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Relationship of the accessory regulator gene (*agr*) with multiresistance in *Staphylococcus aureus* strains isolated from hospitals and dental offices

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ABSTRACT. Inert surfaces favor the persistence of *Staphylococcus aureus*, as they are reservoirs and means of contamination in hospital and clinical environments. The pathogenicity of this bacterium is controlled by the accessory gene regulatory (*agr*) system. We examined virulence and toxin genes in isolated strains of *S. aureus* on inert surfaces and their relationship with the *mecA* gene, responsible for methicillin resistance (MRSA) in 59 *S. aureus* strains isolated from inert surfaces and stored in the molecular biology laboratory of the Catholic University of Cuenca, in which presence of toxin genes (*lukS/lukF-PV,tst*) and the *mecA* gene had been previously detected. Multiplex PCR was used to determine the *agr* types. Of the 59 *S. aureus* strains, 66.1% were positive for *agrI*, 8.5%, *agrII*, and 18.6%, *agrIII*; *agrIV* was not present in any of the

strains. A significant relationship was found between *agrI* and MRSA. Molecular *agr* gene typing is important for monitoring the appearance, dissemination, and persistence of MRSA epidemic strains. In conclusion, the gene with the highest frequency was *agrI* followed by *agrIII* and *agrII*.

Key words: *Staphylococcus aureus*; Genes; Virulence; Hospital surfaces

INTRODUCTION

Staphylococcus aureus, a Gram-positive, opportunistic bacterium, causes a variety of invasive diseases with high morbidity and mortality rates (Dunman et al., 2001). It can adapt to different environments, causing skin, soft tissue, and bloodstream infections (Tuchscherr et al., 2016).

Its ability to spread in hospitals and healthcare institutions has increased the prevalence of methicillin-resistant *S. aureus* (MRSA) strains (Goudarzi et al., 2017). The indiscriminate use of antibiotics has contributed to the development of multi-resistant strains and the failure of antibiotic treatments. In *S. aureus*, the gene responsible for coding the presence of resistance to methicillin is the *mecA* gene (Cechinel et al., 2016).

The accessory regulatory gene (*agr*) is one of the main systems favoring the control of *S. aureus* virulence factors (Tan et al., 2018). The *agr* operon possesses two different promoters P2 and P3. These promoters are responsible for the transcription of RNAII and RNAIII. RNAIII acts mainly at a transcriptional level for target genes and independently regulates the translation of at least one or two exoproteins. RNAII is responsible for encoding the expression of four proteins (AgrA, AgrB, AgrC, AgrD) (Peng et al., 1988; George & Muir, 2007). Each promoter carries out a specific function for the reactivation of the *agr* system. The AIP (autoinducing peptide) signal is produced from the AgrD precursor, which is processed by the transmembrane protein AgrB, whose main function is to export AIP through the membrane to the extracellular space (George & Muir, 2007).

AIP activates the AgrC-AgrA components, and AgrA phosphorylation is provoked, which in turn activates the P2 and P3 promoters, resulting in transcription of the *agr* system (Ji et al., 1995). *S. aureus* isolates are divided into four groups (*agrI*, *agrII*, *agrIII*, *agrIV*); this is the result of a polymorphism of the AIP in its AgrC membrane sensor.

The specificity presented by these groups can influence the ecology of the host, either to enhance, inhibit or compete in relation to another type of *Staphylococcus* (Shopsin et al., 2003). *S. aureus* can encode specific toxins for certain pathologies such as Toxic Shock Syndrome Toxin (TSST-1) and Leukotoxin (Valentine Leukocidin (PVL); however, not all *S. aureus* strains present these toxins (Tahmasebi et al., 2020).

Due to the importance of the accessory gene regulator system (*agr*) in the pathogenesis of *S. aureus*, the objective of this study was to determine the types of *agr* among *S. aureus* strains, as well as to evaluate if there is a relationship between certain toxin genes, *agr* genes (I, II, III, IV), with MRSA.

