ACCESSORY GENE REGULATOR (*agr*) TYPING OF *STAPHYLOCOCCUS AUREUS* ISOLATED FROM HUMAN INFECTIONS

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RESUME

Staphylococcus aureus (S. aureus) est un pathogène majeur de l'homme responsable d'infections communautaires et nosocomiales. Cent souches de S. aureus ont été investiguées. Elles ont été isolées entre Janvier 2004 et Juin 2006 au laboratoire de microbiologie de l'hôpital Charles Nicolle de Tunis. L'identification des souches a été faite selon les méthodes conventionnelles. La résistance à la méticilline a été détectée par des disques d'oxacilline et de céfoxitine et confirmé par la mise en évidence du gène mecA par PCR. Le typage agr a été réalisé par PCR multiplex.

La distribution des groupes agr étaient la suivante: 19 appartenaient au groupe agrI, 16 au groupe agrII et 65 appartenaient au groupe agrIII. Pour les souches de S. aureus résistantes à la méthicilline (SARM) : 9 (16.4%) appartenaient au groupe agrI, 8 (14.5%) au groupe agrII et 38 (69.1%) au groupe agrIII. Pour les souches sensibles à la méticilline (SASM), la distribution des groupes agr était la suivante: seulement 10 (22.2%) appartenaient au groupe agrI, 8 (17.8%) appartenaient au groupe agrII et 27 (60%) appartenaient au groupe agrIII. Un lien significatif entre les groupes agr et le type d'infection a été observé. Les souches ayant un locus agr de type I étaient responsables d'infections invasives ($P = 0.003$) notamment de bactériémies ($P = 10^{-4}$) alors que les souches ayant un locus agr de type II et III étaient responsables d'infections non invasives ($P = 0.003$). Aucune association n'a été observée entre les autres types d'infections et les groupes agr. De même aucune corrélation n'a été trouvé entre les groupes agr, l'age, le sexe et les différents types d'infections.

Mots-clés: *Staphylococcus aureus, PCR multiplexe, systèmes à deux composantes.*

ABSTRACT

Staphylococcus aureus is a major hospital and community acquired pathogen. A total of one hundred strains were investigated. They were collected from January 2004 to July 2006 in the laboratory of microbiology at Charles Nicolle University hospital of Tunis. The isolates were identified by conventional methods. Methicillin resistance was confirmed by amplification of mecA gene by PCR. The agr groups were identified by multiplex PCR.

The agr groups were distributed as follows: 19 strains belonged to group I, 16 to group II and 65 to group III. Among methicillin resistant S. aureus (MRSA), 9 (16.4%) belonged to group I, 8 (14.5%) to group II and 38 (69.1%) to group III. For methicillin susceptible S. aureus (MSSA), only 10 strains (22.2%) belonged to group I, 8 (17.8%) to group II and 27 (60%) to group III. A preferential link was observed between agr group I and invasive infections ($P=0.003$) especially bacteremia ($P=10^{-4}$). Besides, agr groups II and III were closely related with non invasive infections ($P=0.003$). No association was found between other types of infections and agr groups. Likewise, no correlation was observed between agr groups, age or sex of patients and type of infections.

