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OCCURRENCE OF METHICILLIN RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) AMONG CLINICAL SAMPLES IN TEHRAN-IRAN AND ITS CORRELATION WITH POLYMORPHISM OF SPECIFIC ACCESSORY GENE REGULATOR *(AGR)* GROUPS

Amir Azimian[1], Shahin Najar-pirayeh[*1], Siamak Mirab-Samiee [2,5], Mahmood Naderi[3,4]

[1*]Department of Medical Bacteriology, School of medical Science, Tarbiat Modares University, Tehran, P.O.BOX: 14115-331; [2]Food and Drug Laboratory Research Center, Ministry of Health and Medical Education, No. 408, Emam Khomeini Ave., Tehran 11136-15911, Iran; [3]Department of Medical Biotechnology, School of Medical Science, Tarbiat Modares University, Tehran, P.O.BOX: 14115-331; [4]Molecular Biology and Genetic Engineering Department, Stem Cell Technology Research Center, Tehran, Iran; [5]Reference Health Laboratories, Ministry of Health and Medical Education, Tehran, Iran.

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ABSTRACT

Methicillin-Resistant *Staphylococcus aureus* (MRSA) is responsible for an increasing number of serious hospital and community acquired infections. Virulence gene expression in *Staphylococcus aureus* is orchestrated by regulators such as the accessory gene regulator (*agr*). Staphylococcal strains are divided into four major *agr* groups (*agrI-IV*) on the basis of *agrD* and *agrC* polymorphisms. The purpose of this study was to define the prevalence of MRSA strains in appointed Tehran's hospitals and then to define and compare the proportion of *agr* I, II, III, IV polymorphisms between MRSA and Methicillin Sensitive *Staphylococcus aureus* (MSSA) strains. A total of 235 isolates were evaluated by conventional antibiotic susceptibility tests and PCR for *agr* and *mecA* genes. 112 strains were MRSA (47.5%) and the most prevalent *agr* specific group was *agr* I followed by *agr* III, *agr* II and *agr* IV, respectively. The prevalence of *agr* groups amongst MRSA and MSSA strains was not statistically significant ($P \geq 0.05$). This study suggests that *agr* I is not only the most prevalent *agr* type in MRSAs but also the most common one in Methicillin Sensitive *Staphylococcus aureus* (MSSA) strains in Iran.

Key words: Methicillin Resistant *Staphylococcus aureus*, *agr*, PCR.

INTRODUCTION

Staphylococcus aureus is the major pathogen responsible for both hospital and community acquired infections. Based on numerous reports *S. aureus* has become resistant to most available antibiotics (4, 1, 14). In the early 1950s acquisition and spread of beta lactamase producing plasmids thwarted the effectiveness of penicillin for treating *S. aureus* infections. In 1950 methicillin, a semisynthetic penicillin, was introduced, even though in 1960 methicillin resistant *Staphylococcus*

***Corresponding Author. Mailing address:** Department of Medical Bacteriology, School of medical Science, Tarbiat Modares University, Tehran, P.O.BOX: 14115-331.; E-mail: najarp@modares.ac.ir

aureus (MRSA) strains were identified. The mechanism by which *S. aureus* acquires resistance to Methicillin is dependent upon the production of an altered penicillin binding protein (PBP2a) which is encoded by *mecA* gene. Increasing number of isolated MRSA strains has led to complication in treatment of staphylococcal diseases (7, 10, 24).

This pathogen causes a wide range of diseases including septicemia, meningitis, endocarditis, osteomyelitis, septic arthritis, toxic shock syndrome and food poisoning (4, 1, 14). The accessory gene regulator (*agr*) locus was identified as the regulator of virulence factors in *S. aureus*. It controls a large set of genes, including most of those encoding cell wall associated and extracellular proteins (2, 18). The *agr* locus is composed of two divergent transcriptional units, RNAII and RNAIII, driven by P2 and P3 promoters, respectively. The P2 operon encodes four proteins that generate the *agr*-sensing mechanism and as a result of their activation, the effector molecule (RNAIII) is produced and affects the expression of virulence genes. The association between *agr* specific group, the type of infection, and also antibiotic resistance has been reported by many researchers (29, 30). In this study we investigated the occurrence of the Methicillin Resistant *S. aureus* (MRSA) among clinical samples while considering their specific accessory gene regulator (*agr*) groups and the site of infection.