MATERIAL AND METHODS

Bacterial isolation and identification

This study is part of a macro research project of the Catholic University of Cuenca PICCIITT19-40, which aims to identify *S. aureus* strains (virulence genes and resistance genes) isolated from dental offices and hospital areas and dental offices (cell phones and other inert surfaces). In previous investigations, a total of 905 samples were analyzed, of which 59 were isolated; from these strains the toxin genes (*lukS/lukF-PV, tst*) and the *mecA* gene (MRSA) were already determined. These strains were from: 16 cell phones of dental students (Laica et al., 2021), seven cell phone screens of health care personnel working in a public hospital, 12 strains isolated from cell phones of personnel working in dental offices, two strains isolated from inert surfaces of a private clinic (Sánchez Zambrano et al., 2022), eight strains isolated from multipurpose boxes of dental students (Patricia et al., 2022), six strains isolated from cell phones of personnel working in clinics, five strains isolated from inert surfaces of a public hospital (De et al., 2021), and three strains isolated from cell phones of dentists working in private offices (Cornejo Bravo et al., 2022). These strains are preserved at -80°C in the laboratory of molecular biology and genetics of the Catholic University of Cuenca.

Detection of virulence genes

DNA extraction was performed according to the protocol of Andrade and Orellana (2019). Conventional PCR technique (PCR master mix) was used, each sample containing: 10 ul of Green GoTaq Master mix 2x (Promega), 6 µL of nuclease-free water (Promega) 2 µL of sample DNA and 1.5 µL of each primer) for molecular detection of *agrI, agrII, agrIII, agrIV* genes, the sequences of the primers, amplicons, and PCR conditions are detailed in Table 1.

Table 1. Primers used for *agrI, agrII, agrIII and agrIV* gene detection in *Staphylococcus aureus* isolates from inert surfaces in dental offices and hospital areas (Peerayeh et al., 2009).

Gene	Sequence 5'- 3'	PCR	Product size
agrI	**Forward:** ATGCACATGGTGCACATGC **Reverse:** GTCACAAGTACTATAAGCTGCGAT	Initial Denaturalization 94℃ for 300 sg 94℃ for 60 sg 57℃ for 60 sg Elongation 72℃ for 60 sg Extension 72℃ for 300 sg	440 bp
agrII	**Forward:** ATGCACATGGTGCACATGC **Reverse:** GTATTACTAATTGAAAAGTGCCATAGC		572 bp
agrIII	**Forward:** ATGCACATGGTGCACATGC **Reverse:** CTGTTGAAAAAGTCAACTAAAAGCTC		406 bp
agrIV	**Forward:** ATGCACATGGTGCACATGC **Reverse:** CGATAATGCCGTAATACCCG		588 bp

The PCR master mix of each sample was placed in an Agilent Technologies SureCycler 8800 thermal cycler subsequently for separation of the amplicons a 2% W/V agarose gel with 2µl SYBR safe was used and the electrophoretic run was performed at 90 V for a period of 60 minutes. The amplicons of each sample were observed in a UV transilluminator and photographed (Vallejo Pazmiño et al., 2022).

Statistical analysis

The Stata 17 program was used for statistical analysis. Contingency tables of the presence of the *agrI, II, III and IV* genes in the *S. aureus* strains isolated from various inert surfaces in hospital areas, were used for the statistical calculation. Contingency tables were also used to demonstrate the relationship between virulence genes and resistance to methicillin. In addition, the Chi-square statistical test was used to determine statistical relationships between these variables.

RESULTS

agr typing

Of the 59 strains of *S. aureus* isolated from different inert surfaces in dental offices and hospital areas 66.1% *agrI*, 8.5% *agrII* and 18.6% *agrIII* were obtained, as shown in Table 2.

Table 2. Frequency of virulence genes in *Staphylococcus aureus* isolates from inert surfaces in dental offices and hospital areas.

Surface	*agrI*		*agrII*		*agrIII*		*agrIV*		Total	
	N	%	N	%	N	%	N	%	N	%
CE	1	50.0	0	0.0	2	100.0	0	0.0	2	100.0
CJ	7	87.5	0	0.0	1	12.5	0	0.0	8	100.0
CO	10	83.3	1	8.3	2	16.7	0	0.0	12	100.0
CV	5	83.3	1	16.7	0	0.0	0	0.0	6	100.0
EC	9	56.3	2	12.5	2	12.5	0	0.0	16	100.0
HM	5	71.4	0	0.0	2	28.6	0	0.0	7	100.0
PCO	1	33.3	1	33.3	0	0.0	0	0.0	3	100.0
SHM	1	20.0	0	0.0	2	40.0	0	0.0	5	100.0
Total	**39**	**66.1**	**5**	**8.5**	**11**	**18.6**	**0**	**0.0**	**59**	**100.0**
Test Statistic (X^2)	11.213		5.281		13.376		n/a		…	
P-value	0.130		0.626		0.063		n/a		…	