Key words: *Staphylococcus aureus, multiplex PCR, quorum sensing.*

INTRODUCTION

Staphylococcus aureus is both a commensal and an extremely versatile pathogen, causing a wide range of human infections: (i) superficial infections such as skin abscesses and wound infections (ii) deep-seated and systemic infections such as osteomyelitis, endocarditis, pneumonia, bacteremia (iii) and toxemic syndromes such as toxic shock syndrome and staphylococcal scarlet fever, staphylococcal scalded-skin syndrome and staphylococcal food poisoning [1]. With the exception of toxemia, the molecular basis of *S. aureus* pathogenicity depends on the expression of a large class of accessory gene products that comprise cell wall-associated and extracellular proteins [1]. Expression of most virulence factors in *S. aureus* is controlled by the accessory gene regulator (*agr*) locus which encodes a two-component signal transduction system that leads to down-regulation of surface proteins and up-regulation of secreted proteins during *in vitro* growth [2]. A role of *agr* in virulence has been demonstrated by the attenuated virulence of *agr* mutants in different animal infection models [3, 4, 5, 6]. The agr locus consists of 5 genes (*agrA*, *agrC*, *agrD*, *agrB* and *hld*) and codes for two divergent transcripts (RNAII and RNAIII) which are under the control of two distinct promoters, P2 and P3 respectively 1, 2 The P2 operon encodes four proteins (AgrB, AgrD, AgrC and AgrA) that generate the *agr* sensing mechanism. AgrB is a transmembrane protein that appears to be involved in the secretion of an autoinducing peptide (AIP) signal. *AgrA* and *AgrC* form a two component regulatory system in which the transmembrane component, *AgrC* (histidine kinase) binds the extracellular AIP and in turn modulates the activity of *AgrA*, the response regulator that increased transcription of the P3 operon resulting in increase levels of the intracellular RNAIII, favouring the transcription of several secreted virulence factors (enterotoxins, hemolysins, TSST-1). At the same time, the expression of several cell surface virulence factors is decreased. Sequence variation in *agrB, agrD* and *agrC* has led to the identification of at least 4 *S. aureus* agr specificity groups (numbered I to IV), in which AIP production by one group inhibits *agr* expression of the others [1, 2]. Jarraud et al reported that *S. aureus agr* groups were associated with the pattern of *S. aureus* diseases [7].

The aim of this study was to determine the *agr* groups of *S. aureus* isolated at Charles Nicolle hospital of Tunis and to investigate a possible correlation between *agr* groups and human infections.

MATERIALS AND METHODS

Staphylococcal strains and patients

One hundred non replicated *S. aureus* strains were investigated. They were collected from January 2004 to July 2006 at the laboratory of microbiology at Charles Nicolle University hospital of Tunis. They were recovered from cutaneous pus (n=56), blood cultures (n=28), urines (n=10), catheters (n=3), respiratory tract specimens (n=2) and kidney pus (n=1).*S. aureus* isolates were recovered from medicine (64%) especially from dermatology (65.6%) followed by surgery (22%), intensive care unit (6%), pediatrics (4%), gynecology (2%) and others (2%). 56/100 were isolated from cutaneous infections [furuncles (n=12), cutaneous abscesses (n=25)], bacteremia (n=27), urinary tract infections (n=10), catheter related infections (n=3), pneumonia (n=2) and kidney abscess (n=1). The median age was 35 years old ranging from 9 months to 82 years and the sex ratio was 47/53. The isolates were identified as *Staphylococcus* by conventional methods (Gram-positive cocci, catalase positive, mannitol fermenting and DNase-positive) and were confirmed as *S. aureus* by their ability to coagulate rabbit plasma (bioMérieux, Marcy l'Etoile, France) and to agglutinate the Spa (Pastorex staph-plus, Biorad). The biotype was determined on biochemical pattern using Api20 Staph (bioMérieux, Marcy l'Etoile, France).
Demographic and clinical data were collected for all selected patients.

Antimicrobial Susceptibility Testing

The antibiotic susceptibility of the isolates was performed using the disk diffusion method according to the Clinical and Laboratory Standards Institute guidelines (CLSI) [8]. The following antimicrobial disks were tested: penicillin (10UI), oxacillin (1μg), cefoxitin (30μg), amoxicillin + clavulanic acid (20/10μg), kanamycin (30μg), gentamicin (10μg), tobramycin (10μg), tetracycline (30μg), chloramphenicol (30μg), ofloxacin (5μg), trimethoprim-sulfamethoxazole (1.25+23.75μg), erythromycin (15μg), clindamycin (2μg), vancomycin (10μg), rifampin (5μg) and fosfomycin (5μg). Methicillin resistance was detected by oxacillin and cefoxitin disks and confirmed by the detection of *mec*A gene (533pb) using PCR as described previously [9, 10].

