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Accessory Gene Regulator Group Polymorphisms in Methicillin-Resistant *Staphylococcus aureus*: An Association with Clinical Significance

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Purpose: Virulent gene expression in *Staphylococcus aureus* is controlled by regulators such as the accessory gene regulator (*agr*). Strains can be divided into four major agr groups (*agr* I-IV) on the basis of *agrD* and *agrC* polymorphisms. The purpose of this study was to define the proportion of *agr* I, II, and III polymorphisms and to compare the clinical characteristics between group I and non-group I polymorphisms of methicillin-resistant *Staphylococcus aureus* (MRSA) strains in a Korean tertiary care teaching hospital. **Materials and Methods:** A total of 158 clinical isolates were evaluated by RFLPs (restriction fragment length polymorphisms). **Results:** The mean age of the patients was 50.2 ± 21.9 years old. There were 74 (49.3%), 66 (44.0%), 10 (6.7%), 7 (4.4%), and 1 (0.6%) strains in *agr* group I, II, III, I + II, and I + III polymorphisms, respectively. Only ear infections were a statistically significant clinical parameter according to univariate (p= 0.001) and multivariate analysis (OR, 4.721 (1.273-17.508), p= 0.020). **Conclusion:** This study suggests that *agr* group I is the most prevalent in Korea, and ear infections are correlated with the group I polymorphism, which is a different clinical trend from western countries. It can also be inferred that community-acquired MRSA correlates with *agr* group I.

Key Words: *Staphylococcus aureus*, *agr* polymorphism, otitis

INTRODUCTION

Staphylococcus aureus is an important nosocomial pathogen that causes various clinical infections.[1-3] The adaptive response by which bacteria survive before the eventual emergence of more stable antibiotic resistance determinants may be mediated by the up-regulation of efflux pumps and stress-triggered responses, involving various repair systems, global regulators of virulence and housekeeping genes. Until now, as many as 30 potential virulence determinants have been described.[4] Genetic variation among *S. aureus* strains has been shown to be associated with pathogenic potential.[5] Variation occurs at the level of both core genes (present in > 95% of isolates[6]) and accessory (variable) genes. Virulence gene expression in *S. aureus* is controlled by regulators such as *agr*,[4] which is likely to be important for the adaptation and survival of the microorganism in the host. The *agr* locus of *S. aureus* is a quorum-sensing gene cluster of five genes (hld, *agrB*, *agrD*, *agrC*, and *agrA*) that up-regulates the production of secreted virulence factors, including the alpha-, beta-, and delta-hemolysins, and down-regulates the production of cell-associated virulence factors.[3,7-11] Polymorphisms in *agrD* and *agrC* define the four *S. aureus agr* groups.[3] In general, *agr* mutation results in an increased production of cell surface proteins, decreased production of exoproteins, and reduced virulence.[4,12-14]

Methicillin-resistant *S. aureus* (MRSA) has become established outside the hospital environment and is now appearing in community populations without identifiable risk factors.[15] In Korea, the percentage of MRSA in nosocomial infections is as high as 14.4%, according to Korean Society of Nosocomial Infection Control (KOSNIC) data,[16] and Korea is notorious for the overuse of anti-

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biotics and high antibiotic resistance rates. Therefore, the purpose of this study was to define the proportion of *agr* I, II, and III polymorphisms and to compare the clinical characteristics between group I and non-group I polymorphisms of MRSA strains in a Korean tertiary care teaching hospital.

MATERIALS AND METHODS

Clinical isolates

A total of 158 MRSA isolates were analyzed from various clinical specimens at Severance University Hospital. All strains were identified as *S. aureus* by conventional methods.[17] Methicillin susceptibilities were determined by oxacillin disk.[18]

Detection of the *agr* locus restriction fragment length polymorphisms (RFLPs)