CE = Cell Phones – Dental Students. CJ = Multi-Purpose Cases – Dental Students. CO = Cell phone screens of personnel from dental offices. CV = Cell phones of staff working in clinics. HM = Cell phone screens of health personnel working in public hospitals. PCO = Cell phones of dentists who work in private offices. SHM = Inert surfaces of a public hospital. EC = Inert surfaces of a private hospital

Relationship between types of virulence genes and toxin genes with MRSA

When *agr* virulence gene types (*I, II, III and IV*) and toxin genes (*eta, etb, lukS/lukF-PV, tst*) were related to methicillin (MRSA) resistance and susceptibility (MSSA), the following results were obtained: *agrI* MSSA 18.64% and MRSA 13.56%, *agrII* MSSA 66.10% and MRSA 3.39%, *agrIII* MSSA 57.63% and MRSA 5.08%. A P

value of 0.049 for *agrI* indicated a statistically significant correlation between methicillin resistance (MRSA) and the *agrI* virulence gene.

Table 3. Virulence gene and toxin gene frequency related to MRSA and MSSA isolates. P-value associated with the chi-square statistic. * Statistically significant at 5%.

Type	Gene	MSSA (%)	MRSA (%)	X^2	P value
Virulence Control	*agrI*	18.64	13.56	3.865	0.049 *
	agrII	66.10	3.39	0.333	0.564
	agrIII	57.63	5.08	0.016	0.900
	agrIV	71.19	0.00	n/a	n/a
Toxin Genes	*eta*	71.19	0.00	0.000	0.995
	etb	71.19	0.00	0.000	0.995
	lukS/lukF-PV	69.49	1.69	0.453	0.501
	tst	28.81	11.86	1.641	0.200

Figures 1-4 show the results of the PCR used to detect the virulence genes (*agrI*, *agrII*, *agrIII*, *agrIV*) in *S. aureus* strains isolated from samples obtained from inert surfaces.

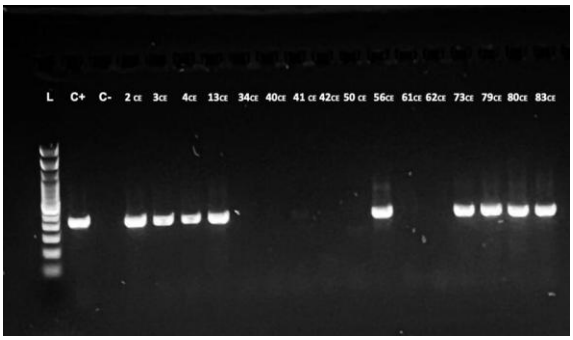

Figure 1. PCR product for the *agrI* gene (440 bp) in *Staphylococcus aureus* strains isolated from Cell Phones of Dental Students (CE), first lane Ladder (L), second lane positive control (C+) *Staphylococcus aureus* ATCC 11632 strains; third lane negative control (C-) *Streptococcus pyogenes* ATCC strain; Positive samples: 02CE, 03CE, 04CE, 13CE, 56CE, 73CE, 79CE, 80CE, 83CE.

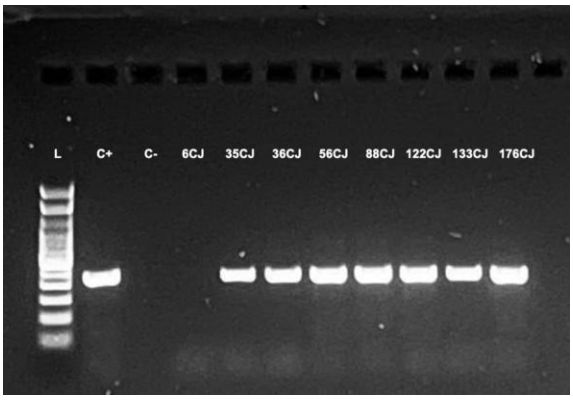

Figure 2. PCR product of the *agrI* gene (440 bp) in *Staphylococcus aureus* strains isolated from dental student multipurpose cases (CJ), first lane Ladder (L), second lane positive control (C+) *Staphylo-coccus* aureus ATCC 11632 strain; third lane negative control (C-) *Streptococcus pyogenes* strain ATCC strain; Positive samples: 35CJ, 36CJ, 56CJ, 88CJ, 122CJ, 133CJ, 176CJ.

