCCGTATTC –3' which amplified a 190 bp and *msrA*/ F: 5' –TCCAATCATTGCACAAAATC –3', *msrA*/ R: 5' –AATTCCCTCTATTTGGTGGT –3' which amplified a 163 bp amplicon (10). PCR reactions were adjusted according to conditions described in previous study with some modifications (10). Each reaction was carried out in a final volume of 25µl with 1X PCR buffer, 0.2U Taq polymerase, 2mM $MgCl_2$, 200µM of dNTP, and 0.4µM of each primer. Amplification conditions were as follows: Initial denaturation, 95°C for 3 min; 35 cycles of 95°C for 30 s, various annealing temperatures (58°C for *erm*C, 62.8°C for *erm*A, 59°C for *erm*B and 55°C for *msr*A) for 30 s and 72°C for 45 s and final extension at 72°C for 7min. PCR products were analyzed by separating on 1.5% agarose gel electrophoresis, then were stained with ethidium bromide solution and finally visualized in gel documentation system (10). One *S. aureus* isolate with *erm*A and another one with *erm*C were sequenced and used as positive control for identification of these genes. We also used another native isolate as positive control for *msr*A gene. Furthermore, a reaction containing all materials except DNA was used as negative control. Distilled water was used instead of DNA in negative control reaction.

Statistical analyses. The results were analyzed using the SPSS for windows software version 19. Fisher's exact test or chi-square, as appropriate, was used to compare frequencies. *P* –value of ≤ 0.017 was considered as statistically significant.

RESULTS

In this study a total of 124 *S. aureus* isolates, which were collected from different hospital wards were examined. The frequencies of *S. aureus* isolated from different clinical samples are shown in Table 1. The results of antimicrobial sensitivity test showed that all isolates were susceptible to vancomycin (100% susceptible) and the majority of them were resistant to penicillin (96.8%). The results of antibiotic

Table 1. Frequency of *S. aureus* isolates in various clinical specimens and different phenotypes

	Phenotypes					
Specimen	**D (%)**	**D^+ (%)**	**S (%)**	**R (%)**	**Negative (%)**	**Number (%)**
Burn	17.7	0	7.3	1.6	0	33 (26.6)
Wound	4.8	0	16.1	4	0	31 (25)
Blood culture	3.2	0	8.1	2.4	0	17 (13.7)
Catheter	4	0	7.3	0.8	0	15 (12.1)
Discharge	0	0	6.5	1.6	0	10 (8.1)
Trachea	0.8	0	3.2	0.8	0	6 (4.8)
Urine culture	0	0	2.4	0	0	3 (2.4)
Corneal lesion	0	0	1.6	0.8	0	3 (2.4)
Abscess	0	0	0.8	0.8	0	2 (1.6)
Nasal swab	0.8	0	0.8	0	0	2 (1.6)
Nail infection	0	0.8	0	0	0	1 (0.8)
Pleural effusion	0	0	0.8	0	0	1 (0.8)
Total	31.5	0.8	54.8	12.9	0	124 (100)

D: Resistant to erythromycin and clindamycin zone like D, D^+: Resistant to erythromycin and D shape zone for clindamycin with small colonies growing within the D zone, S: Sensitive, R: Resistant, Negative: Resistant to erythromycin and susceptible to clindamycin and lack of D shape zone

susceptibility testing for other antibiotics are shown in the Table 2.

As mentioned above, the results of vancomycin E-test showed that all of staphylococcal isolates were sensitive to vancomycin and their MIC to this antibiotic was in range 0.5µg/ml to 2µg/ml, with MIC 50 = 1µg/ml and MIC 90 = 1.5µg/ml. Based on the results of D-Zone test, different phenotypes of *S. aureus* including S phenotype (54.8%), R phenotype (12.9%), D phenotype (31.5%) and D^+ (0.8%) were observed (Fig. 1). The prevalence of different phenotype among each specimen is shown in Table 1.

The electrophoresis results of PCR products showed that 48.4% and 51.6% of isolates were positive and negative for *mec*A gene, respectively. The rate of inducible clindamycin resistance in methicillin-resistant isolates was higher than in MSSA isolates (*P*-value< 0.001). The rate of D, D^+, S and R phenotypes among MRSA isolates were 29%, 0%,6.5% and 12.9% respectively. Among MSSA isolates 2.4%, 0.8%, 48.4% and 0% had D, D^+, S and R phenotypes respectively. According to PCR results, 41.1% isolates had *erm*A (Fig. 2) but 17.7% contained *erm*C (Fig. 3). Twenty isolates (16.1%) were