MATERIALS AND METHODS

Bacterial isolates

A total of 235 *S. aurous* isolates were isolated from patients and healthy individuals. Isolates were taken from blood culture [60], urine [37], skin [43], respiratory tract specimens [55] and miscellaneous specimens such as tissue biopsies, exudates and bone marrow [9]. Also 31 nasal swabs of *S. aureus* were taken from healthy volunteers.

Laboratory methods

S. aureus isolates were identified with the use of conventional tests consisting of gram staining, catalase test, growth in manitol salt agar media, DNase and coagulase test.

Antibiotic susceptibility test

Disk diffusion and MIC agar dilution were performed for all isolates. Disk diffusion and MIC were accomplished according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (28). We used 1 µg Oxacillin disk (HiMedia Code: SD088) for disk diffusion test. Oxacillin powder (Sigma code: O1002) was utilized for MIC (Agar dilution method) while *Staphylococcus aureus* ATCC25923 was used as the control.

Genomic DNA extraction

Bacterial DNA lysates were prepared from 1 ml of an overnight Tripticase Soy Broth (TSB) culture. After centrifugation at 6000 g for 5 min the bacterial pellet was resuspended in 500µl of TE buffer [50mM Tris-Hcl (PH=8), 50mM disodium EDTA] containing 20 unit lysostaphin (Sigma code: L7386) (25), and incubated at 37°C for 30-60 min and then extracted by conventional Phenol-Chloroform method.

DNA amplification

Thermal cycling for amplification of *mecA* gene was performed in an Eppendorf thermal cycler (Mastercycler® gradient). Amplification protocol consisted of 5 min initial denaturation at 94°C, followed by 30 cycles of denaturation (94°C/30 seconds), annealing (55°C/30 seconds) and extension (72°C/60 seconds), and an additional post-amplification extension step at 72°C for 7 min.

The same device was used for *agr* group amplification. Amplification protocol consisted of 5 min initial denaturation at 94°C, followed by 30 cycles of denaturation (94°C/60 seconds), annealing (57°C/60 seconds) and extension (72°C/60 seconds), and a final post-amplification extension at 72°C for 7 min (23). The list of primers used for this experiment is depicted in Table 1. The products of amplified samples were analyzed by electrophoresis in a 1% agarose gel and stained

with ethidium bromide. *S. aureus* strains RN6390 (*agr* groupI), RN6607 (*agr* groupII), RN8465 (*agr* groupIII), RN4550 (*agr* group IV) and RN6911 (*agr* negative) were included as run controls for *agr* group identification.

Table 1. primers

Primer name	sequence	Product size
Forward *mecA*	5'- AAAATCGATGGTAAAGGTTGGC-3'	533 bp
Reverse *mecA*	5'-AGTTCTGCAGTACCGGATTTG-3'	
pan forward *agr*	5'-GTCACAAGTACTATAAGCTGCGAT-3'	-
Reverse *agrI*	5'-GTATTACTAATTGAAAAGTGCCATAGC-3'	440bp
Reverse *agrII*	5'-GTATTACTAATTGAAAAGTGCCATAGC-3'	572bp
Reverse *agrIII*	5'-CTGTTGAAAAAGTCAACTAAAAGCTC-3'	406 bp
Reverse *agrIV*	5'-CGATAATGCCGTAATACCCG-3'	588 bp

Statistical analysis

Statistical significance of differences between groups was analyzed by means of T-student or ANOVA test. Multivariate analysis was performed to assess the independence of the statistically significant variables in unvariate analysis. A ρ-value < 0.05 was considered significant.

RESULTS

A total of 235 strains from patients and healthy individuals (163 men and 72 women; 69% and 31%, respectively) were evaluated. Among the 235 isolates tested by disk diffusion method for detection of oxacillin resistance, 127 strains (54%) were susceptible and 108 strains (46%) showed resistance. By MIC agar dilution method, 130 (55%) strains were susceptible and 105 (45%) strains were resistant. Finally, PCR for *mecA* gene showed that 110 strains (47%) had *mecA* gene while 125 strains (53%) showed no amplification for this target.