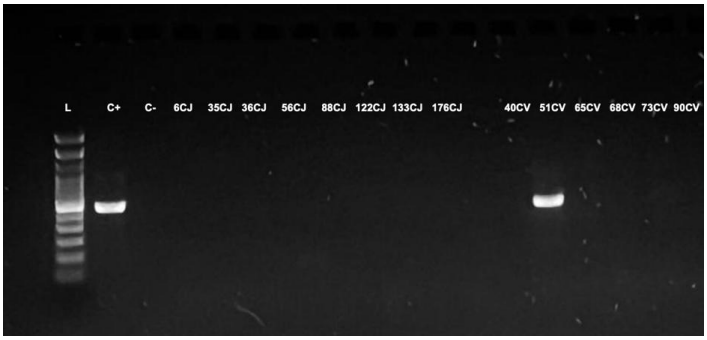

Figure 3. PCR product of the *agrII* gene (572pb) in *Staphylococcus aureus* strains isolated from dental student multipurpose cases (CJ), first lane Ladder, second lane positive control *Staphylococcus aureus* ATCC strain (laboratory strain); third lane negative control *Streptococcus pyogenes* ATCC strain; Negative samples 6CJ ,35CJ ,36CJ ,56CJ ,88CJ ,122CJ ,133CJ ,176CJ. In addition, PCR product of the *agrII* gene (572pb) in *Staphylococcus aureus* strains isolated from cell phones of personnel working in clinics (CV). Positive sample: 51.

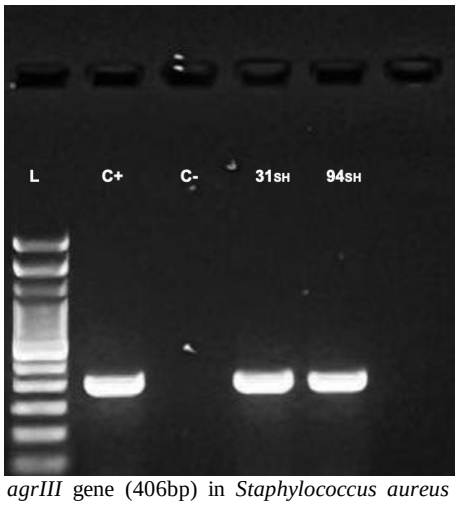

Figure 4. PCR product of the *agrIII* gene (406bp) in *Staphylococcus aureus* strains isolated from Hospital Surfaces (SH), first lane Ladder (L), second lane positive control strain (C+) *Staphylococcus aureus* ATCC 25923; third lane negative control (C-) *Streptococcus pyogenes* ATCC strain; Positive samples: 31SH, 94SH.

DISCUSSION

The gene regulator (*agr*) of *S. aureus* is considered a global regulator that controls virulence factors and its possible relationship in the presence of certain human infections. In relation with *agrI*: Rezk et al. (2022) reported that 50 strains of *S. aureus* were isolated from nasal carriers and *agrI* was present in 54% of all *agr* genes analyzed. Javdan et al. (2019) reported that among 150 positive samples for *S. aureus* obtained from clinical samples (wounds, blood, urine, tissues, etc.) 55% were *agrI*. Goudarzi et al. (2017) point out in their study that *agrI* was the most frequent gene (69.5%) and predominant in samples of *S. aureus* isolated from biological samples (blood). Studies by Van Leeuwen et al. (2000), Peerayeh et al. (2009), and Khan et al. (2014), in biological samples obtained similar results where *agrI* was found more frequently in relation to the others. In addition, the study of the

presence of *agrI* is important since some researchers associate it with diseases such as suppurative infections and endocarditis (Tan et al., 2018).

Pacheco et al. (2021) mentions the high frequency of virulence genes in *S. aureus* strains isolated from mobile phones (9/16 *tst* gene, and 1/16 *lukS-F PV* gene), but there are very few studies of *agr* gene analysis in strains of *S. aureus* isolated from inert surfaces, one of these carried out by Abbasian et al. (2018) , reports 167 strains of *S. aureus* obtained from clinical samples of patients (pus, blood, and nostrils of service employees), inanimate surfaces (computer accessories, telephones, door handles, etc.) and emergency rooms, analyzed the frequency of certain *agr* genes where the frequency of *agrI* was 78% in total of all strains and only 14% on inert surfaces, so it is worth mentioning that compared to our study we obtained a higher percentage of *agrI* on inert surfaces . These dissimilarities may be related to: quantity, geographical location, collection periods, in relation to sampling, etc.

In relation to the presence of *agrII*, the results obtained in the present study were relatively low, compared to the results obtained by Mazloomirad et al. (2021). However, Cechinel et al. (2016) observed that group II is associated with a higher mortality rate in critically ill patients with MRSA bacteremia treated with vancomycin. In addition, some research mentions that the activation of *agrII* system could influence the expression of genes related to antibiotic resistance, which could affect the efficacy of treatments (Sakoulas et al., 2003).