Table 2. The results of antibiogram test for *S. aureus* isolates

Antibiotic	**Sensitive (%)**	**Intermediate (%)**	**Resistant (%)**
Penicillin	4 (3.2)	-	120 (96.8)
Oxacillin	69 (55.6)	-	55 (44.4)
Cefoxitin	64(51.6)	-	60(48.4)
Gentamicin	71 (57.3)	2 (1.6)	51 (41.1)
Trimetoprim-sulfametoxazole	84 (67.7)	-	40 (32.3)
Azithromycin	66 (53.2)	-	58 (46.8)
Vancomycin	124 (100)	-	-
Imipenem	103 (83.1)	2 (1.6)	19 (15.3)
Meropenem	122 (98.4)	-	2(1.6)
Ciprofloxacin	68(54.8)	3(2.4)	53(42.7)
Rifampin	100 (80.6)	13 (10.5)	11 (8.9)
Clindamycin	66(53.2)	-	58(46.8)
Erythromycin	66(53.2)	1(0.8)	57(46)

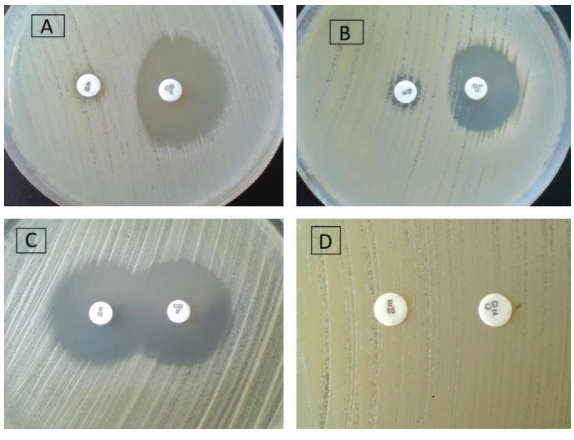

Fig. 1. Four phenotypes observed in the study. A: D phenotype, B: D^+ phenotype, C: S phenotype, D: R phenotype. (E: erythromycin 15µg, CD: clindamycin 2 µg)

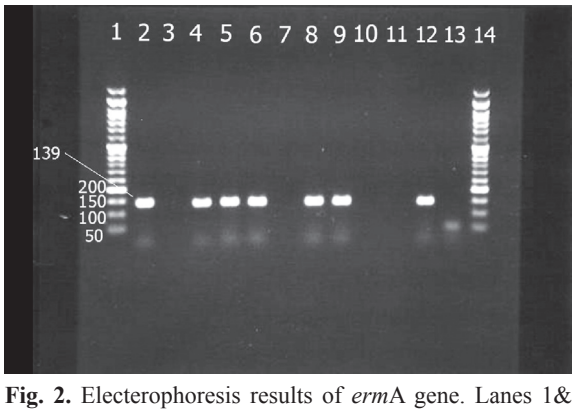

Fig. 2. Electerophoresis results of *erm*A gene. Lanes 1& 14: 50 bp DNA ladder, Lanes 2,4 – 6 and 8-9 isolates with *erm*A gene in 139 bp, Lanes 3, 7, 10-11 isolates with negative *erm*A gene. Lanes 12 & 13, Positive and negative controls respectively.

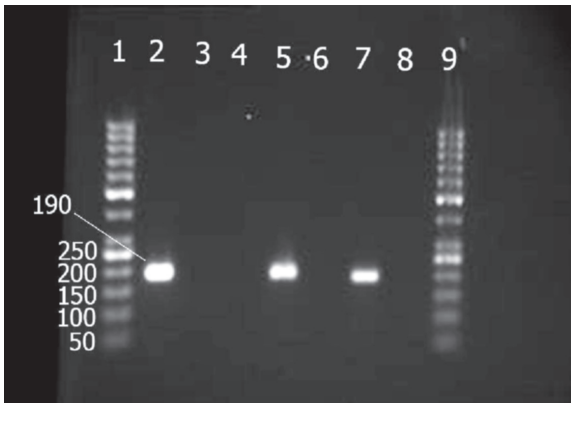

Fig. 3. Electerophoresis results of *erm*C gene. Lanes1 and 9: 50 bp DNA ladder, Lanes 2 and 5: isolates with *erm*C gene in 190 bp, Lanes 3, 4 and 6: isolates with negative *erm*C gene. Lanes 7 & 8 positive and negative controls, respectively.

positive for both *erm*A and *erm*C. Three isolates had D phenotype while the results of *erm* genes PCR were negative. All isolates were negative for *erm*B and *msr*A genes. The result of PCR for *erm* genes according to sensitivity to methicillin is shown in Table 3.

DISCUSSION

For microbiology laboratories there is important to correctly recognize and report an *S. aureus* isolate, which is truly clindamycin susceptible when it's erythromycin resistant, and clindamycin susceptible. This true result may depend obtained by using a simple disk diffusion, described as D –zone test, because of this test can exclude inducible clindamycin resistance (18). Prevalence of *S. aureus* isolates with inducible resistance can be depending on geographic region, patient's age, species of bacteria, sample origin and source of the strains like community or nosocomial. Prevalence of inducible rate is also different from a hospital to another hospital and even among patients (10, 19-20).