DNA extraction was performed using the Qiagen tissue kit (Qiagen, Hilden, Germany). PCR was done in 50-μL volumes containing 3 μL DNA extract, 0.2 mM dNTP mix, 1.5 mM magnesium chloride, 2.5 U AmpliTaq DNA polymerase (Roche Diagnostics), and 20 pmol each of the forward and reverse primers. The nucleotide coordinates of the forward (*agr*1801-1818) and reverse (*agr*3668-3685) primers were derived from the *agr* locus sequence of *S. aureus* RN639019 (GenBank accession no. X52543) and consist of the sequences 5′-ACCAGT TTGCCACGTATC-3′ and 5′-TAAACCACGACCT TCACC-3′, respectively. The target sequences begin 25 nucleotides from the 5′ end of the *agrB*-coding sequence and 93 nucleotides from the 3′ end of the *agrC*-coding sequence. The primer sequences are conserved in each of the three *agr* locus interference groups and flank the polymorphic region that confers group specificity.[5] Thermal cycling was done in a GeneAmp PCR System 2400 (Applied Biosystems, Foster City, CA, U.S.A.) and consisted of 30 cycles of denaturation (94℃, 15 seconds), annealing (55℃, 30 seconds), and extension (72℃, 30 seconds). After PCR, the 1884 base pair amplicons were digested overnight with the restriction enzyme DraI and the fragments resolved by electrophoresis through 1.2% agarose.[19]

Statistical analyses

The factors assessed include the patients' demographics, comorbidities (diabetes mellitus, congestive heart failure, coronary heart disease, hypertension, peripheral vascular disease, dialysis-dependent renal failure, chronic obstructive lung disease, cirrhosis, and malignancy), infection site (central catheter-related bacteremia, bacteremia of unknown origin, device, endocarditis, intraabdominal, respiratory tract, skin, bone and joint, urine, and ear), receipt of mechanical ventilation and operation, the presence of nosocomial infection, colonization, and treatment failure, stay in intensive care unit on day 1, creatinine level, and mortality.

Statistical differences between groups were analyzed by means of χ^2 or ANOVA tests. Multivariate analysis was performed to assess the independence of the statistically significant variables in univariate analysis. A p value < 0.05 was considered significant. The SPSS version 11.0 statistical software package for Windows was used for all statistical analyses.

RESULTS

From March 2002 to July 2004, a total of 158 strains from 158 patients (105 male and 53 female patients) were evaluated. The mean age of the patients was 50.2 ± 21.9 years old. There were more men (65.2%) than women. As shown in Fig. 1, strong specific signals of the expected sizes were obtained with each strain. Among the 158 strains tested, 150 isolates fell into one of the three previously described *agr* groups (alleles) and only one *agr* allele was detected in each carrier: 74 (49.3%) isolates belonged to *agr* group I, 66 (44.0%) belonged to *agr* group II, and 10 (6.7%) belonged to *agr* group III. The remaining eight isolates showed a combination of types: 7 (4.4%) belonged to group I + II, and 1 (0.6%) belonged to group I + III (Table 1).

Among the isolates in 2002, 25 (56.8%) strains were group I, 17 (38.6%) strains were group II, and 2 (4.5%) strains were group III. In 2003, 17 strains were Group I (41.5%), 22 were Group II (53.7%) and 2 were group III (4.9%). In 2004,