agr grouping

DNA was extracted using Instagene Matrix (BioRad Laboratories, Marnes La coquette, France). *agr* specificity groups were identified by PCR amplification of the hypervariable domain of the *agr* locus using oligonucleotide primers specific for each of the four major specificity groups [11, 12]. A forward primer, pan *agr* 5'-ATGCACATGGTCCACATGC-3' corresponding to conserved sequences from the *agr*B gene, and four reverse primers, each one specific for amplification of a single *agr* group based on *agr*D or *agr*C gene nucleotide polymorphism were as follows: *agr*I, 5'-GTCACAAGTACTATAAGCTGCTGCGAT-3', *agr*II, 5'GTATTACTAATTGAAAAGTGCCATAGC3', *agr*III, 5'CTGTTGAAAAAGTCAACTAAAAGCTC-3' and *agr*IV, 5'-CGATAATGCCGTAATAC-3'. PCR was performed by adding 5µl of chromosomal template DNA to a 45 µl PCR mixture that includes 2.5U of GoTaq DNA polymerase (Promega, Lyon, France), 2mM MgCl2, 350 µM of each dNTP and 20µM of each primer. Amplification was performed with a GeneAmp2400 instrument (Perkin Elmer, Norwalk, CT, USA): 30 cycles of PCR were done (denaturation (1min at 94°C), annealing (1min at 55°C) and extension (1min at 72°C)), with one cycle of denaturation (1min at 94°C). PCR products were separated by electrophoresis on a 1% agarose gel. The lengths of the PCR products were estimated by comparison with the ϕx174 DNA Ladder molecular size markers (Promega, Lyon, France) [12].

S. aureus reference strains RN6390 (*agr* group I), RN6607 (*agr* group II) and RN8462 (*agr* group III), kindly provided by Novick RP were used as a control for *agr* group identification (Shirball Institute, New York).

Statistical analysis

Fisher's exact test, a statistical significance test applied in the analysis of categorical data where sample sizes are small, was used to examine the significance of the association between two variables in a 2.2 contingency tables. It was done to determine a possible relationship between the *agr* dichotomic groups: *agr*I and non *agr* groupI (II and III) and the invasivity of the infections (invasive and non invasive infections).

RESULTS

Antimicrobial susceptibility testing

Fifty five strains were resistant to methicillin (Figure1). The rates of resistance to other antibiotics were: kanamycin (n=80), tobramycin (n=33), gentamicin (n=6), chloramphenicol (n=2), ofloxacin (n=9), trimethoprim-sulfamethoxazole (n=7), tetracyclines (n=60), erythromycin (n=53), clindamycin (n=9), fosfomycin (n=6) and rifampin (n=37). All the strains were susceptible to vancomycin.

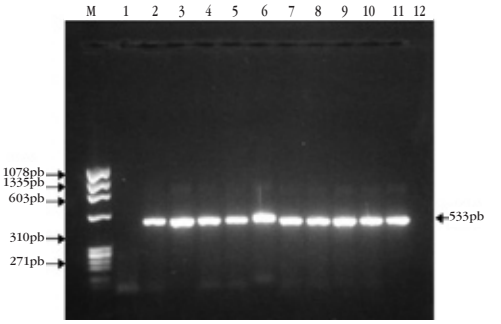

Figure 1. *PCR assay for the detection of mecA gene. Lane M: molecular size marker øx174 digested by HaeIII, Lane 1: S. aureus 25923 (mecA-), Lane 2: S. aureus 43300 (mecA+), Lane 3-12: positive strains.*

agr grouping

All isolates were classified by PCR as part of one of three *agr* groups (Figure 2): 19 strains belonged to group I (14/19 were represented by invasive infections), 16 to group II and 65 to group III (11/16 and 40/65 were from non invasive infections respectively). No *agr* group IV strains were found in our study (Table I). Among methicillin resistant *S. aureus* (MRSA), 9 (16. 4%) belonged to group I, 8 (14. 5%) to group II and 38 (69. 1%) to group III. For methicillin susceptible *S. aureus* (MSSA), only 10 strains (22.2%) belonged to group I, 8 (17.8%) to group II, and 27 (60%) to group III (Table II).