Results inconsistent with those obtained in this study were reported by Maleki et al. (2019); out of a total of 48 strains for *S. aureus* obtained from pediatric clinical samples, indicated *agrIII* was the most prevalent with 56.3%. In our study obtained negative results for all 59 strains of *S. aureus* coinciding with Pereira et al. (2022) and Mazloomirad et al. (2021), where *agrIV* was not observed in any of the isolates.

In other investigations, which related the *agr* group and the type of disease: the strains of *agrIII* group were associated with non-invasive infections (P = 0.02) and *agrI* group strains with invasive infections, especially bacteremia (P = 0.002) (Cheraghi et al., 2017), several studies have found that there is an overly complex relationship between *agr* genes and antibiotic resistance (Painter et al., 2014).

Studies carried out for the presence of toxin genes in *S. aureus* MRSA strains, report 58.6% of *tst* (TSST-1 toxic shock syndrome), being the toxin gene with the highest prevalence (Goudarzi et al., 2017). These results coincide with those of the present study in which *tst* presented a higher frequency in relation to the other toxin genes. In the present study, there is a relationship between *S. aureus* carrying the *lukS/lukF-PV* toxin gene with resistance to MRSA methicillin, although low, which coincides with some studies (Elbargisy, 2022; Kmiha et al., 2023).

In our study, significant association was found between *agrI* and MRSA (13.56% with a value of P=0.049). Similar results were found in studies carried out by Goudarzi et al. (2017), who obtained from a total of 128 MRSA strains 69% *agrI*. Tahmasbi et al. 2020) obtained 71.8% *agrI* from 85 MRSA strains, in addition some MRSA *agrI* strains have been isolated from skin infections and hospital environments.

Contrasting data were reported by (Ben Ayed et al., 2006) who mentions that out of a total of 57 MRSA isolates, obtained from clinical samples (pus, blood cultures, urine, materials, respiratory tract, fluid punctures), the *agr* groups were distributed as follows: 9 (15.7%) belonged to group I, 2 (3.5%) belonged to group II and 23 (40.3%) belonged to group III and no strains were found from group IV.

Latifpour et al. (2022), analyzed 55 MRSA strains obtained from clinical samples of urine, wounds, and cerebrospinal fluid, where 54.54% *agrII* was obtained. (Rezk et al., 2022), in their study identified MRSA strains obtained from clinical samples and nasal carriers there was a statistically significant association between *agrIII* and MRSA ($p = 0.006$), these dissimilarities in the results may be due to differences in geographic location and source of isolation, but from all these findings it is clear that there is a relationship between *agrI*, *agrII*, *agrIII*, and MRSA.

Tuchscherr et al. (2020) mentioned in their study on the small colony clinical variants (SCV) of *S. aureus* phenotype, the main characteristics of *S. aureus* SCV include reduced membrane potential, low virulence due to alterations in the *agr* system through interactions with other molecules, extended survival within host cells, high resistance to specific antimicrobials, and efficient evasion of the host immune response. These characteristics contribute to the failure of clinical treatment of chronic staphylococcal infections, thus the imperative need for a future analysis of this phenotypic variant and its relationship with *agr*.

Le et al. (2015) and Jenul et al. (2019), indicate that the regulation of quorum sensing in *S. aureus* is due to several master virulence regulators, the most important being the quorum sensing *agr* system due to its strong impact on many types of staphylococcal diseases. In acute disease, *agr* generally enhances pathogenesis by increasing the expression of aggressive virulence determinants, such as toxins and degradative exoenzymes. Whereas *agr* has a more complicated role during chronic infections, as *agr* mutants show increased biofilm formation, but a lower potential for dissemination, as well as correlating with greater success during persistent bacteremia. In the future, a better understanding of the often-divergent roles of *agr* in various types of staphylococcal infection is needed to establish a solid scientific basis to underline the applicability of quorum sensing blockers for the treatment of staphylococcal disease.

CONCLUSIONS

In conclusion, the gene variant with the highest frequency was *agrI* followed by *agrIII*, *agrII* and no *agrIV*, in addition to a significant relationship between *agrI* and MRSA, which may demonstrate a relationship between virulence and resistance. Consequently, we conclude that *S. aureus* is a bacterium that prevails on different inert surfaces and that in certain strains different virulence genes and toxins preside. In addition, it can influence antibiotic resistance, so it presents a challenge. Therefore, molecular typing of the *agr* gene can be used to control the emergence, spread and persistence of endemic MRSA strains.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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