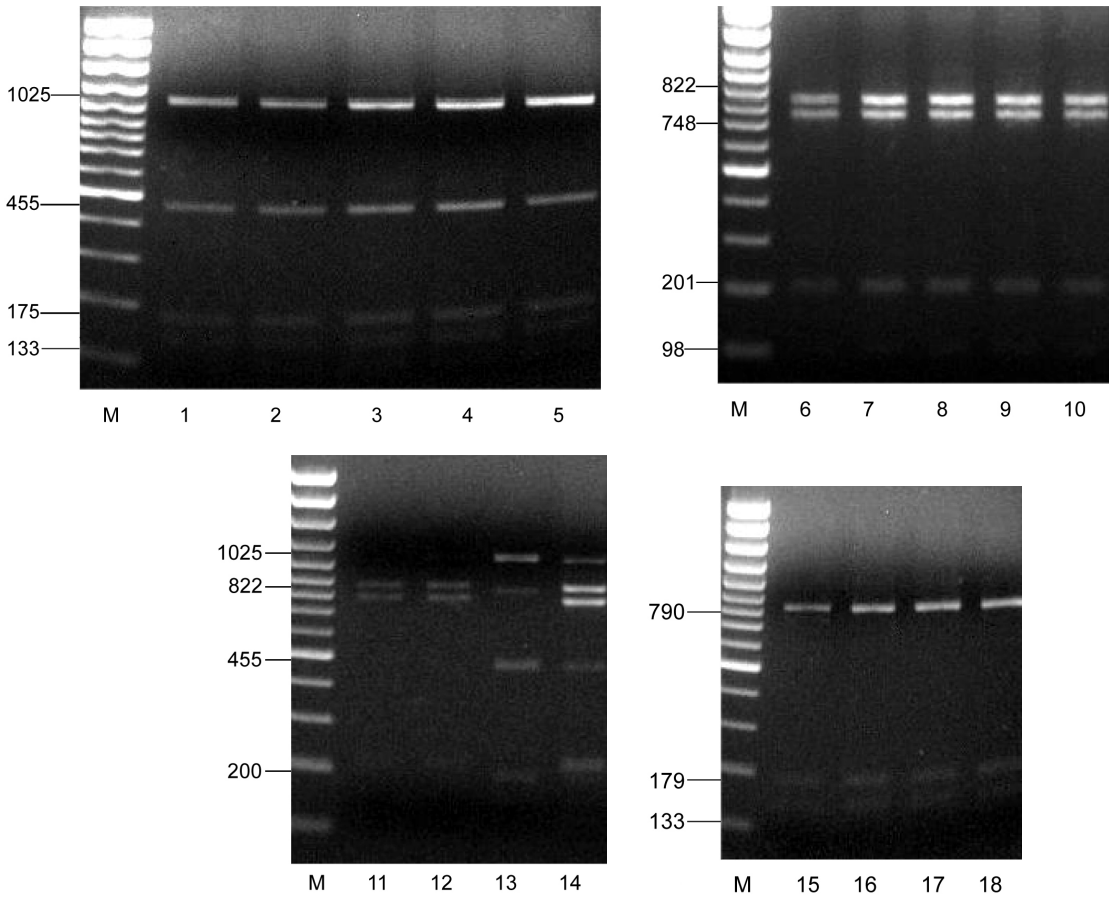

Fig. 1. Representative RFLP analysis with *Dra*I of an amplified portion of the *agr* locus in MRSA strains on 1.2% agarose gel. M: DNA ladder size marker, A1-5: MRSA strains of *agr* polymorphism group I, B6-10: group II, C11-12: group II, 13, group I+III, 14, group I+II, D15-18: group III.

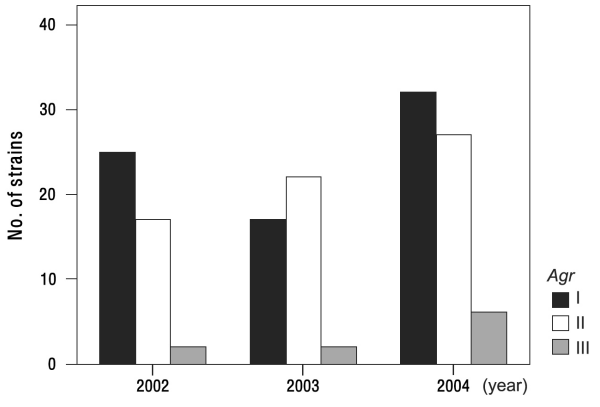

Fig. 2. The numbers of each *agr* group from 2002 to 2004.

Group I had 32 (49.2%), Group II, 27 (41.5%), and group III, 6 (9.2%). There was no significant statistical difference between those years (Fig. 2).

Table 1. The Genetic Characteristics of MRSA Strains

Agr group	No. of clinical isolates (%)
Group I	74 (46.9)
Group II	66 (41.8)
Group III	10 (6.3)
Group I+II	7 (4.4)
Group I+III	1 (0.6)
Total	158 (100)

Many patients had significant comorbidities, such as diabetes (38 patients), congestive heart failure (6), coronary artery disease (15), hypertension (23), peripheral vascular disease (6), chronic obstructive lung disease (3), cirrhosis (6), and malignancy (41). There were also many sites of infection: central catheter-related bacteremia (3