Our strains were isolated from blood, urine, coetaneous samples, respiratory tract, nasal swabs and miscellaneous samples. Prevalence of MRSA strains in different samples was depicted in Table 2.

Our results showed good correlation between phenotypic and genotypic methods for detection of antibiotic susceptibility tests. According to Table 2, the highest percentage of MRSA strains were isolated from respiratory tract specimens (49%) followed by blood cultures (48%), miscellaneous specimens such as tissue biopsies , exudates and bone marrow (45%), urine (43%), cutaneous specimens (41%) and nasal swabs (34%), respectively. The observed differences were not statistically significant (p>0.05).

Table 2. Resistance against oxacillin in different specimens with various phenotypic and genotypic tests

resistant% / sample	MIC* n(%)	Disk diffusion n(%)	*mecA* gene positive n(%)
Blood (n=60)	29(48)	50(30)	30(50)
Urine (n=37)	16(43)	16 (43)	17(46)
Coetaneous (n=43)	18(42)	18(42)	19 (44)
Respiratory tract (n=55)	27 (49)	27(49)	28(51)
Other (n=9)	4(45)	5(46)	5(46)
Nasal swab (n=31)	11 (35)	12(39)	11(35)

* According the NCCLS guidelines isolates with MIC≥4μg were resistant to oxacillin.

The majority of *S. aureus* strains isolated from clinical and healthy cases belonged to *agr* group I (128 strains), followed by *agr* group III (41 strains), *agr* group II (39 strains) and finally *agr* group IV (22 strains). Five isolated strains were untypable by our assay (Table 3).

There was a difference in prevalence of specific *agr* groups between MRSA and MSSA isolates. In MRSA isolates, *agr* group I had the highest prevalence (57%) followed by group III (19%), group II (14%) and group IV (8%). Two percent of MRSA isolates were untypable. In MSSA isolates, most of the strains belonged to *agr* group I (52%) followed by group II (19%), group III (16%) and group IV (11%). Two percent of MSSA isolates were untypable. Results are depicted in Table 4. The differences were not statistically significant (P>0.05).

Table 3. Genetic polymorphism of the *agr* locus in staphylococcus aureus isolates from different specimens

Sample / group	Blood n(%)	Urine n(%)	Coetaneous n(%)	Respiratory tract n(%)	Other n(%)	Nasal swab n(%)	Total n(%)
agr group I	32(53)	24(65)	24(56)	27(49)	6(67)	15(48)	128(54.5)
agr group II	10(17)	5(13.5)	6(14)	8(14.5)	1(11)	9(29)	39(17)
agr group III	10(17)	6(16)	5(12)	13(23.5)	2(22)	5(16)	41(17.5)
agr group IV	6(10)	2(5.5)	6(14)	6(11)	0	2(7)	22(9)
nontybable	2(3)	0	2(4)	1(2)	0	0	5(2)
Total	60(25.5)	37(16)	43(18)	55(23.5)	9(4)	31(13)	235(100)

Table 4. Genetic polymorphism of the *agr* locus in MRSA and MSSA strains

	***agr* I** n(%)	***agr* II** n(%)	***agr* III** n(%)	***agr* IV** n(%)	**N** n(%)	**Total** n
MRSA	64(57)	16(14)	21(19)	9(8)	2(2)	112
MSSA	64(52)	23(19)	20(16)	13(11)	3(2)	123
Total	128(54.5)	39(16.5)	41(17.5)	22(9.5)	5(2)	235

DISCUSSION

Since the introduction of semisynthetic penicillins such as methicillin and oxacillin for the therapy of infections caused by *S. aureus*, the occurrence of resistant strains to methicillin has steadily increased and MRSA strains have become the major nosocomial pathogens (19, 27). Infections with MRSA strains require treatment with glycopeptide antibiotics which could be nephro- and ototoxic (9). *Staphylococcus aureus* is the major pathogen in both community and hospital acquired infections (26). The ability of this organism to cause a multitude of human diseases such as endocarditis, pneumonia, bacteremia and Toxic Shock Syndrome (TSS) suggests that the pathogenesis of *Staphylococcus aureus* infections is highly complex. The growth phase is not only affected by many cell surface proteins as well as exotoxins but also influenced by the environmental and host signals which contribute to the regulation of virulence factors (18).