The results of our study have shown that incidence of inducible clindamycin resistance was 32.3% among all isolates. Rahbar *et al.* in Iran showed that 10.8% of *S. aureus* isolates had $iMLS_B$(21) . Jethwani *et al*. in India showed that 43% of *S. aureus* isolates were $iMLS_B$ (22). Dizbay *et al*., in Turkey, reported that 90% erythromycin resistant, clindamycin sensitive *S. aureus* showed inducible clindamycin resistance (23).

As mentioned, the rate of inducible resistance may vary depending on the resistance bacteria to methicillin (21). In our study $iMLS_B$ was most prevalent in MRSA (29%) compared to MSSA (3.2%) isolates (*P*-value <0.001). The prevalence of $iMLS_B$ resistance in MRSA has been previously reported as highly variable, from12.3% to 35.9%, in different parts of the world (7, 11, 15, 19, 21, 24-25). Similar to MRSA, the prevalence rate of $iMLS_B$ is variable among MSSA isolates. In present study 3.2% of MSSA had $iMLS_B$ phenotype. This rate has been reported variously from different countries. Some of these reports showed $iMLS_B$ rates from 4% to 68% (7, 11, 15, 19, 21, 25).

Our results showed that S phenotype rate with 48.4% was the most prevalent among MSSA isolates, while, its rate was 6.5% among MRSA (*P*-value < 0.001). Similar to D phenotype, the prevalence of S phenotype is very variable among MSSA and MRSA in different countries. The prevalence rate of S phenotype in

Table 3. Results of *erm* genes PCR according sensitivity to methicillin for the *S. aureus* isolates

	Genotype	Results	Sensitivity to methicilin	
			MRSA (%)	MSSA (%)
PCR results	*erm*A	positive	39.5	1.6
		negative	2.4	1.6
	*erm*C	positive	16.9	0.8
		negative	25	2.4
	*erm*B	positive	0	0
		negative	0	0
	*msr*A	positive	0	0
		negative	0	0

MSSA has been reported 14% to 90.9% and among MRSA isolates from 0% to 26.3% (7, 15, 21-22).

We detected constitutive resistance (12.9%) only in MRSA, but it was not found in MSSA isolates. This type of resistance has been reported from 8 to 64.6% in MRSA and 1.6 to 13% in MSSA isolates in different parts of the world (11, 15, 21-23, 26).

The results of PCR in our study showed that only *ermA* with 41.1% and *ermC* with 17.7%were found among studied isolates. No *ermB* or *msrA* was detected in this study. Westh *et al*. in Denmark showed that among *S. aureus* strains isolated from 1959 to 1988, *ermA* and *ermC* were responsible for 98% resistance to erythromycin (27). Cetin *et al*. in Turkey found that 62% and 17% of *S. aureus* isolates were positive for *ermA* & *ermC* genes respectively (26). Saderi *et al*. in Tehran reported 60.3% and 54.8% of genes belonged to *ermA* and *ermC* respectively in *S. aureus* strains (28).

We have not found any *msrA* gene, although different rates of *msrA* genes among *S. aureus* isolates had been reported (10, 26). Lina *et al*. showed that *msrA* was more prevalent in coagulase-negative staphylococci (29). Prevalence of *ermB* is low and few studies reported this gene in *S. aureus*. Coutinho *et al*. reported that between 45 isolates of *S. aureus* only, 1 isolate had *ermB*(20), while in Aktas *et al*. report, this rate was 8.3% (10). Lina *et al*. showed only one isolate with *ermB* among 144 isolates of *S. aureus* (29). Cetin *et al*. reported the same as present study, found no *ermB* gene in 47 *S. aureus* isolates (26). We detected that 16.1% of isolates had both *erm A* & *erm* C. Some of the studies also found both *erm A* and *erm C* among *S. aureus* isolates (10, 26).

In our study three *S. aureus* isolates showed inducible clindamycin resistance (D phenotype), while the results of *erm* genes PCR were negative. Similar findings have been previously reported. Aktas *et al*. found that 16.6% of *S. aureus* isolates were PCR negative (10). Saderi *et al*. in Tehran studied *S. aureus* strains for *ermA* & *ermC* and reported that 33.3% of strains were negative for both genes (28). Other phenotypes including Hazy D (HD) or Negative (Neg) that previously described (14), were not found in our study. In the present study, all the erythromycin resistant isolates and clindamycin susceptible showed inducible resistance and no negative phenotype was identified among them. In conclusion, we recommend that microbiology laboratories in hospitals perform the D test for any *S. aureus* isolate that is resistance to erythromycin and sensitive to clindamycin.

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