Table 2. A Univariate Analysis of Risk Factors for the Agr Group I Polymorphism

Variables	Group I (n = 74) No. (%)	Non-group I (n = 76) No. (%)	*p* value
Age (yr)	47.7 ± 19.9	52.3 ± 24.1	NS
Sex (M/F)	49/25	48/28	NS
Comorbidity			
Diabetes mellitus	11 (14.9)	25 (32.9)	0.010
Congestive hear failure	3 (4.1)	3 (3.9)	NS
Coronary artery disease	3 (4.1)	12 (15.8)	0.017
Hypertension	7 (9.5)	15 (19.7)	NS
Peripheral vascular disease	3 (4.1)	1 (1.3)	NS
Dialysis-dependent renal failure	5 (6.8)	8 (10.5)	NS
Chronic obstructive lung disease	-	3 (3.9)	NS
Cirrhosis	1 (1.4)	5 (6.6)	NS
Malignancy	20 (27.0)	17 (22.4)	NS
Site of infection			
Central catheter-related bacteremia	-	3 (3.9)	NS
Bacteremia of unknown origin	1 (1.4)	4 (5.3)	NS
Device	3 (4.1)	3 (3.9)	NS
Endocarditis		1 (1.3)	NS
Intraabdominal	2 (2.7)	6 (7.9)	NS
Respiratory	28 (37.8)	37 (48.7)	NS
Skin and skin structure	18 (24.3)	15 (19.7)	NS
Bone and joint	1 (1.4)	1 (1.3)	NS
Urine	5 (6.8)	4 (5.3)	NS
Ear	16 (21.6)	3 (3.9)	0.001
Receipt of mechanical ventilation	20 (27.0)	36 (47.4)	0.010
Receipt of operation	51 (68.9)	45 (59.2)	NS
ICU stay on day 1	4 (5.4)	12 (15.8)	NS
Nosocomial infection	50 (67.6)	57 (75.0)	NS
Treatment failure	42 (56.8)	38 (50.0)	NS
Colonization	31 (41.9)	24 (31.6)	NS
Creatinine	1.3 - 1.7	1.6 - 2.3	NS
Mortality	8 (10.8)	12 (15.8)	NS

ICU, intensive care unit; NS, statistically not significant ($p > 0.05$).

patients), bacteremia of unknown origin (5), device (6), endocarditis (1), intraabdominal infection (10), respiratory infection (68), skin and skin structure infection (34), bone and joint infection (3), urinary infection (9), and ear infection (20) were observed. Fifty-nine (37.3%) of the 158 patients received mechanical ventilation, 103 (65.2%) received operations, and 17 (10.8%) were in an intensive care unit. Nosocomial infection, treatment failure, and colonization were found in 107 (67.7%), 84 (53.2%), and 58 (36.7%) patients, respectively. Twenty-one (13.3%) patients died of unknown causes during hospitalization. The mean creatinine level was 1.42 ± 1.98 mg/dL.

The demographic data, clinical data, and univariate analysis are shown in Table 2 for the patients who had only one *agr* type. In the univariate analysis, comparison of group I isolates with non-group I isolates, ear infection, use of mechanical ventilation, presence of diabetes mel-

litus and coronary artery disease remained significant ($p < 0.05$). In the multivariate analysis, group I was more prevalent only in ear infections (OR, 4.721 (1.273-17.508); $p = 0.020$).

DISCUSSION

Falkow et al. said "The basic unit of bacterial pathogenicity is the clone or lineage that expands due to the possession of unique combinations of virulence genes".[20] It is generally held that no single virulence factor is responsible for the pathogenicity of staphylococci and that disease occurs *in vivo* due to a complex series of processes with the appropriate pathogenic factors being present at each stage.[21] Attenuation of bacteria to eliminate the production of gamma-hemolysin or several other toxins, by mutations in the *agr* two-component signal regulatory system, can result in significant reduction of infection severity.[22]