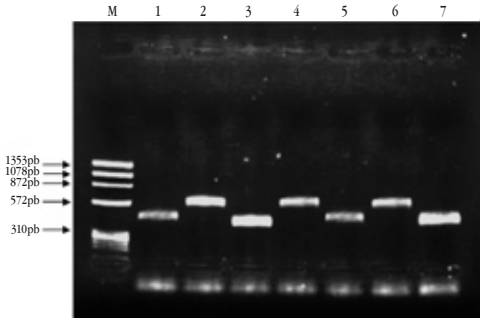

Figure 2. *PCR assay for the identification of agr specificity groups. Lane M: molecular size marker øx174, Lane 1: RN6390 (agrI), Lane 2: RN6607 (agrII), Lane 3: RN8462 (agrIII), Lane 4: agrII strain, Lane 5: agrI strain, Lane 6: agrII strain, Lane7: agrIII strain.*

Table I: agr group distribution according the types of infections.

Types of infections	Total number	*agr* groupI	*agr* groupII	*agr* groupIII	P value
Non invasive infections	**56**	**5 (9 %)**	**11(19.6%)**	**40 (71.4%)**	***P* =0.003**
Cutaneous abcesses	25	1 (4 %)	4 (16 %)	20 (80%)	NS
Furuncles	12	1 (8.33%)	4 (33.33%)	7 (58.33%)	NS
Other cutaneous infections	19	3 (15.8%)	3 (15.8%)	13 (68.4%)	NS
Invasive infections	**44**	**14 (31.83%)**	**5 (11.36)**	**25 (56.81%)**	***P* =0.003**
Bacteremia	27	13 (48.14%)	4 (14.81%)	10 (37.03%)	$P = 10^{-4}$
Urinary tract infection	10	0 (0%)	1 (10%)	9 (90%)	NS
Catheter related infection	3	0 (0%)	0 (0%)	3 (100%)	NS
Pneumonia	2	1 (50%)	0 (0%)	1 (50%)	NS
Kidney infection	1	0 (0%)	0 (0%)	1 (100%)	NS
Toxic Shock Syndrome	1	0 (0%)	0 (0%)	1(100%)	NS

Statistical analysis

The frequency of invasive infections among *agr* group I was high (73%) contrarely to the frequency of non invasive infections among *agr* group II and III (37%). A preferential link was observed between *agr* groupI and invasive infections (30/44=68, 18% versus 51/56=91, 07%, $P=0.003$) especially bacteremia (13/27=48, 14% versus 5/51=9, $P=10^{-4}$) (Table I). *agr* group II and III were significantly related with non invasive infections with $P=0.003$ (30/44= 68,18% versus 51/56=91.07%). No association was found between other types of infections and *agr* groups. Likewise, no correlation was observed between *agr* groups, age or sex of patients and type of infections.

100% (all strains) of *agr* group I MRSA strains caused invasive infections (9/1=52, 9% invasive infections versus 0/38=0% non invasive infections, $P=4.10^{-6}$) but *agr* group II and III MRSA strains were associated with invasive infections in only 9.8 % of cases. All *agr* group II MRSA isolates caused non invasive infections (Table II). For MSSA, the isolates from invasive infections were not significantly associated among the different *agr* groups.

Table II: Relationship between agr groups, susceptibility to methicillin and types of infections.

agr groups	Invasive	Infections (n=44)	Non Invasive	Infections (n=56)	All isolates
	MRSA (n=17)	**MSSA (n=27)**	**MRSA (n=38)**	**MSSA (n=18)**	**n=100**
GroupI	n=9 (53%)	n=5 (18.5%)	n=0	n=5 (28%)	n=19 (19%)
GroupII	n=0	n=5 (18.5%)	n=8 (21%)	n=3 (17)	n=16 (16%)
GroupIII	n=8 (47%)	n=17 (63%)	n=30 (79%)	n=10 (55%)	n=65 (65%)
GroupIV	n=0	n=0	n=0	n=0	n=0

DISCUSSION

S. aureus expresses many potential virulence factors [1, 2]. For the majority of diseases caused by *S. aureus*, pathogenesis is multifactorial, so it is difficult to determine precisely the role of any given factor. To improve their ability to cause variety of human diseases, staphylococci have developed quorum-sensing systems that enable cell-to-cell communication and regulation of numerous colonization and virulence factors [1, 2].