The *agr* operon involves in the coordinated regulation of a number of *Staphylococcus aureus* virulence factors. *Staphylococcus aureus* strains exhibit well-defined genetic polymorphisms within the *agr* locus. Four *agr* genotypes, group I to IV, have been described to date (4, 6). Although there is massive amounts of data relating *agr* type and specific infections, Jarraud et al. have shown that specific *agr* genotype strains may be associated with particular infectious syndromes, with enterotoxin disease linked to *agr* group I, endocarditis linked to *agr* groups I and II, toxic shock syndrome linked to *agr* group III and exofoliative disease linked to *agr* group IV (12). The *agr* group III has been overrepresented among strains isolated from community-acquired MRSA infections, whereas *agr* group II is predominant in isolated MRSA strains from

hospitals (17, 21).

In our study, resistance to oxacillin between four *agr* groups was almost similar. *S. aureus* strains belonging to *agr* groups II and IV were equally resistant to oxacillin (41%) whereas strains carrying *agr* group I and *agr* group III were more resistant with resistance rates of 50% and 51%, respectively. However, the differences were not statistically significant (P>0.05). Other studies showed a correlation between induction of Glycopeptide Intermediate-resistant *Staphylococcus aureus* (GISA) phenotype and autolytic deficiency, especially in the context of *agr* genotype II (13). Some reports stated that there are clinical trends according to each *agr* group**.** For example, *agr* group I was prevalent in a collection of 192 *S. aureus* strains in which 71% were methicillin resistant (11, 26). Recently, Jarraud et al. reported an overrepresentation of *agr* genotype II in *S. aureus* isolates from patients with infective endocarditis (12). Pamela et al. showed that *agr* group II polymorphism in MRSA predicts the failure of vancomycin therapy (16). Moreover, it has been reported that community-acquired MRSA, Methicillin Sensitive *S. aureus* (MSSA) (3, 20) and Toxic Shock Syndrome Toxin (TSST-1) producing isolates belong to *agr* specificity group III (6).

In our study most of MRSA strains belonged to *agr* group I and III, respectively, and most of MSSA strains belonged to *agr* groups II and IV (%59). Van Leeuwen et al. screened a collection of 55 MSSA isolates, mostly taken from healthy nasal carriers, but did not find any *agr* III isolate (26). Most exofoliatin producing strains responsible for *Staphylococcal* Scalded Skin Syndrome (SSSS) belongs to group IV (11). The *agr* group IV was absent in many previously reported articles (23, 26, 15, 22), nevertheless, we detected *agr* group IV (9.5%) in our experiments that was more likely due to ecological and geographical differences. Goerke et al. reported that the majority of *S. aureus* strains, taken from patients undergoing intubations, belonged to group III (5). Manago et al. found that most of *agr* I strains show poor biofilm formation, compared with other *agr* groups. They also found a lower prevalence of group I strains and a higher prevalence of group II strains in the nosocomial infections (15). Most of the *agr group I* clones which had been previously reported by the Brazilian, Portuguese, Hungarian and Berlin Research Groups. Group II strains were mainly isolated in Japan and North America. On the other hand, strains of group III were mainly isolated in Europe (8). Recent data demonstrate that the vast majority of MRSA in France and around the world belongs to *agr* group III (20, 3). Our experience revealed that group I is the most prevalent group in Iran, followed by groups III, II and IV. Iran is one of the several countries with high antibiotic resistance rate, including methicillin resistance. Therefore, it is important to emphasize on the verification of characteristics of MRSA in this country. This report has evaluated the correlation between *agr* groups and antibiotic resistance in Iran population. This result will be helpful to encourage verification of the characteristics of MRSA in other Asian countries. In addition, this study may also aid in evaluating the global spread of MRSA strains based on *agr* locus polymorphisms.There seems to be a geographic distribution difference between *agr* groups.

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