Some reports state 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, 71% of which were methicillin resistant.[23,24] Jarraud et al. recently reported an overrepresentation of *agr* genotype II in *S. aureus* isolates from patients with infective endocarditis.[25] Another group indicated that all glycopeptide-intermediate *S. aureus* (GISA) isolates from diverse geographic origins belonged to *agr* group II,[26] and Pamela et al. said *agr* group II polymorphisms in MRSA predicts the failure of vancomycin therapy.[27] One study reported that community-acquired MRSA belonged to *agr* group III and methicillin-sensitive *S. aureus* (MSSA),[28-30] and toxic shock syndrome toxin (TSST-1) producing isolates belonged to the *agr* specificity group III.[7] van Leeuwen et al., however, screened a collection of 55 MSSA isolates mostly taken from healthy nasal carriers and did not find any *agr* class III isolates.[24] Most exfoliation-producing strains responsible for staphylococcal scalded skin syndrome (SSSS) belonged to *agr* group IV.[23] In our study, diabetes mellitus, coronary artery disease and the receipt of mechanical ventilation were factors associated with non-*agr* group I in the univariate analysis. Goerke et al.[31] reported that the majority of *S. aureus* strains recovered from patients undergoing intubation was type *agr* group II, which is consistent with our findings. Manago et al.[32] found that most of the *agr*-1 strains showed poor biofilm formation, compared with *agr*-2 and *agr*-3 strains. They also found a lower prevalence of *agr*-1 strains and a higher prevalence of *agr*-2 strains in the nosocomial infection group. Since diabetes mellitus is a risk factor for nosocomial infection,[33,34] it may be related to *agr* group II. The non-*agr* I group correlation with coronary artery disease is uncertain so far. However, the above three factors appeared to be non-significant in multivariate analysis. It may because the non-*agr* group I included both group II and III. There are still only a few studies on the correlations between clinical factors and *agr* groups at present, so these factors should be re-evaluated in further studies. Lastly, ear infections were shown to be more greatly associated with *agr* group I clones than other clones in both univariate and multivariate analysis. Generally, the bacteria that cause ear infections are thought to originate from the community, so these results suggest that group I clones are prevalent in the Korean community. Furthermore, it has been inferred they have some unknown beneficial mechanism for survival in the community. On the basis of the results obtained, it is proposed that the dynamics observed in the population of MRSA in Korea is due to different *agr* group specificities and that this trend in the oriental countries is different from that in western countries.

It is believed that strains within a given *S. aureus agr* group are related genetically and share similar biological properties.[3] However, these relationships need to be further defined, especially in the context of commonly used typing methods such as pulsed field gel electrophoresis (PFGE) and multi-locus sequence typing (MLST). The highly discriminatory method of MLST shows that the sequence of type 5 (ST-5) MRSA is the genetic progenitor of most GISA strains throughout the world, including Mu50, US GISA, and the New York/Japanese MRSA clone. Some investigators reported that the *agr* type had no direct responsibility for disease initiation and speculated that the preferential association between certain *agr* alleles, certain toxin genes, and a particular genetic background may reflect an ancient evolu-

tionary division of *S. aureus* in terms of the fundamental biology of the species.[25] Although our report suggests the relationship between the *agr* group I clones and the ear infections, there may be more ancient fundamental biology beyond this association. This was a limitation of our study since we had no MLST typing data.

There seems to be a geographic difference between *agr* groups. Most clones belonged to *agr* group I, represented by the Brazilian, Portuguese, Hungarian, Berlin, and EMRSA-15 clones, which are predominant in Europe and some South American countries.[35] Group II strains, represented by the Pediatric and NY/Japan clones, have been isolated mainly in Japan and North America (but also in some European countries).[35] Strains of group III, which were represented only by the EMRSA-16 clone, are also isolated mainly in Europe.[35] More recent data demonstrate that the vast majority of community-acquired MRSA in France and around the world belong to *agr* group III.[28-30] Our isolates revealed that group I clones are prevalent in Korea, followed by group II and group III, which was relatively small compared to the previous two groups. We hypothesize that, due to differences in genomic characteristics associated with a given *agr* type, MRSA epidemic clones belonging to three *agr* types may be competing for dominance in hospital settings throughout the world.

It has been proposed that *agr* II *S. aureus* strains hinder umbilical stump colonization by *agr* I strains.[36] The biological mechanism of this interference is unknown but might be caused by molecular cross-interference between *agr* alleles. *Agr* alleles, with the exception of *agr* I and IV, all mutually inhibit *in vitro* RNA III expression.[7,23] Groups II and III both share domains with group I but do not share domains with each other, except for sequences which are present in all three *agr* groups.[24] In our results, group I+II were found in 7 cases and I+III in one case, which is consistent with the previous findings.[24] This phenomenon of *agrD*-dependent cross-inhibition suggests significant variability of the domain encoding the *agrD* signal peptide.[7]

Korea is one of several countries in the world with a high antibiotic resistance rate, including MRSA. Therefore, it is important to verify the characteristics of MRSA in this country. This report is significant in that it is the first to group *agr* data and compares clinical characteristics according to *agr* groups in Korea. Our results will be helpful in verifying the characteristics of MRSA in other Asian countries. This study may also aid in finding an appropriate method to eradicate MRSA clones because *agr* is a potential target for therapy and the response can be modulated by synthetic peptides.[37]

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