Among our isolates, the most predominant *agr* group, in both MRSA and MSSA, was the group III (69% and 60% respectively), followed by group I (19%) and group II (16%). This was also reported by Ben Nejma et al in MRSA strains [15]. It seems that *agr* group III was the predominant group in Tunisia, contrarely that was observed in other regions worldwide where the most frequent group was *agr*I. In fact, Van Leewen et al reported that 92.2% of isolates were *agr* group I [16]. Yoon et al reported that 49.3% of MRSA in a Korean

tertiary care teaching hospital were *agr* group I, followed by *agr* group II (44%) and III (6.7%). This study suggests that *agr* groupI is the most prevalent in Korea [17]. In USA, a study done by Shopsin et al reported that 42% of their collection belonged to *agr* groupI, followed by group III (34%) and groupII (24%). Similar results were found in Belgium and Germany studies [18, 19, 20]. More recent data demonstrate that the vast majority of community-acquired MRSA in France and around the world belong to *agr* group III [21]. It was clearly demonstrated that *agr* group I and III are closely related (80% sequence homology) suggesting that strains circulating worldwide possess a unique genetic characteristic [16]. *agr* group II strains, which was relatively small in our study compared to the group I, have been isolated mainly in Japan, North America and also in some European countries [17].

The balanced recovery of three of the four *agr* groups differs from countries [12, 13] perhaps reflecting ecological and geographical structuring (or sampling bias).

agr group IV was not found among our isolates. The absence of *agr* group IV from this study and other studies suggests that competition does not favor these strains [12, 15]. The cognate peptide produced by some strains inhibits the *agr* expression in other strains. Cross inhibition of gene expression represents is a form of bacterial interference excluding other strains of the same species from the infection or colonization site.

The *agr* system has historically been assigned a central role in the model of *S. aureus* pathogenesis [1, 2]. In fact, expression of *agr* was found to contribute to staphylococcal pathogenesis in several infection models, including murine subcutaneous abscesses and arthritis, as well as rabbit endocarditis. Expression of *agr* also appears to be involved in the invasion and apoptosis of epithelial cells. Interestingly, different *agr* groups, as defined by their production and recognition of distinct secreted signals, are associated predominantly with certain diseases [17].

In our study, a good association was observed between *agr* groupI and invasive infections, especially bacteremia and between *agr* group II and III and cutaneous infections. Goerke et al [18], reported that the majority of *S. aureus* strains recovered from patients undergoing intubation was *agr* group II type, which is consistent with the findings of Yoon et al. [17]. Jarraud et al [22] reported that *agr* group I and II strains caused preferentially enterotoxin mediated diseases and that *agr* group III strains were involved in TSST-1 mediated diseases [7]. In our study, the only strain producing TSST-1 belonged to *agr* groupIII (unpublished data). This toxin was detected by PCR from a patient with bacteremia.

More recently, Jarraud et al reported a good correlation between *agr* group II and infective endocarditis [7]. Other studies observed a good association between *agr* II and the bicomponent toxin gene *lukD-lukE* isolate from invasive and non invasive infections [23]. A recent Korean study suggests that *agr* group I and ear infections were correlated [17]. The reasons for the association between *agr* groups and infection types are not yet clear, but a better understanding of this phenomenon may contribute to our understanding of the epidemiology of staphylococcal diseases [24]. So, further studies should re-evaluated theses associations.

CONCLUSION

agr group III was the most prevalent group in our study. Moreover, there was a very strong relationship between *agr* group I and invasive infections especially bacteremia but *agr* II and III were closely related to non invasive infections.

ACKNOWLEDGES

This study was done with the financial support of the Ministry of Scientific Research, Technology and Competence Development of Tunisia.

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